Scrub typhus in northern Thailand

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Scrub typhus, a neglected infectious disease caused by obligate intracellular bacteria *Orientia tsutsugamushi*, is a major cause of acute non-malarial fever in the tropics. Difficulties surrounding diagnosis have hampered our understanding of disease burden, which in turn negatively impacts awareness. Using national surveillance data, I highlighted the high burden of scrub typhus in Thailand with a substantial rise in cases over time. Spatially, disease burden was greatest in the northern region and geographical and meteorological factors may contribute to disease prevalence. Scrub typhus contributes significantly to the febrile disease burden in Chiangrai, northern Thailand, with 22.5% of adults admitted to hospital with acute undifferentiated fever diagnosed with the disease. I described the disease in adults and found presence of eschar and elevated liver enzymes to be predictive of scrub typhus. Scrub typhus in children remains understudied and in this thesis, I characterised paediatric scrub typhus in Chiangrai, showing that the disease was frequently severe and potentially fatal with complication and treatment failure rates of 40% and 23%, respectively. Severe hepatitis was found to be predictive of treatment failure in this cohort.

In the 1990s, reports of putative drug resistant scrub typhus emerged from northern Thailand. This proved controversial at the time as doxycycline resistant *Orientia tsutsugamushi* had not been described. Studies on treatment outcome and its determinants were not pursued, leading to uncertainty regarding optimal scrub typhus treatment. I reviewed the evidence on

drug resistant scrub typhus extensively and concluded that doxycycline resistance may have been misconceived. Finally, I described my ongoing role as principal investigator for the Scrub Typhus Antibiotic Resistance Trial, a randomised controlled trial comparing the efficacy of 7 days of doxycycline versus 3 days of doxycycline versus 3 days of azithromycin in northern Thailand. Detailed immunological, microbiological and pharmacological analyses are embedded, which should provide clarity on the determinants of treatment outcome and whether drug resistance is illusory.

Table of Contents

1 Preface

1.1 Acknowledgements

I would like to formally acknowledge those who helped and supported me during my DPhil studies. Often, their support began well before matriculation at the University of Oxford in October 2017 and without them, this work would not have been possible. I thank Prof Nicholas Day (Director, Mahidol-Oxford Tropical Medicine Research Unit – MORU) for not only being my main supervisor and sponsor, but also my mentor. I first met Nick in Bangkok in January 2012 and I could not have envisaged all that has been subsequently achieved both personally and collaboratively. His enthusiasm for scrub typhus and rickettsial diseases research is highly contagious and I hope to continue building on the foundations laid by Nick and others in future. I am grateful to Prof Paul Newton (formerly Lao-Oxford-Mahosot-Wellcome Trust Tropical Medicine Research Unit – LOMWRU and Oxford) for agreeing to supervise me for this DPhil project and for his wisdom and support whenever I needed it. I am indebted to both of them for their insights into the historical context of scrub typhus, which remains fascinating, and continues to be a treasure trove of ideas and potential answers into the issues encountered today. I also thank my other supervisors – Prof Joel Tarning (MORU) for his insights and support for the pharmacological aspects of the Scrub Typhus Antibiotic Resistance Trial (START) and Prof Mavuto Mukaka (MORU) for not only teaching me about statistics, but for his patience while I learn to apply it to clinical research.

I thank Prof Daniel Paris (Swiss Tropical and Public Health Institute) for laying the foundations for many aspects of my work and for supporting me through those tentative early years in Thailand. Many aspects of this thesis would not have been as successful if the paths had not been trodden and straightened by Danny previously. I am grateful for his ideas, support and friendship and in the relatively small world of rickettsial diseases

research, I hope to continue our collaborations in the near future. My gratitude too to Prof Sir Nicholas White (MORU), Prof Stuart Blacksell (MORU), Dr Al Richards (formerly Naval Medical Research Center – NMRC, retired) and Prof Jeanne Salje (Rutgers and Oxford) who have provided helpful and insightful discussions and collaborations over the years along with financial, laboratory and intellectual support.

I am grateful for the support, camaraderie and friendship of the team at Chiangrai Clinical Research Unit, Thailand. It was a joy to lead and work with them and I am proud to see their growth and development over the 5 years of my tenure. The team included study nurses Suthathip Kaewta, Nidanuch Tasak, Piangnet Jaiboon and Nipaphan Kantawang and laboratory technicians Areerat Thaiprakhong, Nattapon Pinthong and Panumas Konlam. My thanks to Dr Carlo Perrone who took over my role in leading the unit and running the studies. I am certain that my connection with the Chiangrai unit will continue for the foreseeable future.

I would like to thank all the staff and patients at Chiangrai Prachanukroh Hospital who welcomed our team and graciously participated in our studies. In particular, I would like to thank Dr Pacharee Kantipong (retired), Dr Achara Laongnualpanich (past Deputy Director), Dr Samroeng Seekaew (Deputy Director), Dr Chulapong Chanta (Paediatrician), Dr Supalert Nedsuwan (Family Medicine physician), Dr Daranee Intralawan (Family Medicine physician) and Dr Suwimon Khusuwan (Infectious Diseases physician). I am also grateful for the help and support provided by staff at Chiangrai Provincial Public Health Office for our studies and also for our public engagement work. They generously allowed us to utilise and seek support from the network of primary care units and their staff throughout the province.

At MORU, I would like to thank the Clinical Trials Support Group (CTSG) and specifically, Zoe Doran and Tanya Cope – past and current Heads of CTSG who were always helpful and supported me in running our studies. Also, Naomi Waithira (CTSG's Head of Data),

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I am thankful of the team at Shoklo-Malaria Research Unit (SMRU) for their enthusiastic support and participation with START especially Prof Francois Nosten (Director, SMRU), Prof Rose McGready (Deputy Director, SMRU) and research physicians Dr Cindy Chu and Dr Tobias Brummaier. My thanks also to Dr Clare Ling (Molecular Microbiologist, SMRU) who coordinated study sample management and diagnostic assays on site.

Thank you to my colleague and friend Dr Ivo Elliott (LOMWRU and Oxford) who allowed me to participate in the field trips in Chiangrai as part of his scrub typhus ecology project and DPhil. We had a great time in the field trapping rodents and trying to find the ever-elusive free-living mites while enjoying delicious food and philosophical discussions on scrub typhus, rickettsial diseases, tennis, climate and other aspects of life in the tropics. I am grateful for the wisdom and friendship of Prof Serge Morand (National Center for Scientific Research-French Agricultural Research Centre for International Development, CNRS-CIRAD) who was ever patient with me while I tried to understand the basics of ecology and modelling. I also thank Dr Kittipong Chaisiri (Entomologist, Mahidol University) and Dr Rawadee Kumlert (Entomologist, Ministry of Public Health, Thailand) for their input and

collaboration. All four participated in my study estimating the burden of scrub typhus in Thailand (Chapter 3).

I am thankful for my colleagues and friends in the MORU Clinical Microbiology Network who provided a sounding board for ideas and allowed me to participate in collaborative projects across the MORU Tropical Health Network. They were a source of wisdom, particularly as I was starting out, and I found their advice and support to be invaluable. The network included, Dr Clare Ling, Prof Paul Turner, Prof Elizabeth Ashley, Prof David Dance and Dr Matthew Robinson. I would also like to thank Dr Weerawat Phuklia (LOMWRU) for his crucial input and work on *Orientia tsutsugamushi* antibiotic susceptibility testing which allowed me to formulate a working hypothesis and explanation for why scrub typhus treatment with doxycycline may sometimes fail (Chapter 2).

My time in northern Thailand would not have been possible without financial support from Wellcome (part of MORU's core funding). I would also like to thank Marty Stokes at Defense Threat Reduction Agency (US Department of Defense) who sponsored the 2nd Asia Pacific Rickettsia Conference (APRC2) which contributed to its success and allowed us to support early career researchers from low-to-middle income countries to attend.

In February 2020, I returned to clinical work at Oxford University Hospitals NHS Foundation Trust. It has been a challenging time, looking after patients throughout the COVID-19 pandemic. I would like to thank my wonderful colleagues in general medicine and microbiology and infectious diseases for their friendship, kindness and support as I transitioned back to the NHS. I am grateful to them for allowing me time to complete my DPhil thesis and their constant encouragement.

Finally, I dedicate this thesis to my family. I would like to thank my parents, Krassanai and Kanya Wangrangsimakul who, despite the sacrifices they went through, supported me

throughout my formative years including sending me abroad to study in pursuit of a better education and future. They instilled in me from an early age, the drive to excel and to help others who are suffering, be it through poverty or illness. They continue to support me in other ways today and despite the physical distance between us, I know they are proud of this achievement.

I would like to end by thanking my wife and best friend Rachel whom I met 11 years ago in a lecture theatre at the Liverpool School of Tropical Medicine and Hygiene. This journey to the tropics and back again would not have been possible without her love, endless patience and support and I look forward to future adventures together. I am grateful to my children, Lydia and Ethan, whose arrivals bookended this work. They have borne my stumbling of fatherhood, provided a constant life anchor and continue to be a source of unfettered joy.

1.2 Funding

I was financially supported by a Nuffield Department of Medicine Prize Studentship (University of Oxford) which covered University and College fees.

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1.3 Ethics statements

In chapter 3, publically available disease reporting data were obtained from the National Disease Surveillance System (R506), Bureau of Epidemiology (BoE), Department of Disease Control (DDC), Ministry of Public Health (MoPH), Thailand. All data analysed were anonymised, collected as part of routine national public health measures and therefore, did not require ethics committee review.

In chapter 4, ethical approval for the study was obtained from Chiangrai Prachanukroh Hospital Ethical Committee (MoPH, Thailand) and the Faculty of Tropical Medicine Ethics Committee, Mahidol University, Bangkok, Thailand (MUTM 2006-035).

Ethical approval for the study contributing to chapter 5 was obtained from the ethics committees of Chiang Rai Prachanukroh Hospital and Chiang Rai Provincial Public Health Office (MoPH, Thailand), Faculty of Tropical Medicine (MUTM 2014-059-04, Mahidol University, Bangkok, Thailand), and University of Oxford (OxTREC 52-14, Oxford Tropical Research EC, Oxford, United Kingdom). This study was registered at ClinicalTrials.gov (identifier NCT02398162).

In Chapter 6, ethical approval for START was obtained from the ethics committees of Chiang Rai Prachanukroh Hospital (MoPH, Thailand), Faculty of Tropical Medicine (MUTM 2016-064-04, Mahidol University, Bangkok, Thailand), and University of Oxford (OxTREC 4-17, Oxford Tropical Research EC, Oxford, United Kingdom). This study was registered at ClinicalTrials.gov (identifier NCT03083197).

1.4 Declarations and attributions

The work detailed in this thesis encompasses tropical medicine, microbiology, epidemiology and statistical modelling which could only be achieved through collaboration. I shall outline in detail, work attributed to others below. However, each chapter is based on my own work during the course of my DPhil studies under the supervision of Professors Nick Day, Paul Newton, Joel Tarning and Mavuto Mukaka. All figures and tables were made by me unless stated below or in the individual figure/table legends.

In chapter 3, I designed the study with input from my collaborators, Dr Ivo Elliott and Prof Serge Morand. I collected and collated disease reporting national surveillance data and local meteorological data for Chiangrai province. Additional high resolution disease surveillance data and meteorological data for Chiangrai province along with satellite imaging data were obtained by Prof Morand and Dr Rawadee Kumlert. Descriptive analyses and assessment of seasonality were performed by me. Prof Morand wrote the R codes and performed spatio-temporal analyses and modelling for scrub typhus cases. I wrote the manuscript describing our results with support and input from Dr Ivo Elliott, Dr Kittipong Chaisiri, Prof Serge Morand and Prof Nick Day. I saw the paper through the submission process to publication with support from my co-authors.

The study contributing to chapter 4 was carried out before my time in Thailand. It was designed by Dr Wirongrong Chierakul, Prof Daniel Paris and Prof Nick Day. Recruitment, sample collection and data collection was carried out by local healthcare staff at Chiangrai Prachanukroh Hospital under the supervision of Dr Pacharee Kantipong and Dr Achara Laongnualpanich. Diagnostic assays were performed by laboratory staff at MORU including Tippawan Anantatat, Dr Pruksa Nawtaisong, Suthatip Jintaworn, Ampai Tanganuchitcharnchai, Aunchalee Thanwisai and Kemajittra Jenjaroen. CRP and procalcitonin assays were performed by Dr Thomas Althaus at MORU. I completed data

input, collated and cleaned the database and attributed final diagnosis based on the results available. All statistical analyses and modelling were performed by me with support from Prof Mavuto Mukaka. I wrote the manuscript with support from Prof Daniel Paris and Prof Nick Day, responded to peer reviewers after submission and successfully published our findings.

In chapter 5, the study was conceived and designed by Prof Daniel Paris and Prof Nick Day with a significant contribution from me. I was the acting principal investigator involved in finalising the study protocol and study documents, obtaining ethical approval, study initiation and running the study to its completion. I was assisted by my team at Chiangrai Clinical Research Unit (CCRU) including Rachel Greer, Nidanuch Tasak, Piangnet Jaiboon, Suthathip Kaewta, Areerat Thaiprakong and Nattapon Pinthong. MORU's CTSG staff supported us throughout the study. Diagnostic assays were performed by Ampai Tanganuchitcharnchai, Manutsanun Sumonwiriya and Tippawan Anantatat with oversight from Prof Stuart Blacksell. All statistical analyses and modelling were performed by me with advice from Prof Mavuto Mukaka. I wrote the manuscript with support from my co-authors and saw the paper through the peer-review process to publication.

With regards to chapter 6, I am the principal investigator for START and was involved in study design along with Prof Daniel Paris, Dr Al Richards and Prof Nick Day. Prof Paris, Dr Al Richards and I were central to obtaining a funding grant from NMRC. I am involved in all aspects of the trial and was ably supported by the team at CCRU, Dr Tobias Brummaier and Prof Rose McGready at SMRU and staff at the diagnostic team at MORU, mainly Artharee Rungrojn and Pimpan Sujariyakul. Prof Mavuto and I formulated the data analysis and interim analysis plan and I wrote the STATA data cleaning and analysis codes with his support. We also tested the codes for errors using jumbled study data. Interim analysis using these codes was performed by Dr Carlo Perrone with oversight by Prof Mukaka.

Communication with the Data Safety and Monitoring Committee (DSMC) was carried out by me with support from Dr Perrone and Prof Mukaka.

I plan to collate and submit the START study protocol for peer review and publication prior to the completion of the study. I will obtain support and input from all my co-investigators in this endeavour.

1.5 Publications arising

1. 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 neglected tropical diseases. May 2018; 12(5):e0006477. https://doi.org/10.1371/journal.pntd.0006477.

2. Wangrangsimakul T, Elliott I, Nedsuwan S, Kumlert R, Hinjoy S, Chaisiri K, *et al*. The estimated burden of scrub typhus in Thailand from national surveillance data (2003-2018). PLoS neglected tropical diseases. April 2020; 14(4):e0008233. Epub 2020/04/15. https://doi.org/10.1371/journal.pntd.0008233.

3. 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. Journal of the Pediatric Infectious Diseases Society. Volume 9, Issue 2, June 2020, Pages 202–209, https://doi.org/10.1093/jpids/piz014.

4. Wangrangsimakul T, Phuklia W, Newton PN, Richards AL, Day NPJ. Scrub typhus and the misconception of doxycycline resistance. Clinical infectious diseases: an official publication of the Infectious Diseases Society of America. Volume 70, Issue 11, 1 June 2020, Pages 2444–2449, https://doi.org/10.1093/cid/ciz972.

5. Wangrangsimakul T, Phuklia W, Newton PN, Richards AL, Day NPJ. Drug-resistant scrub typhus – Reply to Watt. Clinical infectious diseases: an official publication of the Infectious Diseases Society of America. Volume 71, Issue 6, 15 September 2020, Pages 1580–1581, https://doi.org/10.1093/cid/ciz1192.

1.6 Abbreviations

ACF – Auto-correlation function

ADEM – Acute disseminated encephalomyelitis

AE – Adverse event

AG – Ancestral group

AIR – Annual incidence rate per 100,000 population

ALP – Alkaline phosphatase

ALT – Alanine aminotransferase

APRC2 – 2nd Asia Pacific Rickettsia Conference

ARDS – Acute respiratory distress syndrome

AST – Antibiotic susceptibility testing

AUC – Area under concentration/time curve

AUF – Acute undifferentiated fever

BSL-3 – Biosafety level-3

BUN – Blood urea nitrogen

CAI – Culture-attributed infection

CART – Classification and regression tree

CI – Confidence interval

CNS – Central nervous system

CONSORT – Consolidated Standards of Reporting Trials

COVID-19 – Coronavirus Disease 2019

CRF – Case record form

CRP – C-reactive protein

CSF – Cerebrospinal fluid

CTSG – Clinical Trials Support Group, MORU Tropical Health Network

CXR – Chest x-ray

DFA – Direct fluorescent antibody assay

DIC – Disseminated intravascular coagulation

DNA – Deoxyribonucleic acid

DSMC – Data and Safety Monitoring Committee

DTRA – Defense Threat Reduction Agency, US Department of Defense

EC/IRB – Ethics Committee/Institutional Review Board

EB – Elementary bodies

ELISA – Enzyme-linked immunosorbant assay

ELISpot – Enzyme-linked immunospot

ESR – Erythrocyte sedimentation rate

FACS – Fluorescence activated cell sorting

FCT – Fever clearance time

FDA – Food and drug administration

GAM – General additive modelling

GCP – Good clinical practice

GLM – General linear modelling

Hb - Haemoglobin

HIV – Human immunodeficiency virus

HLH – Haemophagocytic lymphohistiocytosis syndrome

ICF – Informed consent form

ICH – International Conference on Harmonization

IFA – Indirect immunofluorescence assay

IIP – Indirect immunoperoxidase assay

IMR – Institute for Medical Research

IP – Intra-peritoneal

IQR – Interquartile range

ITT – Intention to treat analysis

IV – Intravenous

JEV – Japanese Encephalitis Virus

LC-MS/MS – Liquid chromatography-mass spectrometry/mass spectrometry

LD – Loading dose

LOMWRU – Lao-Oxford-Mahosot Hospital-Wellcome Trust Research Unit

LPS – Lipopolysaccharide

MIC – Minimum inhibitory concentration

MIDRP - Military Infectious Diseases Research Program, US Department of Defense

MLST – Multi-locus sequence typing

MOI – Multiplicity of infection

MOPH – Ministry of Public Health (Thailand)

MORU – Mahidol-Oxford Tropical Medicine Research Unit

MRC – Medical Research Council (UK)

NHP – Non-human primates

NIH – National Institutes of Health (Department of Health and Human Services, USA)

NMRC – Naval Medical Research Center

OD – Optical density

OR – Odds ratio

PABA – Para-aminobenzoic acid

PBMC – Peripheral blood mononuclear cell

PCR – Polymerase chain reaction

PCT – Procalcitonin

PD – Pharmacodynamic

PI – Principal investigator

PK – Pharmacokinetic

PIS – Patient information sheet

PO – Per oral

RB – Reticulate bodies

RCT – Randomised controlled trial

RDT – Rapid diagnostic test

RMSF – Rocky Mountain spotted fever

RNA – Ribonucleic acid

ROC – Receiver operating characteristic

rRNA – Ribosomal ribonucleic acid

SAE – Serious adverse event

SD – Standard deviation

SF – Spotted fever

SFG – Spotted fever group

SMRU – Shoklo Malaria Research Unit

START – Scrub Typhus Antibiotic Resistance Trial

STG – Scrub typhus group

STIC – Scrub Typhus Infection Criteria

TB – Tuberculosis

TBV – Total blood volume

TG – Typhus group

TRG – Transitional group

TSA – Type specific antigen

ULN – Upper limit of normal

VIF – Variance inflation factor

WBC – White blood cells

WGS – Whole genome sequencing

WHO – World Health Organisation

WWII – World War II

1.7 Figures

1.8 Tables

2 Background and literature review

Scrub typhus is a neglected infectious disease and a major cause of acute non-malarial fever in the rural tropics. It is caused by the obligate intracellular bacterium *Orientia tsutsugamushi* along with the closely related and recently described *Candidatus* Orientia chuto. It is endemic over a 13,000,000 km^2 area of the Asia Pacific region, traditionally called the "Tsutsugamushi triangle", extending from Pakistan in the West to the Russian Far East in the North and to the South, northern Australia and the western Pacific islands [1, 2]. Reports over the last decade describing scrub typhus-like illnesses or evidence of the pathogen from further afield challenge our long-held perceptions regarding disease epidemiology [3]. Prior to the discovery of effective antibiotic treatment, mortality ranged from 0.6-41% [4, 5]. More recently, mortality in patients receiving appropriate treatment has been estimated at 1.4% (range 0-33.3%), highlighting the potential severity of the disease [6, 7]. Worldwide, it is probably the most important and significant rickettsial disease.

It is perplexing then that scrub typhus remains severely neglected despite growing evidence of its global reach and potentially severe nature. It is not included on the official list of neglected tropical diseases of the World Health Organization (WHO) [8]. A search on PubMed using "scrub typhus" as the search term in September 2018 revealed 2,118 articles compared to other officially recognised neglected tropical diseases such as 19,844 for "dengue", 4,368 for "chikungunya", 25,904 for "leishmaniasis", 3,889 for "human African trypanosomiasis", 16,764 for "Chagas" disease, 18,376 for "human schistosomiasis", 4,728 for "onchocerciasis", 3,905 for "lymphatic filariasis", 14,781 for "rabies" and 4,301 for "scabies" [9]. The criteria for neglected tropical diseases – communicable, occurs in tropical and sub-tropical environments, affect those living in poverty in remote rural areas, urban slums or conflict zones, inadequate sanitation, contact with disease vectors and other animals, low profile of people affected, low public health priority, absence of reliable

statistics – could readily apply to scrub typhus; arguably, it is more compliant than many on the WHO list yet the disease remains overlooked [8].

In this chapter, I shall give an overview of the disease and review the major outstanding challenges of scrub typhus research, many of which formed the basis for this thesis. I will focus particularly on disease epidemiology, the difficulties surrounding diagnosis and the detrimental impact this has had on our understanding, and the conundrum of drug resistance in scrub typhus which remains unresolved more than two decades after it was first reported from northern Thailand.

2.1 History

Historical accounts prior to the 20th century

Scrub typhus as a clinical entity was first described in China by Ge Hong in *Zhouhofang*, an ancient Chinese clinical manual, as *sha shi du* or “chigger fever” in A.D. 313. It was referenced and described further in the Compendium of Materia Medica or *Pen Ts’oa Kang Mu* by Li Shih-Chen, published posthumously in 1596 during the Ming dynasty [10, 11]. The text referred to small red insects, probably the larval stage of the mites, which may have been involved in the disease process.

In Japanese folklore “river fever” was known to be associated with jungle mites, and the disease or *tsutsuga* was first described by Hashimoto in Japan in 1810 as reported by Kawamura over a century later (although he used the term Japanese “flood fever” to describe the entity) [5]. The term *tsutsugamushi* is made up of two distinct Japanese words: *tsutsuga* meaning disease, illness or harm and *mushi* for bug. Theobald Palm, a protestant medical missionary based in Niigata prefecture, Japan, wrote the first accounts of the disease, known locally as *shima-mushi* or island-insect disease, to be published in the West in 1878. In his letter, a clear clinical description of scrub typhus is given which included the classical symptoms of fever, eschar formation, lymphadenopathy, rash, headache and malaise with death occurring from exhaustion within three to four weeks in fatal cases [12]. He also outlined important ecological observations including disease seasonality, predisposition in agricultural workers, higher risk in demarcated areas along the river bank where new soil and vegetation are present, and a brief description of a mite observed on a patient, although unlikely to be the vector itself [12]. The first scientific investigations of the disease was carried out in the same region by Nagino, Baelz and Kawakami, as narrated by Audy, although the conclusions that the disease was caused by a “miasma” rather than the farmers’ theory of toxins from mites proved incorrect [13]. During this period, no effective

treatment was available and the potential severity of the disease was highlighted by the high mortality, reaching 41.4% in 1917, reported from Niigata prefecture by Kawamura [5].

20th century history until World War Two

Despite the well-described clinical entity, there was controversy surrounding the identity of the pathogen. In the late 19th century, the aetiological agent of scrub typhus was thought to be a protozoan parasite, similar to that of malaria and described as “the plasmodia of tsutsugamushi” [14]. In 1920, the name *Theileria tsutsugamushi* for the tentative protozoan pathogen was proposed by Hayashi. However, similarities between scrub typhus and Rocky Mountain spotted fever suggested an alternative aetiology [15]. Further studies in the 1920s by Nagayo *et al*, using infectious material from patients and experimentally infecting rabbits and subsequently monkeys, provided further information about the identity of the pathogen as a rickettsia [16-18]. In 1930, the name *Rickettsia orientalis* was proposed [16]. Around the same time, Ogata recommended the name *Rickettsia tsutsugamushi* which became the accepted nomenclature for the pathogen until the end of the 20th century [19]. In 1995 Tamura, in homage to Nagayo and Ogata, proposed to re-classify the pathogen into a new genus as *Orientia tsutsugamushi* based on the phenotypic and genotypic differences with other species of the *Rickettsia* genus [20].

At the beginning of the 20th century the term tropical typhus, adapted from a description of a typhus-like illness occurring in towns in eastern USA by Brill, was used to describe similar diseases being reported throughout the Asia Pacific region [21]. It was only after 1916 when Weil and Felix described a phenomenon whereby sera from typhus patients agglutinated selected serotypes of *Proteus* spp. that the task of differentiating the causes of tropical typhus became possible [22]. Serum from patients with epidemic typhus caused by

Rickettsia prowazeckii positively agglutinated the *Proteus vulgaris* OX19 strain while the same also occurred with serum from patients suffering with the urban form of tropical typhus, later termed murine typhus. The rural form of tropical typhus differed by its epidemiology and by the results of the Weil-Felix reaction; sera from patients agglutinated the *Proteus mirabilis* OXK (Kingsbury) strain and not the *Proteus vulgaris* OX19 strain [23].

In the 1920s, studies by researchers at the Institute for Medical Research (IMR) in Kuala Lumpur, Malaya (now Malaysia), on patients with tropical typhus led to the conclusion that there were two distinct diseases: an urban or shop form and a rural form for which the name scrub typhus was proposed due to its propensity to cause outbreaks in areas of previously cleared jungle where scrub grew [23]. Tsutsugamushi disease had previously been reported in 1915 in Malaya and in Sumatra as Kedani river fever [24, 25]. The clinical and epidemiological similarities between scrub typhus and tsutsugamushi disease from the region were noted including the occurrence of outbreaks on plantations (oil-palm in Malaya and rubber in Sumatra), which suggested a shared aetiology and trombiculid mite vector [23, 25, 26]. However, some distinctions were noted between rural or scrub typhus and tsutsugamushi disease in patients: differential fever profiles, variability in the development of the primary bite lesion (the eschar), and inconsistencies in the agglutination titres of the Weil-Felix reaction [23]. A similar clinical syndrome in northern Australia called Mossman fever and Queensland coastal fever were also described around this time [27-30].

During this period, the larval stage of *Leptotrombidium akamushi* was shown to be the mite vector of scrub typhus or tsutsugamushi disease in Japan while *Leptotrombidium deliense* was described as the vector species in Sumatra and Malaya [26, 31, 32]. Both species have been found on rodents, their natural host, although Walch and Keukenschrijver, as reported by Fletcher *et al*, also described detection of the vector in other mammals and more intriguingly, in local and migratory birds in Sumatra [26]. In 1921, Megaw, who was bitten on

the neck by a tick while in the Himalayan foothills, developed tick typhus and subsequently recovered, proposed a system of classifying typhus fevers according to their vectors; louse typhus (inclusive of epidemic typhus), tick typhus (e.g. Rocky Mountain spotted fever, Indian tick typhus) and mite typhus (inclusive of tsutsugamushi disease) [33, 34]. This system was not only apt but provided the impetus for future work to dissect the disease group encapsulated by “tropical typhus” and “typhus fever”.

The abundance of names for a clinically and epidemiologically analogous tropical disease generated much confusion and it was through a series of extensive and systematic investigations by Lewthwaite and Savoor at the IMR in the 1930s that the situation was clarified. Through the study of human cases and investigations in laboratory animals (guinea pigs, white rats, rabbits and primates) with pathogenic strains from Malaya, Sumatra and Japan, multiple conclusions were reached:

a) The pathological characteristics of *Rickettsia orientalis* (as isolated by Nagayo in Japan) and pathogens isolated from patients with rural typhus and tsutsugamushi disease in Malaya were identical [35].
b) The pathological appearances of the rickettsial agent of urban/shop typhus (now known to be endemic (or murine) typhus caused by *Rickettsia typhi*) were similar to *Rickettsia prowazeckii*, the causative agent of epidemic typhus [36].
c) The rickettsial agents for rural typhus and tsutsugamushi disease were detected in two wild rats in Malaya [32]. However, the conclusion reached that rats were the main reservoir of disease was premature.
d) Cross-immunity studies using rickettsial strains of rural typhus and tsutsugamushi disease in animals showed complete cross-protection and the variability in incidence of eschar formation (bubo followed by primary ulcer in the original descriptions) was concluded to be a natural spectrum of the disease [37].

e) Rural/scrub typhus and tsutsugamushi disease were immunologically distinct from urban/shop typhus as well as from Rocky Mountain spotted fever [38].
f) Sumatran mite fever was identical to tsutsugamushi disease in pathological appearances and cross-immunity studies [39].

Thus, rural typhus, scrub typhus, tsutsugamushi disease, Sumatran mite fever, Kedani (or hairy mite) river fever and Japanese river fever were proven to be the same disease with a common aetiological agent (*Rickettsia tsutsugamushi* or *Rickettsia orientalis*, which was renamed *Orientia tsutsugamushi*) and trombiculid mite vector. Despite being responsible for the paradigm shift in our understanding of tropical typhus, Lewthwaite and Savoor were unsuccessful in their attempts to promote adoption of the name "tsutsugamushi disease" and eradicate the term "scrub typhus" [40, 41]. Subsequently, it was concluded that Queensland coastal fever and Mossman fever were likely to be tsutsugamushi disease based on serological evidence (Weil-Felix OXK test) and isolation of the pathogen through inoculation of patients' blood into mice [27, 29, 30, 42].

The impact of World War Two (WWII)

The history of typhus is often synchronous to that of war, conflict, famine and disaster (natural or man-made). Examples include the destruction of Napoleon's army during the invasion of Russia and subsequent retreat, to which louse-borne infectious diseases such as epidemic typhus and trench fever (caused by *Bartonella quintana*) contributed, and the large outbreaks of epidemic typhus during the two world wars affecting large areas of Europe, Mesopotamia (now Iraq) and North Africa [43-45]. The spectre of epidemic typhus led to the establishment of the US Typhus Commission in 1942 which successfully limited the impact of epidemic typhus on the US military during WWII through effective preventative

measures such as the extensive application of dichlorodiphenyltrichloroethane (DDT) for louse control and compulsory immunization [45, 46].

However, the impact of scrub typhus as the major cause of fever beyond malaria in SE Asia and the Pacific was unanticipated. Around 18,000 cases of scrub typhus occurred in Allied forces between 1942 and 1945 with an average mortality of 4% (range 0.6-37.5%) while an estimated 20,000 Japanese troops were affected in the Asia Pacific region [47, 48]. Two major theatres of operation, the Southwest Pacific including northern Australia, New Guinea and Borneo and the Assam-Burmese border, accounted for a majority of Allied cases (mainly American, British, British-Indian, Australian and African regiments) [45]. The greatest burden of disease was witnessed during the Burmese campaign by British and Commonwealth forces, with 5,000 cases and an associated 7% mortality reported from 1944 alone [48-50]. It was a disease that instilled fear in the troops based on the lack of treatment, the significant risk of death and frequent prolonged convalescent periods of many months following recovery [51]. Additionally, two major outbreaks following brief exposure involving US troops in West Papua (now Papua in Indonesia) and British troops in Sri Lanka helped to alert the military hierarchy to the alarming nature of scrub typhus. On the Schouten Islands and beach head at Sansopor of West Papua, approximately 1,255 cases in 4 months and 931 cases in the first 2 months, respectively, were hospitalised, placing significant strains on frontline medical facilities and staff [52, 53]. In Sri Lanka (then Ceylon), following 4 days of exercises in jungle environment near the hamlet of Embilipitiya, 756 patients were hospitalised [47]. These outbreaks and others led to the assignment of specialist staff by Allied forces to aid in the control and prevention of scrub typhus and to study the clinical, diagnostic, epidemiological, ecological and pathological aspects of the disease at appointed field sites. Of note was the field laboratory set up by the US Typhus

Commission at Myitkyina in northern Burma in October 1944 and the Scrub Typhus Research Laboratory established by the British South East Asia Command in mid-1945 [13].

Successes during this period included detailed clinical accounts of scrub typhus from diverse environments [49, 50, 54, 55], advances in pathology through post-mortem studies [56], descriptions of the variety of ecological settings where outbreaks occurred [13, 57], the first suggestions of highly diverse strain heterogeneity of *Orientia tsutsugamushi* throughout the endemic region [58, 59], and advances in entomology including the use of acaricide-impregnated clothing (e.g. dimethyl- and dibutyl-phthalate) as an effective form of prevention [13, 47].

Negative findings were equally enlightening and included accounts of therapeutic failure with penicillin and immune blood and plasma therapy [50]. Of greater significance was the failure of a formalin killed vaccine of the Karp strain of *Orientia tsutsugamushi*, prepared from the lungs of cotton-rats [60]. This required extensive logistical preparation including the construction of new buildings and laboratories as part of the Special Operational Store "Tyburn", Wellcome Research Laboratory, Frant, Sussex [61]. The gravity of the situation demanded haste and this was exemplified by the efficiency and laudable effort in completing the specialist buildings and laboratories within 109 days and completion of vaccine production (of which 300 litres were produced) by October 1945, within six months of production initiation [61]. The field trial, carried out between July 1945 and February 1946 in India and Burma, was impaired due to the cessation in hostilities, unpredictability of troop movements, and the establishment of acaricide-impregnated clothing [62]. A total of 10,000 troops were vaccinated on a controlled basis (although as many as 50,000 others may have received inoculations) and incidence rates of 1.8 versus 3.1 per thousand per annum in fully protected and non-protected troops, respectively, were observed [62]. Statistical significance was not achieved. A second vaccine field trial carried out in Japan in 1947 using a killed

Orientia tsutsugamushi Volner strain (isolated from the Philippines) produced in lungs and spleens of rats also proved to be unsuccessful [63].

For scrub typhus WWII would prove to be a defining moment with the impact of the disease driving research and advances in our understanding and establishing the disease firmly in the memory of various military institutions affected. One of the most enduring images from the war was taken by George Silk, a photographer from New Zealand working for the Australian Department of Information and stationed at the time on New Guinea. On Christmas Day, 1942, he captured a moment when Australian Private George "Dick" Whittington, having been wounded by sniper fire, was being guided back to a casualty station by Raphael Oimbari, a Papuan native, through kunai grass (Figure 1). Although, Private Whittington recovered from his wounds, he died 7 weeks later from scrub typhus. He is buried in Port Moresby (Bomana) War Cemetery. Shortly after the war, the discovery of chloromycetin (chloramphenicol) in 1947 and the establishment of its potency in treating scrub typhus in 1948 opened a new chapter in the history of the disease [64, 65]. Many of the American and British researchers at the IMR, where the first clinical trials for the treatment of scrub typhus with chloromycetin took place, felt at the time that the "bogey of scrub typhus" could be laid to rest (Figure 2) [13]. This seemed apt with the discovery of the highly efficacious tetracyclines in the subsequent decades. However, as I shall outline in subsequent sections, the story of scrub typhus and its treatment remain unfinished and more complicated than originally thought.

Other 20th century conflicts

Scrub typhus was also an important health problem in the Vietnam War but apparently less so in the Korean War. In Korea, what was striking was the low number of cases (may be as

low as 8 cases) reported amongst US or UN troops in a country we now know is highly endemic for scrub typhus [45, 66]. Although the report of 2 cases among British and Commonwealth forces in 1951 may be the first from Korea with supportive evidence from a diagnostic assay (serological testing with the Weil-Felix OXK antigen), a description of suspected scrub typhus cases was given in 1915 [67, 68]. From 1980 onwards, reports of scrub typhus among the general population and military personnel stationed in South Korea increased, suggesting that a lack of awareness may have contributed to the previous low rates of reporting [45]. However, it is unlikely that this explanation alone accounted for the low rates of reporting. The temporal proximity of the Korean War to WWII and the presence of military personnel with prior experiences of scrub typhus should have ensured that the disease was not forgotten. Advances in preventative measures originating from the final years of WWII such as the use of acaricide-impregnated clothing may have played a major role in reducing the number of infections. This is in contrast to the Vietnam conflict where this preventative measure was dropped due to tentative safety concerns regarding developmental toxicity associated with exposure to the synthetic chemicals utilised [45, 69].

Consequently, scrub typhus was once again a significant cause of medical casualties in Vietnam. Malaria remained the dominant cause of fever but once it had been excluded, undiagnosed fever posed a major diagnostic challenge. Scrub typhus was responsible for between 3-17% of fever in such patients and was unsurprisingly more common in combat troops exposed to jungle environments than in those stationed in urban settings [70-73]. Malaria and leptospirosis were also diagnosed more frequently in this at-risk group, while arbovirus infections (e.g. chikungunya and dengue) and murine typhus were commonplace in urban areas [70-73]. The availability of effective antibiotics such as chloramphenicol and tetracycline made a real impact on disease outcome with no known American deaths due to typhus occurring throughout the entire war [45, 74, 75]. Despite these studies, it was

suggested that the true burden of disease is likely to have been much greater due to the difficulties surrounding clinical and laboratory diagnosis [45].

Figure 1. Private George “Dick” Whittington (Australian Army) being guided by Raphael Oimbari through tall kunai grass, New Guinea, Christmas Day, 1942. [Photograph by George Silk, taken from Australian War Memorial Archives 014028 [76]]

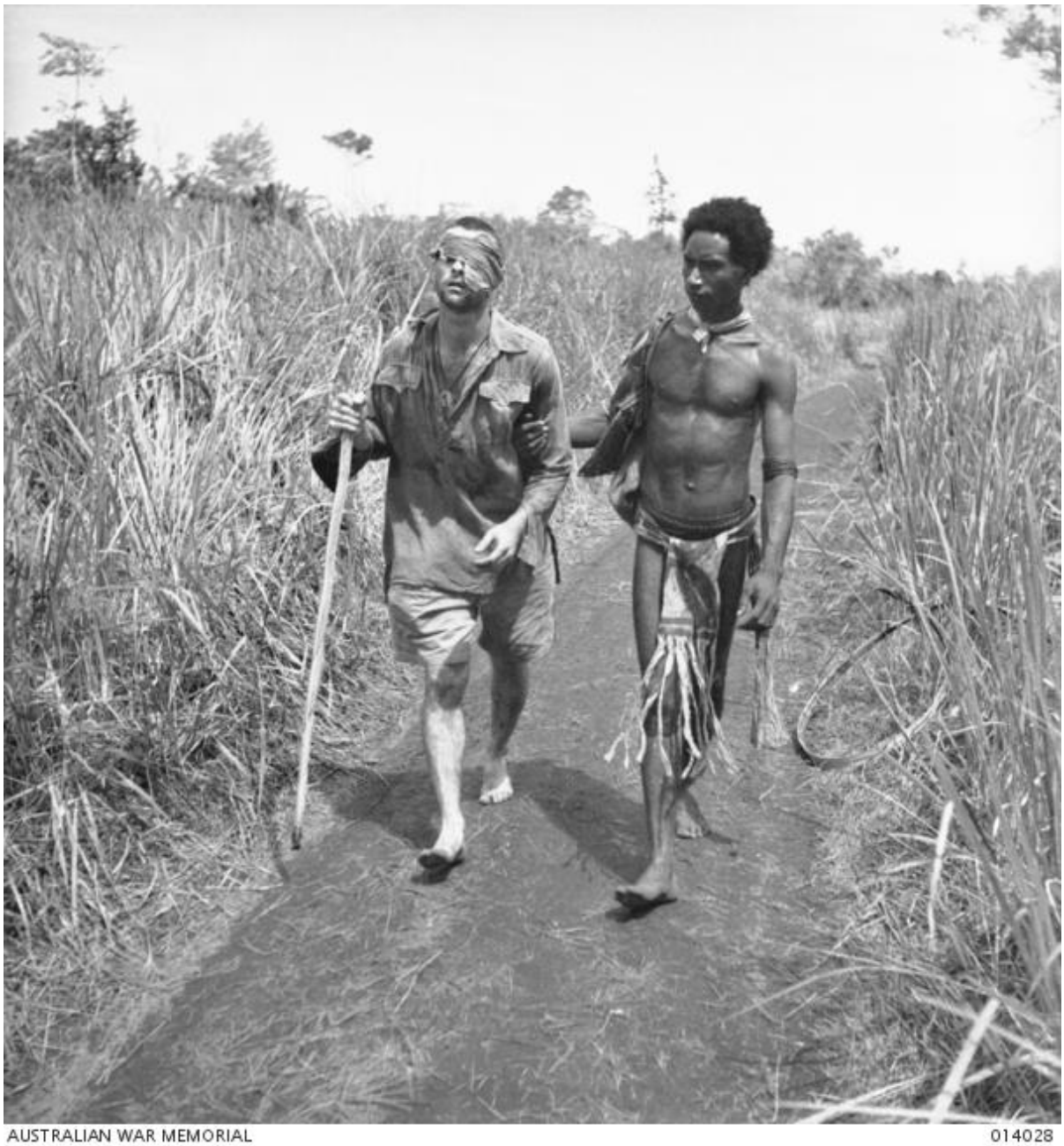


Figure 2. Laying the bogey of scrub typhus to rest in Malaya in 1949. [Cartoon by Austin Dorall, Institute of Medical Research, Kuala Lumpur, Malaya; taken from [13], originally published in [77]]

2.2 Epidemiology and ecology

Traditionally, scrub typhus has been reported within the “tsutsugamushi triangle”, a vast area covering 13,000,000 km^2 comprising many countries of the Asia Pacific region and containing over 1 billion people at risk of the disease [2, 78]. The causative agent, *Orientia tsutsugamushi*, is transmitted when the infected larval stage of the trombiculid mite, known as a ‘chigger’, feeds on vertebrate hosts, usually rodents, other small mammals and occasionally humans. The bacteria is maintained in mite populations within the environment through transstadial and transovarial transmission which allows the mites to act as both natural reservoir and vector [13]. Humans are accidental and dead-end hosts, infected through contact with infected mites. This may occur when venturing through “mite islands”, which are characteristic discrete foci within the environment. Reports of scrub typhus over the last few decades from countries beyond the established endemic region have challenged us to re-examine our beliefs regarding the epidemiology of the disease [3].

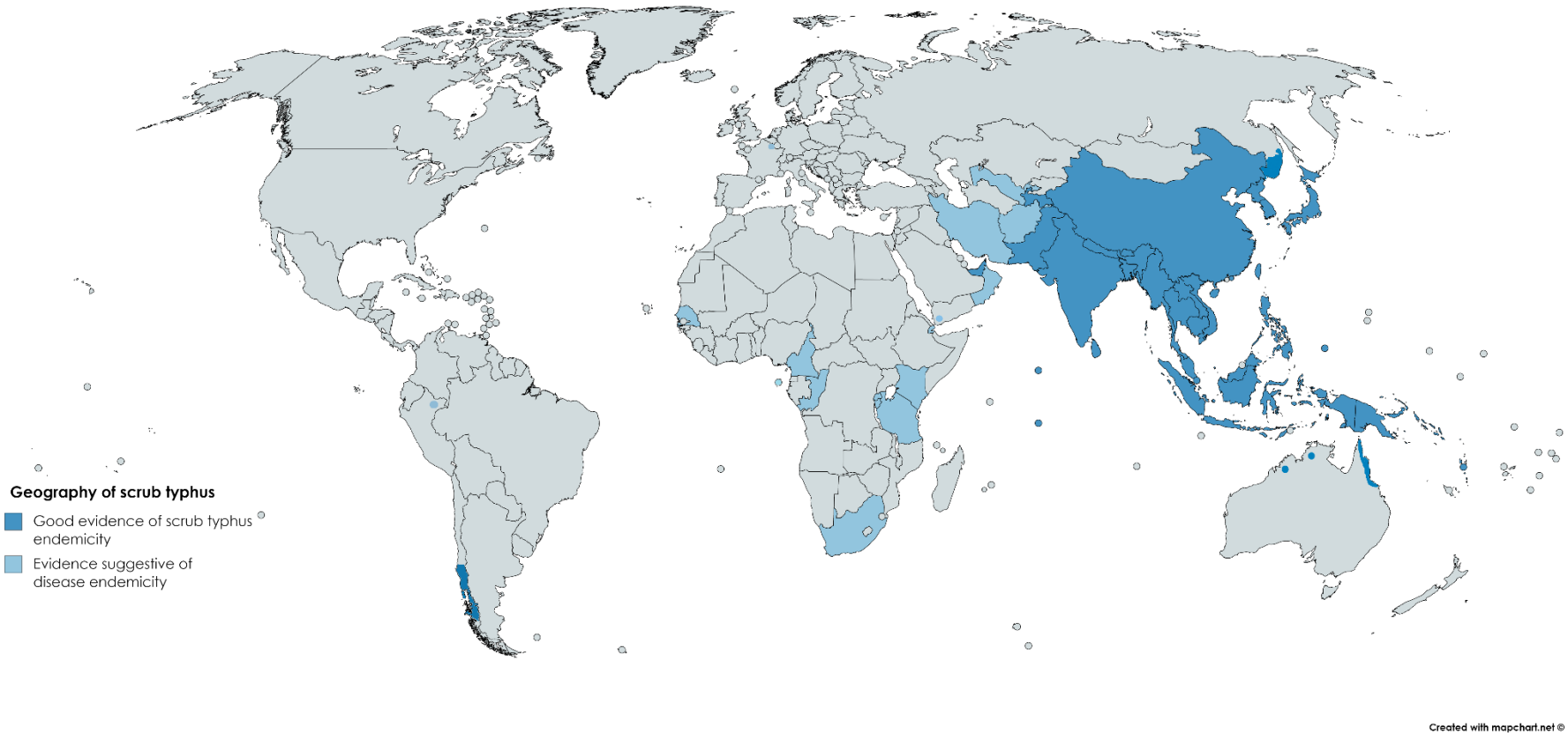
In this section, I shall review the global distribution of scrub typhus, the epidemiology in Thailand, and disease ecology.

Global distribution of scrub typhus

A chronological summary of the distribution of scrub typhus is tabulated in Appendix 1. The focus is on human cases of scrub typhus rather than on *Orientia tsutsugamushi* detected in other vertebrate hosts or invertebrate vectors, unless these findings are notable (e.g. beyond or on the boundaries of the established endemic region). Wherever possible, the earliest account (year of disease occurrence used if available) and a more recent report(s) on disease burden in a location are included. Additionally, a detailed world map outlining the countries or regions where the disease has been described is depicted in Figure 3.

Figure 3. The geography of scrub typhus (created with mapchart.net ©, https://mapchart.net/detworld.html).

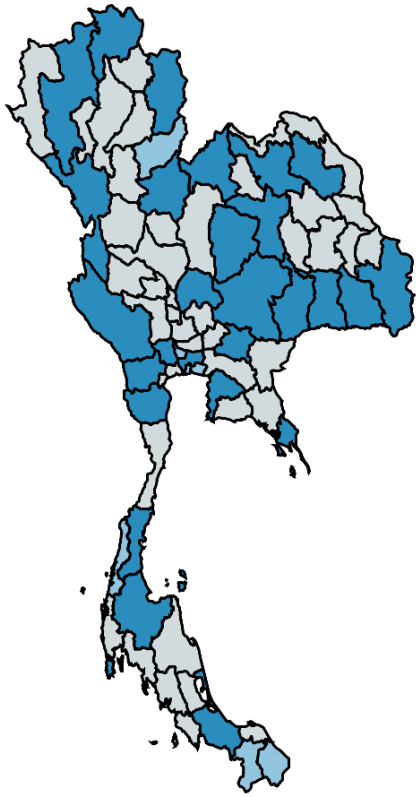
[Good evidence of endemicity – laboratory confirmed clinical cases via serology, PCR or culture. Evidence suggestive of endemicity – single or few serologically confirmed clinical cases, seroprevalence in humans or animals, detection of *Orientia* spp. DNA in animals or vectors]


Geography of scrub typhus
Good evidence of scrub typhus endemicity
Evidence suggestive of disease endemicity
Created with mapchart.net ©

Epidemiology of scrub typhus in Thailand

Appendix 2 provides a summary of published evidence on the distribution of scrub typhus in Thailand. The focus is on clinical cases or exposure data in humans with only significant data on natural hosts and vectors included. The year of disease occurrence or when the study was performed was used when available. An overview map of scrub typhus in Thailand, is shown in Figure 4. Reported studies alone are unlikely to be fully representative of disease burden. However, the pattern of disease is suggestive of the ubiquitous nature of scrub typhus throughout Thailand.

Figure 4. Distribution of human scrub typhus in Thailand according to published reports (created with mapchart.net ©, https://mapchart.net/asia-detailed.html).


Scrub typhus in man, Thailand
Provinces with clinical cases reported
Provinces with seroprevalence data reported
Created with mapchart.net ©

Ecology

A detailed review of the ecology of scrub typhus is beyond the scope of this thesis. However, I shall give a brief overview, focusing on the environments/habitats, the vectors and natural vertebrate hosts of the disease, drawing on the extensive reviews by Audy, Oaks Jr. *et al*, Traub and Wisseman, and Kelly *et al* [2, 4, 13, 79-81]. The key ecological features include: disease localisation by geography, the influence of climate, seasons, and terrain, and the life-cycle of the trombiculid mite vectors and their interactions with vertebrate hosts.

As described above, the scrub typhus endemic area has expanded well beyond the traditional tsutsugamushi triangle. Evidence is growing of the presence of scrub typhus in Africa and South America and the endemic area now stretches to 47° and 43° above (Primorye region, Russia) and below (Chiloé Island, Chile) the equator, respectively. This large area encompasses tropical regions around the equator characterised by wet and dry seasons of tropical climate to humid subtropical regions to areas beyond this with oceanic or continental climates.

Scrub typhus infection is associated with exposure to outdoor habitats. Audy describes 3 main types of terrain [13]: a) man-made waste land, b) water-meadows (e.g. along meandering streams in virgin forests), and c) fringe habitats and secondary scrub. All of these areas are ecotones, characterised by growth of new or transitional vegetation, mainly grass or scrub. In addition, many of these areas contain an abundance of food and shelter for rodents while moist soil provides favourable conditions for mite vectors. These conditions can lead to the development of “mite-islands” and when populated by mite colonies infected with *Orientia tsutsugamushi*, can result in the development of a *yudokuchi* or “poisonous place” [13]. Traub and Wisseman used the term “zoonotic tetrad” to describe the close association between rats, mites, transitional vegetation and the pathogen, *Orientia*

tsutsugamushi [79]. The habitats described are diverse; ranging from virgin forests, gardens, fringe habitats or scrubland, water-meadows, beachheads, rice paddies, bamboo patches and oil palm or rubber plantations. Evidence of *Orientia tsutsugamushi* in unusual habitats, such as semi-arid deserts (e.g. Pakistan, Inner Mongolia) and alpine conditions above 3,000 metres (e.g. Pakistan, Eastern Himalayas) highlights the very wide range of scrub typhus [82-84].

Disease seasonality was clearly described by Palm in 1878 in Niigata Prefecture, Japan, occurring only in July and August while more recent scrub typhus national reporting data from the Japanese National Institute of Infectious Diseases for 2007-2016 showed 2 distinct peaks, May-June and November-December [12, 85]. Reported data from national surveillance systems from South Korea, China and Taiwan also showed seasonal trends in reported patients [66, 86, 87]. One possible explanation posited (in Japan and S. Korea) is that in areas where cold-tolerant *Leptotrobidium pallidum* is prevalent, over-wintering larvae are responsible for the spring peaks while areas with a preponderance of *Leptotrombidium scutellare* led to autumn and early winter peaks in cases [66, 85]. Significant correlation between average monthly temperature (not rainfall) and chigger abundance was shown in the Pescadores (Penghu) Islands of Taiwan [88]. In contrast, disease seasonality was not observed in Malaya (Malaysia) and was not as marked in Sumatra, which may reflect the geographical proximity to the equator where temperature variability is less marked and where the effect of rainfall on chigger activity may be greater [25, 41, 89].

There are a couple of key points to highlight with regards to the life cycle of trombiculid mite vectors (see Figure 5). First, only the 6-legged larvae (chiggers) feed on vertebrate hosts and they feed only once before continuing their development free-living in the soil. The 8-legged nymph and adult mites are non-parasitic and feed on other smaller insects or insect eggs. Adult male and female mites are fertile, with females able to deposit around 400 eggs over a

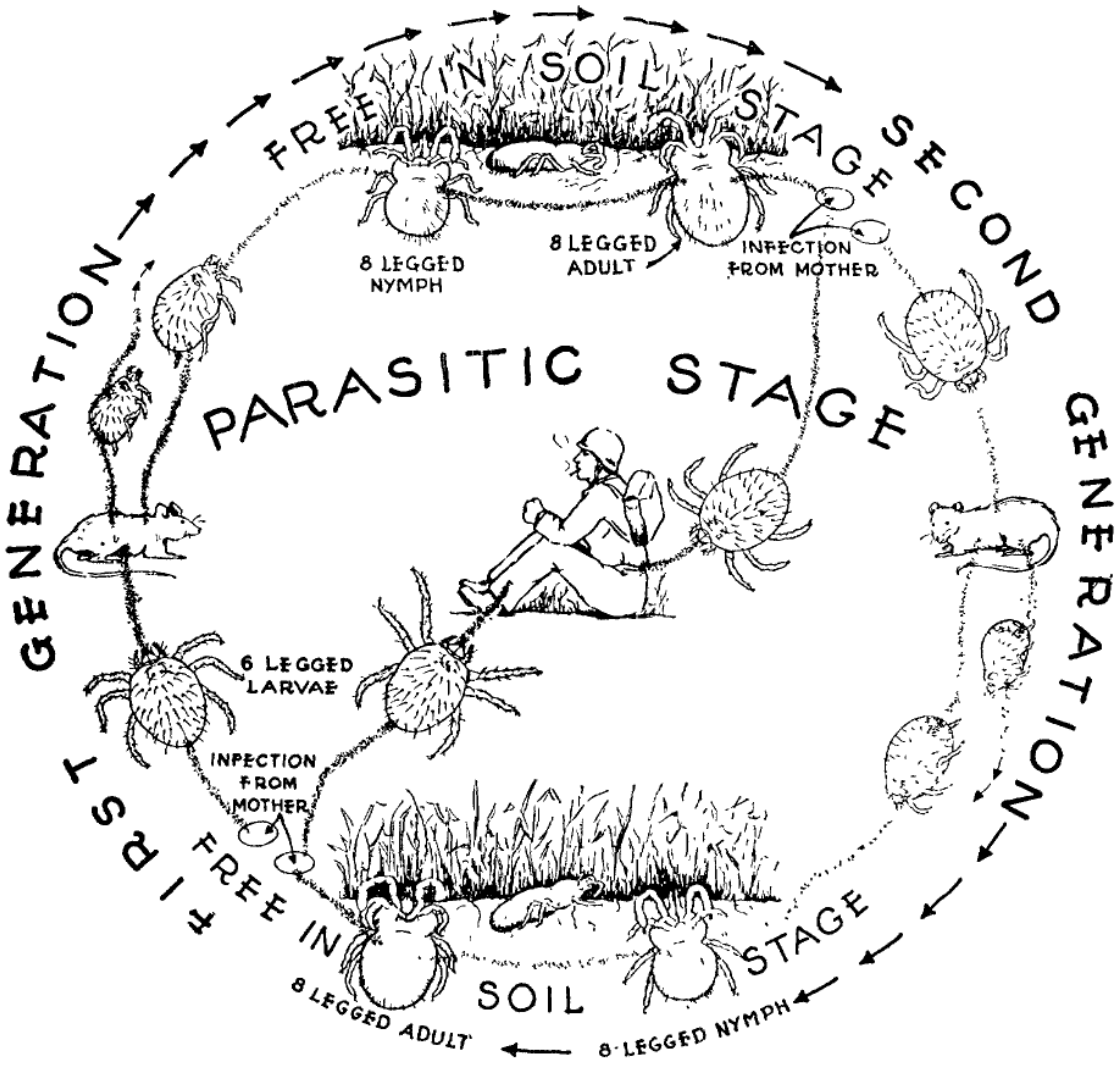
period of several months. Lifespan of adults may be 15 months or more while the developmental life cycle of *Leptotrombidium* spp. may take 2-3 months in warmer climates, ranging to over 8 months in colder climates [4]. The second key point is that transstadial and transovarial transmission naturally occurs within the mites; an infected female passing on *Orientia tsutsugamushi* to its progeny which is then maintained through each of the developmental stages [18, 90]. Experiments to infect uninfected chiggers by feeding them on infected mice with *Orientia tsutsugamushi* (either through inoculation or infected chiggers) showed that only a very small proportion of larvae become infected, with some evidence of subsequent transstadial transmission, but no evidence of transovarial transmission in subsequent generations [91, 92]. Thus, it is likely that the mite act as both vector and natural reservoir of the disease, rather than rodents [79]. Another potential modality for bacterial transmission to uninfected chiggers is through co-feeding with infected chiggers [93, 94]. Multiple strains of *Orientia tsutsugamushi* can infect a single mite and are usually maintained in subsequent generations [95].

A recent review by Kelly *et al* (with information drawn from Oaks Jr. *et al*) included an informative table on *Leptotrombidium* spp. with good evidence of transmission of *Orientia tsutsugamushi* to man [2, 4]. The list includes *L. deliense*, *L. akamushi*, *L. scutellare*, *L. chiangraiensis*, *L. arenicola*, *L. imphalum*, *L. pallidum*, *L. pavlovskyi*, *L. fletcheri*, and *L. gaochuensis*. *L. deliense* and *L. scutellare* have been shown to be prevalent in Thailand while *L. chiangraiensis* and *L. imphalum* have also been reported from Thailand.

Humans are accidental dead-end hosts while small mammals, particularly rodents, are the usual hosts for chiggers. However, larger mammals and birds (both ground-feeding and migratory) have been shown to harbour chiggers and could play a role in spreading infected chiggers to other areas. An extensive list of animals known to harbour *L. akamushi* and *L.deliense* was compiled by Harrison and Audy in 1951 which included [96]: rats, mice, voles,

gerbils, squirrels, porcupines, shrews, hedgehogs, macaque monkeys, deer, civets, weasels, mongooses, bandicoots, kangaroos, dogs, cats, cows, buffaloes, oxen, and various birds including chickens. *Orientia tsutsugamushi* has been isolated mainly from wild rodents, with the majority from *Rattus* spp., although evidence of the bacteria infecting migratory birds has also been reported [4].

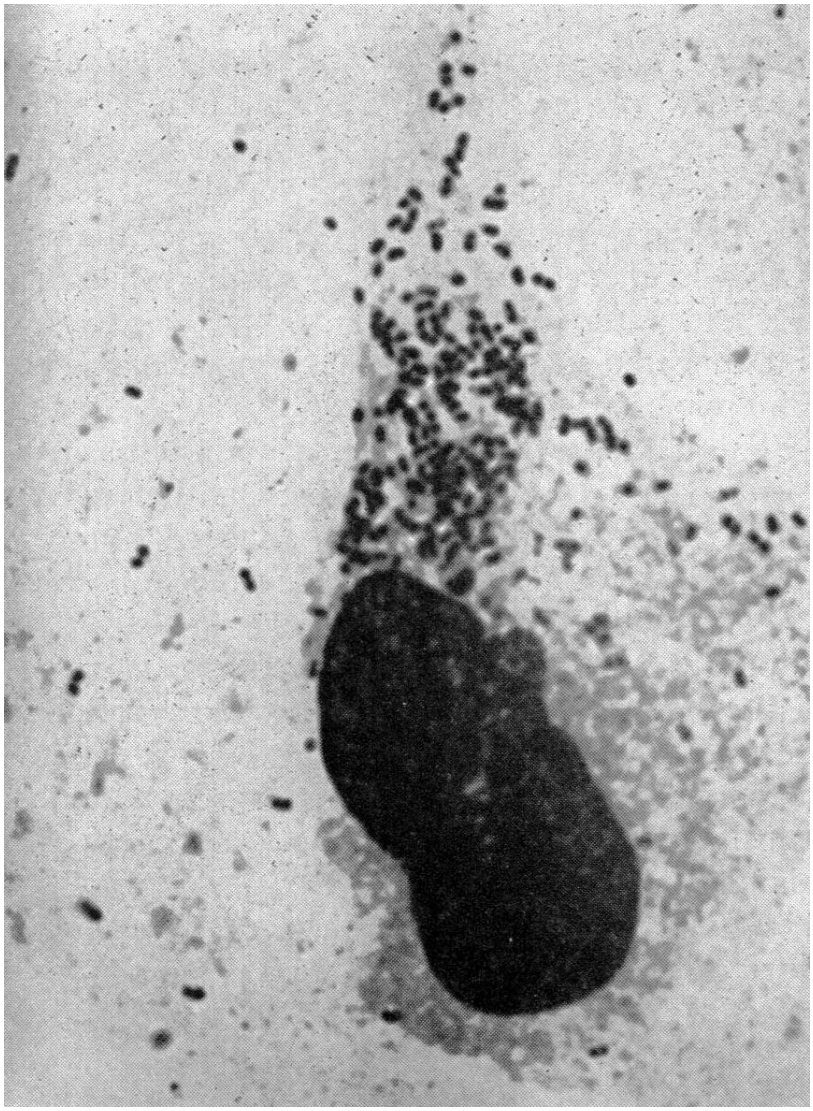
Figure 5. Life cycle of trombiculid mites. [from [97], courtesy of US Army Medical Museum]



2.3 Microbiology

Orientia tsutsugamushi are pleomorphic, Gram negative, non-motile, obligate intracellular bacteria that appear coccobacillary on microscopy following staining (Giemsa stain, blue to purple appearance) and occasionally bacillary. They appear more coccobacillary than *Rickettsia* spp. and are small in size (0.3-0.5 by 0.8-1.5 µm) compared to the majority of bacteria (Figure 6).

Figure 6. *Orientia tsutsugamushi* in infected cell taken from infected animal tissue, Giemsa-stained, black and white (x1500). [from [97]]

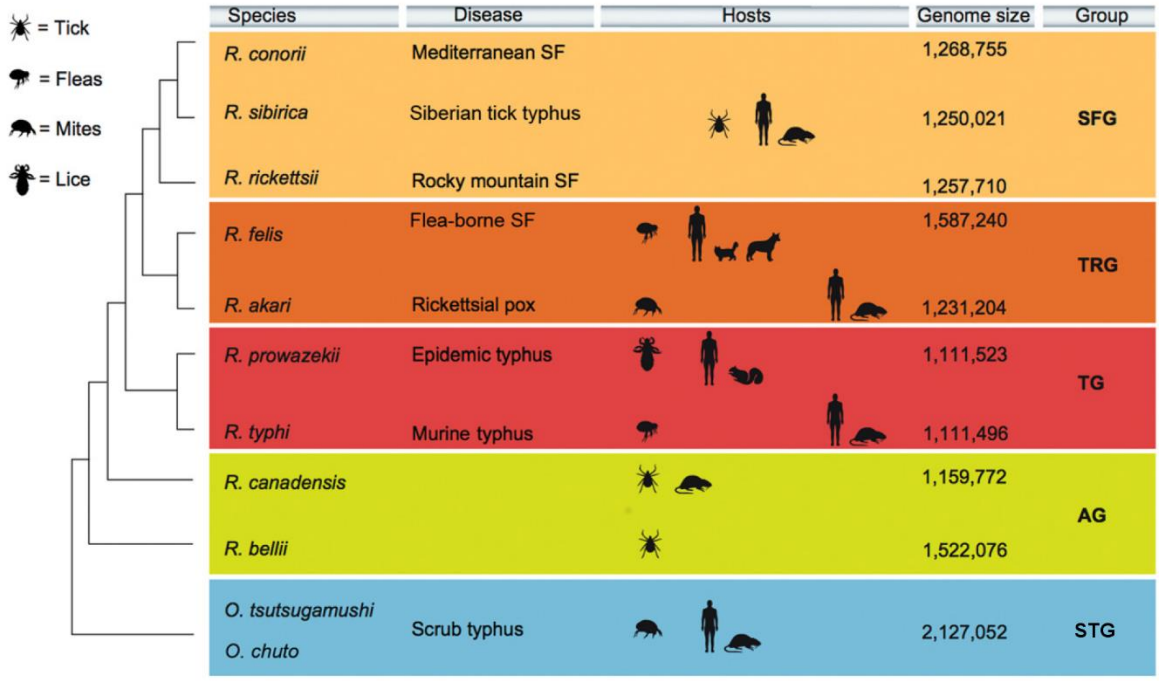


Nomenclature and classification

It was only in 1995 that sequencing of the 16S rRNA gene led to the reclassification of *Rickettsia tsutsugamushi* as *Orientia tsutsugamushi*. Prior to this, evidence of structural differences on electron microscopy and differing protein composition, particularly of the outer envelope/cell wall, suggested that *Rickettsia tsutsugamushi* was distinct to other *Rickettsia* spp. 16S rRNA sequences of *Rickettsia tsutsugamushi* (Gilliam and Kato strains) had levels of similarity of 90.2-90.6% when compared with *R. prowazekii*, *R. rickettsii* and *R. sibirica* and 81.0-82.9% when compared with selected *Ehrlichia* and *Bartonella* species [20]. Levels of similarity between different strains of *Rickettsia tsutsugamushi* (Gilliam, Kato, Karp, Kawasaki, Kuroki and Kimoshoki strains) were >98.5%, providing further evidence supporting the creation of the genus *Orientia* [20].

Orientia tsutsugamushi belongs to the family Rickettsiaceae, order Rickettsiales, Class Alphaproteobacteria, phylum Proteobacteria and kingdom Bacteria. Until the last decade, it was the only species of the genus *Orientia*. In 2006, an Australian patient developed an acute febrile illness after returning from a trip to the UK via a period of transit in Dubai, UAE, where a visit to a local stable and contact with horses, dogs and cats was reported [98]. An eschar was noted 11 days later while other symptoms concurrent with scrub typhus such as myalgia, headache and rash were also present. Diagnosis was confirmed by serology (IFA), PCR and culture. 16S rRNA, 47kDa, and partial 56kDa gene sequencing of the isolated *Orientia* spp. was performed. Phylogenetic comparison to other reference strains revealed significant divergence, particularly of the 47kDa (17.7-18.2%) and partial 56kDa (47-58%) gene sequences. Given this and the unique epidemiological background, a new species, *Candidatus* Orientia chuto, was proposed (chuto – Middle East in Japanese) [98]. A summary of the genetic groups of *Orientia* and *Rickettsia* spp. is shown in Figure 7.

Figure 7. Comparison of the genomes of *Orientia* and *Rickettsia* spp. [SF – spotted fever, SFG – spotted fever group, TRG – transitional group, TG – typhus group, AG – ancestral group, STG – scrub typhus group; inferred phylogeny based on 15 proteins [99]; adapted from [100] and [101]]. Although *Rickettsia africae* (the cause of African tick typhus) is not included, this species is phylogenetically closely to *R. conorii* with a genome size of 1,278,540 base pairs.



Structure, cell biology and genome

The cell wall of *Orientia tsutsugamushi* has a thick outer membrane and a thin inner membrane while the reverse is true of *Rickettsia* spp. It was previously believed that the cell wall lacked both peptidoglycan and lipopolysaccharide (LPS) but recent studies have shown that the genome of *Orientia tsutsugamushi* contains many genes required for the biosynthesis of peptidoglycan (e.g. *murA* to *murG*, PBP2 and PBP3) with a few key absences (e.g. PBP1) [102]. Evidence of a peptidoglycan-like structure within the cell wall

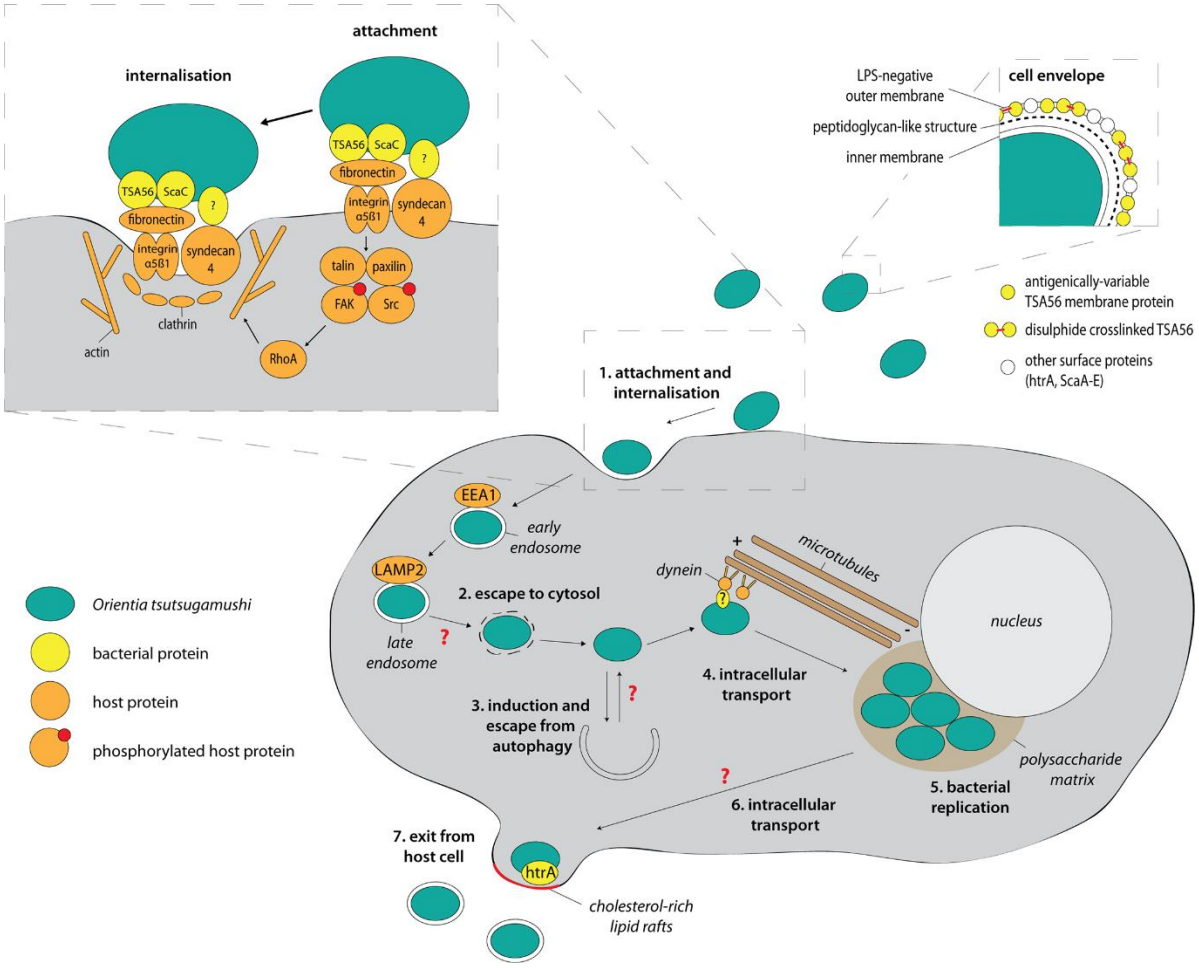
and presence of disulphide cross-linked outer membrane proteins, including the Type Specific Antigen (TSA) 56kDa protein unique to this bacteria, as integral to the stability and infectivity of *Orientia tsutsugamushi* have also been described [102]. The similarities to another group of obligate intracellular bacteria, the Chlamydiae, are marked [102, 103].

Many surface proteins have been identified and studied, usually in the context of diagnostics or immunogenicity. These include the 56kDa TSA, 47kDa high temperature requirement A (htrA) protein, ScaC antigen, OmpA and others as reviewed by Paris *et al* [100]. The 56kDa TSA is the major variable immunogenic protein and the main source of antigenic heterogeneity when comparing different strains of *Orientia tsutsugamushi*, so much so that the 56kDa TSA gene is the major target used for genotyping. Both 56kDa TSA and ScaC are integral to the attachment and invasion of the bacteria to host cells. This process and subsequent steps of the infection cycle are depicted in Figure 8 and reviewed by Salje and Paris *et al* [100, 103].

The genome of *Orientia tsutsugamushi* is larger than those of *Rickettsia* spp. (Figure 7) and is characterised by a proliferation of repeat sequences and conjugative elements. Until recently, only the whole genome sequences of the Boryong and Ikeda strains have been determined of which approximately 37% consisted of identical repeats, one of the highest proportion of repeated DNA elements of any bacteria to date [104, 105]. This quirk has hindered the sequencing and assembling of additional *Orientia tsutsugamushi* genomes, mainly due to the problems surrounding assembling and managing contaminating sequences. A recent study overcame these issues by using Pacific Biosciences long-read sequencing and Illumina sequencing for polishing and reducing errors. Six new genomes (*Orientia tsutsugamushi* strains – Karp, Kato, Gilliam, TA686, UT76 and UT176) were assembled, sizes ranging from 1.93Mb (UT176) to 2.47Mb (Gilliam) and number of predicted genes ranging from 2,086 (UT176) to 2,709 (Gilliam) [106]. A putative core

genome of 657 genes conserved in all 6 strains plus the Boryong and Ikeda strains was identified and the total proportion of repetitive genome ranged from 33% (UT176) to 51% (Gilliam). Minimal synteny was seen in all 6 assembled and 2 previously reported genomes with a pattern emerging of islands of core genes separated by transposable and repeat elements, suggestive of repeat-mediated chromosomal rearrangement of which the mechanisms and timing of this process remain undetermined [106].

Figure 8. Infection cycle of *Orientia tsutsugamushi*. [taken from [103]]



Orientia tsutsugamushi strain heterogeneity

It became apparent quite early on in the study of scrub typhus that dramatic antigenic variation and strain heterogeneity existed and that the spectrum of virulence in humans and animals may, in part, be explained by this characteristic of *Orientia tsutsugamushi*. During WWII and soon after, this was shown by cross-immunisation or cross-vaccination studies in laboratory animals along with serological studies in patients using complement fixation and antigens from 3 reference strains (Karp, Gilliam and Serangayee strains) [58, 59, 107, 108]. The serological responses of patient sera to the Karp and Gilliam strains (originally isolated from Allied soldiers infected in New Guinea and the Assam-Burmese border, respectively) were distinctive enough to allow these strains to become established as 2 out of the 3 original type strains; the other being the Kato strain, isolated from Niigata Prefecture, Japan, and similarly characterised by complement fixation over a decade later [59].

In the 1970s, an additional 5 type strains were determined serologically using direct fluorescent antibody assays (DFA). These were isolated from rodents (TA678, TA763 - Fan, TA686, TA716 - Chon) and a patient (TH1817) from Thailand in the 1960s [2]. Additional serological methods to characterise *Orientia tsutsugamushi* isolates and to diagnose scrub typhus have been developed with the indirect immunofluorescence (IFA) and indirect immunoperoxidase (IIP) assays being the most important. However, serological characterisation methods have inherent weaknesses including cross-reactivity, the reliance on a panel of antigens from reference strains suitable to the question at hand (serotyping or diagnosis), and the need to maintain and propagate reference strains in a biosafety level-3 (BSL-3) laboratory.

Molecular characterisation of *Orientia tsutsugamushi* mainly based on specific genes began in the 1990s and the 56kDa TSA gene has become the main target used to infer phylogenetic relationships between strains. A review and study by Kelly *et al* showed that

among the 135 sequences studied based on the 56kDa TSA gene, 9 distinct clades could be defined including Karp-related, Saitama, Kuroki, TA763, Gilliam, Kawasaki, JG, Kato and a group of divergent strains including Shimokoshi [2]. In this review on genotypic diversity of *Orientia tsutsugamushi*, Kelly *et al* outlines over 20 antigenically distinct strains [2]. In Thailand, Karp and strains from the Karp-related clade appear to be predominant. Whole genome sequencing should permit more accurate analyses of the phylogenetic differences between strains than current methods based on multi-locus sequence typing (MLST) or single gene genotyping (e.g. 16s rRNA, 56kDa genes).

2.4 Pathogenesis and immunity

Pathogenesis

Our understanding of the pathogenic process of scrub typhus has improved over the last few decades but remains incomplete. Previously, the general belief was that scrub typhus, akin to epidemic typhus and Rocky Mountain spotted fever (RMSF), led to a vasculitis-like systemic disease affecting multiple organs. Although some of the evidence outlined below supports this notion, we now know this is an oversimplification with scrub typhus displaying distinctive pathophysiology. Inoculation of *Orientia tsutsugamushi* at the chigger feeding site on vertebrates commonly leads to the formation of a localised skin lesion, beginning with a papule, eschar, ulcer and finally a small scar that disappears over time. The subsequent dissemination pathways are ill-defined. The eschar is not omnipresent, present in 1-97% of cases in reported studies, and is dependent on the degree of pre-existing immunity and thoroughness of clinical examination [100, 109, 110].

Historical studies from WWII on the histology of eschars revealed polymorphonuclear leucocyte infiltration in the upper zone with dense collections of mononuclear cells (lymphocytes, plasma cells, mast cells and macrophages) around structures of the dermis (hair follicles, sebaceous glands, nerves and blood vessels) [56]. There was evidence of acute thrombophlebitis and arteritis in the upper acute inflammatory zone but veins at a considerable distance from the eschar also showed intimal damage via infiltration by mononuclear cells [56]. This pattern of phlebitis was evident in other organs, suggesting an important role for mononuclear cells in the dissemination of *Orientia tsutsugamushi* via the vasculature, lymphatics, or both. In this large post-mortem study of scrub typhus, only 1 out of 6 cadavers where an extensive search for the bacteria was made revealed endothelial infection in the eschar section [56]. More recent work has demonstrated cellular tropism of *Orientia tsutsugamushi* to activated inflammatory monocytes and dermal dendritic cells in

eschar biopsies from scrub typhus patients and not endothelial cells [111]. It was also shown to be present in peripheral blood mononuclear cells from patients' blood using immunoalkaline phosphatase and direct immunofluorescence methods [112]. This is in contrast to 2 other post-mortem studies (5 cases total) where endothelial cells in the brain, heart, lung, kidney, pancreas and skin along with macrophages in the liver, spleen and lymph nodes (immunohistochemistry – both studies, electron microscopy – 1 study) were shown to be the main cell type infected with *Orientia tsutsugamushi* [113, 114]. These discrepancies may reflect the different stages of disease studied (early stage *ex vivo* samples vs autopsy samples).

Much can be gleaned from the historical autopsy studies (Allen and Spitz) from WWII which compared scrub typhus pathology to RMSF and epidemic typhus [56]:

- Evidence of extensive vascular damage in scrub typhus is sparse when compared to epidemic typhus and RMSF. A general perivascular and interstitial reaction seen.
- Interstitial pneumonitis (morphologically acute necrotizing bronchiolitis) much more common in scrub typhus (55% of cases studied) and more severe.
- Myocarditis common in all 3 diseases (90.5% of cases studied for scrub typhus) but severity increased in scrub typhus.
- Acute diffuse glomerulonephritis common in all 3 diseases (30% of scrub typhus cases studied).
- Acute splenitis common with erythrophagocytosis and cytophagocytosis almost always present and often severe in scrub typhus. Necrosis of the pulp seen in 5-7%.
- Lymph nodes were hyperplastic and distinct in scrub typhus by their necrosis. Macrophages and histiocytes exhibited marked erythrophagocytosis and cytophagocytosis.

- Focal encephalitis of the grey matter similar in scrub and epidemic typhus whereas RMSF had evidence of encephalitis mainly affecting the white matter.
- Bone marrow showed phagocytosis of red and white blood cells by macrophages in all 3 diseases
- Pancreas and adrenals had foci of peri-vascular and interstitial reaction composed of lymphocytes, plasma cells and macrophages. Similar for all 3 diseases although vascular damage in the adrenals present in some patients with epidemic typhus.
- Other minor findings included evidence of acute diffuse cholecystitis and acute gastritis with ileitis in 1-2 scrub typhus cases.

Many of these findings may help explain some of the clinical features and complications associated with scrub typhus as reviewed in the next section.

Soluble adhesion molecules (soluble E-selection, intercellular adhesion molecule-1, vascular adhesion molecule-1 and L-selectin) have been shown to be elevated during acute scrub typhus infection in Lao and Thai patients, suggesting both endothelial and mononuclear cell activation [115]. This also concurred with findings from India where elevated monocyte/macrophage and endothelial related markers were described in scrub typhus patients with YKL-40, a glycoprotein linked to inflammation and expressed in macrophages, found to be associated with disease severity and mortality [116]. Comparison of coagulation and inflammatory markers between scrub typhus and murine typhus patients revealed significant differences. Scrub typhus was associated with raised levels of coagulation markers (thrombin-antithrombin complexes and soluble tissue factor) and pro-inflammatory cytokines (IL-6, IL-8 and IL-10) while murine typhus was distinguished by markers of endothelial perturbation (von Willebrand factor, soluble thrombomodulin, tissue plasminogen activator and plasminogen activator inhibitor-1) [117]. Additionally, increased neutrophil activation in scrub typhus has been associated with disease severity and

complications through increased plasma levels of neutrophil-derived nucleosomes and neutrophil-elastase complexes [118].

Another important aspect of scrub typhus, the issue of persistence, has been suspected but not appreciably investigated. It has been known that *Orientia tsutsugamushi* can be detected in laboratory animals previously infected months after apparent recovery. A recent study in mice experimentally infected via the intra-peritoneal route with *Orientia tsutsugamushi* Karp strain and successfully treated showed that reactivation could be achieved 647 days post-infection on immunosuppression with cyclophosphamide [119]. *In vitro*, *Orientia tsutsugamushi* growth in ECV304 cell culture was inhibited by rifampicin, azithromycin, doxycycline and chloramphenicol applied to the media at high doses for 4 days, after which antibiotic-free media was used [120]. Bacterial growth was detected again at 13 and 28 days, regardless of whether the antibiotic was considered bacteriostatic (azithromycin, doxycycline, chloramphenicol) or bactericidal (rifampicin).

In humans, *Orientia tsutsugamushi* has reportedly been recovered from the lymph node of a volunteer infected and successfully treated 15 months previously as part of a chemoprophylaxis trial [121]. In the same report, two other cases who had undergone fever therapy in Japan (one for paralytic dementia and the other for an unknown reason) with deliberate scrub typhus infection and had clinically recovered were referenced and described. One case had evidence of *Orientia tsutsugamushi* persistence in a lymph node excised 5 months after infection while in the other case, bacteraemia was allegedly successfully induced 1-2 months post-infection through the intravenous injection of a typhoid vaccine [121]. More recently, a study from Korea reported compelling evidence of persistence: 6 patients previously infected with scrub typhus and successfully treated had evidence of bacteraemia (PCR and culture) 1 to 18 months following primary infection after attending hospital for follow-up or treatment of other underlying chronic illnesses [122]. The

genotypes of the cultured isolates from latent samples matched the original genotypes obtained during acute infection from culture (4 patients were culture positive during the acute infection). Clearly, further studies are required to verify the nature of *Orientia tsutsugamushi* persistence and whether recrudescence, akin to Brill-Zinsser disease in epidemic typhus, is a significant clinical entity for scrub typhus.

Immunity

One of the major challenges in scrub typhus is the short-lived immunity effected through natural infection. It allows repeated infections in at-risk individuals living within the endemic region and can only be partially explained by the heterogeneity of *Orientia tsutsugamushi* strains. Immunisation studies in humans and non-human primates (NHPs) provide some key learning points regarding the natural immune response. Using live *Orientia tsutsugamushi* Karp and Gilliam strains, Smadel *et al* demonstrated homologous immunity (same strain) for most study volunteers for at least 1 year with one individual protected for up to 3.5 years [110]. Heterologous immunity (different strains) was weaker and more transient, ranging from 1 month to 1 year [110]. During this period of waning heterologous immunity, the clinical picture of disease occurring following re-challenge was modified; the incubation period was longer, the symptoms milder, and the eschar absent, suggestive of partial immunity [110]. These findings were also seen in NHPs in which *Orientia tsutsugamushi*-specific IFN-γ producing peripheral blood mononuclear cells (PBMCs) were shown to correlate with immunity parameters such as sterile immunity, low frequency of eschar formation, less bacteraemia and longer incubation periods [109].

Humoral responses are particularly important for homologous protection. Unsurprisingly, antibodies targeted against strain-specific epitopes of the 56kDa outer membrane protein

play a major role in conferring immunity. They increase the uptake of *Orientia tsutsugamushi* by neutrophil polymorphs and macrophages while reducing the infectivity of other target cells such as endothelial cells in a strain-specific manner [100, 123]. Antibodies can reduce cytoplasmic trafficking crucial for the survival of *Orientia tsutsugamushi* while cytokine activation of macrophages are also required to completely suppress infection [123]. Longitudinal studies have shown that antibodies to *Orientia tsutsugamushi* have an annual reversion rate of 61% and the specific anti-TSA56 IgG response persisted for up to 1 year after recovery, similar to the time-period of homologous immunity from cross-immunisation studies [124, 125].

For heterologous protection, cellular immunity is likely to be of major importance with humoral immunity playing a subsidiary role. Mice who survived infection with a live Gilliam strain of *Orientia tsutsugamushi* were able to resist re-challenge with a Karp strain with passive transfer of lymphocytes and not sera shown to infer heterologous protection [126]. Specifically, it was shown that IFN-γ producing T cells were key in providing resistance against challenge in mice, similar to results from NHPs [109, 127]. In humans, apart from elevated levels of pro-inflammatory cytokines such as IL-6, IL-8 and IL-10, higher levels of IFN-γ have also been reported during the acute phase of illness, suggesting that activation of T lymphocytes is part of the early host response [128]. As described above, monocytes/macrophages and monocyte-derived dendritic cells may also play a key role in the early immune response in man. Cellular immunity has also been shown to be short-lived in humans with CD4 and CD8 T cells secreting IFN-γ upon stimulation with *Orientia tsutsugamushi* antigens appearing to be significantly elevated in the immediate period following scrub typhus infection before declining rapidly by the 1 year mark [125].

2.5 Clinical features

Clinical symptoms and signs

The incubation period of scrub typhus is usually between 1-2 weeks but can range from 6-21 days. The main feature is fever, which is almost always present, and is usually accompanied by other less consistent symptoms such as malaise, headache, cough, myalgia, rash and lymphadenopathy. If present, the eschar provides the best diagnostic clue (Figure 9). However, eschar prevalence can vary (1-97%) depending on the thoroughness of clinical examination and the degree of pre-existing immunity to the infecting *Orientia tsutsugamushi* strain [100]. The prevalence may also be reliant on study design with cause of fever studies likely to report more accurate eschar prevalence than studies where diagnostic tests were based on clinical judgement [129]. The eschar begins as a small papule, then ulcerates and undergoes central necrosis. Common locations include the groin, thigh, external genitalia, axilla, waist, neck, abdomen or chest. Generally, eschars are located in warm, moist and covered areas such as skinfolds and areas covered by underwear. Other diagnoses associated with eschar such as spotted fever rickettsia infection, cutaneous anthrax or spider bites may confound the diagnosis.

In the absence of an eschar, clinical diagnosis is challenging as other causes of acute undifferentiated fever in the tropics may present similarly (e.g. dengue or other flavivirus infections, malaria, leptospirosis, murine typhus or other rickettsial infections and typhoid fever). This is compounded by the difficulties surrounding laboratory diagnosis that I will explore in the following section. As displayed in Figure 10, the absence of eschar and rash leaves the clinician with only two realistic options; be reliant on laboratory assays that may not be available or are sub-optimal, or treat patients empirically. Unfortunately, the diagnosis of scrub typhus is often missed or delayed due to a lack of awareness, often with fatal

consequences. Recent systematic reviews have concluded that the median mortality in treated patients is 1.4% while the figure rises to 6.0% in untreated disease [7, 130].

Figure 9. Eschar from adult scrub typhus patients, Chiangrai, northern Thailand.

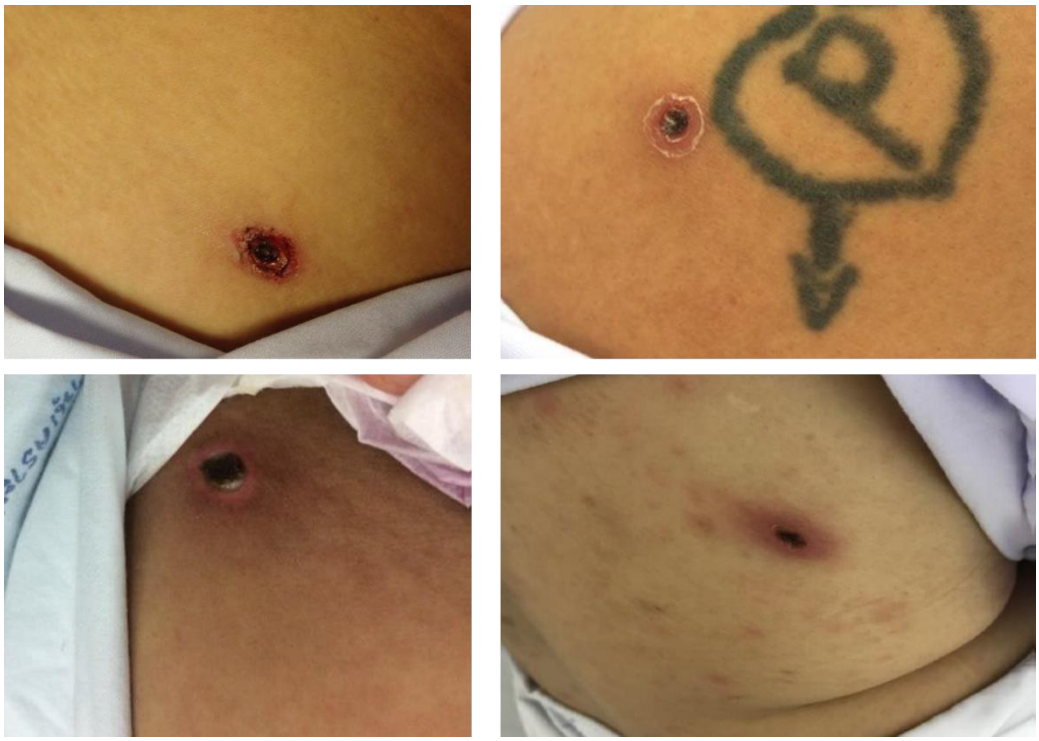
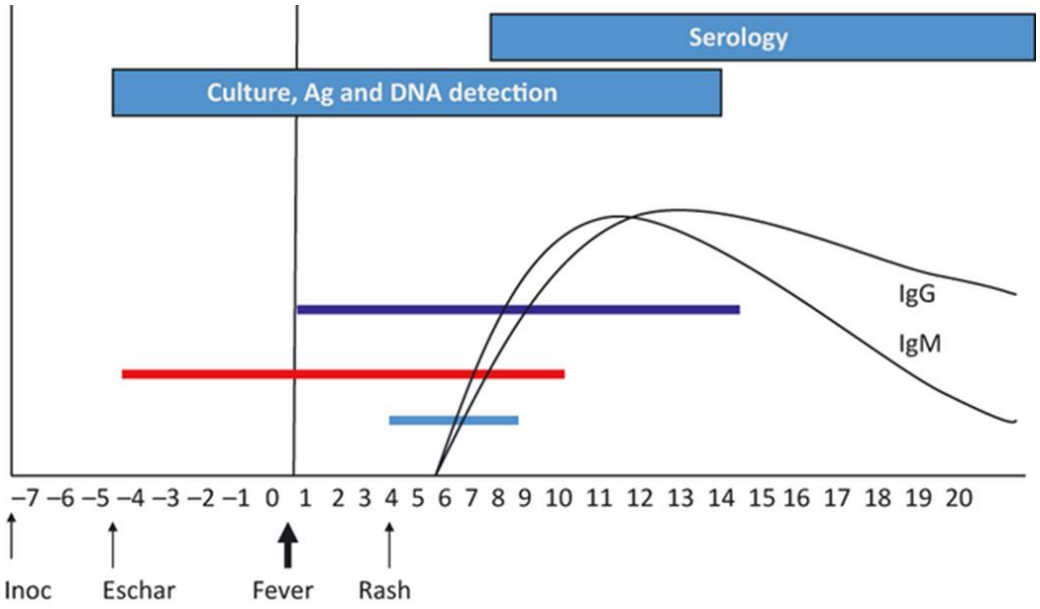


Figure 10. Timeline of scrub typhus infection with reference to clinical features and diagnostic windows. [time in days on x-axis; dark blue bar – fever, red bar – presence of eschar, light blue bar – rash; Inoc – inoculation; from [131]]



Complications

The multitude of systemic manifestations and complications of scrub typhus are in keeping with the vasculitis-like nature of the disease. In the absence of treatment or protective immunity, they tend to occur during the second or beginning of the third week of illness. A recent systematic review was published by Rajapakse *et al* [132]. A summary is given in Appendix 3, with complications classified by organ systems and prevalence.

In addition, there is evidence, albeit limited, of adverse pregnancy outcomes (miscarriages, stillbirths, premature births and low birth weight) in patients affected by scrub typhus during gestation (abortion in 18.2%, poor neonatal outcome in 44.4%) [133]. Suspected vertical transmission leading to neonatal scrub typhus has been reported in 2 cases [134, 135]. Multi-organ failure is a common feature of severe scrub typhus with 85.2% of patients admitted to an intensive care unit in southern India suffering with dysfunction of 3 or more

organ systems [136]. The mortality in this study was reported as 24.1%. Higher mortality has also been noted in older age groups and patients with pulmonary complications (e.g. ARDS), myocarditis, haemorrhagic complications, central nervous system (CNS) involvement and renal failure [7, 130, 137].

2.6 Diagnosis

The diagnosis of scrub typhus is a preeminent challenge facing healthcare workers and researchers in endemic areas today. The inability to diagnose acute scrub typhus infection accurately and cost-effectively has had a detrimental impact on our understanding of the epidemiological, clinical, treatment and preventative aspects of the disease as well as contributing to the lack of awareness among healthcare workers and the general population. In this section, I shall review the diagnostic assays currently used and outline the strengths and limitations of each modality.

Antibody detection-based assays

Serological assays detecting antibodies targeting *Orientia tsutsugamushi* have many advantages over other modalities. They tend to be sensitive, if paired acute and convalescent plasma or serum are used, and specific, if suitable antigen targets are selected to detect scrub typhus-specific antibodies and do not cross-react with antibodies targeting other diseases. Most assays use an antigen panel consisting of at least 3 reference type strains (Karp, Kato and Gilliam) with the addition of a fourth strain (Boryong, TA716) in some assays. Antigens are either whole cell antigens derived from *in vitro* culture or are recombinant proteins containing epitopes from representative strains. Serological assays also tend to be relatively cost-effective when compared to molecular assays or *in vitro* culture.

However, diagnosis of acute disease remains a major weakness and in Figure 10, we can see that antibodies may not be detectable in the first few days of fever. The necessity of paired samples for accurate diagnosis limits the usefulness of antibody-based assays in the acute stages of disease. Additionally, the delayed sero-reversion rates of both IgM and IgG

after recovery is problematic in populations living in endemic areas where repeated infection is commonplace [124, 125, 138]. Here, serological cut-offs used for diagnosis need to be validated for each region, particularly if testing on acute samples remains the only practical option for diagnosis, or the potential for false-positive results will increase [139]. Despite favourable reports by assay manufacturers and developers, cross-reactivity remains an issue in real-world situations. Of the antibody isotypes studied, detection of scrub typhus-specific IgM is most useful for acute diagnosis while the relative longevity of IgG makes this isotype more suited for studies of seroprevalence [138].

The indirect immunofluorescence assay (IFA) performed on acute and convalescent plasma or serum samples remains the gold standard reference assay despite being in use for over half a century [139]. The inherent weaknesses associated with antibody-based assays remain. The lack of standardisation, variable cut-off titres applied, relatively high costs (require trained staff and a fluorescence microscope), and the qualitative and subjective endpoints with significant inter- and intra-operator variability are additional concerns [139, 140]. For example, a review found that diagnostic cut-off IFA titres ranged from 1:10 to 1:400 with 1:400 being the most commonly used [139]. Given the imperfect nature of IFA, Scrub Typhus Infection Criteria (STIC) combining IFA, PCR and culture results were proposed [141]. Although STIC was an improvement, it remained imperfect, leading to sub-optimal biased results in evaluating new diagnostics [142]. The diagnostic cut-offs for the IFA component were further refined using Bayesian latent class modelling to provide unbiased sensitivity and specificity estimates. Optimal cut-off titres for northern Thailand were an IgM IFA titre of ≥1:3,200 in an acute sample and/or 4-fold rise to at least 1:3,200 in paired samples [143]. The accuracy of antibody-based assays are also dependent on the antigen panel used. IFA-like assays, such as the micro-immunofluorescence (MIF) and indirect immunoperoxidase assays (IIP), are also in use but have similar weaknesses.

The use of enzyme-linked immunosorbant assays (ELISAs) is becoming more widespread and have inherent advantages over IFA. These include the relatively lower cost, standardisation, ease of use, reproducibility and objective outputs (i.e. net optical density (OD) values). The accuracy is comparable to IFA although establishing validated diagnostic cut-off OD values remains of prime importance [144-146]. A recent example highlighting the dangers of ignoring this particular challenge comes from India where the Indian Council of Medical Research recommended using 0.5 OD cut-off value for the detection of *Orientia tsutsugamushi*-specific IgM using ELISA (InBios) with a caveat that "baseline titres need to be established" [147]. However, the majority of scrub typhus observational studies from India published subsequently that used the InBios IgM ELISA as the main diagnostic modality applied the 0.5 OD cut-off value broadly without in-depth validation [146]. A recent longitudinal study exploring the kinetics of antibodies following scrub typhus infection in adults using ELISA from southern India is revealing [138]. IgM antibody levels were high initially (OD 1.5-2.0), decreased to OD 1.0 around 7-10 months post-infection, and fell to the OD 0.5 level around 15-18 months post-infection [138]. The OD values for IgM antibodies remained around the OD 0.5 mark up to 46 months post-infection, suggesting that in highly endemic areas the 0.5 OD cut-off value is wholly inadequate for diagnosis of acute disease.

The introduction of affordable point-of-care rapid diagnostic tests (RDTs) has had a positive impact on disease awareness as well as providing a means to diagnose scrub typhus in areas without access to reference diagnostic assays. A recent meta-analysis of studies on the accuracy of scrub typhus RDTs revealed a pooled sensitivity of 66% and specificity of 92%, albeit with significant heterogeneity between studies [148]. The results suggest that performance of current antibody detection-based RDTs is dependent on the pre-test probability of scrub typhus infection.

Despite being the first serological test for scrub typhus, the Weil-Felix assay remains in use today due to its availability, low cost and simplicity. However, the diagnostic accuracy is low and the test has been supplanted by other diagnostic assays in most settings.

Bacteria detection-based assays

Bacteria detection-based assays (i.e. molecular assays and culture) are advantageous in being specific and allows more accurate differentiation of *Orientia tsutsugamushi* strains through genotyping or WGS. The assays are also useful for the diagnosis of acute scrub typhus infection when antibodies are not yet detectable. However, they are dependent on the bacterial load, quality of the samples used, time-point of disease and whether treatment has been initiated.

Polymerase chain reaction (PCR) used to detect *Orientia tsutsugamushi* DNA was first introduced in 1990 and was based on the detection of the 56kDa TSA gene from DNA obtained from Karp, Gilliam and Kato strains as well as blood from infected mice [149]. It was subsequently used to detect 56kDa TSA gene from blood clots in patients in 1991 [150]. Other common gene targets include the 47kDa *htra*, 16S rRNA *rrs* and *groEL* genes [151]. Conventional and nested PCR commonly target the 56kDa TSA and *groEL* genes while real-time quantitative PCR (qPCR) target the 47kDa *htra*, 16S rRNA *rrs* and *groEL* genes [100]. Loop-mediated isothermal PCR assays are an alternative which foregoes the need for a thermocycler (single temperature utilised) and the ease of use lowers the requirement for specially-trained staff although the sensitivity is lower when compared to other standard PCR assays [141].

Clinical samples used include whole blood, buffy coat, eschar and CSF [137, 151]. Buffy coat is more sensitive than whole blood due to tropism of *Orientia tsutsugamushi* for PBMCs

but remains dependent on the degree of bacteraemia, stage of disease and whether antibiotics have been administered [112]. In cases where an eschar or eschars are present, the scrapings, crust, or swabs can provide an alternative material for PCR diagnosis, which may be more sensitive than blood samples, and remains positive for *Orientia tsutsugamushi* DNA up to 2 weeks following antibiotic treatment and recovery [152].

Culture remains the most specific diagnostic test but lacks sensitivity due to the intracellular and fastidious nature of *Orientia tsutsugamushi* and confounding effects of pre-test antibiotics. It is also time consuming (3-4 weeks) and reliant on laboratory expertise and specialist facilities. Isolation from clinical samples (usually whole blood) can be performed in embryonated chicken eggs, *in vitro* mammalian cell culture (e.g. Vero or L929 cell lines) or laboratory animals [100]. Historically, intra-peritoneal injection of patients' blood into mice yielded high retrieval rates but is laborious, costly and poses higher risk to laboratory staff. In addition, the risk of laboratory acquisition has meant that isolation of *Orientia tsutsugamushi* has been limited to biosafety level 3 facilities. In contrast, a recent review suggested that biosafety requirements should reflect the lower risk associated with *in vitro* isolation using cell culture, particularly in the early stages when the infectious load remains low, where work can be carried out safely in a biosafety level 2 laboratory within the confines of a biological safety cabinet [153]. Immunohistochemical staining of tissue (e.g. eschar biopsy) can be used to diagnosed scrub typhus, particularly in the early stages of disease, but is mildly invasive and requires trained staff and equipment [154].

Other diagnostic modalities

The presence of an eschar can be relatively specific for scrub typhus in settings where it is the dominant eschar-associated disease. In northern Thailand, presence of an eschar in

adults had a sensitivity of 42.7% and a specificity of 98.9% for scrub typhus based on Bayesian latent class analysis [142]. Other potential diagnostic modalities include immunological assays (enzyme-linked immunospot – ELISpot, flow cytometry), gene expression profiling and proteomic analysis [125, 155-157]. The latter is particularly encouraging as it provides an avenue for the discovery of *Orientia tsutsugamushi*-specific antigens. This could in turn form the basis for combined antigen/antibody RDTs, akin to dengue NS1/IgM RDTs, and transform our ability to diagnose acute scrub typhus in an accurate and cost-effective way.

2.7 Treatment, drug resistance and outcomes

Prior to the discovery of potent antibiotic treatment after the end of WWII, various treatments were trialed but ultimately proven to be ineffective. These treatments included the established antibiotics penicillin and sulphonamides, methylene blue, and immune or convalescent blood, plasma or sera therapy [49-51]. Para-aminobenzoic acid (PABA), despite initial encouraging results in mice and patients, showed lower efficacy when later compared with chloramphenicol and tetracyclines [158, 159]. Despite being supplanted by these newly discovered antibiotics, it must be recognised that PABA was the first scrub typhus treatment described that had some evidence of efficacy and its significance would have been greater if Chloromycetin had not been discovered soon afterwards by Ehrlich *et al* in 1947. A summary of the prospective clinical trials on scrub typhus treatment reviewed below is outlined in Appendix 4.

Chloramphenicol

In 1947, a *Streptomyces* spp. was isolated from soil collected near Caracas, Venezuela, and filtrates of the bacteria was found to possess remarkable antibiotic properties, inhibiting the growth of a variety of Gram negative and Gram positive bacteria [64]. The filtrates were concentrated and crystallised which became the antibiotic Chloromycetin (Parke, Davis and Company). It was also shown to be effective against a variety of rickettsial species including *Orientia tsutsugamushi* (at the time *Rickettsia orientalis*) in experimentally infected embryonated eggs and mice [160].

In 1948, following encouraging results when Chloromycetin was used to treat a few patients with epidemic typhus in Mexico and confirmation of suitable pharmacokinetic properties of the oral formulation, a small treatment trial was performed in Malaya by a team led by

Joseph Smadel [65]. 25 patients confirmed to have scrub typhus by isolation in mice or serologically (Weil-Felix OXK reactions) were treated with Chloromycetin while 12 other patients were managed symptomatically. Patients treated defervesced rapidly (10-96 hrs after treatment initiation), suffered no complications and none died. 2 patients suffered with complications and 1 died in the untreated group with fever lasting up to 29 days after symptom onset. A range of treatment regime was used ranging from 1 to 7 days of treatment and a total dose of 6 to 15.5g of drug split into 6-12 doses per day [65]. Further work established that a total dose of 6 g administered orally over 24hrs was adequate in the majority of patients with a repeat course used in cases of recrudescence [161].

Chloromycetin was also tested as a chemoprophylactic agent and while it was effective during the exposure period (9 days), around half of the volunteers went on to develop clinical scrub typhus after cessation of the drug (given at 1 g daily during exposure and for 13 days afterwards) [162]. The clinical picture was altered when prophylaxis was given with a more prolonged incubation period and absence of eschar formation (akin to partial immunity described in previous vaccine studies), possibly reflecting the development of fledgling host immunity which was inadequate to prevent clinical disease following cessation of prophylaxis [162]. Another key phenomenon observed was that of relapse. The authors concluded that treatment (1 day course) in the volunteer group occurred at a much earlier disease stage than naturally infected patients due to the nature of the trial which may have not allowed time for sufficient immunity to develop [161]. Follow-up chemoprophylaxis studies in Malaya utilised higher doses with a more prolonged post-exposure course (3-4 g every 4-7 days for 4-6 weeks post-exposure) which proved to be highly efficacious in preventing scrub typhus [159].

In 1949, Chloromycetin's chemical structure was ascertained (through degradation studies) and a method to artificially manufacture the drug (chloramphenicol) determined which, being

more cost-effective than extraction from *Streptomyces venezuelae*, led the drug to be the first antibiotic to be artificially manufactured rather than extraction from a microorganism [163, 164]. Early comparisons of efficacy between chloramphenicol and the newly discovered tetracyclines chlortetracycline (Aureomycin) and oxytetracycline (Terramycin) revealed similar responses in scrub typhus patients [159, 165]. With greater availability and clinical experience, longer courses of chloramphenicol became more commonplace. In 1966, a treatment trial carried out during the Vietnam War compared the efficacy of oral chloramphenicol (3 g daily for at least 3 days) versus tetracycline (2 g daily for at least 3 days), based on which hospital patients were transported to [166]. Diagnosis was based on clinical findings with overall eschar prevalence rates of 85% and rising titre with the Weil-Felix OXK test in a proportion of patients (32%). Fever and other symptoms resolved more rapidly in patients receiving tetracycline than chloramphenicol with relapses often occurring in both groups when treatment was limited to 3-4 days [166]. Current guidance recommend chloramphenicol treatment PO or IV for 7 days in both adults and children >28 days old at 500 mg every 6 hrs and 12.5-25 mg/kg every 6 hrs (maximum 2-4 g/day), respectively [44, 167].

Chloramphenicol inhibits bacterial protein synthesis by binding to the 50S subunit of the 70S ribosome, preventing attachment of transfer RNA, and mainly has a bacteriostatic effect [168]. Reversible side-effects include fever, rash, bone marrow suppression (dose-related), diarrhoea, headache and confusion. Rare side-effects include aplastic anaemia (in 1:21,600 courses), grey baby syndrome in premature infants, anaphylactoid reactions, optic atrophy or neuropathy, digital paresthesias and mild disulfiram-like reactions [167]. Fears surrounding rare side-effects and suggestions of lower efficacy have led to chloramphenicol being superseded by the tetracyclines as the recommended treatment for scrub typhus.

Tetracyclines

Although chloramphenicol was the first highly effective antibiotic for the treatment of scrub typhus, tetracycline antibiotics, particularly doxycycline, grew in prominence during the remainder of the 20th century. The first of these was chlortetracycline or Aureomycin (Cyanamid), a potent antibiotic compound produced by the soil bacterium *Streptomyces aureofaciens*, discovered by Benjamin Duggar, and reported in 1948 [169]. Bio-prospecting for new antibiotics was now progressing rapidly and in 1950, Alexander Finlay *et al* at Pfizer discovered oxytetracycline or Terramycin, produced by another soil bacterium, *Streptomyces rimosus* [169]. It had the advantage over chlortetracycline in being more water soluble and thus had a greater oral bioavailability. The structures of these drugs were published in 1954 by Robert Woodward *et al* of Pfizer, showing that a naphthacene core provided the main structure of this drug class (Figure 11). In the same year, chemical modification of chlortetracycline (Lloyd Conover, Pfizer) led to the synthesis of tetracycline, a semi-synthetic drug with a much higher potency, better solubility and improved pharmacological activity [169].

Between 1948 and 1951, staff at the US Army Medical Research Unit based at the IMR in Kuala Lumpur, Malaya (Malaysia) continued to record experiences in treating scrub typhus, diagnosed by positive culture in mice or rising Weil-Felix- OXK titres, with short 1-2 day courses of chloramphenicol, chlortetracycline, oxytetracycline and PABA [159]. PABA was shown to be much less effective than the other 3 drugs, which had comparable efficacy. Gastric irritation was a common feature with chlortetracycline and oxytetracycline with relapses occurring in all groups. A similar study was performed during a 1952 outbreak of scrub typhus in Nationalist Chinese soldiers stationed in the Pescadores (Penghu, Taiwan) Islands [165]. Chloramphenicol, chlortetracycline and oxytetracycline were all highly effective treatment (although comparison of efficacy was correctly withheld due to

inadequate power) with transient nausea and vomiting being observed in some of the patients. Results from the trial performed during the Vietnam War described above suggest that tetracycline may be more efficacious than chloramphenicol in patients [166]. The current recommended dose for scrub typhus treatment PO with tetracycline is 500 mg every 6 hrs for 7 days in adults and 25-50 mg/kg divided 6 hourly in children age >8 yrs [44, 167].

Second generation tetracyclines are semi-synthetic and include doxycycline, demeclocycline and minocycline (Figure 12) with doxycycline proving to be the most important antibiotic for the treatment of rickettsial diseases today [169]. They were discovered by Lederle and Pfizer and approved during the late 1960s and early 1970s. These drugs possessed much greater antibacterial potency, stability and more favourable pharmacokinetic profiles, allowing for twice-daily dosing. The recommended regime for PO or IV doxycycline for the treatment of rickettsial diseases including scrub typhus is 100 mg twice a day for 7 days in adults and 2-4 mg/kg divided 12 hourly in children age >8 yrs [44, 167]. An initial loading dose of 200 mg in adults or 4 mg/kg in children age >8 yrs is often used. Likewise, PO or IV minocycline is given for 7 days as a 200 mg loading dose followed by 100 mg 12 hourly in adults or 4 mg/kg loading dose then 2 mg/kg 12 hourly in children [167]. Evidence for the clinical use of demeclocycline is lacking.

A randomised trial was performed in adults in Malaysia from 1976-1977 to compare efficacy of 7 days of tetracycline with a single 200 mg dose of doxycycline in scrub typhus patients diagnosed by isolation of *Orientia tsutsugamushi* or positive serology (IFA or Weil-Felix OXK titres) [170]. Both treatments were effective and comparable with no reported relapses although follow-up was limited to 14 days. A randomised, double-blinded, placebo-controlled trial was performed on the Pescadores Islands comparing a single 200mg dose of doxycycline vs 200mg doxycycline followed by a second dose after 1 week vs 7 days of oxytetracycline [171]. Response to treatment was similar in the three arms. A third trial

performed in S. Korea in the 1990s compared 3 days of doxycycline vs 7 days of tetracycline in the treatment of adult scrub typhus patients diagnosed serologically (IFA) with similar results in both arms [172]. There were no relapses during the 4 week follow-up period and although 6.1% of patients in the doxycycline arm had fever beyond 72 hrs, this difference did not reach statistical significance when compared to the tetracycline arm.

In the 2000s, 3 comparative randomised-controlled trials (RCTs) comparing doxycycline with macrolide or macrolide-related antibiotics in the treatment of scrub typhus (diagnosed by IFA) in adults were performed. Doxycycline for 7 days was compared to the azalide (sub-class of the macrolide group) azithromycin in 2 trials (S. Korea – single 500 mg dose, Thailand – 1 g loading dose followed by 500 mg daily for a total duration of 3 days) with no significant difference in fever-based outcomes reported [173, 174]. Similarly, outcomes were comparable in another RCT performed in S. Korea comparing 7 days of doxycycline with 5 days of the ketolide telithromycin (800 mg once a day) [175]. Gastrointestinal side-effects were more frequent with doxycycline for all 3 trials. In the only RCT published in English performed in children with scrub typhus (northern Thailand, diagnosed by RDT), azithromycin was as effective as doxycycline or chloramphenicol (age <8 yrs received IV chloramphenicol, age ≥8 yrs received PO doxycycline) [176].

Efficacy of doxycycline has also been compared to rifampicin in 2 trials in adults with scrub typhus (northern Thailand – IFA; S. Korea – IFA, PCR). Rifampicin (600 mg and 900 mg daily, 7 days) was shown to be more effective than doxycycline (200 mg loading dose followed by 100 mg 12 hourly, 7 days) in northern Thailand where poor response to doxycycline treatment had been reported [177, 178]. In S. Korea, comparable outcomes and side-effect profiles were observed in patients receiving 5 days of doxycycline (100 mg 12 hourly) or rifampicin (600 mg daily) [179].

In contrast, evidence supporting the clinical use of minocycline is limited to case reports, case series and retrospective studies. One such study (in Taiwan) compared fever clearance in adult patients who had received IV minocycline vs PO doxycycline (both at 100 mg 12 hourly, duration not specified) with FCT comparable in both treatment arms [180]. Another retrospective study (in China) suggested that treatment with 7 days of PO minocycline (200 mg loading dose then 100 mg twice daily) led to shorter FCT than 5 days of IV azithromycin (500 mg daily) [181].

First generation tetracyclines are associated with significant side-effects, particularly in young children and pregnant women. These include adverse teratogenic effects, permanent dental staining, reversible bone growth retardation and hepatotoxicity. Until recently, newer tetracyclines such as doxycycline have been classified with the first generation tetracyclines, as medicines contraindicated in pregnancy and children <8 yrs. In 2015, the US FDA replaced the category classification with an evidence-based system which led to the revisiting of doxycycline in these demographic groups [182]. A systematic review of the current evidence revealed that the risk of hepatotoxicity, dental and bone-associated adverse effects and teratogenicity from doxycycline use during pregnancy and early childhood are negligible although better quality data is required [182]. More common side-effects of doxycycline include gastrointestinal irritation, oesophagitis, gastritis, photosensitivity rash and onycholysis [167]. Minocycline's lipid solubility and CNS penetration can lead to vestibular symptoms [167].

Tetracyclines are mainly bacteriostatic and work by reversibly binding to the 30S ribosomal subunit, blocking the binding of aminoacyl-transfer RNA to the ribosomal A site and inhibiting protein synthesis [168]. Further review of the issue of putative doxycycline resistance will follow below.

Azithromycin and telithromycin

In the 1990s, tentative reports of *Orientia tsutsugamushi* strains potentially resistant to doxycycline and chloramphenicol emerged from Chiangrai, Thailand, stimulating the search for alternative antibiotics for scrub typhus [178]. The first antibiotic to be studied in detail was azithromycin, an azalide antibiotic belonging to the macrolide group. The mode of action includes the inhibition of protein synthesis by interfering with the chain elongation process at the peptidyl transferase site and the formation of the 50S ribosomal subunit [168].

Azithromycin was shown to be effective against *Orientia tsutsugamushi* Karp strain (referenced control strain) and strains reported as doxycycline-resistant in cell culture (AFSC-4 and AFC-3) and in mice (AFC-3) [183, 184]. Case reports highlighted its effectiveness in treating scrub typhus in pregnant women, a hitherto understudied group in whom standard antibiotic treatment for scrub typhus (doxycycline or chloramphenicol) were avoided due to concerns surrounding side-effects, mainly to the fetus [184, 185]. Two prospective RCTs carried out in adults in Korea and Thailand comparing scrub typhus treatment between azithromycin (Korea – single 500 mg dose; Thailand – 1 g loading dose then 500 mg daily for a total of 3 days) and doxycycline (standard 7 day regime) showed no apparent differences in efficacy [173, 174]. Additionally, similar outcomes were observed in children with scrub typhus in northern Thailand who were randomised to receive doxycycline or chloramphenicol (based on age) for 5 days or more vs azithromycin for 3 days (weight-dependent doses) [176]. Two additional retrospective studies (Korea and China) were also reported, comparing PO doxycycline for 7 days (100 mg 12 hourly) with 5 days of IV azithromycin (500 mg daily) and PO minocycline for 7 days (200 mg loading dose then 100 mg 12 hourly) with 5 days of IV azithromycin (500 mg daily) [181, 186]. Outcomes were comparable in the former study and minocycline was associated with shorter FCT in the latter study. Side-effects commonly include nausea, vomiting and diarrhoea and rarely, rash,

photosensitivity, neutropenia, thrombocytopenia, transient reversible hearing loss and QTc prolongation potentially increasing the risk of ventricular arrhythmias [167].

Telithromycin, a ketolide antibiotic related to the macrolide group with similar mode of action to azithromycin, was derived from erythromycin A and can be effective in some bacteria that have developed resistance against macrolides [168]. Evidence is limited to 1 RCT in adult scrub typhus patients that showed comparable efficacy when compared to standard 7 day doxycycline therapy [175]. It can also be administered once daily (800 mg daily) and has similar gastrointestinal side-effects as azithromycin along with headache. Rare adverse effects include slowed visual accommodation leading to blurred vision, severe hepatotoxicity (23 cases per 10 million prescriptions) and potential for prolonged QTc and ventricular arrhythmias [167].

Rifampicin

On the basis of preliminary findings of efficacy in mice and *in vitro* cell culture, rifampicin has also been investigated as a potential treatment for scrub typhus [187]. Two RCTs (northern Thailand and Korea) compared treatment efficacy in adult patients receiving doxycycline (200 mg loading dose then 100 mg 12 hourly, 7 days, Thailand; 100 mg 12 hourly, 5 days, Korea) vs rifampicin (300 mg or 450 mg twice a day, 7 days, Thailand; 600 mg daily, 5 days, Korea) [177, 179]. There were contrasting findings with rifampicin shown to be more effective in clearing fever in northern Thailand than doxycycline while no significant difference was observed in the more recent RCT from Korea. The Korean study was powered appropriately and utilised both serology (IFA) and PCR for diagnosis while the older Thai study was underpowered and relied solely on IFA for diagnosis. Intriguingly, protocol change occurred with the Thai RCT as 1 of the 3 original treatment arms

(combination of doxycycline and rifampicin) was discontinued early due to 3 out of 8 patients failing treatment – defined by fever lasting more than 1 week [177]. With only 5 and 9 patients in the other 2 arms (rifampicin only and doxycycline only) and an under-powered target of 20 patients per arm (22/60 recruited, 36.7%), termination of the combination arm by the study monitor may have been premature [188].

One major drawback with rifampicin therapy is the significant epidemiological overlap between scrub typhus endemic regions and areas with high prevalence of HIV and tuberculosis (TB). Rifampicin resistance could develop within weeks in patients with acute TB treated with monotherapy although the risk is reduced with relatively short treatment courses (e.g. 5-7 days) [189]. Common adverse effects include orange discolouration of urine, faeces, saliva, tears and sweat along with gastrointestinal irritation and potential multiple drug-drug interactions. Rare adverse effects include hepatotoxicity, flu-like syndrome, thrombocytopenia, vasculitis and interstitial nephritis [167].

Ineffective antibiotics and antibiotics with limited evidence of efficacy

Betalactams, sulphonamides and aminoglycosides have been shown to be ineffective *in vitro* and *in vivo* for rickettsial infections including scrub typhus [50, 51, 100, 190, 191]. Other macrolide antibiotics have limited evidence to support their routine use. Reports of erythromycin efficacy are inconsistent with clinical evidence of treatment failure as well as alleged success [192, 193]. Evidence suggestive of efficacy came from a RCT in children with scrub typhus from China which was underpowered, had unclear randomisation and inclusion/exclusion procedures and included the Weil-Felix OXK assay in the diagnostic panel, weakening its validity [193]. *In vitro* susceptibility testing of reference *Orientia tsutsugamushi* strains suggest a minimum inhibitory concentration of erythromycin (in

culture media) in the 2.5-10 µg/ml range [187, 194]. Pharmacokinetic studies revealed a maximum plasma concentration of around 2.5 µg/ml in healthy volunteers at day 3 of the standard treatment course of 500 mg 6 hourly, suggesting that standard erythromycin regimens may not be effective for scrub typhus [195].

Clarithromycin use was not associated with a better clinical outcome in adults diagnosed with scrub typhus in Hong Kong while it may be as effective as chloramphenicol and azithromycin in children in S Korea [196, 197]. Both of these studies were retrospective and contained small numbers of patients diagnosed serologically by IFA. One case of an infant with severe scrub typhus (S Korea) complicated by haemophagocytic lymphohistiocytosis and ARDS was reported as successfully cured by clarithromycin [198]. Roxithromycin was reported to be comparable with doxycycline or chloramphenicol for paediatric scrub typhus in another small retrospective study from S Korea although haemagglutination was used for serological testing instead of IFA [199]. However, a small prospective study in children in northern Thailand revealed that roxithromycin was ineffective (patients diagnosed with IFA or RDT) [200].

Patient outcomes with fluoroquinolones (ciprofloxacin, perfloxacin and levofloxacin) have been reported in patients with scrub typhus. A majority of studies revealed disappointing efficacy although a few reported treatment success. However, *in vitro* susceptibility testing revealed resistance in the Kato reference strain and *in silico* analysis of the *gyrA* gene (bacterial DNA gyrase, a target of fluoroquinolones) of multiple reference and clinical strains revealed intrinsic Ser83Leu (substitution mutation, leucine for serine) *gyrA* mutation in all strains studied, suggestive of natural resistance to fluoroquinolones in *Orientia tsutsugamushi* [201, 202].

Other potential antibiotics or adjunctive treatments

Other antibiotics have been investigated, mainly *in vitro* or animal experiments, as potential scrub typhus treatments. Tigecycline, a glycylcycline antibiotic structurally related to tetracyclines, has been shown to have *in vitro* activity against *Orientia tsutsugamushi* in cell culture [203]. However, a cautious approach to the clinical use of tigecycline is required as pooled analysis of its use in the treatment of severe infections caused by multi-drug resistant Gram negative bacterial infections revealed higher mortality than comparator antibiotics [168]. *In vitro* culture of *Orientia tsutsugamushi* with other test antibiotics have revealed that fosfomycin and D-cycloserine may also be effective as they both inhibited growth to a similar extent as chloramphenicol [102]. Another antibiotic agent, a novel α-pyrone compound corallopyronin A (CorA), produced by the myxobacterium *Corallococcus coralloides*, is a non-competitive inhibitor of the bacterial RNA polymerase switch region with a different binding site to rifamycins (e.g. rifampicin) [119]. It was shown that *Orientia tsutsugamushi* Karp strain was highly susceptible to CorA *in vitro* and in experimentally infected mice. Together with its lipophilic properties, effectiveness against other intracellular bacteria and apparent ineffectiveness to *Mycobacterium* spp., CorA is an intriguing therapeutic prospect with further pre-clinical studies now required.

Combination therapy is commonly used for various infectious diseases but evidence is almost wholly absent for scrub typhus, the exception being the terminated study arm combining rifampicin and doxycycline in one RCT from northern Thailand [177]. A RCT for the treatment of severe scrub typhus in India is underway with one treatment arm combining IV doxycycline and IV azithromycin (Varghese G., personal communication, May 2018). Additionally, *in vitro* experiments performed in S Korea were suggestive of both synergistic and antagonistic relationships between antibiotics with proven effect for scrub typhus and other drugs. The antimalarial drug chloroquine, which has marginal inhibitory effects on

Orientia tsutsugamushi in isolation, was shown to exhibit synergism when combined with doxycycline, rifampicin or azithromycin [204]. Conversely, cefotaxime exerted antagonism when combined with the same antibiotics [205]. The latter study is of particular interest as a combination of 3rd generation cephalosporin and another antibiotic is often used in the tropics. Clearly, further studies are required to confirm or refute these findings, both of which were reported by the same group.

Evidence on adjunctive treatment for scrub typhus is limited, perhaps due to the satisfactory responses achieved by antibiotics in the majority of cases. However, corticosteroids have been used by clinicians sporadically in scrub typhus patients with severe life-threatening disease. An early study performed on patients experimentally infected with scrub typhus observed that the 8 cases who received chloramphenicol with adjunctive cortisone (both for 1-2 days) defervesced quicker but around half relapsed compared to 1 case out of 7 in the chloramphenicol-only group [206]. *In vitro* experiments using L929 cells and *Orientia tsutsugamushi* Gilliam strain revealed no significant effect on bacterial growth with the addition of dexamethasone to the cell culture [207]. However, a RCT comparing short-course adjunctive corticosteroid treatment for severe scrub typhus was apparently performed in northern Thailand, a region where severe disease is commonplace and tentative drug resistance was previously reported (Kantipong P., personal communication, March 2018). The results reportedly showed a clinical benefit and has been translated to everyday practice at the hospital where the study was performed. Unfortunately, the trial was neither registered nor published and my attempts to obtain further details of the trial (e.g. study design, patient characteristics, power calculation, outcomes) have been unsuccessful. Further studies into the antibiotic treatment of severe scrub typhus and the use of adjunctive therapy are required.

Figure 11. Chemical structure of the first generation tetracycline antibiotics. [year – approval by Food and Drug Administration (FDA); taken from [169]]

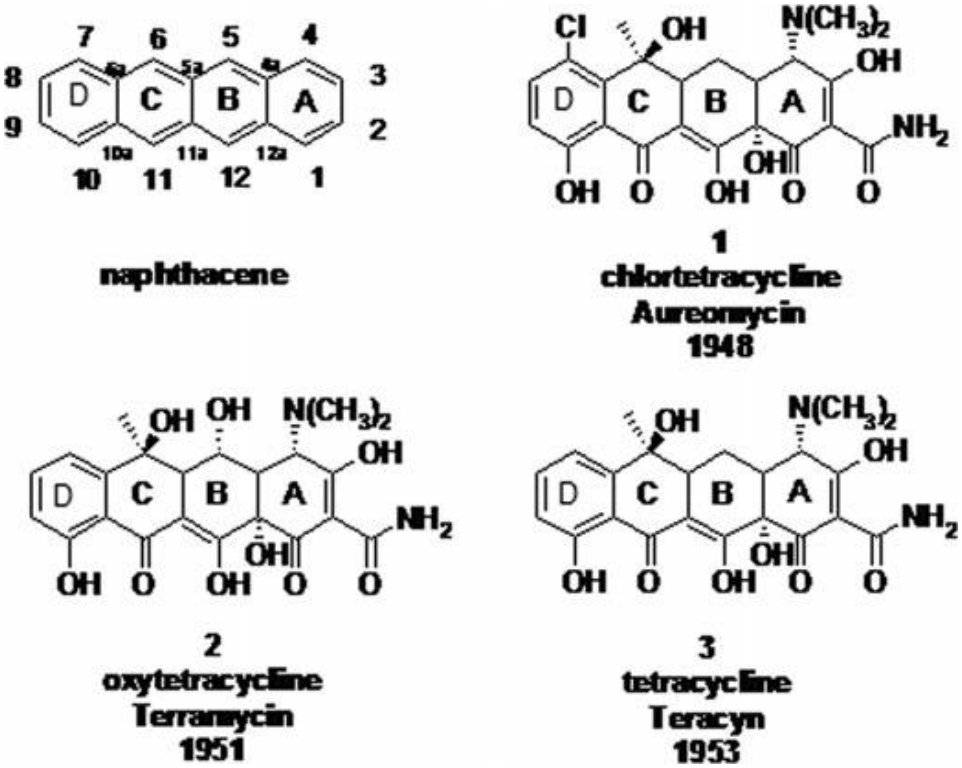
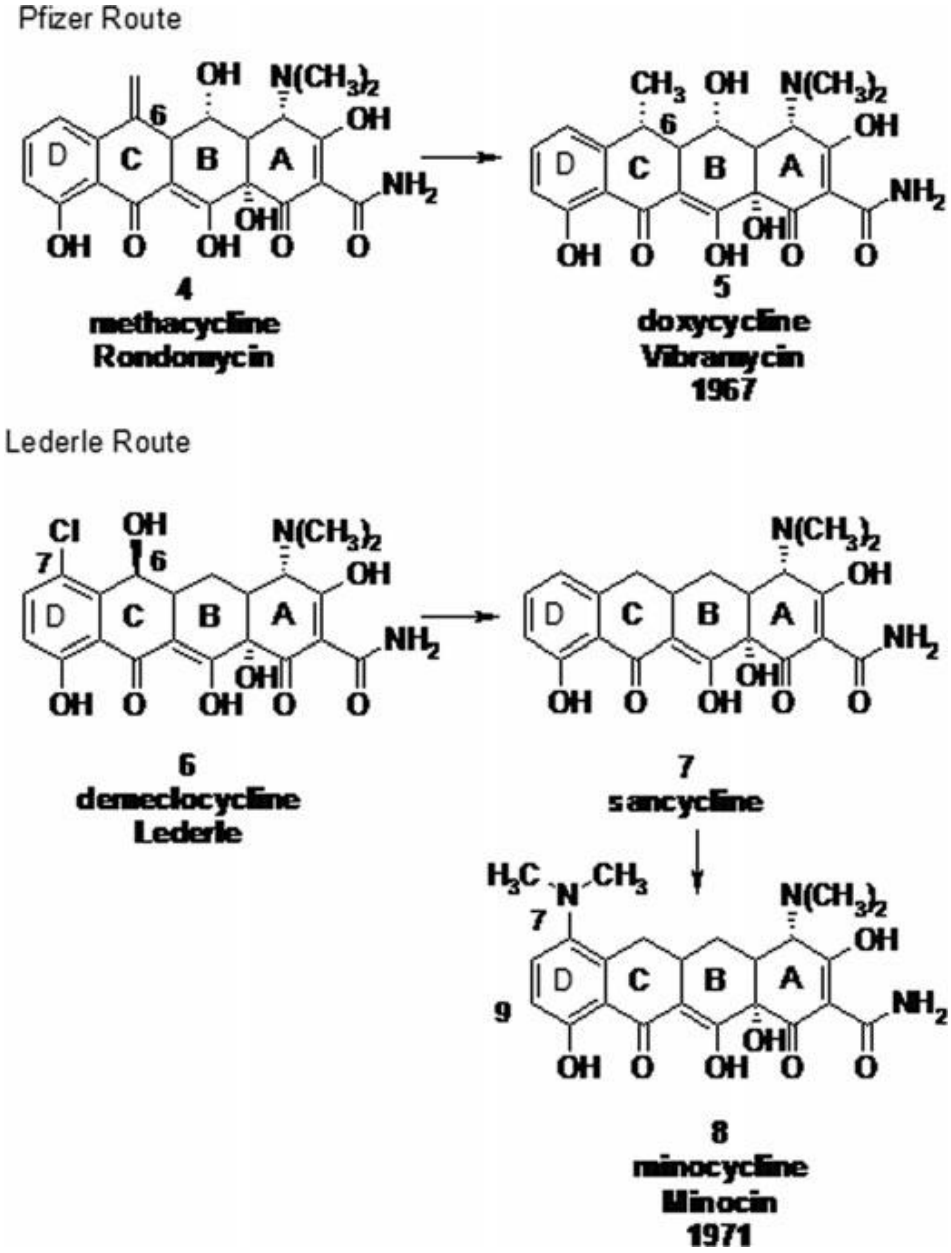


Figure 12. Chemical structure of the second generation tetracycline antibiotics. [year – approval by Food and Drug Administration (FDA); taken from [169]]



Reports of drug resistance

In the 1990s, researchers were informed by clinicians in northern Thailand of cases of scrub typhus that were severe and sometimes fatal despite the use of appropriate antibiotic treatment. Subsequent studies performed by US Army researchers confirmed these findings, which at the time were attributed to drug resistance based on supportive evidence from *in vitro* susceptibility assays and survival models in mice [178]. Independent verification was lacking and despite the fact that the evidence for drug resistance was weak, drug resistance in scrub typhus has repeatedly been referenced and speculated upon as the reason for failures of treatment and prophylaxis [208-212].

In the clinical study performed in northern Thailand (performed 1991, published 1996, Watt *et al*), 12 scrub typhus patients from Chiangrai had prolonged fever-clearance time (FCT) when compared to 7 patients from Mae Sot; median FCT of 80 hrs (range 15-190) and 30 hrs (range 4-58), respectively [178]. All patients were treated with a 7 day course of oral doxycycline and diagnosis was confirmed by serology (IgM titre of ≥1:400 or IgG titre of ≥1:1,600 in a single acute blood sample by IIP). Blood from patients included in the study were injected intra-peritoneal into mice to obtain isolates of *Orientia tsutsugamushi* (C1, C3 and C27) which proceeded to antibiotic susceptibility testing using a mouse survivability model and cell culture. Clinical isolates appeared to be less susceptible to chloramphenicol (C1 and C3) and doxycycline (C3 and C27) in the mouse model and doxycycline (C3) in cell culture when compared to the reference Karp strain. The minimum inhibitory concentration (MIC) of AFC-3 (C3) isolate to doxycycline was estimated at >4 μg/ml. These results prompted the authors to conclude that the *Orientia tsutsugamushi* AFC-3 isolate was resistant to doxycycline.

In an *in vitro* susceptibility study (published 1995, Strickman *et al*), an *Orientia tsutsugamushi* isolate, AFSC-4, obtained from a patient in 1990 from Kanchanaburi

Province in western Thailand was shown to be less susceptible to doxycycline (MIC 0.25 µg/ml to 0.5 µg/ml) than the reference Karp strain (MIC 0.0625 µg/ml) with azithromycin appearing to be effective for both strains [183].

More recent clinical reports from southern India, S Korea and northeastern Thailand of severe intractable disease and reports of failure of prophylaxis in military personnel in Southeast Asia and Australia have speculated on the role of doxycycline resistance [208-212]. However, none were substantiated and only the Australian study performed culture and susceptibility testing, which ruled out doxycycline resistance [212]. The recommended prophylactic regimen of 200mg doxycycline weekly was based on two RCTs performed in the 1970s, before PK validation became commonplace [213, 214]. Plasma concentrations of doxycycline following a 200mg dose falls below 0.5 mg/l at 48 hrs, suggesting that the current recommended regime may provide sub-optimal protection, especially when doses are missed or delayed [215].

Assessing the evidence for and against doxycycline resistance

These initial reports were controversial at the time and remain so today. The lack of independent verification and a scientifically-sound explanation for the findings in these studies and indeed, the determinants of poor treatment outcome, have contributed to the ongoing uncertainty. At the time, researchers debated the validity of these findings and struggled to explain why, if true, drug resistance to doxycycline, an antibiotic with proven efficacy, developed [78]. Above, I explored inherent resistance to fluoroquinolones in *Orientia tsutsugamushi* but for doxycycline, any resistance must have been acquired. As chiggers (the natural disease reservoir) are thought to feed only once, antibiotic selective pressure is unlikely to come from humans or other natural hosts. The initial clinical report

speculated that doxycycline in animal feed or *Streptomyces* spp. in soil may have provided the selective pressure for doxycycline resistance to develop [178]. However, neither of these conditions are unique to northern Thailand and viable evidence of doxycycline resistance from elsewhere are lacking. Furthermore, if we review the treatment trials summarised in Appendix 4, prolonged fever clearance times occurred in most settings, suggesting that treatment outcome, as defined by fever clearance, is a spectrum.

In the former, clinical Chiangrai study, the number of patients was small and details of the recruitment process were not described [178]. Thus, selection bias may have been an issue. The patient characteristics were also different between the two sites. In Chiangrai, patients were, on average, febrile for a day longer than Mae Sot while they were generally older; factors which may have affected outcome despite statistical significance not being reached (p values were unavailable). Cut-off diagnostic titres of ≥1:400 and ≥1:1,600 on admission samples for *Orientia tsutsugamushi*-specific IgM and IgG, respectively, performed using the indirect immunoperoxidase (IIP) assay were used. We have seen from subsequent validation studies that these serological cut-offs are likely to be too low, reducing the specificity of diagnosis [143].

Both antibiotic susceptibility assays used in the AFC-3 study were flawed. In the mouse model, 1,000 units of mouse 50% lethal dose of the clinical *Orientia tsutsugamushi* isolates were injected intra-peritoneal (IP) into mice prior to the administration of high doses of chloramphenicol or doxycycline through the same route 5-8 days later. Compared to natural infection, where dermal inoculating doses are miniscule and the bacterial replication and early dissemination phase corresponds to the incubation period of 5-7 days (see Figure 10), the balance of infection and antibiotic effect is shifted significantly in favour of infection in this model. It has taken a further two decades for scrub typhus animal models reflecting natural infection to be developed (C57BL/6 mice and cynomolgus macaques, intradermal

inoculation) [109, 216]. Furthermore, the mouse survivability assay does not account for the differences in virulence of *Orientia tsutsugamushi* isolates and ignores the differences in pharmacokinetics when compared to humans [217, 218]. The half-life of IP doxycycline is significantly shorter in mice (3-6 hrs) than in humans (15-30 hrs) suggesting multiple doses a day and higher doses in mice – approximately 15mg/kg – are required to correspond to a 100mg dose in humans [218].

In cell culture, mouse fibroblast (L929) cells were used and infected with clinical *Orientia tsutsugamushi* isolates. Infected cells were subsequently incubated with media containing no antibiotics, 4 µg/ml and 16 µg/ml of doxycycline, or 8 µg/ml and 32 µg/ml of chloramphenicol. However, only after 30 hrs incubation time, the cells were removed, fixed in methanol, Giemsa-stained and examined by microscopy to assess the percentage of cells infected. In essence, methodology for antibiotic susceptibility testing in extracellular bacteria (including shorter incubation period and higher antibiotic doses) were utilised for the obligate intracellular *Orientia tsutsugamushi* which has vastly different growth dynamics. It was thus unsurprising that the AFC-3 isolate had a high doxycycline MIC of >4 µg/ml. Assays used to determine antibiotic susceptibility in *Rickettsia* spp. had an incubation period of at least 4 days with subsequent studies incubating between 5-10 days depending on the specific assay [219, 220].

It is important to recognise that there are still no internationally agreed reference standards for antibiotic susceptibility testing (AST) of *Orientia tsutsugamushi*. It is also crucial to be aware of the fundamental differences in growth cycles between obligate intracellular bacteria and other bacteria and how this impacts AST results. Studies of growth cycle-dependent pharmacodynamics (PD) of antibiotics in *Orientia tsutsugamushi* have not been published. However, in a sophisticated *in vitro* study by Siewert *et al*, this phenomenon was examined in the closely related obligate intracellular bacteria, chlamydiae [221]. The results

were epiphanous. Firstly, the growth cycle revealed that soon after infection, the bacteria transitioned from the extracellular infectious phase – termed elementary bodies (EB) – to the early intracellular non-infectious phase – called reticulate bodies (RB). In the mid-phase, replicating intracellular RBs dominate while the late-phase is characterised by the recondensation of RBs to EBs, thus regaining infectivity [221]. Furthermore, differences in the lengths of the growth cycle were shown between *C. trachomatis* (short) and *C. pneumoniae* (long). Significantly, they also report that in the extracellular phase (EBs), antibiotic treatment (rifampicin, doxycycline, erythromycin and ciprofloxacin) had minimal effect on infectivity. Susceptibility was maximal in the intracellular phases where the metabolically active replicating RBs dominate with rifampicin shown to be the most effective. This milestone study revealed that AST in obligate intracellular bacteria is complex and dependent on the characteristics of the growth cycle of the pathogen along with the particular pharmacokinetics (PK) and PD of the tested antibiotics. For *Orientia tsutsugamushi* AST, an incubation period that allows for all tested extracellular phase bacteria (of all known strains) to transition through to the metabolically active, replicative intracellular phase and, for the antibiotics tested to have sufficient time to concentrate intracellularly and to exert their effects is a necessity for accurate results to be achieved.

In the study by Strickman *et al*, L929 cells were used for cell culture and a greater range of antibiotic concentrations (doxycycline and azithromycin) were studied. Incubation was for 3 days after which, cells were examined using Giemsa staining and microscopy as well as flow cytometry [183]. Using this assay, it appears that azithromycin was more effective than doxycycline at inhibiting growth in both Karp and AFSC-4 strains. However, the incubation period remained short and this was suggested by the screening test performed with very high concentrations of both drugs (16 µg/ml of doxycycline and 8 µg/ml of azithromycin). At these drug concentrations, if peak bacterial concentrations had been achieved after 3 days

incubation, all extracellular phase bacteria had transitioned to the intracellular phase and the antibiotics tested have had sufficient time to concentrate intracellularly and exert their effects, the proportion of infected cells should be zero or close to zero. This was not the case for either antibiotics for both Karp and AFSC-4 strains. The doxycycline MIC for AFSC-4 was estimated to be 0.25 to 0.5 µg/ml while for Karp, it was 0.0625 µg/ml. These differences led the authors to conclude that AFSC-4 is doxycycline resistant even though the incubation period was likely sub-optimal, the plasma drug concentrations achieved in humans with standard doses are significantly higher than 0.5 µg/ml, and other more feasible pharmacological explanations exist (explored below) [178, 215].

These two studies conclude that *Orientia tsutsugamushi* AFC-3 and AFSC-4 isolates are doxycycline resistant and contributed to treatment failure. However, the flaws and limitations outlined call into question whether this is, in fact, true. A third strain, AFSC-7, isolated in the same year as AFSC-4 (1990), has also been reported as doxycycline resistant but the initial characterisation of antibiotic susceptibility has not been published [222]. Additional *Orientia tsutsugamushi* strains collected from patients from Chiangrai and Mae Sot, northern Thailand, with delayed responses to therapy were studied by the US military but the methodology and findings never published [223].

More recently, two independent groups have performed AST on AFC-3 and AFSC-4 isolates. In the first study, AFC-1 (C1 isolate from Chiangrai), AFC-3, AFSC-4 and Karp strains were tested for doxycycline susceptibility using the same methodology (including 3 days incubation and microscopy to assess growth) as Strickman *et al* [223]. Growth appeared to have been inhibited at a concentration of 0.1 µg/ml in all the studied strains, suggesting susceptibility to doxycycline. In the second study (from SE Asia), a novel, PCR-based method to determine antimicrobial susceptibility in *Orientia tsutsugamushi* was developed and validated using multiple reference strains (Karp, Kato, Gilliam, UT76 and

TA763). The optimal conditions for antimicrobial susceptibility testing included an *Orientia tsutsugamushi* bacterial load of $1x10^6$ copies of DNA per $1x10^4$ L929 cells giving a multiplicity of infection (MOI) of 100:1, a 10 day incubation period which corresponded to peak bacterial concentrations in all studied reference strains and trypsinisation for cell harvesting [224]. The doxycycline MICs were 0.125 mg/l for AFC-3 and 0.250 for AFSC-4 while the MICs for the reference strains ranged from 0.016 mg/l to 0.125 mg/l, concentrations well below the achieved peak drug serum concentrations in the 1996 Chiangrai study (2-3 mg/l and 4-7 mg/l following 100mg and 200mg oral doses, respectively) [178, 224]. In addition, a further 51 *Orientia tsutsugamushi* isolates from Thai and Lao patients underwent AST to determine antibiotic screening concentrations for azithromycin, doxycycline and chloramphenicol. All appeared to be susceptible to the tested antibiotics. The doxycycline MICs for AFC-3 and AFSC-4 fall within the expected wild type distribution of doxycycline MICs (median of 0.125 mg/l +/- one to two two-fold dilution steps) in the 5 reference strains (including Karp and Gilliam, prototypical strains considered doxycycline susceptible) and 51 clinical isolates. The importance of incubation period on AST was further highlighted by unpublished data from this second study [224]. Doxycycline MICs for Karp, AFC-3 and AFSC-4 isolates were compared at day 3 and day 7 with lower MICs observed at the longer incubation period for all studied strains (Figure 13).

These two recent studies suggest that both AFC-3 and AFSC-4 *Orientia tsutsugamushi* isolates are doxycycline sensitive. Together with the significant shortcomings of the AST assays used in the original studies, doxycycline resistance appears not to be real. Mutations of AFC-3 and AFSC-4 isolates through multiple passages and long term storage contributing to the loss of doxycycline resistant phenotype have been suggested as a potential explanation. However, recent analysis of the genetic stability of *Orientia tsutsugamushi* prototype strains Gilliam, Karp and Kato and unpublished work on the genomic stability of

UT76 and TM4942 strains through multiple passages suggest this is unlikely [225] (Salje J., personal communication, April 2019). The presence of undiscovered naturally doxycycline-resistant *Orientia tsutsugamushi* isolates remains a possibility but only a theoretical one.

In summary, the presence of doxycycline-resistant *Orientia tsutsugamushi* is predicated on limited evidence and AST assays with significant shortcomings. Recent studies have not supported the presence of drug resistance and these findings along with alternative explanations for poor treatment outcomes are explored in the section below.

Determinants of treatment outcome

If there is little or no evidence for drug resistance in scrub typhus, how can we explain the poor responses to appropriate antibiotic treatment in some patients from northern Thailand and beyond? The answer is likely to be multifactorial. Delays in disease recognition, seeking medical help and administration of effective treatment are likely to contribute and increases the likelihood of the infection becoming established and disseminated. Patients presenting late with severe disease and evidence of multi-organ failure have higher mortality despite appropriate treatment being instigated [136]. Older age is also associated with increasing mortality [7].

Bacterial factors including the diversity of virulence of *Orientia tsutsugamushi* have been described [226]. Infectivity and growth rate of AFSC-4 in comparison to native and other reference *Orientia tsutsugamushi* strains have been studied by Korean investigators [227]. ECV304 cells (human cell line) were infected with the studied bacterial strains and incubated for 3 days. At 4 hrs, AFSC-4 showed higher infectivity than other tested *Orientia tsutsugamushi* strains (Figure 14). The higher proportion of infected cells for AFSC-4 compared to other strains were maintained to 72 hrs and the growth curve shape did not

differ significantly. This key study suggests that higher infectivity of *Orientia tsutsugamushi* strains allows the active, intracellular, replicative phase of the bacterial life cycle to begin earlier than other strains with lower infectivity, influencing its virulence. Previous studies in mice concluded that the pathogenicity of an *Orientia tsutsugamushi* strain may be dependent upon the growth rate [217]. The Korean study informs us that it is not the growth rate per se, but rather the infectivity which is the crucial element in determining virulence.

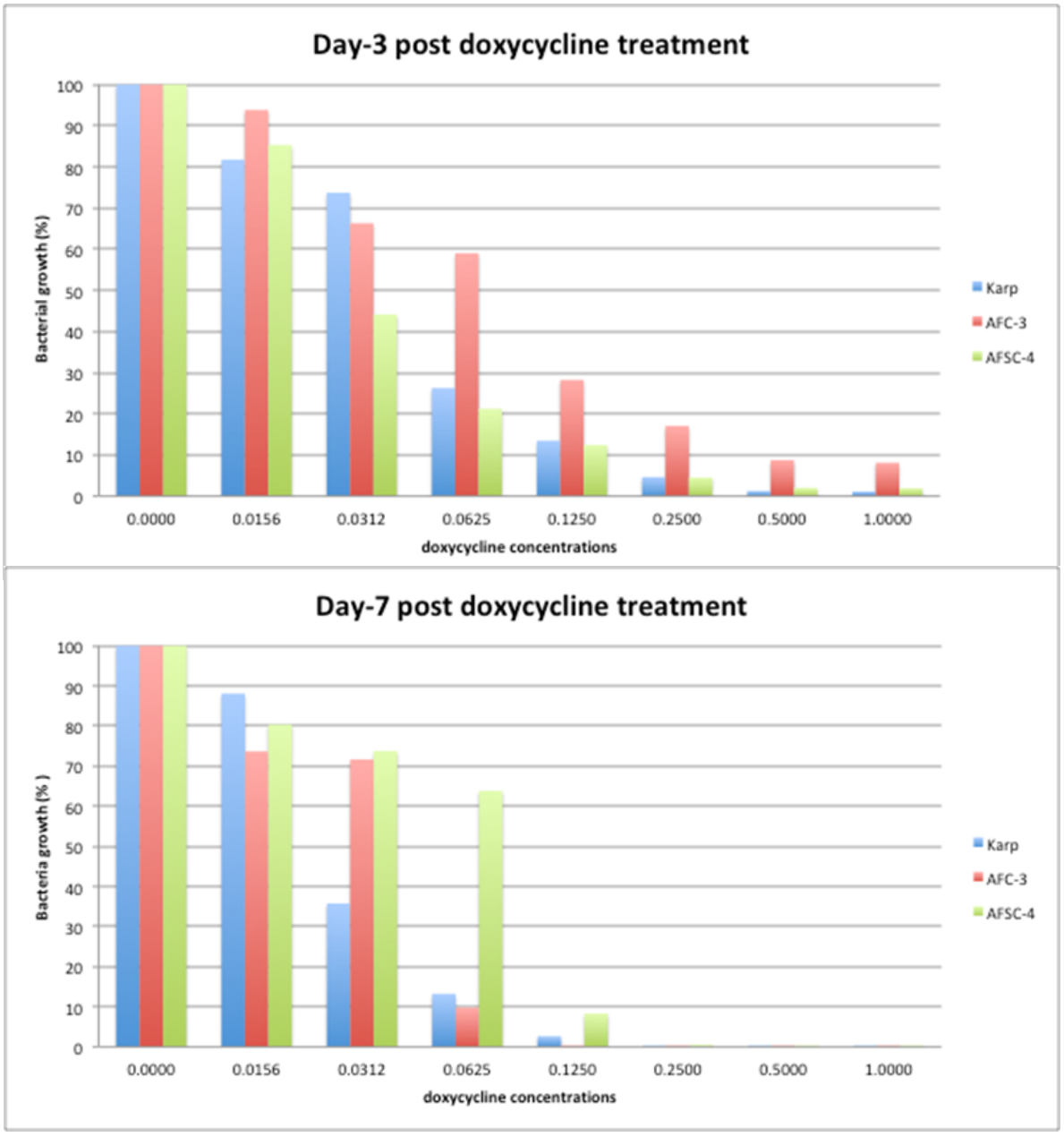
Additional evidence to support the high infectivity rates of AFC-3 and AFSC-4 strains have been published as part of the technical AST study from SE Asia described above [224]. In this study, the doubling time observed for AFC-3 and AFSC-4 strains, 9.50 and 10.53 hrs, respectively, were clearly shorter than the other five reference strains (range 12.03 to 19.85 hrs). The growth curves reveal that AFC-3 and AFSC-4 reach a set bacterial load much faster than other strains although growth rates in the first 3 days were not studied. This is clinically significant as high *Orientia tsutsugamushi* load is associated with more severe disease and treatment failure is much more likely in this setting [228]. The slightly higher MICs for AFC-3 and AFSC-4 are reflective of their increased infectivity compared to other reference strains. For doxycycline, the MICs of the tested strains ranged over 5 dilutions from 0.0156 to 0.250 mg/l. For azithromycin, the MICs ranged over 4 dilutions from 0.0039 to 0.0313 mg/l. The relatively higher MICs for doxycycline in comparison to azithromycin may reflect the differing PK characteristics of the two drugs.

Pharmacological factors, specifically the drug concentrations achieved with current antibiotic doses within the intracellular compartment of *Orientia tsutsugamushi*-tropic cells such as PBMCs, will also contribute to treatment outcome [229]. Both doxycycline and azithromycin inhibit protein synthesis, both are likely to affect the active intracellular phase of the *Orientia tsutsugamushi* growth cycle and both likely to be ineffective in the extracellular phase. PK data suggest that azithromycin concentrations in the intracellular compartment (peripheral

blood mononuclear cells – PBMCs, polymorphonuclear leucocytes – PMNs) are over 100 times higher than plasma in healthy human volunteers [230]. Likewise, doxycycline concentrates intracellularly (in PMNs) when compared to plasma but to a lesser degree [231]. Azithromycin may appear to be more effective than doxycycline at inhibiting *Orientia tsutsugamushi* growth in Karp and AFSC-4 strains in cell culture susceptibility assays due to the greater intracellular drug concentrations achieved, especially if the incubation period is shortened [183]. Rifampicin also concentrates well in the intracellular compartment, perhaps more so than azithromycin, which could explain the growing evidence for its efficacy in scrub typhus treatment [232]. Limited data from clinical and PK/PD studies of doxycycline suggest that in patients presenting with more severe disease, the current doses may be suboptimal and further investigations are warranted [233]. Furthermore, it is unclear which antibiotic, each with its own distinct mechanism of action, will be most effective against *Orientia tsutsugamushi*. Currently, it is difficult to draw any firm conclusions regarding the optimal choice of antibiotic due to the heterogeneous nature of the trials previously performed and the fact that the majority were underpowered [234] (Appendix 4).

Finally, host immunity will contribute to treatment outcome as previously explored. Historical data showed that indigenous populations, who were likely to have a degree of immunity, were more likely to suffer from a milder disease phenotype than expatriate populations [4]. In the pre-treatment era, despite the higher mortality and prolonged recovery, the majority of patients with scrub typhus survived, suggesting a key role played by the immune response. To conclude, treatment outcome in scrub typhus is likely to be determined by multiple factors including the timeliness of recognition and treatment, the presence or absence of protective immunity in the host, the virulence (particularly the infectivity) of the infecting *Orientia tsutsugamushi* strain and the characteristics of the antibiotics used for treatment.

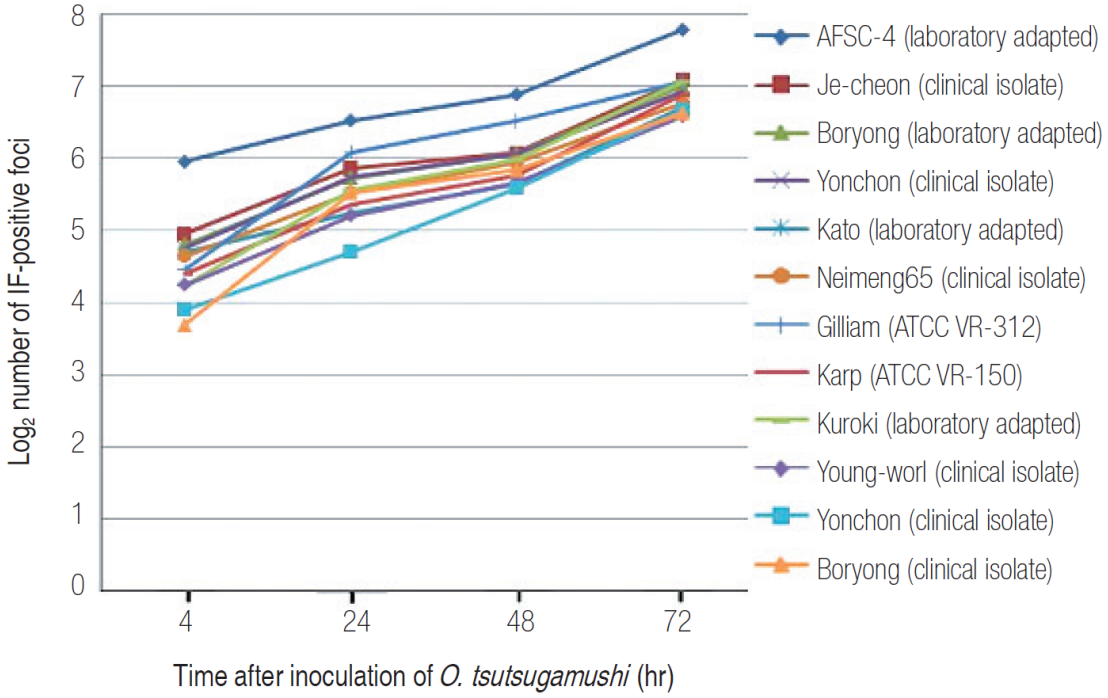
Figure 13. Doxycycline susceptibility testing of *Orientia tsutsugamushi* Karp, AFC-3 and AFSC-4 strains at incubation post-treatment. MICs represent drug concentrations that inhibit >90% of bacterial growth. [Phuklia W., personal communication, June 2018]



Day 3: MIC – Karp = 0.2500 mg/l, AFC-3 = 0.5000 mg/l, AFSC-4 = 0.2500 mg/l

Day 7: MIC – Karp = 0.1250 mg/l, AFC-3 = 0.0625 mg/l, AFSC-4 = 0.1250 mg/l

Figure 14. Infectivity and growth rate of *Orientia tsutsugamushi* AFSC-4 strain in ECV304 cell culture when compared to Korean and other reference strains. [taken from [227]]



2.8 Prevention

Preventative measures for scrub typhus are currently limited. There are currently no vaccines providing sterilising, long-lasting and cross-protective immunity as previously discussed. Past vaccines against *Orientia tsutsugamushi*, the barriers to developing an effective vaccine, and suggestions for future approaches have been reviewed recently by Valbuena and Walker [235]. Over the last 70 years, the most efficacious immunogens in humans have been live strains (with subsequent treatment) although the problem of waning immunity, as seen following naturally acquired infections, remained. Formalin-killed *Orientia tsutsugamushi* vaccines were not effective in humans with limited protection seen in mice. Experience with recombinant protein immunogens (56kDa, 47kDa) also yielded limited success in mice and NHPs. It was hypothesised that immunodominant antigens are mainly encoded by non-conserved genes among the different *Orientia tsutsugamushi* strains while conserved genes encode subdominant antigens that yielded suppressed immune responses. Thus, the key to an effective vaccine may be to utilise a combination of conserved *Orientia tsutsugamushi* antigens that stimulate a significant humoral and cellular immune response which will hopefully be sustained [235].

Evidence for the effectiveness of prophylaxis are limited to historical studies using chloramphenicol and doxycycline. For chloramphenicol, a regime of 3-4 g every 4-7 days for 4-6 weeks post-exposure was deemed protective of severe disease following a series of studies [110, 159, 162]. For doxycycline, 200mg once a week was tested in both naturally exposed military personnel in Taiwan and volunteers challenged with infected chiggers in Malaysia [213, 214]. Although subjects who received prophylaxis were generally protected from severe disease, there was limited protection from infection as mild illness and seroconversion remained quite common in the prophylaxis group. In another retrospective study, military personnel deployed to Southeast Asia who reportedly took 100mg

doxycycline daily as part of malaria prophylaxis were screened for seroconversion using pre- and post-deployment blood samples [211]. 14/347 (4%) subjects had seroconverted (based on detection of *Orientia tsutsugamushi* antibodies using ELISA) and 3 of these subjects reported some non-specific symptoms of low-grade fever, maculo-papular rash and arthralgia. The authors concluded that these findings were suggestive of drug resistance associated with scrub typhus even though evidence of compliance was not presented and past studies clearly showed that infection per se remained common even in those receiving prophylaxis. A more recent outbreak in military personnel was reported from Australia where 45/124 (36%) personnel developed clinical scrub typhus (60% with evidence of seroconversion) despite protocols stipulating the use of 200mg doxycycline weekly [212]. In one case, *Orientia tsutsugamushi* was successfully cultured and susceptibility testing revealed an MIC of ≤0.04 μg/ml. The authors concluded that poor compliance was likely to blame for the outbreak rather than doxycycline resistance.

From these studies and previous discussions on doxycycline PK, it can be concluded that weekly 200mg doxycycline doses provide a degree of protection from severe scrub typhus although protection from infection is sub-optimal. Limited evidence suggests that 100mg daily doses of doxycycline may provide greater protection from severe scrub typhus and infection but seroconversion and mild disease may still occur. It remains unclear why breakthrough infection occurs with the latter regime although poor compliance and the characteristics of the infective *Orientia tsutsugamushi* life cycle may contribute.

Acaricide treated clothing and insect repellent were effective at preventing scrub typhus during 20th century conflicts. However, these measures along with the use of antibiotic prophylaxis remain impractical for the majority of the population living within endemic areas. Bite avoidance and chigger removal remains the most feasible measures currently available in these settings and some evidence exist to show that it is effective.

The burden of scrub typhus in South Korea is much higher than Japan despite similar climate, geographical and agricultural factors [85, 236]. Ma *et al* explored the differences in agricultural activities between Jeollabuk-do (high risk, high incidence) in Korea and Fukuoka prefecture (high risk, low incidence) in Japan [237]. Differences included more time spent in the field, work postures that increased contact with grass and the ground and resting around farmland during work days in S Korea when compared to Japan. Japanese farmers tended to exhibit protective behaviour when compared to their counterparts in S Korea including use of hats, neck towels, long-sleeves or sleeve covers and boots. They also tend to avoid lying or sitting on grass, avoid laying clothes on grass, wash clothes after farming and shower or bathe after farming. These findings were somewhat surprising as the level of knowledge of scrub typhus and preventative behaviours were higher in S Korean farmers than Japanese farmers in this study. In a small case-control study from S Korea, patients with scrub typhus had low awareness of the disease and were less knowledgeable regarding the use of protective clothing when compared to controls [238]. Knowledge of other preventative behaviour such as avoiding sitting or lying on grass, removing work clothes immediately after outdoor work, keeping work and daily clothes separate and bathing or showering immediately after working outdoors were also lower in scrub typhus patients compared to controls. Use of protective clothing in less temperate climates are unlikely to be as successful so the focus in these tropical regions should be on protective behaviour and chigger removal (e.g. by bathing) following a day in the forest or field.

2.9 Current challenges in scrub typhus and thesis outline

In this chapter, I have reviewed scrub typhus and highlighted some of the major unresolved challenges. The lack of an accurate and cost-effective test to diagnose acute scrub typhus and the absence of standardisation of current diagnostic modalities continue to hinder our understanding of the disease. As a result, the burden of disease remains unclear and lack of awareness among healthcare professionals and the general population persists, contributing to the ongoing neglect by funders, policy makers and the wider scientific community. Treatment outcome and its determinants remain poorly understood and studied. The old dictum, that scrub typhus treatment was straightforward and fever clearance was almost always rapid, was an oversimplification. Treatment outcome as a spectrum, dependent on multiple factors, is the more realistic and scientific representation. The conclusion of drug resistant *Orientia tsutsugamushi* as the explanation for poor treatment outcome was based on flawed methodology and ignored some fundamental factors regarding bacterial infection and growth characteristics and pharmacological properties of the antibiotics tested. This has led to ongoing uncertainty regarding the optimal treatment of scrub typhus, particularly in patients with severe disease. Treatment in children and pregnant women has also been neglected despite the more limited treatment options in these groups. Other challenges include the lack of feasible preventative measures (including vaccines) for scrub typhus and the uncertainty surrounding bacterial persistence and potential for clinically significant relapse.

These challenges can be summarised in a series of research questions below:

- What is the burden of scrub typhus at the national, regional and global level?
- How can the diagnosis of scrub typhus be improved and standardised?
- What are the determinants of treatment outcome in scrub typhus?

- Is poor treatment outcome in scrub typhus related to drug resistance?
- How can awareness of scrub typhus be raised?
- What feasible measures can be used to prevent scrub typhus?
- Does persistence of *Orientia tsutsugamushi* and clinically significant relapse following infection pose a substantial risk to patients?

It is impossible to answer all of these questions within the framework of a single DPhil thesis. However, I shall endeavour to answer some of these questions in the remaining chapters and highlight areas for future work. In chapter 3, I will study the burden of scrub typhus in Thailand at the national to the provincial level and provide a more detailed case study of Chiangrai Province in northern Thailand. In chapter 4, I shall investigate how scrub typhus contributes to the febrile disease burden in adults in Chiangrai and study which factors help to differentiate it from other causes of fever. Chapter 5 will focus on the clinical characteristics and treatment outcomes in paediatric scrub typhus. Chapter 6 will outline how I plan to characterise the determinants of treatment outcome and investigate whether drug resistant *Orientia tsutsugamushi* is a contributing factor. In chapters 3, 4 and 5, I will also focus on epidemiological factors such as sex, age, ethnic group and occupation associated with scrub typhus. Diagnosis will be another cross-cutting theme revisited in all results chapters. Personal development, conclusions and future work will be discussed in chapter 7 and 8, respectively.

3 Estimating the burden of scrub typhus in Thailand

3.1 Introduction

In the previous chapter, I highlighted one of the major challenges in scrub typhus; how the burden of disease remains unclear with the difficulties surrounding diagnosis of acute infection a major contributing factor. An estimated figure of one million cases annually was first mentioned in a review article in 2003 and featured prominently but was not referenced [239]. A WHO Recommended Surveillance Standards document from 1999 contains the often quoted sentence [1]: “Scrub typhus is probably one of the most underdiagnosed and underreported febrile illnesses requiring hospitalization in the region.” It is damning, then, that this remains applicable two decades later but critically, this document did not include an estimate of the global burden of disease. Where did the figure of one million cases a year come from? The figure was probably derived, in part, from the results of a review of scrub typhus in the Western Pacific region published in 1997 [240]. Here, seroprevalence data from Malaysia, Thailand, India, Nepal, Indonesia, Philippines, Korea and Taiwan were reviewed along with cause of fever studies from Malaysia, Thailand and in US military personnel during the Vietnam War [240].

Seroprevalence data are useful to gauge the extent of disease exposure in a population and to assess disease distribution. However, they cannot reliably be used to determine the clinical burden of disease as exposure does not equate to symptomatic illness. The seroprevalence of scrub typhus IgM and IgG as measured by IFA was as high as 59.5% in a 1994 study of 200 febrile patients attending 6 rural malaria clinics located in 3 central provinces of Thailand, suggesting high disease exposure [241]. Subsequent cause of fever studies have confirmed that scrub typhus is an important cause of non-malarial fever in South and Southeast Asia in febrile adults, pregnant women and children (range 5.2%-

19.9%) [242-248]. These fever aetiology studies also have their limitations. They are geographically limited to sites associated with a research unit, or an established link to a research organisation, and often suffer from high degree of heterogeneity; mainly related to the study population or the diagnostic criteria used. Furthermore, they are costly and challenging to perform, making it impractical for the burden of scrub typhus to be determined by fever studies alone. Case reports, case series and observational studies, while clinically useful, cannot provide sufficient information on disease burden at a national level.

The most comprehensive set of data on the burden of scrub typhus currently available comes from national disease surveillance systems of countries with well-established public health systems and where scrub typhus is a notifiable disease. These countries include Japan, South Korea, China, Taiwan and Thailand [66, 85-87, 236, 249, 250]. More recently, Bhutan and Nepal have established scrub typhus as a notifiable disease and initiated disease reporting in 2010 and 2014, respectively, but the systems require further strengthening [251, 252].

In Japan, the clinical reporting criteria include fever, eschar and other associated symptoms and signs such as rash, lymphadenopathy, malaise, anorexia and headache [85]. Coverage by the national health insurance scheme ensures good accessibility to diagnostic serology (detection of IgM by IFA) while diagnosis by PCR is becoming more common. All cases reported have clinical features of scrub typhus and have had at least one confirmatory diagnostic test performed which includes detection of *Orientia tsutsugamushi* by culture, detection of *Orientia tsutsugamushi* genome by PCR or detection of specific IgM by IFA or IIP [85]. From 2007 to 2016, there were 4,185 scrub typhus cases reported in Japan with the disease incidence relatively stable at around 400-500 cases a year. There were more male (54%) than female cases (46%) and patients had a median age of 68 years. Cases reported exhibited two seasonal peaks: a smaller peak from May to June and a larger peak from

November to December. Seasonality was explained by the differences in prevalence of the vector, *Leptotrombidium* spp., in each prefecture. *Leptotrombidium pallidum* is cold tolerant and larvae surviving the winter are thought to be responsible for the spring peaks of cases while areas where *Leptotrombidium scutellare* were prevalent had later autumn and early winter peaks [85]. Disease prevalence varied by prefecture and is mainly related to agricultural activity (Figure 15) [85, 237].

Since 1994, scrub typhus has been a notifiable infectious disease in South Korea [236]. Details of the reporting criteria from published reports in English have been limited although both suspected clinical cases, based on clinical features alone, and confirmed cases, with clinical features and laboratory evidence of scrub typhus are reported [253, 254]. Confirmatory laboratory tests include IFA and PCR assays. From 2001 to 2013, there were 70,914 cases reported in South Korea with an increasing trend over the study period [236]. In contrast to Japan, there were more female (63%) than male scrub typhus patients (37%) although the older 60-79 year age group had the highest number of reported cases. Agricultural workers had a higher disease incidence than other occupational groups. Unlike Japan, there was one large peak in reported cases from October to November (92%) with the secondary spring peak absent [236]. Unsurprisingly, scrub typhus burden was higher in rural areas and in provinces where agriculture was the main industry (Figure 16). However, as the disease burden increased from 2001 to 2013, the number of cases from urban and suburban areas also rose and here, patients were less likely to be farmers [236, 253]. An observation was also made that as latitude decreased, the peak incidence tended to shift to the latter months of the year [66].

In mainland China, data from the national reporting system for 2006-2014 have been published [86]. Details of the reporting criteria were outlined including epidemiological risk factors (travel to endemic area, contact with chiggers or rodents) and clinical features

consisting of fever, rash and eschar. At least one laboratory criteria must also be met: positive agglutination titre ≥ 1:160 Weil-Felix OX-K test, ≥ 4 fold or more rise of antibody titre against *Orientia tsutsugamushi* using IFA, detection of *Orientia tsutsugamushi* genome by PCR, or *in vitro* isolation of *Orientia tsutsugamushi* from clinical samples. 54,558 scrub typhus cases were reported with more female (53%) than male (47%) patients [86]. The 50-59 year old age group had the highest number of reported cases (22%), followed by 40-49 year old (18%) and 60-69 year old (18%). Farmers once again made up the majority of scrub typhus cases reported (59% in 2006, rising to 74% in 2014). Similar to South Korea, the disease incidence rose significantly during the study period from 1,248 cases in 2006 to 16,050 cases in 2014. The burden of disease was highest in the southern provinces and a few eastern provinces with differences in seasonality also observed (Figure 17) [86]. In the south, the scrub typhus “season” began in spring and continued through to the end of the year while the provinces further north exhibited a narrower peak with cases reported between September and December.

In Taiwan, online access to the scrub typhus reporting data of Taiwan National Infectious Disease Statistics System, Centers for Disease Control, and published reports based on this dataset are available [87, 249, 255]. The system requires clinically suspected cases to be reported to a regional centre with samples sent to the reference laboratory for testing. Cases are confirmed by PCR or IFA with cut-off IgM titre of 1:80, IgG titre of 1:320 or a 4 fold increase of IgG titre in paired sera applied. From January 2010 to September 2019, 4,215 cases of confirmed scrub typhus were reported [255]. The number of reported cases per year averaged around 400 cases and was stable during this period. The highest incidence was seen in the 55-69 year old age group but disease incidence was also moderately high in the 20-24 year old age group [255]. Generally, there was a higher incidence of scrub typhus among men than women and the proportion of farmers in the population was

predictive of higher disease incidence [87]. Disease burden was highest in the central, eastern and island counties (Figure 18) [255]. Seasonality of confirmed cases in Taiwan was observed with the majority of cases occurring between May and January, similar to the pattern seen in southern China.

In all four countries above, scrub typhus was the most frequent rickettsial disease observed. In Bhutan, available data from the reporting system are limited. The case definition used include clinical or suspected cases with acute fever and one or more of eschar, headache, rash, cough, malaise, myalgia and lymphadenopathy [256]. Probable cases are patients suspected of scrub typhus with a positive rapid diagnostic test result while confirmed cases are clinical or probable cases with one or more confirmatory diagnostic test including positive scrub typhus IgM by ELISA, 4 fold increase in antibody titre detected by IFA or IIP in paired samples and detection of *Orientia tsutsugamushi* genome by PCR. Data from 2015 showed that there were 470 scrub typhus cases reported and the majority were reported from June to November [256]. The distribution of disease is shown in Figure 19. Prior to 2015, the picture is unclear but the reviewed evidence suggest the number of reported cases were increasing, likely due to growing awareness and improving access to diagnostic tests [251].

Similarly, Nepal has a fledgling reporting system for scrub typhus which began in 2014 [252]. Clinical or suspected cases have fever with or without eschar while probable cases have symptoms plus some serological evidence of disease (e.g. IgM titre > 1:32 in acute sample). Confirmed cases are defined by detection of *Orientia tsutsugamushi* DNA by PCR or 4-fold rise in antibody titres in paired acute and convalescent samples detected via IFA or IIP methods. In 2015, 101 scrub typhus cases with 8 deaths were reported while the number increased in 2016 to 831 cases with 14 deaths [252]. Geographical distribution of cases is shown in Figure 20.

In Thailand, even though the first case of scrub typhus was reported in 1952, it was not until the last decade of the 20th century and the beginning of the 21st century that further studies revealed the disease as a major contributor to the acute febrile illness burden [257]. The disease is notifiable to the National Disease Surveillance System (R506), Bureau of Epidemiology (BoE), Department of Disease Control (DDC), Ministry of Public Health (MoPH) [250]. Healthcare facilities in each province are responsible for reporting scrub typhus cases to the provincial public health office. Thailand has 4 administrative regions, 76 provinces and 2 special administrative areas (Bangkok and Pattaya). Each province is further sub-divided into districts, sub-districts and villages. The population in each province is served by a central provincial hospital, district hospitals and primary care units located in each sub-district (larger sub-districts may have more than one primary care unit). Scrub typhus reporting data and case definitions are accessible online and records are available from the last four decades [250]. Despite this, analyses and reporting of this dataset have not been performed and information on the national burden of scrub typhus are not widely shared. There remains an issue with lack of data sharing and amalgamation between research studies and surveillance data [258].

In this chapter, I shall estimate the scrub typhus burden in Thailand using detailed national surveillance data from 2003-2018. I will also investigate the potential effect of geography, population, rainfall, temperature, habitat complexity and diversity of land use/land cover on disease incidence in conjunction with collaborators as specified.

Figure 15. Number of (a) scrub typhus and (b) Japanese spotted fever cases by reported prefecture, 2007-2016, Japan. [taken from [85]]

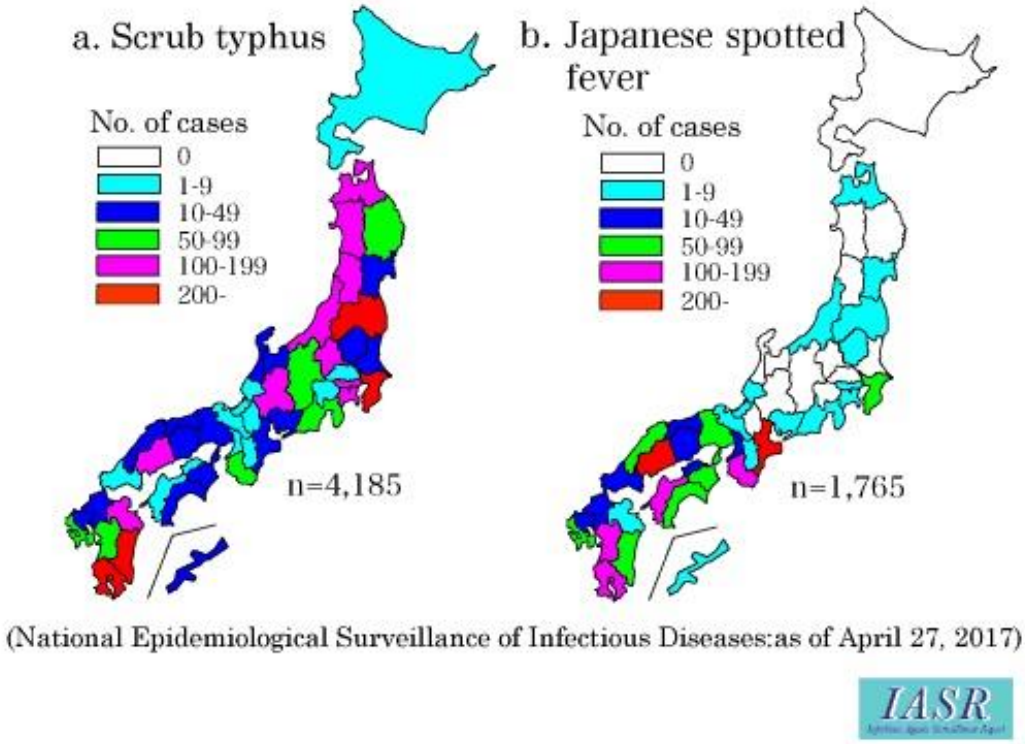


Figure 16. Scrub typhus in South Korea with changes in disease incidence at the county and provincial level over time. [APR = annual prevalence rate or cases/100,000 population, S = Seoul, GG = Gyeonggi Province, GW = Gangwon Province, CB = Chungbuk Province, CN = Chungnam Province, JB = Jeonbuk Province, JN = Jeonnam Province, GB = Gyeongbuk Province, GN = Gyeongnam Province, JJ = Jeju Province; taken from [236]]

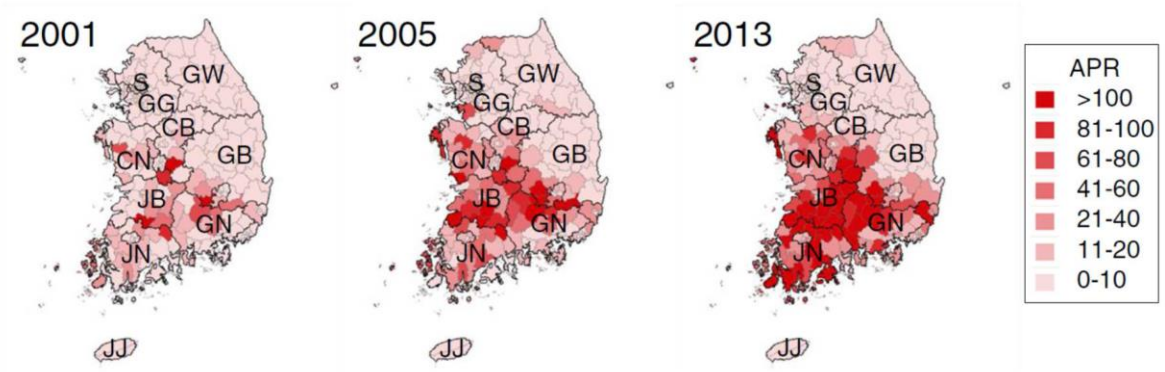


Figure 17. Scrub typhus distribution by incidence, mainland China, 2006-2014. [taken from [86]]

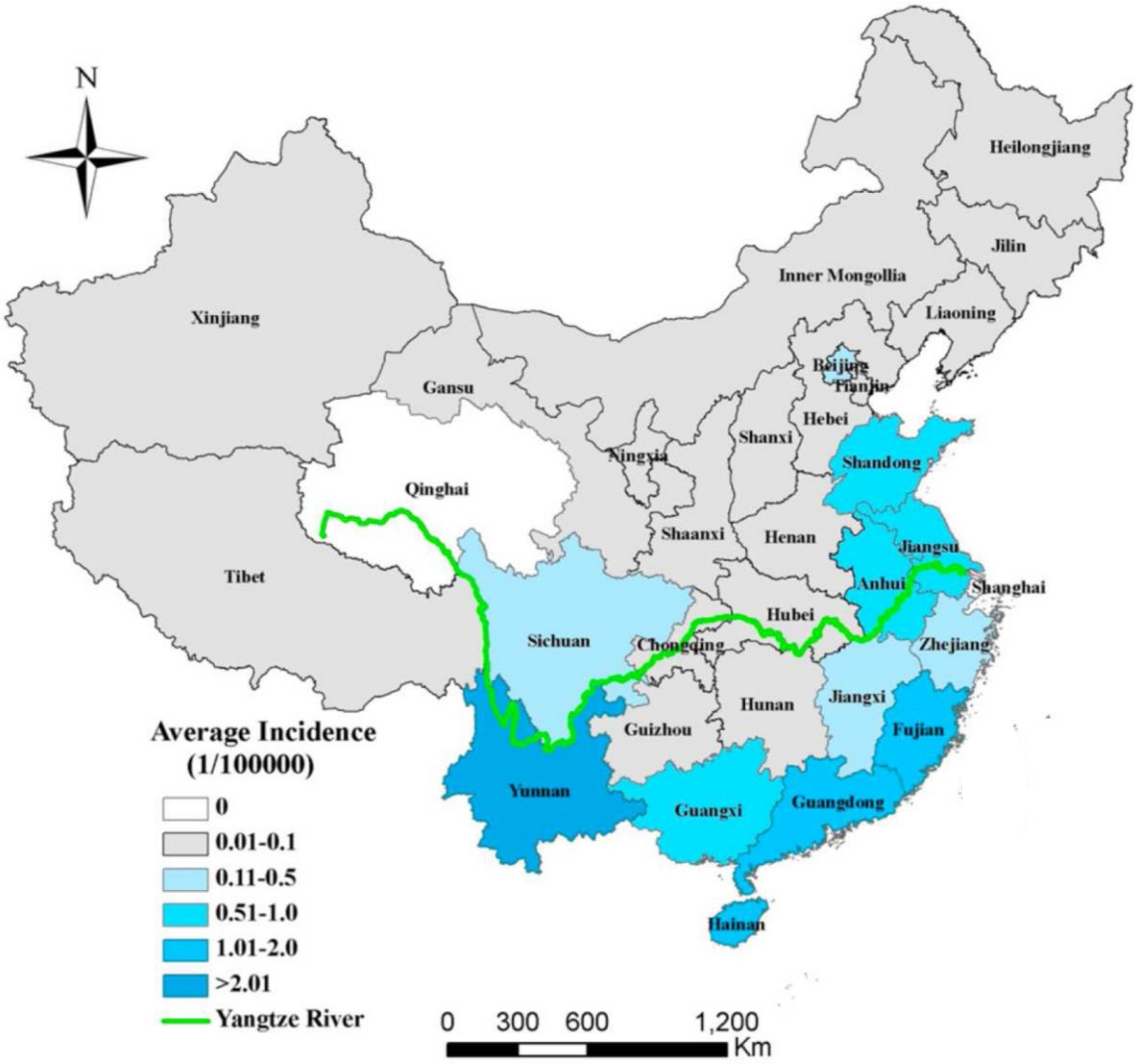


Figure 18. Distribution of scrub typhus cases by county in Taiwan, 2010-2019. [taken from [255]]

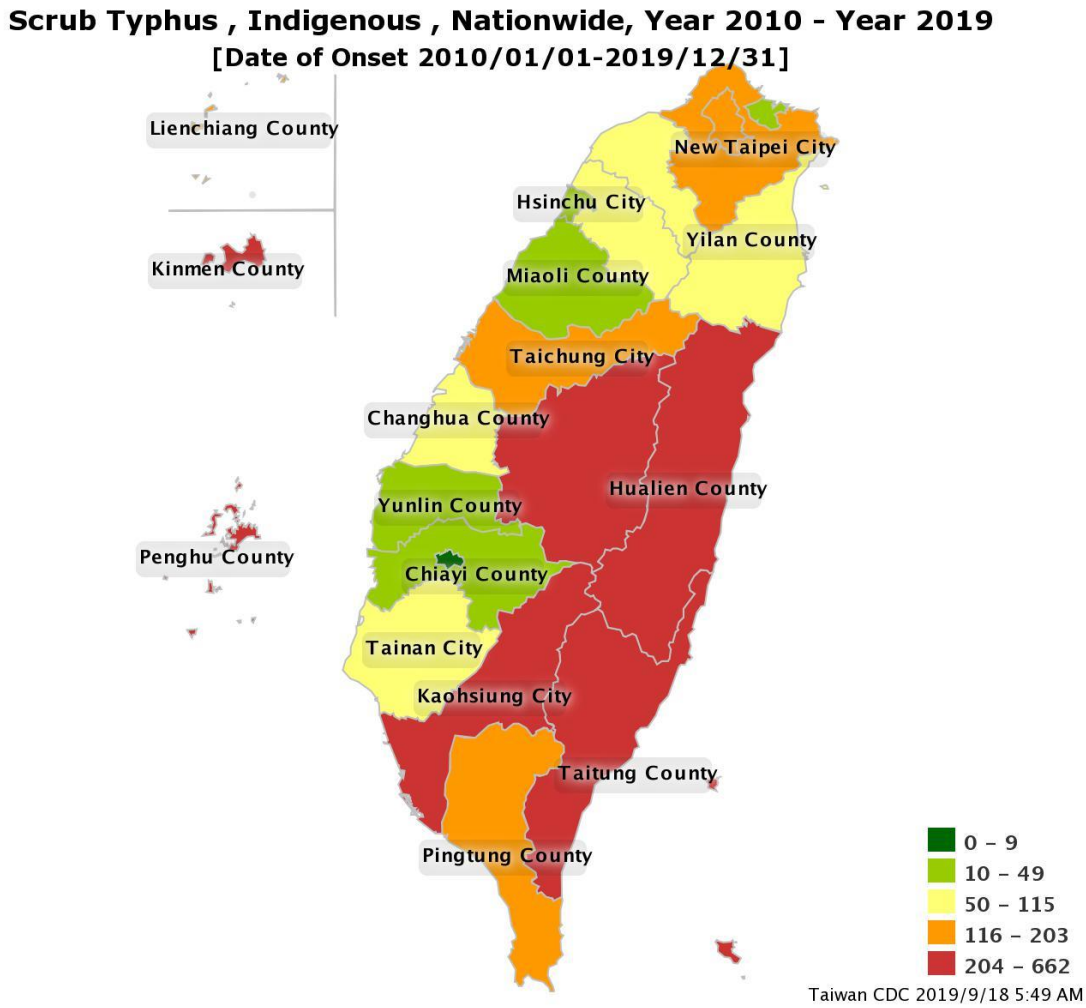


Figure 19. Distribution of scrub typhus cases in Bhutan by district, 2015. [taken from [256]]

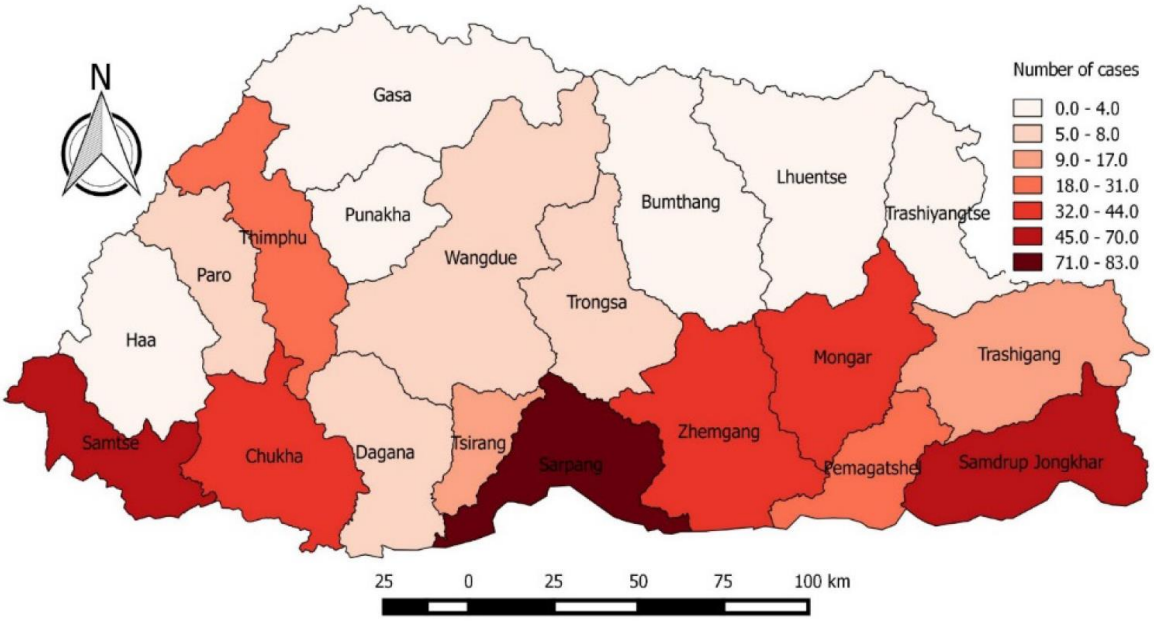
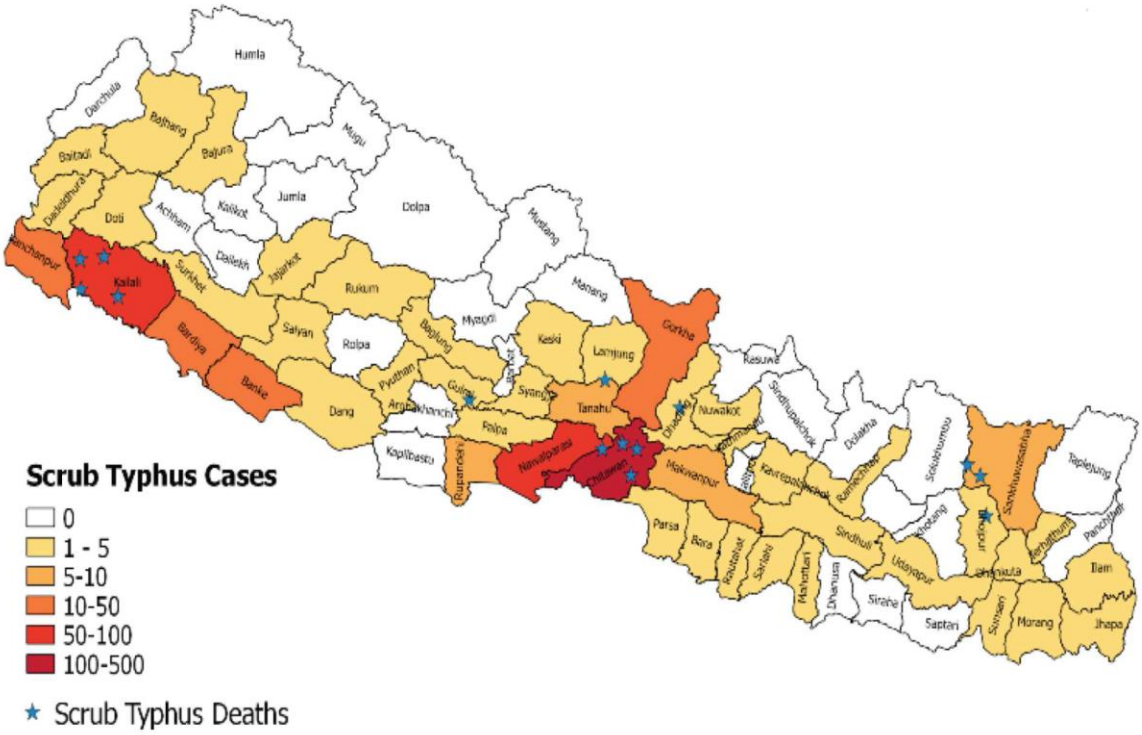


Figure 20. Distribution of scrub typhus cases in Nepal by district, 2016. [taken from [252]]



3.2 Method

3.2.1 Data collection

Detailed scrub typhus reporting data from 2003-2018 were obtained from the National Disease Surveillance System (R506) [250]. Annual summary reports were available for 1980-2012 which were collected and collated with the main dataset. Probable and confirmed cases were reported and the case definition used (ICD-10: A75.3) are described in Table 1.

Table 1. Scrub typhus reporting criteria

Clinical criteria	Acute febrile illness and an eschar with at least one other symptom including: - Headache - Myalgia - Arthralgia - Ocular or orbital pain - Petechial rash
Laboratory criteria	General findings suggestive of scrub typhus: - Low white count - Normal or low platelet count Disease-specific: - Detection of a four-fold rise in scrub typhus antibodies in paired sera by haemagglutination inhibition assay *or* antibodies detected at a cut-off titre of >1:1,280 in a single sample *or*, - Detection of scrub typhus IgM antibodies by ELISA *or*, - *Orientia tsutsugamushi* PCR *or* culture positive from blood
Case classification: • Probable case	- Fulfil clinical criteria and has general laboratory findings suggestive of scrub typhus *or* epidemiological link to confirmed cases
• Confirmed case	- Fulfil clinical and disease-specific laboratory criteria

[ELISA – enzyme-linked immunosorbant assay, PCR – polymerase chain reaction]

Cases were mainly reported from governmental healthcare facilities and included provincial hospitals, district hospitals and primary care units. Private healthcare facilities also notified cases but less consistently and to a lesser extent. Detailed scrub typhus reporting data from 2003-2018 consisted of the number of cases per province per month, population, annual incidence rate per 100,000 population (AIR), age groups, sex and occupation. As a single province case study, we obtained further data for Chiangrai Province from 2003-2018, the province with the highest number of reported cases during this period. Included were patient demographics, location and type of healthcare facilities, and residential address to the sub-district and village level. Boundaries of the administrative divisions (districts and sub-districts) and populations at these levels for Chiangrai province were obtained from the Ministry of Natural Resources and Environment, Thailand (2012).

A central representative weather station in Chiangrai province was identified and average monthly temperature (°C) and total monthly rainfall (mm) data were collected from the Thai Meteorological Department, Ministry of Information and Communication Technology. Satellite imaging data at a resolution of 1km was obtained from the European Space Agency (GlobCover 2009, http://due.esrin.esa.int/page_globcover.php) including land use/land cover data.

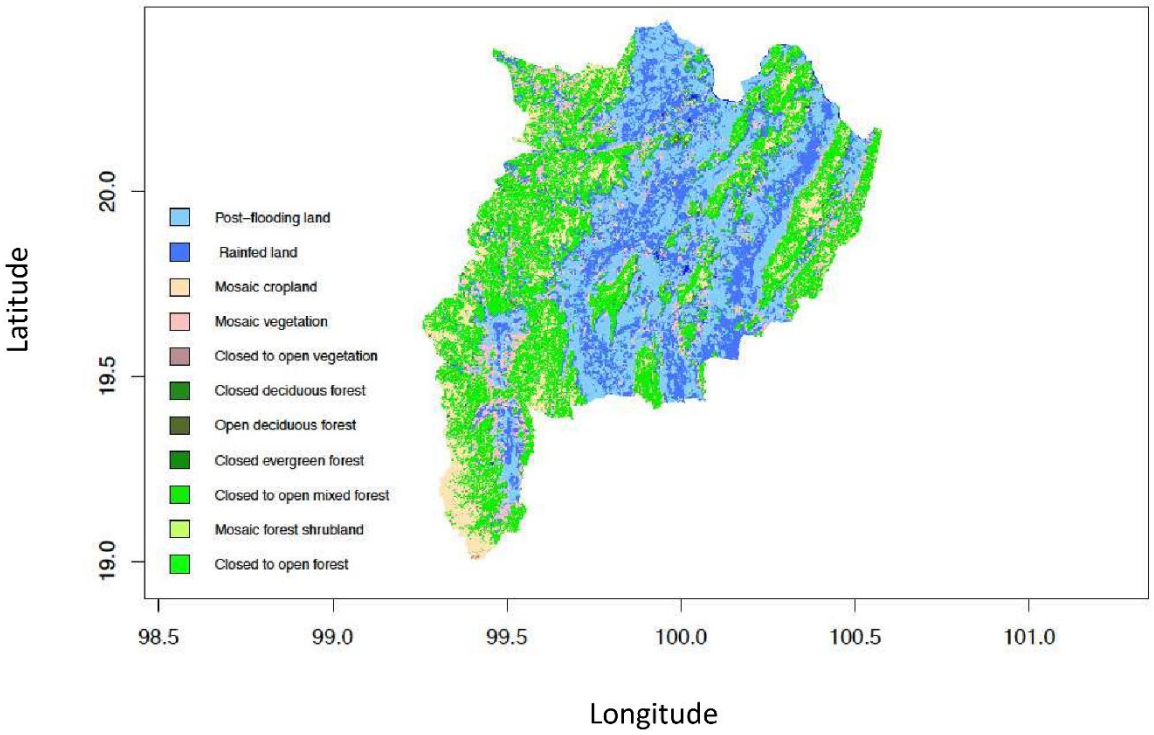
Classification of land use/land cover data is shown in Figure 21. The classification categories are defined below:

- Post-flooding land – include irrigated or aquatic croplands e.g. rice paddies, floodplains of streams or rivers.
- Rainfed land – areas of cropland reliant on rain.
- Mosaic cropland – 50-70% cropland and 20-50% vegetation (grassland/shrubland/forest).

- Mosaic vegetation – 50-70% vegetation (grassland/shrubland/forest) and 20-50% cropland.
- Closed to open vegetation – >15% grassland or woody vegetation on regularly flooded or waterlogged soil - fresh, brackish or saline water.
- Closed deciduous forest – >40% broadleaved deciduous forest (>5m in height).
- Open deciduous forest – 15-40% broadleaved deciduous forest or woodland >5m in height.
- Closed evergreen forest – >40% needleleaved evergreen forest (>5m in height).
- Closed to open mixed forest – >15% mixed broadleaved and needleleaved forest (>5m in height).
- Mosaic forest or shrubland – 50-70% forest and 20-50% grassland/shrubland.
- Closed to open forest – >15% broadleaved or needleleaved forest (>5m in height).

Data were obtained at different administrative levels for Chiangrai province. Scrub typhus incidence was at the village level, population at the sub-district level, rainfall at the provincial level and land use/land cover at 1km resolution. Data subsets were integrated at the sub-district level, aggregating human cases obtained at village level and cropping raster data (land use) using sub-district boundaries.

Figure 21. Classification of land use/land cover of GlobCover 2009 with a resolution of 1 km for Chiang Rai Province. [taken from http://due.esrin.esa.int/page_globcover.php]



3.2.2 Statistical analyses

Proportions, percentages and averages (median and interquartile range [IQR] or mean and standard deviation [SD] as appropriate) were calculated controlling for any missing data. Seasonality was assessed by calculating proportions of cases (and 95% confidence intervals) reported during discrete time-periods and assessing for overlap as well as performing two-sample test of proportions. Descriptive analyses and assessment of seasonality were performed using STATA 15 software (College Station, Texas, USA). Spatio-temporal analyses were performed by Prof Serge Morand using R software (R Core Team, 2018) [259].

The following spatio-temporal analyses were performed on the dataset obtained for Chiangrai province from 2003-2018:

1. Spatial autocorrelation of scrub typhus cases was investigated using correlogram analysis and Moran's I test within the R packages spatialEco and spData [260, 261].
2. Spatial interpolation of scrub typhus cases over time was performed using semivariogram and Gaussian process regression or kriging. Kriging is a geostatistical interpolation method that uses a fixed number of nearest neighbour points within a fixed radius and semivariograms that quantify spatial autocorrelation among all pairs of data according to distance [262, 263]. Semivariograms were estimated by calculating the squared difference of scrub typhus cases between all pairs of sub-district centroid. Semi-variance values were grouped and averaged according to separation distance (lags). Semi-variogram model and kriging were computed using the R packages FRK and INLA [264, 265].
3. Temporal incidence and autocorrelation of scrub typhus cases were analysed using time-series analysis. Temporal trends in the number of reported scrub typhus cases were assessed using exponential smoothing model and the ncf R package [266]. The

plot of the residual autocorrelation function (ACF) was examined to determine the general form of the model to be fitted. After analysing the ACF graph, different Autoregressive Integrated Moving Average (ARIMA) models were identified for selection. The series were then decomposed with a moving average taking into account a period of one year. Temporal variations in rainfall and temperature were analysed in the same way.

Additionally, wavelet analysis was performed to analyse the variations in scrub typhus cases, rainfall and temperature over time. This method transforms function to decompose a time-series and can reveal periodic signals at each time-point in a series. The coefficients obtained reveal magnitudes of correlation of scrub typhus cases/rainfall/temperature for each year and period length and subsequently displayed using a power spectrum over the full study period. The R packages biwavelet and WaveletComp were used [267, 268].

4. Cross temporal correlation analysis between scrub typhus cases, rainfall and temperature was performed. Initially, results from the time-series analysis were used to study the patterns of scrub typhus cases from 2003-2018. Using ACF, the correlation lag and the correlation values at the best lag period (months) for rainfall, temperature and scrub typhus cases were calculated. The core function ccf was used to compute the cross-correlation or cross-covariance between rainfall and scrub typhus cases and temperature and scrub typhus cases.
5. Global land use/land cover information for each sub-district was extracted using the R packages raster, rgdal, dismo and rgeos [269-272]. The size of each land use/land cover class was extracted and the following classification variables (as above) were computed: forest cover (%): as the sum of closed broadleaved deciduous forest, and closed needle-leaved evergreen forest, divided by the total area of the sub-district;

forest open cover (%): as the sum of closed to open broadleaved evergreen or semi-deciduous forest, and closed to open mixed broadleaved and needle-leaved forest, and open broadleaved deciduous forest, divided by the total area of the sub-district;

grassland open cover (%): as the sum of closed to open herbaceous vegetation, and closed to open grassland, divided by the total area of the sub-district;

mosaic habitat cover (%): as the sum of mosaic cropland, mosaic grassland, and mosaic vegetation, divided by the total area of the sub-district;

rain-fed cropland cover (%): as rain-fed croplands divided by the total area of the sub-district;

flooded-irrigated land cover (%): as post-flooding or irrigated croplands divided by the total area of the sub-district.

Complexity and fragmentation of the land use/land cover for each sub-district were estimated by calculating two metrics determined using the PatchStat function of the R package SDMTools [273]:

habitat complexity: which measures complexity of the land use and computed as the sum of each patch perimeter divided by the square root of patch area;

habitat fragmentation: which reflects shape complexity across a range of spatial scales and computes as the logarithm of patch perimeter (m) divided by the logarithm of patch area.

6. The relationship between scrub typhus cases and agro-environmental variables were investigated using general linear modelling (GLM). The distribution of scrub typhus cases per sub-district per month was analysed using the R package fitdistrplus [274]. General linear modelling (GLM) was then performed to determine potential explanatory factors for scrub typhus cases using a negative binomial link function. These factors included land use characteristics (habitat complexity, habitat fragmentation), forest cover (%), forest open cover (%), grassland open cover (%), mosaic habitat cover (%), rain-fed

land cover (%), flooded-irrigated land cover (%), population size, rainfall and temperature. The best model was selected using the R package MuMIn and Akaike Information Criteria (AIC) selection procedure for model strength [275]. The Akaike weight (wr) was calculated to determine the probability that a given model is the best approximating model (wr is a value between 0 and 1 with the sum of all models in the candidate set being 1) [276]. Potential collinearity was assessed using Variance Inflating Factor (VIF) with the R package car [277]. Odds ratios were calculated using the package oddsratio in R [278]. Performance of models were assessed by estimating the percentage of deviance explained using maximum likelihood R^2.

7. General additive modelling (GAM) was then performed as an extension of the GLMs with the adaptability for non-parametric variables. The assumption is made that scrub typhus cases per sub-district per month is dependent on the univariate smooth-terms of independent variables [279]. Model fitting was analysed using the MGCV package in R [280]. The function gam.check was used to choose the basis dimension for each predictor variable according to estimated degrees of freedom in the main effect. GAM model outputs/graphs were obtained via the gratia R package [281].

3.3 Results

3.3.1 National burden of scrub typhus in Thailand, 2003-2018

From 2003 to 2018, there were a total of 103,345 scrub typhus patients reported to the national disease surveillance system (R506). Cases per year ranged from a low of 2,928 in 2005 to a high of 10,952 in 2013. Similarly, the annual incidence rate/100,000 population (AIR) ranged from 4.71 in 2005 to 17.09 in 2013. Median male to female ratio (IQR) was 1:0.74 (0.71-0.76) and the main occupational groups affected (median, IQR) were agricultural workers (44.80% [42.65-45.88]), labourers (17.95% [15.77-19.22) and students (14.83% [13.89-16.04]). Summarised annual reports for the preceding years from 1980 to 2002 revealed that there were 27,043 scrub typhus cases reported (data from 2002 missing) with cases per year ranging from 17 in 1980 to 5,094 in 2001. The AIR rose from 0.53 in 1985 to 8.2 in 2001 (AIR data pre-1985 unavailable). Despite the inconsistent reporting of sex and occupational groups from 1980-2002, there were more cases reported in men (median [IQR] male to female ratio was 1:0.67 [0.56-0.76]) and burden was highest in agricultural workers (median percentage [IQR] 51.35% [49.01-55.72]). The trend in total annual reported cases and AIR are depicted in Figure 22 A and B, respectively.

Age group data for reported cases were available from 2004-2018 (Figure 23). The majority of cases (72,144/99,543, 72.48%) were in adults of working age – 15-64 years old. The proportion of scrub typhus cases in children (<15 years old) has been falling from 825/3,290 (25.08%) of cases in 2004 to 1,396/9,756 (14.31%) of cases in 2018. On closer inspection, the actual number of children with scrub typhus has remained relatively stable, particularly in the last decade. What has contributed to the reduction in the proportion of cases in children has been the disproportionate growth in the number of cases seen in adults (Figure 24).

When cases per month are plotted, it is apparent that disease seasonality occurs (Figure 25). Scrub typhus burden (mean, SD) was highest in the following months: October 809 (361) cases, July 772 (315) cases and September 757 (342) cases. The proportion of cases (95% CI) from June to November and December to May were: 0.678 (0.675-0.681) and 0.322 (0.319-0.325), $p<0.001$. The period from June to November corresponds to the rainy and early cool/dry seasons in Thailand while December to May covers the cool/dry and hot seasons.

Figure 22. A – Annual number of reported scrub typhus cases in Thailand from 1980-2018. B – Annual incidence rate per 100,000 population from 1985-2018. [NB – number of cases and AIR for 2002 missing]

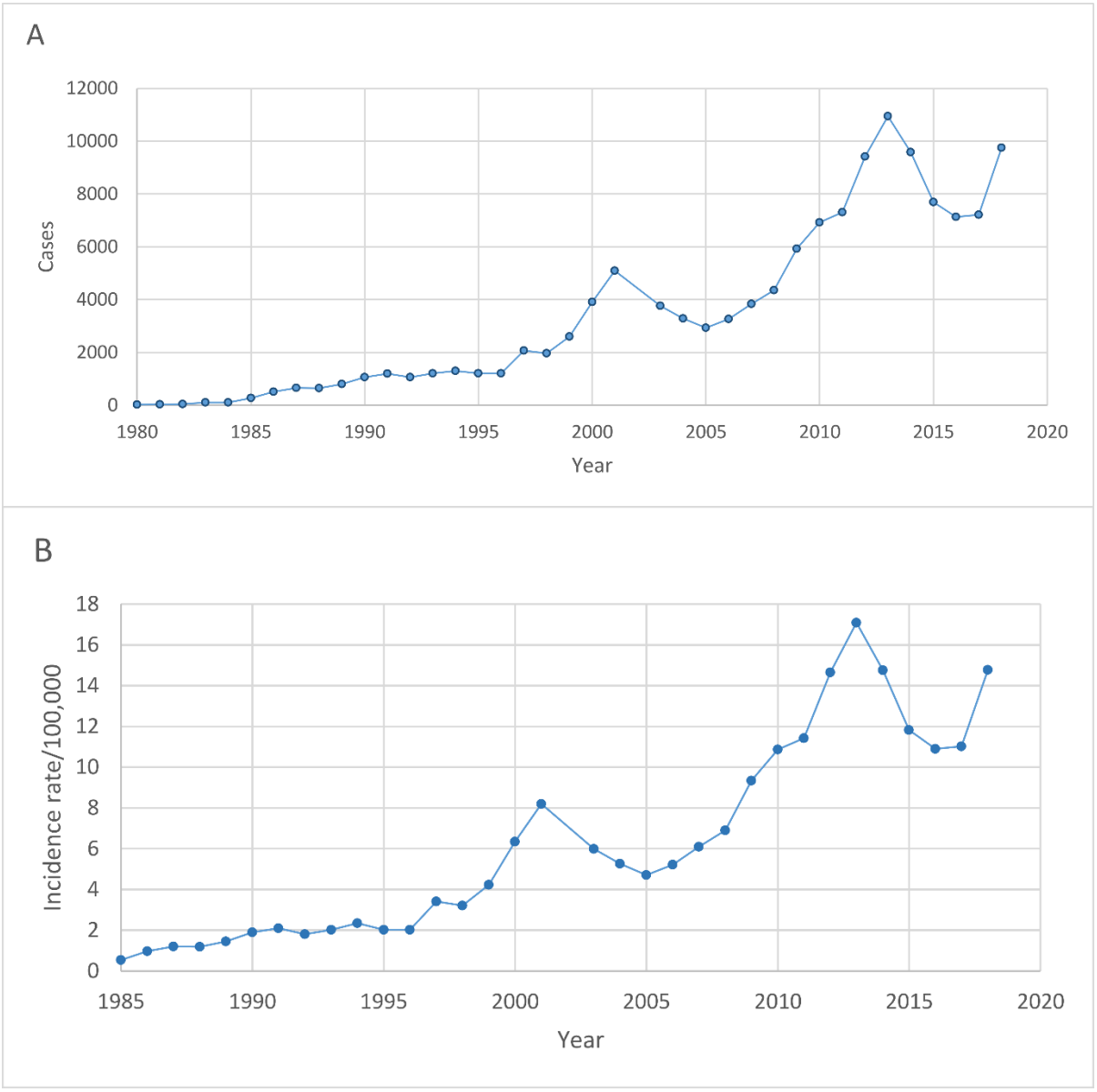


Figure 23. Scrub typhus cases by age group 2004-2018, Thailand.

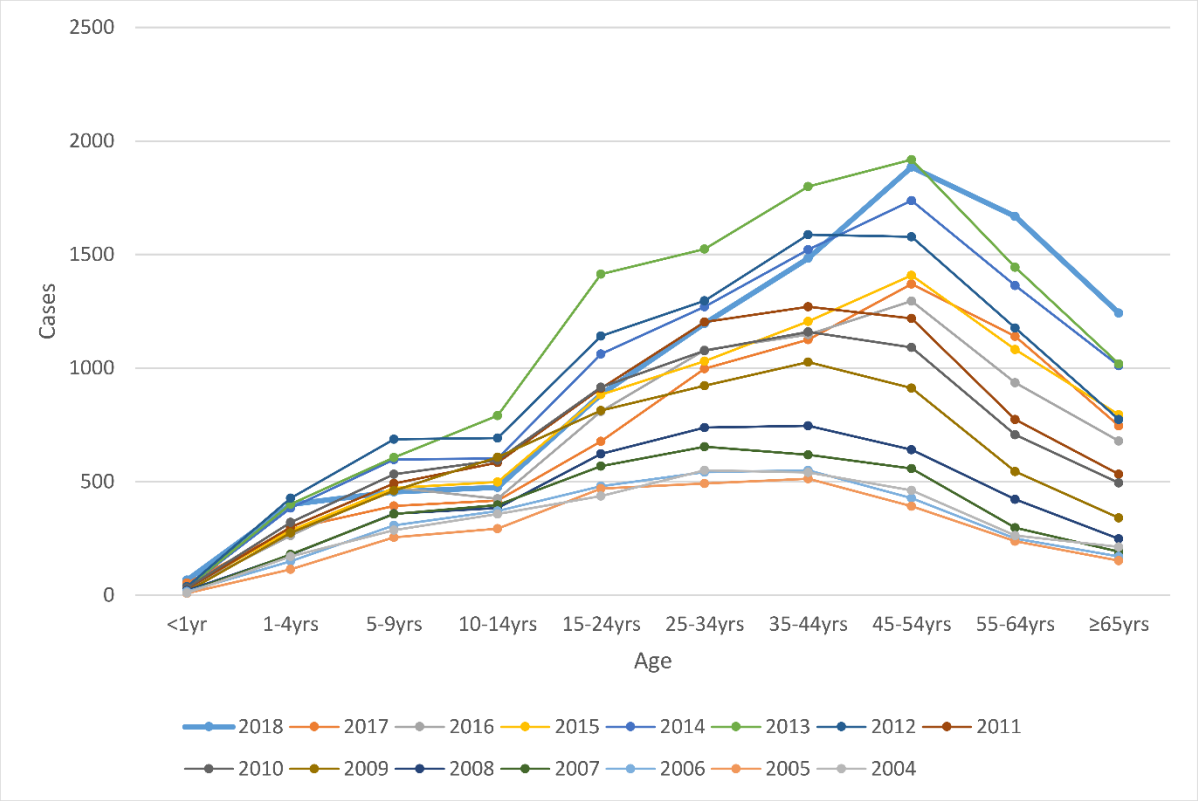


Figure 24. Proportion of reported scrub typhus cases in children <15 years old and adults from 2004-2018 for Thailand.

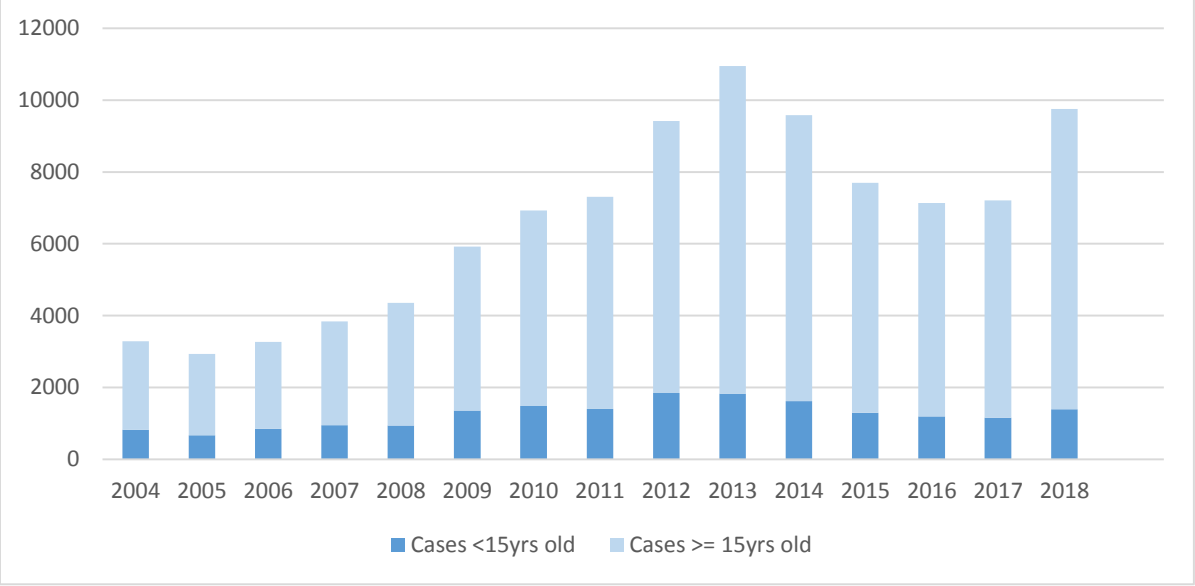
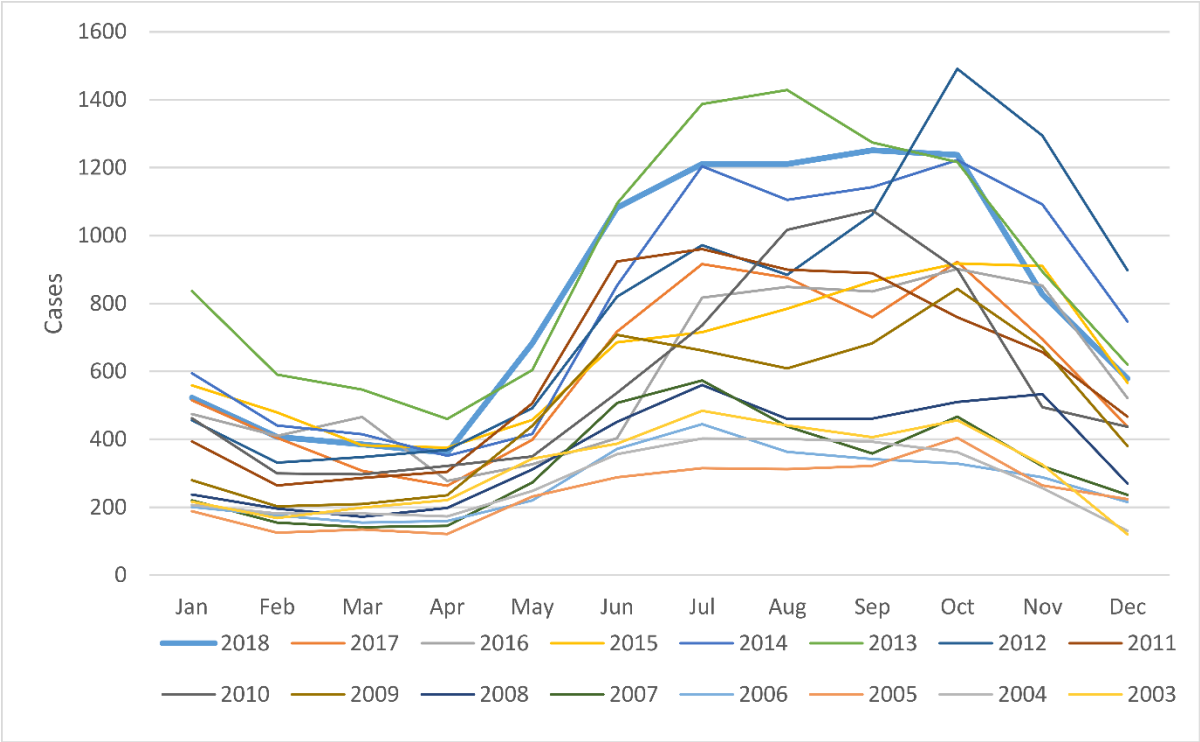


Figure 25. Scrub typhus cases per month from 2003-2018, Thailand.



3.3.2 Regional and provincial burden of scrub typhus in Thailand, 2003-2018

Thailand is divided into 4 administrative regions: North, Northeast, Central and South (Figure 26). Disease burden was greatest in the northern region, followed by the northeastern, southern and central regions as reflected in the percentage of cases per year (mean, SD) and AIR (mean, SD): North 52.55% (7.49%), 29.40 (13.39); Northeast 30.92% (6.97%), 9.01 (3.94); South 12.40% (2.54%), 8.94 (4.04); and Central 4.12% (2.49%), 0.99 (0.30). The central and northeastern regions are the most populated with the northern region contributing only 18.60% (SD 0.3%) of the total national population during the study period.

Occupational data at the regional and provincial levels were available from 2006-2018. In the North, Northeast and South, agricultural workers made up the largest percentage of cases, followed by labourers and students (median and IQR):

- North – 40.35% (39.72-42.29), 19.36% (18.79-20.94) and 16.45% (15.10-19.10), respectively.
- Northeast – 57.78% (52.82-60.93), 11.31% (10.36-12.57) and 10.47% (9.56-13.79), respectively.
- South – 33.33% (31.67-33.70), 22.72% (20.74-25.37) and 15.71% (13.68-17.65), respectively.

In central Thailand, disease burden was highest in labourers, followed by students and agricultural workers [40.59% (38.06-42.51), 18.54% (16.48-21.36) and 12.57% (10.71-13.95), respectively].

Regional differences in seasonality of reported cases are depicted in Figure 27. From 2003-2018, the average monthly reported cases were at their lowest between February and April in all regions. There were significant differences observed between the proportions of cases (95% CI) from June to November and from December to May in the four regions:

- North – 0.687 (0.683-0.691):0.313 (0.309-0.317), p<0.001.
- Northeast – 0.726 (0.721-0.731):0.274 (0.269-0.279), p<0.001.
- Central – 0.618 (0.601-0.634):0.382 (0.366-0.399), p<0.001.
- South – 0.538 (0.530-0.547):0.462 (0.453-0.470), p<0.001.

However, seasonality of scrub typhus cases was less marked in the southern region than other regions at higher latitudes. There is approximately a 15° difference in latitude between southern and northern Thailand. It is important to note that the equatorial climate in the southern region is different to the more temperate climate of the northern region – the South generally has wet and dry seasons which is influenced by the monsoon seasons in the Gulf of Thailand (east coast) and Andaman Sea (west coast) whereas the northern provinces tend to experience rainy, cool and hot seasons.

The burden of disease at the provincial level was unsurprisingly dominated by northern provinces although cases were reported from all provinces during the study period (Figure 28 and 29). The five provinces with the highest average number of cases per year (mean, SD) were Chiangrai – 716 (378), Chiangmai – 684 (435), Tak – 609 (361), Nan – 488 (292) and Mae Hong Son – 437 (221). Together, they accounted for 46,927 cases from 2003-2018, representing 46,927/55,872 (83.99%) of cases from the northern region, or 46,927/103,345 (45.41%) of cases nationally. Disease incidence (AIR – mean, SD) were highest in Mae Hong Son – 173.14 (85.62), Tak – 111.40 (64.54), Nan – 102.07 (61.20), Chiangrai – 58.61 (30.80) and Phang Nga - 47.98 (27.85) provinces.

Figure 26. Administrative regions of Thailand [created using mapchart.net under a CC BY license, with permission from Minas Giannekas original copyright 2019].

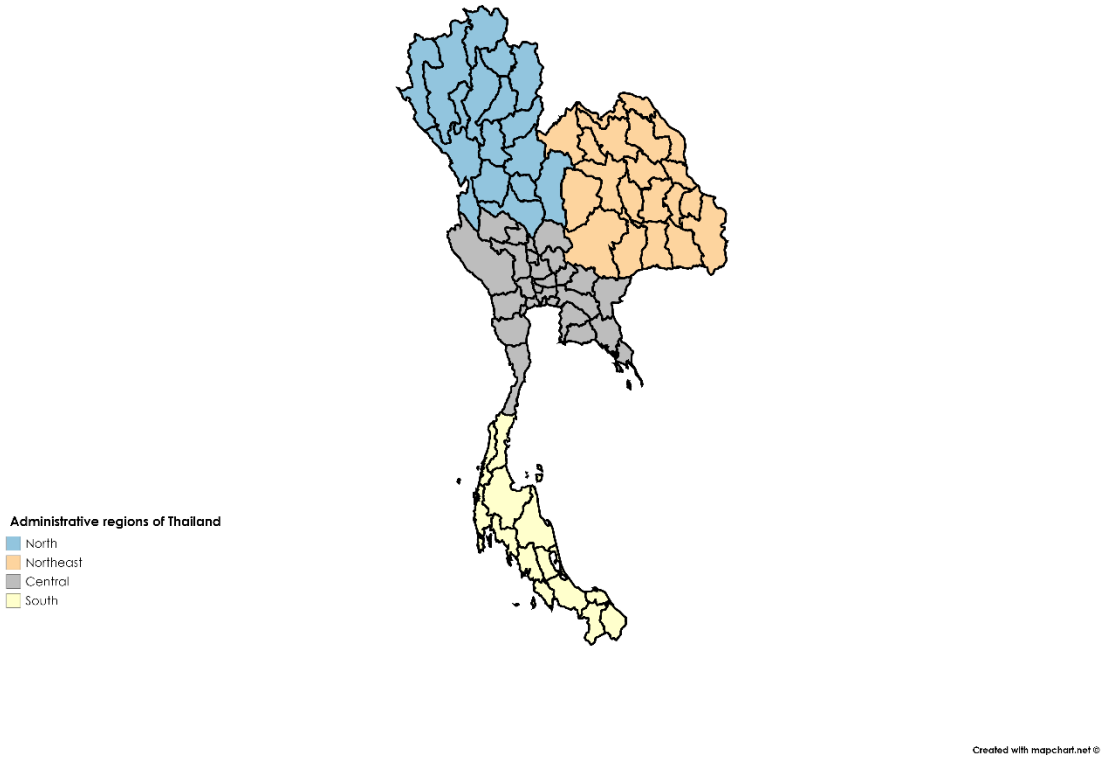


Figure 27. Seasonality of scrub typhus cases per region from 2003-2018, Thailand.

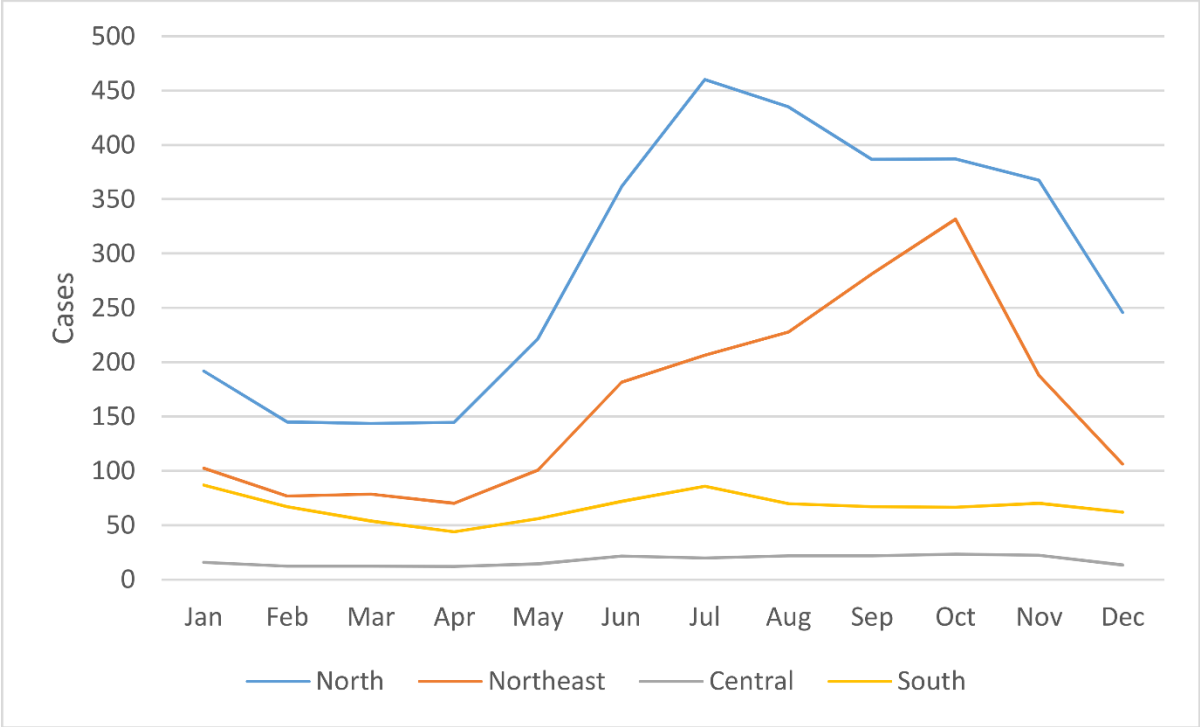


Figure 28. Map of Thailand depicting the average number of scrub typhus cases per province per year from 2003-2018. High burden provinces (top 8) labelled.

[CR = Chiang Rai, CM = Chiang Mai, T = Tak, N = Nan, MHS = Mae Hong Son, SSK = Si Sa Kaet, NR = Nakorn Ratchasima, PN = Phang Nga; created using mapchart.net under a CC BY license, with permission from Minas Giannekas original copyright 2019]

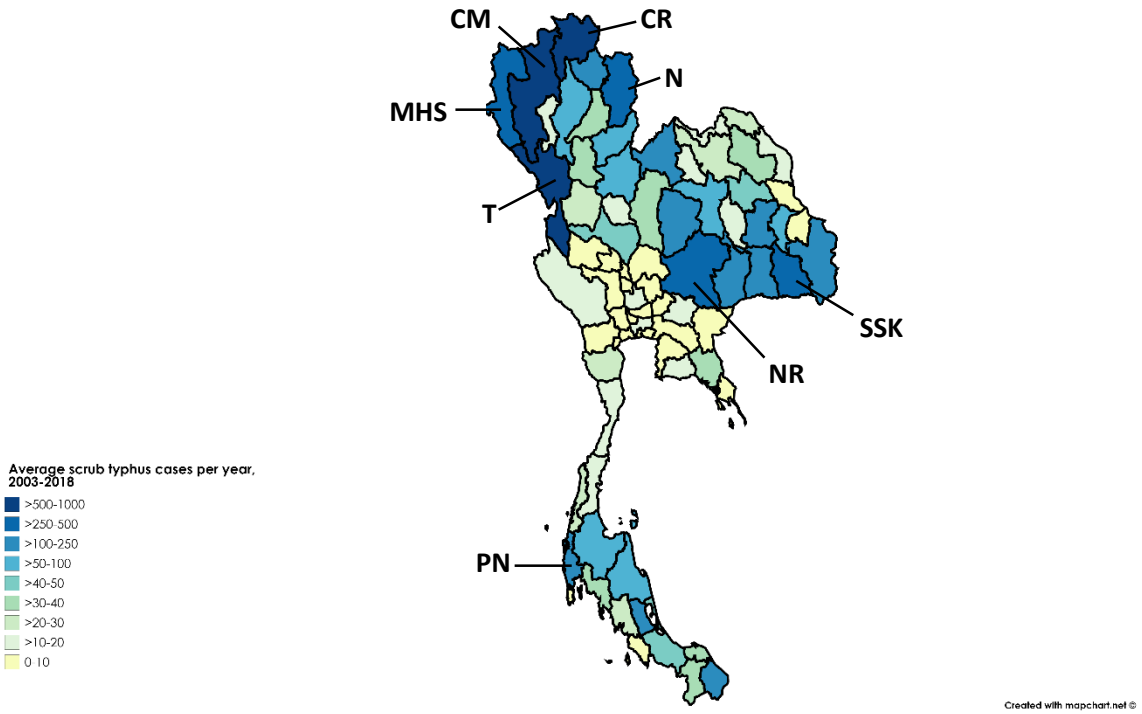
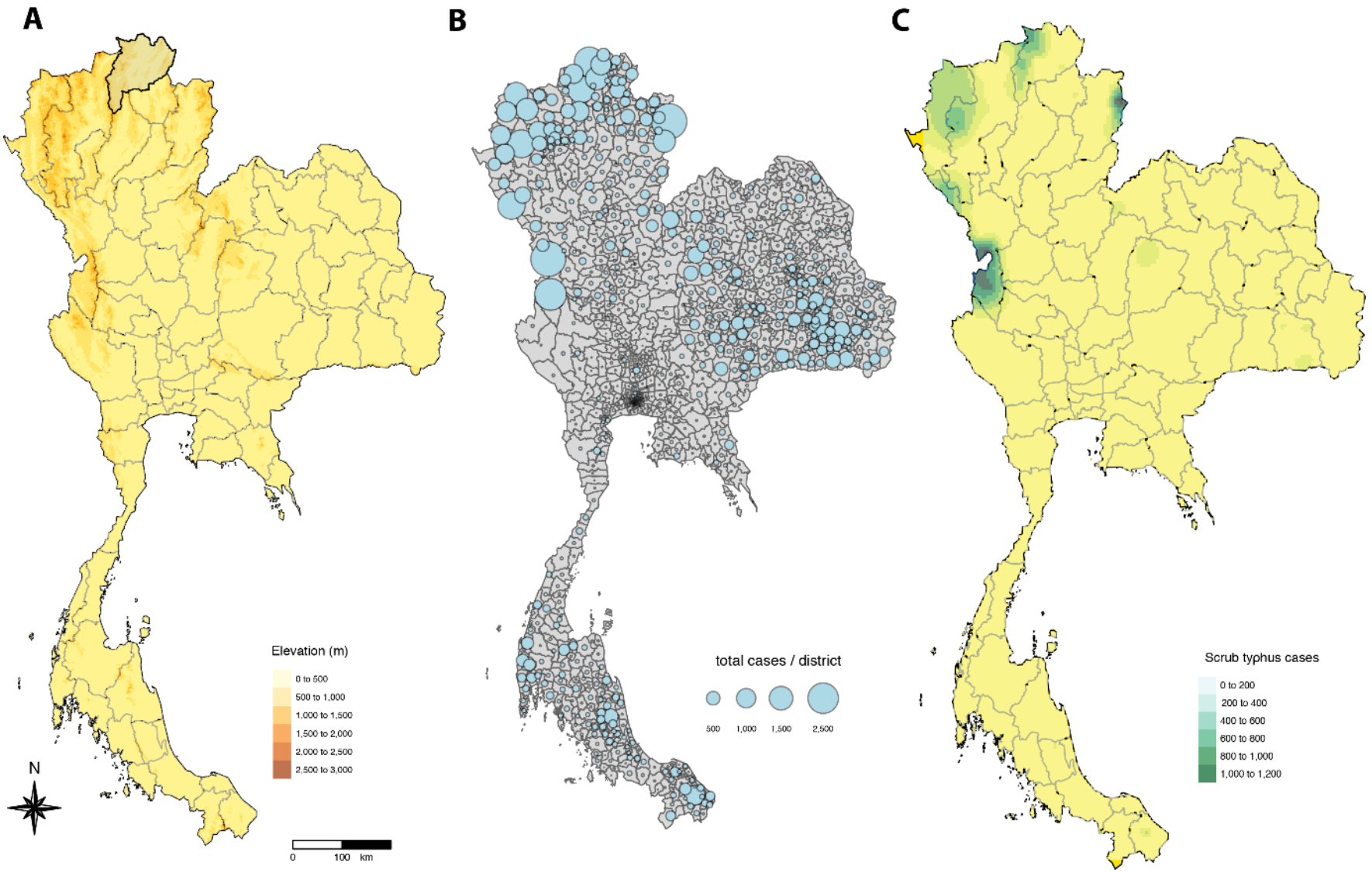


Figure 29. Map of Thailand depicting: (A) the geography with elevation, provincial boundaries and location of Chiangrai province; (B) total scrub typhus cases from 2003-2018 per district; and (C) interpolation of scrub typhus cases at sub-district level by kriging using semi-variogram based on the centroids of geographical coordinates of each sub-district (for clarity, only provincial boundaries are shown). [created using R software by Prof Serge Morand]


A
Elevation (m)
0 to 500
500 to 1,000
1,000 to 1,500
1,500 to 2,000
2,000 to 2,500
2,500 to 3,000
N
0
100 km
B
total cases / district
500
1,000
1,500
2,500
C
Scrub typhus cases
0 to 200
200 to 400
400 to 600
600 to 800
800 to 1,000
1,000 to 1,200

3.3.3 Single province case study – Chiangrai province

Chiangrai province had the highest average number of scrub typhus per year as well as the fourth highest AIR. It is the northernmost province in Thailand and is divided further into 18 districts, 124 sub-districts and 1,816 villages. From 2003-2018, there were 11,444 scrub typhus cases reported with a male to female ratio of 1:0.81. Adults of working age (15 to 64 years old) contributed 7,731/11,444 (67.56%) of the total reported cases from 2003-2018 while the proportion of paediatric cases has generally fallen over the study period (Figure 30). Disease burden (mean monthly cases and SD, 2003-2018) was highest in July, August and June at 110 (73), 95 (49), and 90 (55) cases, respectively. There was a significant difference between the proportion of cases (95% CI) from June to November and December to January: 0.716 (0.708-0.7205) and 0.284 (0.275-0.292), $p<0.001$, respectively.

Of the 11,444 scrub typhus patients reported from 2003-2018, agricultural workers, labourers and students were the main occupational groups affected at 35.63%, 22.76% and 20.06%, respectively. 75.26% of cases were reported from district hospitals, 22.51% from the main provincial hospital and 1.69% from private healthcare facilities. 66.89% of cases presented as outpatients.

The scrub typhus burden for the top five districts from 2003-2018 along with the highest burden sub-districts per district and top five villages per sub-district are depicted in Table 2 (cases) and Table 3 (AIR). In terms of total number of cases, Mae Fah Luang, Mae Suai, Mueang, Mae Chan and Wiang Pa Pao districts had the highest burden. All 5 districts are located in the western part of the province. Districts with the highest scrub typhus burden by incidence rate (AIR) were similar with the exception of Wiang Kaen District (eastern part of the province) which replaced Mueang district, the central and most populated district in the province. The western and eastern parts of Chiangrai contain more mountainous, rural and less developed areas than the rest of the province.

Table 2. Chiangrai Province, top five districts 2003-2018 – Number of reported scrub typhus cases broken down by highest burden sub-districts and top 5 burden villages. [where number of cases per village are identical, village names separated by "/"; where village names are the same, village code added; where a group of villages are under one administrative code, "," used to separate individual villages in the group]

District	Cases	Sub-district	Cases	Village	Cases
Mae Fah Luang (4 sub-districts, 77 villages)	2,413	Mae Salong Nai	1,119	Hin Taek	129
				Huay Yuak Pa So	108
				Hua Mae Kham	101
				Huay Phung	98
				Pong Hai	93
		Thoet Thai	722	Thoet Thai	114
				Huay Uen	113
				Ah Goo Ah Hai	69
				Mae Mor	58
				Phaya Phrai Loa Ma	45
		Mae Salong Nok	314	Santikhiri	96
				Jiang Ja Sai	33
				Pah Kha Sook Jai	24
				Ah Bae	21
				Tong Ja Sai	20
		Mae Fah Luang	258	Samakkhi Kao	39
				Ja Lor	27
				Li Che	25
				Ee Kor Pah Gluay	22
				Pa Sang Nah Ngoen/Musur La Ba	15
Mae Suai (7 sub-districts, 128 villages)	2,014	Wawi	671	Doi Chang	116
				Pong Klang Nam	70
				Saen Charoen	49
				Doi Lan	41

				Huay Nam Yen/Wawi	39
		Tha Ko	502	Huay Nam Kun	62
				Ba La	59
				Pa Gea	52
				Pana Seri	41
				Doi Ngam	38
		Pa Daet	308	Ja Ha	65
				Huay Salak	46
				Huay Makaeng	37
				Huay Ya Sai	36
				Sri Don Moon	24
		Si Thoi	217	Ayi Koh	43
				Phaya Gong Di	39
				Mae Yang Min	36
				Mai Mae Yang Min	34
				Huay Hear	21
		Mae Suai	124	Nong Pha	22
				Mae Suai	14
				Pa Bong	13
				Pong Pa Kham	10
				Pong Fu Fueang	9
Mueang (16 sub-districts, 291 villages)	1,405	Huay Chompu	356	Mae Mon	62
				Huay Ma Liam	56
				Pang Khon	51
				Huay Sarn	45
				Huay Kaew	37
		Mae Yao	341	Huay Mae Sai	74
				Panasawan	56
				Huay Khom	47
				Ruam Mitr	34
				Huay Khom Nai	32
		Doi Hang	101	Pa Surd Pattana	30
				Yang Kham Noo	21

				Pong Nam Ron	20
				Huay Poo Pattana	12
				Rong Or	8
		Rop Wiang	96	Doi Khao Kwai	10
				San Khong Noi	9
				Koh Thong	8
				Nong Hiang	7
				Roi Phraputtabaht	7
		Rim Kok	65	Pa Yang Luang	25
				Rim Ngam	12
				Sampantakit Farm (01)	7
				Mueang Ngim	6
				Sampantakit Farm (06)	5
Mae Chan (11 sub-districts, 139 villages)	1,128	Pa Tueng	473	Santisuk	116
				Huay Gang Pla	69
				Lao Fu	57
				Huay Tang	53
				Pang Sa	35
		Mae Chan	139	Jor Pa Ka	21
				Sala	18
				Den Pa Sak	17
				San Ton Haen, Pong Tong, Suan Sak	16
				Thammajarik	15
		Pa Sang	123	Mae Salong Nai	20
				Pa Du	18
				Pang Pu Loei	15
				Rong Ki/Mae Ki Luang	8
				San Khue/Mae Salong	8
		Mae Rai	100	Huay Rai	57
				Dong Ma Tuen	13
				San Gong	6
				Pa Kwao	5

				San Gong Mai	5
		Tha Khao Plueak	65	Pa Taek	11
				Pa Rai	10
				Mae Paeng (04)	8
				Tha Khao Plueak	7
				Mae Lak	5
Wiang Pa Pao (7 sub-districts, 93 villages)	1,081	Wiang	329	Mae Poon Lang	98
				Mae Poon Luang	79
				Dong Lai Na	39
				Hua Wiang	34
				Pong Nong	19
		San Sali	185	Pong Nok	49
				Mae Poon Noi	30
				Mae Tala	24
				Pong Nuea	21
				Loh	16
		Mae Chedi Mai	150	Mueang Noi	34
				Huay Muang	26
				Jum Bon	19
				Buak Khon	11
				Rong	10
		Mae Chedi	126	Pang Ma Gat	22
				Mae Ka Jarn	14
				Huay Nam Gin	14
				Pa Ngae	12
				Tung Yao	12
		Pa Ngio	126	Nong Kiew	35
				Mae Hang	12
				Khun Mueang Ngam	11
				Huay Ma Kliang	10
				Hang Tam	9

Table 3. Chiangrai Province, top five districts 2003-2018 – Average annual scrub typhus incidence/100,000 population (AIR) by highest incidence sub-districts and top 5 incidence villages.

District	AIR	Sub-district	AIR	Village	AIR
Mae Fah Luang (4 sub-districts, 77 villages)	212.84	Mae Salong Nai	279.55	Ma Hin Gong	710.56
				Huay Yuak Pa So	643.47
				Ah Ham	584.28
				Pa Noi Akha	577.96
				Huay Yo	568.18
		Thoet Thai	208.98	Ah Goo Ah Hai	791.28
				Tu Mor Ahney	410.58
				Huay Mor	369.62
				Saen Mueang Go	356.88
				Thoet Thai (13)	352.82
		Mae Fah Luang	177.14	Li Che	688.33
				Pa Sang Nah Ngoen	571.65
				Ja Lor	452.41
				Musur La Ba	347.22
				Pa Kha	336.02
		Mae Salong Nok	129.59	Ah Lae	678.29
				Ah Bare	602.06
				Pa Kha Samakkhi	406.50
				Lao Sip	402.23
				Pa Kah Sook Jai	247.12
Mae Suai (7 sub-districts, 128 villages)	131.45	Si Thoi	215.18	Mae Yang Min	827.21
				Ayi Koh	289.60
				Phaya Gong Di	253.38
				Mai Mae Yang Min	224.16

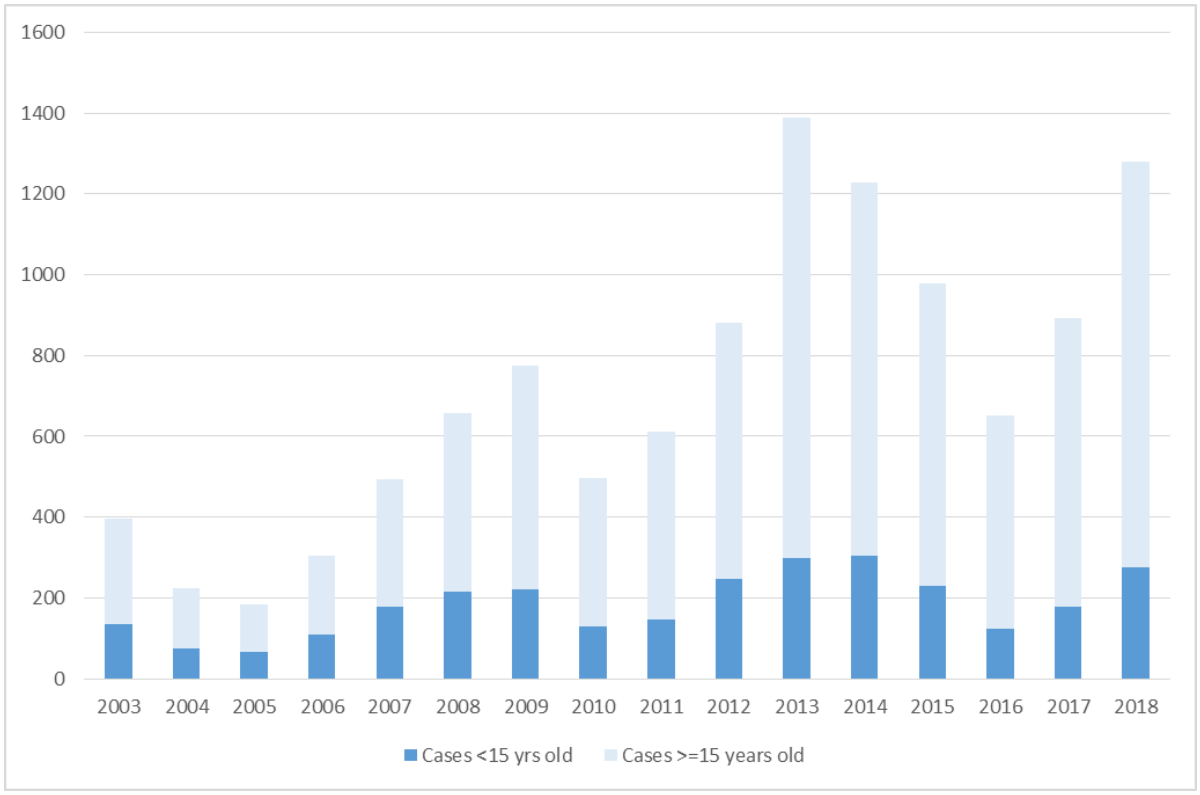
				Huay Hear	193.30
		Pa Daet	147.62	Huay Ya Sai	346.15
				Ja Ha	246.96
				Huay Salak	247.84
				Huay Makaeng	240.89
				Thung Ruang Tong	238.64
		Tha Ko	147.45	Mae Tum Noi Lang	465.43
				Pang Nam Hai	287.72
				Ba La	277.67
				Pana Seri	251.47
				Pa Gea	246.03
		Wawi	119.38	Huay Poo	694.44
				Huay Nam Yen	526.46
				Pang Klang	308.30
				Mai Pattana	287.58
				Doi Lan	281.28
		Chedi Luang	104.79	Mae Ta Maew	278.66
				San That	221.24
				Huay Som	140.90
				Nong Lom	129.96
				Rong Bong	102.46
Wiang Pa Pao (7 sub-districts, 93 villages)	96.25	Mae Chedi Mai	129.97	Huay Khun Phra	373.13
				Mueang Noi	237.17
				Huay Muang	221.99
				Huay Pong	159.35
				Jum Bon	153.23
		Ban Pong	113.43	Pong Thevee	227.15
				Huay Hin Lad	164.47
				Pong	127.84

				San	61.12
				San Tor	51.76
		Wiang	113.35	Dong Lai Na	199.96
				Mae Poon Luang	172.40
				Mae Poon Lang	141.19
				Hua Wiang	113.15
				Pong Nong	108.85
		San Sali	102.49	Mae Poon Noi	322.72
				Pong Nok	224.85
				Mae Ta La	136.49
				Pang Makham Pom	87.05
				Pong Nok Nuea	71.08
		Pa Ngio	87.66	Mae Hang	535.71
				Huay Ma Kliang	525.21
				Nong Kiew	339.67
				Hang Tam	82.60
				Khun Mueang Ngam	80.79
Wiang Kaen (4 sub-districts, 41 villages)	64.97	Tha Kham	117.07	Kwak Nuea	330.15
				Kwak Tai	140.13
				Huay Laeng	105.39
				Wang Pa	77.54
				Loh	69.21
		Po	59.53	Rom Po Ngoen	300.00
				Nong Tao	134.89
				Rom Fah Pa Mon	111.61
				Sai Thong	102.57
				Po Klang	84.98
		Mueang Yai	52.05	Lu	108.53
				Thai Charoen	102.65

				Thai Samakkhi	71.84
				Thai Pattana	64.43
				Huay Luek	49.37
		Lai Ngao	46.60	Thung Kham	65.33
				Jam Pong	52.30
				Lai Ngao	50.58
				Tha Kam	40.41
				Thung Sai	29.76
Mae Chan (11 sub-districts, 139 villages)	61.85	Pa Tueng	105.56	Huay Tang Santisuk	202.35
				Pa Miang	188.58
				Pang Sa	180.93
				Lao Fu	150.32
				Tung Tang	144.93
		Mae Chan	78.63	Jor Pa Ka	259.90
				Huay Jo	176.77
				Thammajarik	120.97
				Sala	115.38
				San Mongkol	85.15
		Pa Sang	60.39	Pa Du	238.35
				Mae Salong Nai	135.28
				Pang Pu Loei	113.64
				Rong Ki	112.61
				Pa Miang	82.42
		Mae Rai	70.97	Huay Rai	130.54
				Dong Ma Tuen	89.88
				San Gong Mai	71.67
				Hong Hae	65.27
				San Gong	35.61
		Si Kham	47.13	Nikom (San Suk)	193.52

				Mae Kham Pattana	58.41
				Mae Salong Nok	51.65
				San Sali Luang	48.89
				Mueang Klang	28.22

Figure 30. Proportion of reported scrub typhus cases in children <15 years old and adults from 2003-2018 for Chiangrai Province.



Spatial autocorrelation, semi-variogram and kriging interpolation of scrub typhus cases, Chiangrai, 2003-2018

Weak spatial autocorrelation was observed up to 30 km after which significance was lost (Figure 31). Figure 32 depicts the geography of Chiangrai province (A), scrub typhus cases per sub-district (B) and interpolation of scrub typhus cases at sub-district level based on centroids of the geographical coordinates of each sub-district (C). Spatial interpolation clearly shows more cases in the mountainous areas of the province.

Figure 31. Spatial autocorrelation of scrub typhus cases, Chiangrai Province, 2003-2018.

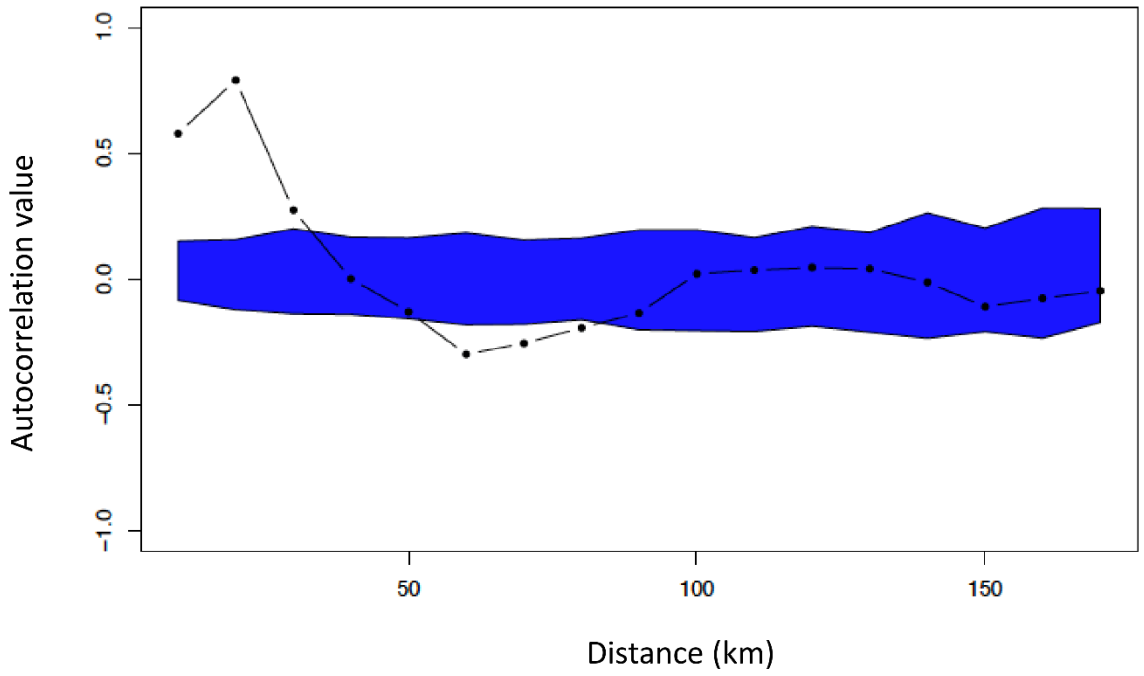
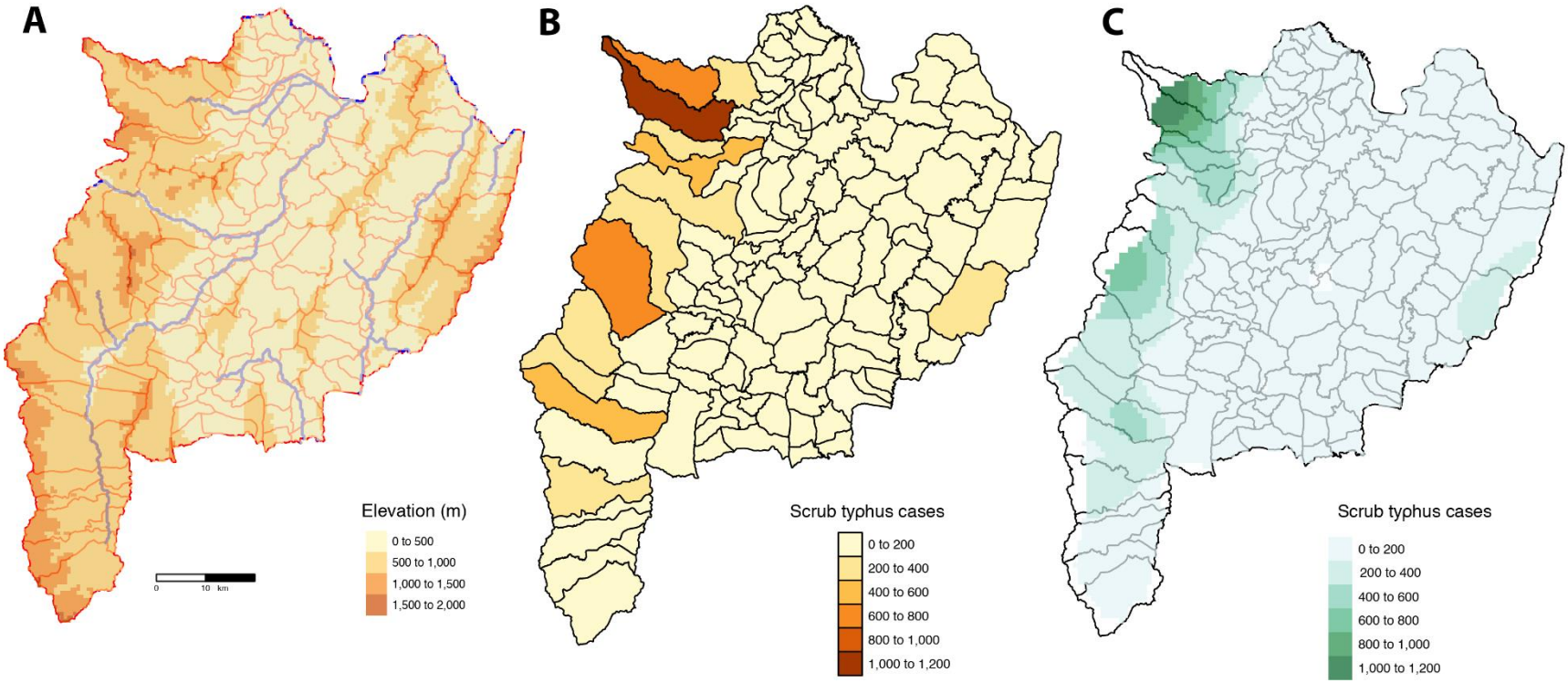


Figure 32. Chiangrai province with: (A) the geography with elevation, main rivers and sub-district boundaries; (B) scrub typhus cases from 2003-2018 per sub-district; and (C) interpolation of scrub typhus cases at sub-district level.

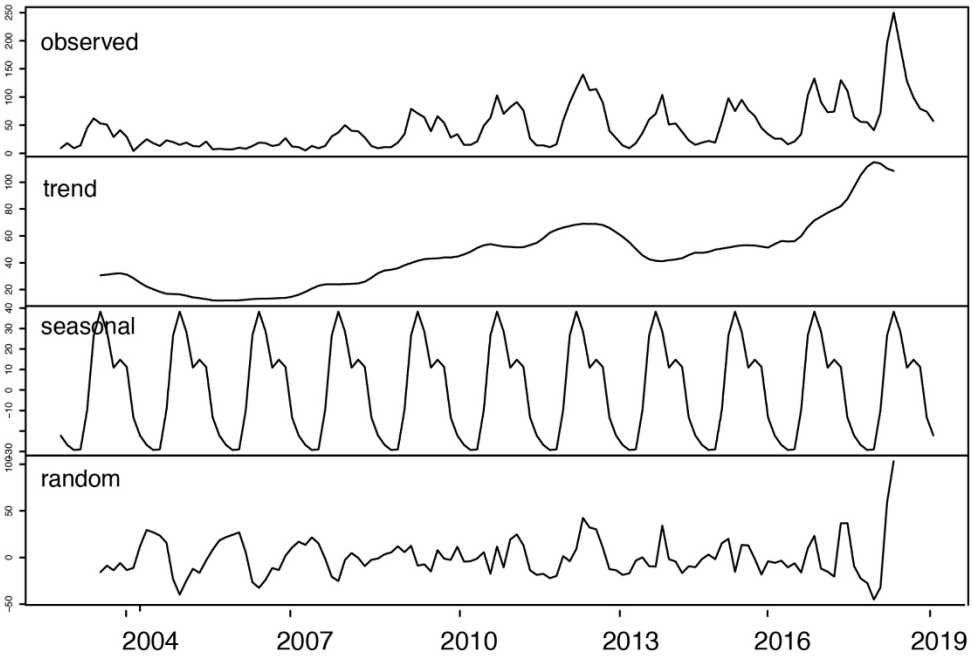

A
Elevation (m)
0 to 500
500 to 1,000
1,000 to 1,500
1,500 to 2,000
0
10 km
B
Scrub typhus cases
0 to 200
200 to 400
400 to 600
600 to 800
800 to 1,000
1,000 to 1,200
C
Scrub typhus cases
0 to 200
200 to 400
400 to 600
600 to 800
800 to 1,000
1,000 to 1,200

Temporal analysis of scrub typhus cases, rainfall and temperature

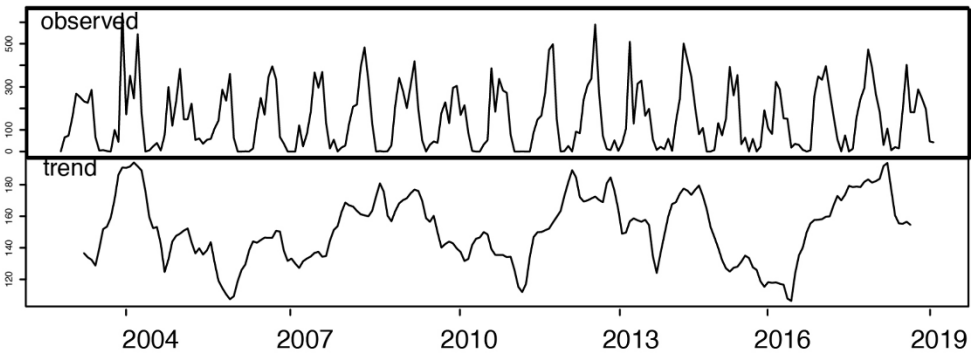
The number of reported scrub typhus cases increased over the study period with a marked increase after 2010. A strong seasonal pattern was observed with more cases during the rainy season while both rainfall and temperature exhibited strong seasonal patterns as expected (Figure 33). For temperature, an increase from an average of 24.5°C in 2003 to 25.5°C in 2018 was observed. Wavelet analysis confirmed a seasonal pattern in the number of cases from 2008 onwards while rainfall and temperature displayed significant 12-month periodicity over the study period (Figure 34).

Figure 33. Temporal analysis of (A) reported monthly scrub typhus cases, (B) total monthly rainfall in mm and (C) average monthly temperature in °C for Chiangrai province. [Temporal analysis involves the decomposition of time series data into systematic (level or average values observed, trend and seasonality) and unsystematic components (noise or random variation in the series)]

A Scrub typhus cases



B Rainfall



C Temperature

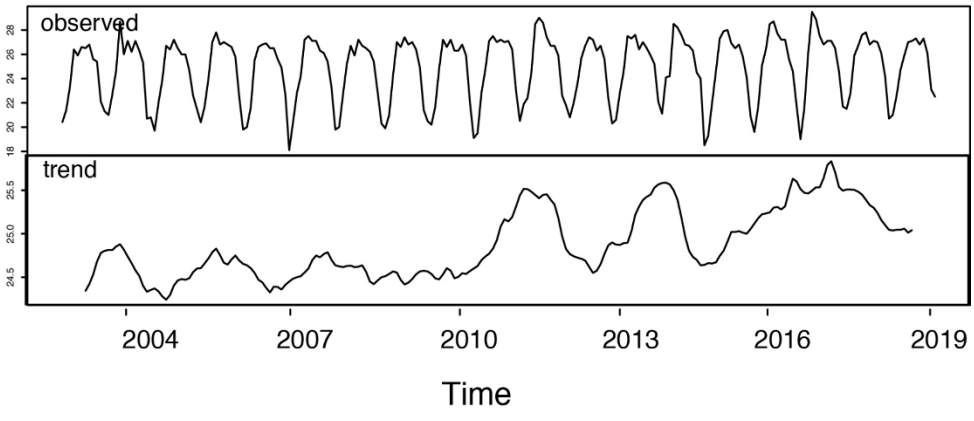
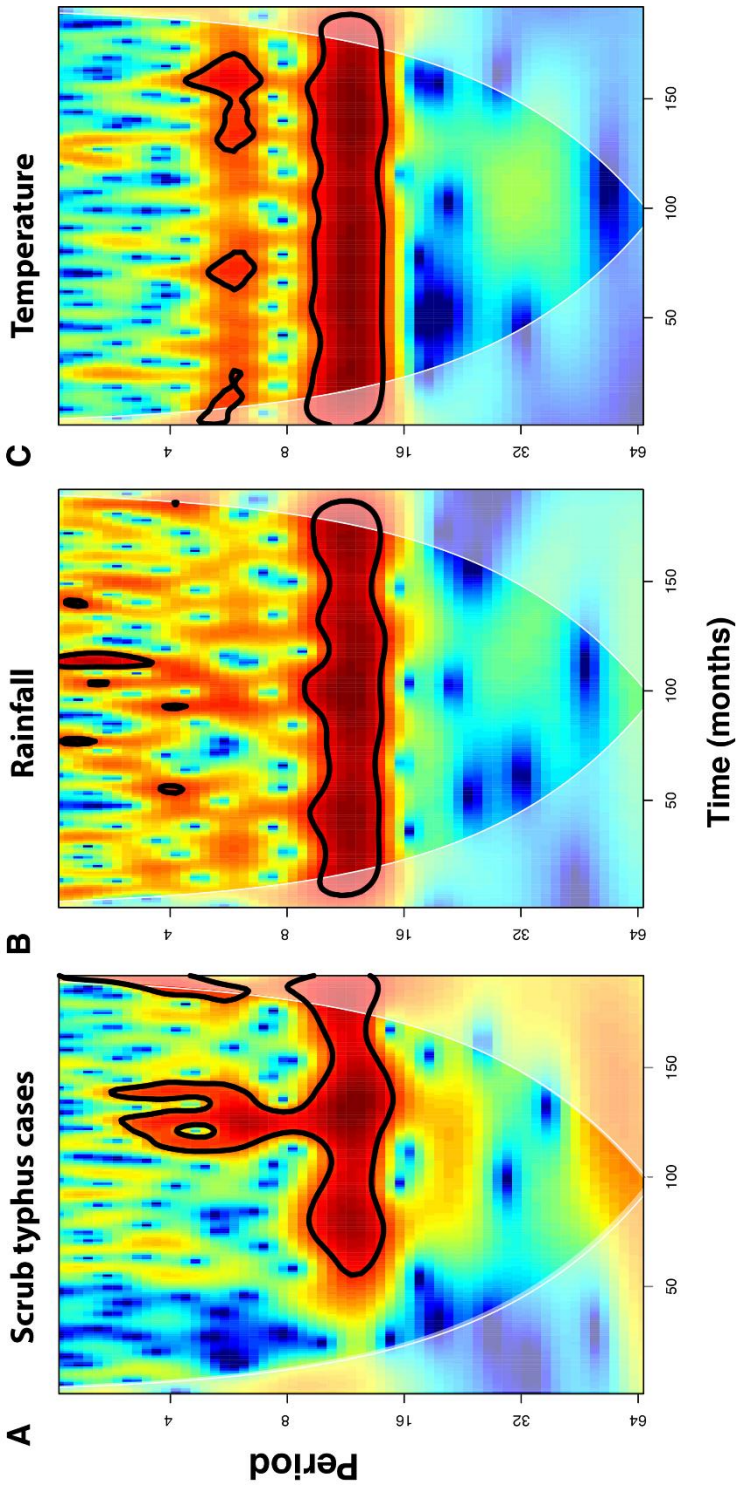


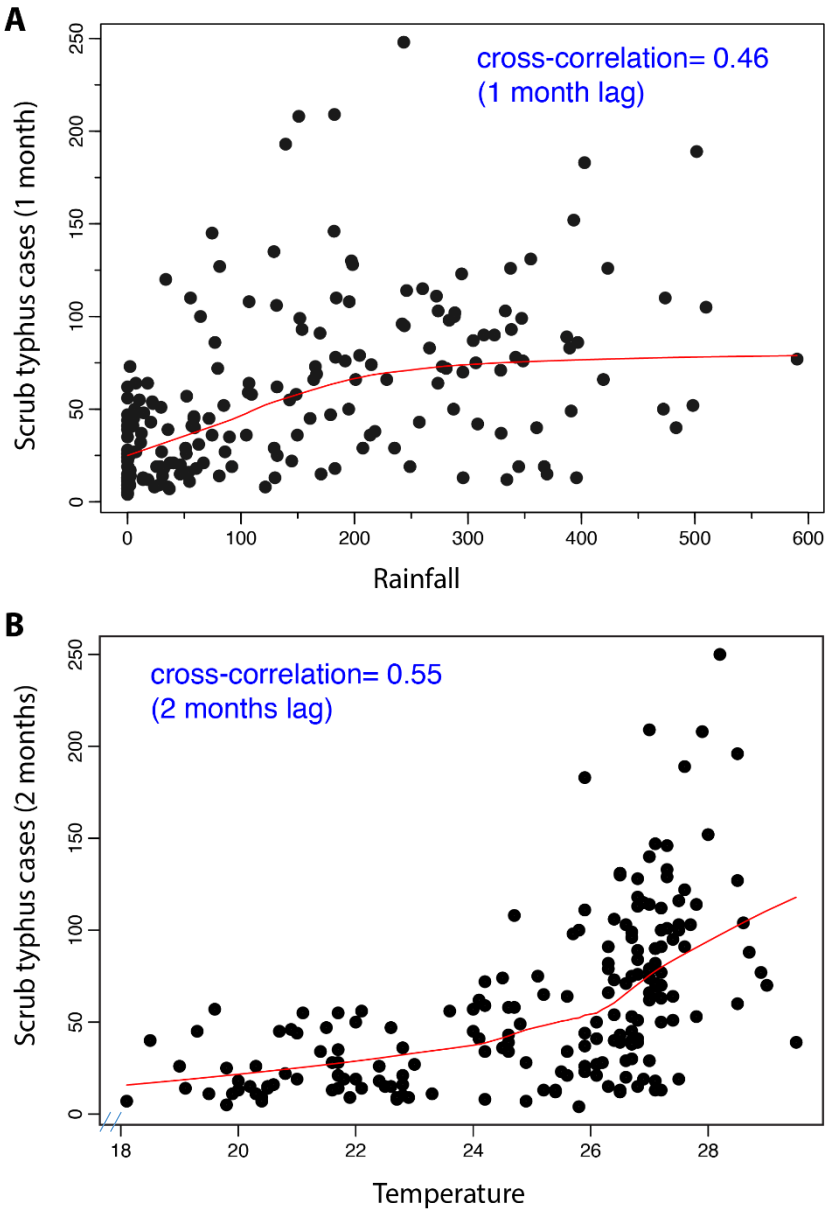
Figure 34. Wavelet analysis for Chiangrai province from 2003-2018: (A) seasonality of scrub typhus cases from 2008 onwards, (B) significant 12-month periodicity of total monthly rainfall, (C) significant 12-month periodicity of average monthly temperature.



Cross temporal correlation analysis

Cross-correlation analysis between rainfall and scrub typhus cases revealed moderate correlation with a lag time of one month (R=0.46, Figure 35A) while there was also moderate cross-correlation between temperature and cases with a lag time of two months (R=0.55, Figure 35B).

Figure 35. Cross temporal correlation analysis for scrub typhus cases, total monthly rainfall (mm) and average monthly temperature (°C) for Chiangrai province.



Association between scrub typhus cases and predictive variables using General Linear Modelling (GLM)

GLM analysis was performed and included the following variables: habitat complexity, habitat fragmentation, forest cover (%), forest open cover (%), grassland open cover (%), mosaic habitat cover (%), rain-fed cropland cover (%), flooded-irrigated land cover (%), population size, rainfall and temperature. The best model selected revealed that scrub typhus cases were significantly associated with all variables included, supported by an Akaike weights value (w_r) of 0.36. Variance Inflation Factor (VIF), a measure of multi-collinearity, was used and the values for most variables were less than 10, suggesting a lack of collinearity. VIF for forest open cover was 22.55, suggesting high collinearity with other land use/land cover variables. The maximum likelihood R^2, which estimates the percentage of variance explained by this model, was 0.25 suggesting good prediction. These results along with the odds ratios generated are shown in Table 4.

Significant relationships between scrub typhus cases and other variables are shown in Figure 36. Scrub typhus burden was positively associated with population, temperature, rainfall and habitat complexity characterised by large cover of open forested habitat. It was negatively associated with habitat fragmentation, mosaic, grassland, forested, rain-fed and flooded land covers.

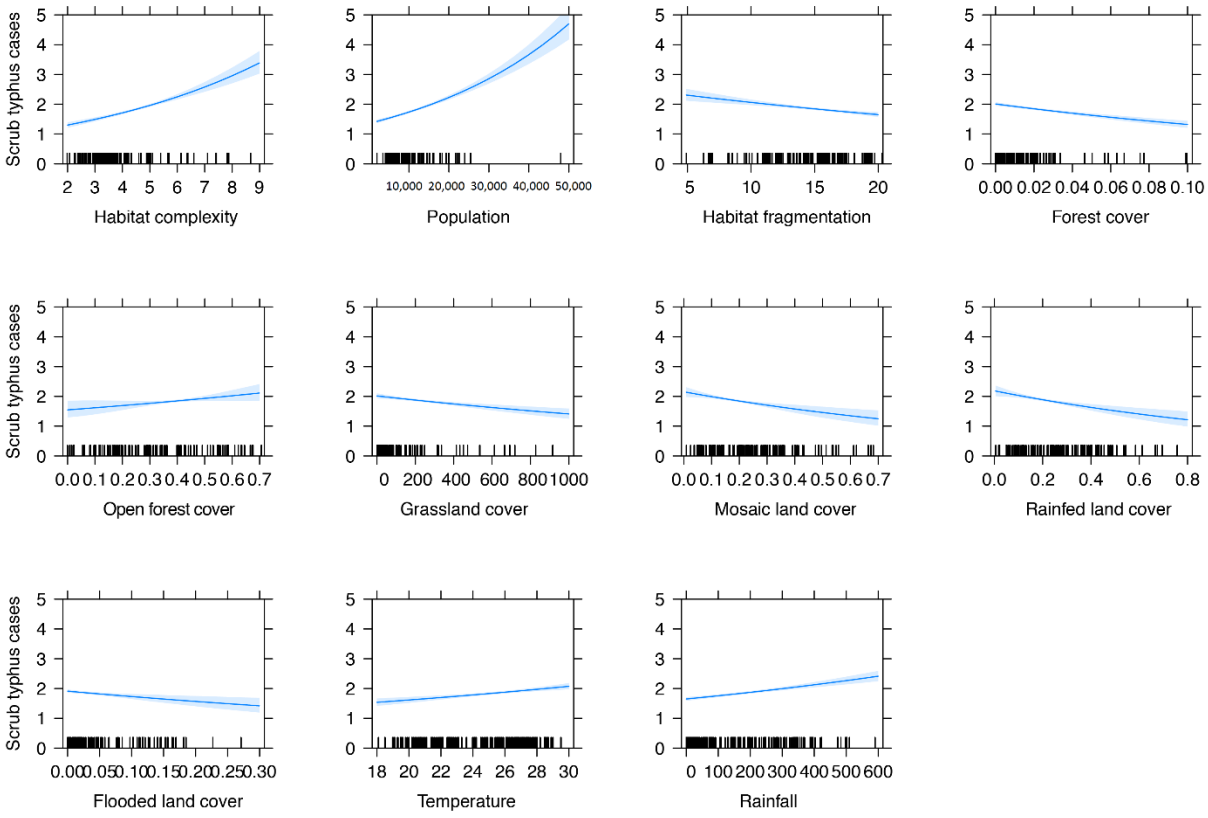
Table 4. Results of the best global GLM with binomial negative link function (p<0.001), with theta = 2.12 (standard deviation = 0.026), explaining the number of scrub typhus cases in Chiangrai province from 2003-2018 by sub-district and by month with explanatory geographical and meteorological variables in the initial model. Estimate and standard deviation (SD) were given for each selected explanatory variables, with P value and VIF (Variance Inflation Factor). For the best selected model the log likelihood = -8925.35 with degree of freedom (DF) = 12, null deviance = 3497.2, R^2 estimated by maximum likelihood (R^2ML) = 0.25, w_r = 0.36 and Akaike Information Criteria (AIC) = 17875.

Explanatory variables	Estimate (SD)	*P* value	VIF*	Odds ratio (CI 2.5%- 97.5%)
Population size	0.00002 (0.000001)	<0.0001	1.45	1.45 (1.38 - 1.53)
Total monthly rainfall	0.001 (0.0001)	<0.0001	1.53	1.02 (1.01 - 1.02)
Average monthly temperature	0.025 (0.005)	<0.0001	1.53	1.87 (1.44 - 2.42)
Forest cover (%)	-4.19 (0.53)	<0.0001	1.44	0.81 (0.77 – 0.86)
Forest open cover (%)	0.45 (0.22)	0.046	22.55	1.56 (1.01 - 2.41)
Grassland open cover (%)	-0.0004(0.0001)	<0.0001	4.91	0.84 (0.78 - 0.90)
Mosaic habitat cover (%)	-0.78 (0.20)	0.0001	7.60	0.79 (0.70 - 0.89)
Rain-fed land cover (%)	-0.73 (0.18)	<0.0001	8.89	0.80 (0.72 - 0.89)
Flooded-irrigated land cover (%)	-0.99 (0.32)	0.002	2.09	0.88 (0.81 - 0.95)
Habitat complexity	0.14 (0.013)	<0.0001	5.11	1.51 (1.40 - 1.62)

Habitat fragmentation	-0.02 (0.004)	<0.0001	1.28	0.80 (0.74 - 0.87)

(*value of VIF > 10 for continuous variables may indicate problems with collinearity)

Figure 36. Results of the best General Linear Modeling (GLM) explaining the number of scrub typhus cases in Chiangrai province over 2003-2018 by sub-district and by month, using a binomial negative link function (with theta = 2.12). The smoothed variables selected in the best GLM were (A) habitat complexity, (B) population, (C) habitat fragmentation, (D) forest cover, (E) forest open cover, (F) grassland open cover, (G) mosaic habitat cover, (H) rain-fed land cover, (I) flooded-irrigated land cover, (J) average monthly temperature in °C and (K) total monthly rainfall in mm.



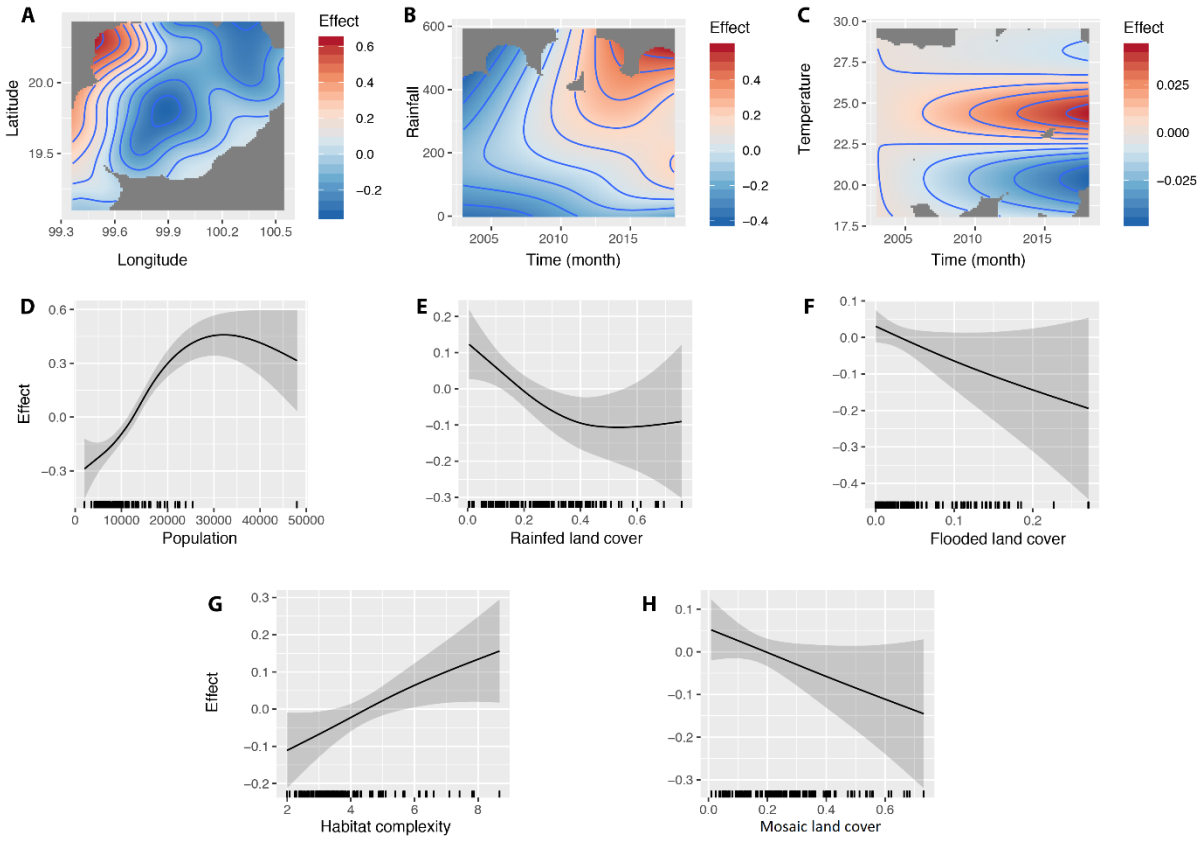
Association between scrub typhus cases and predictive variables using General Additive Modelling (GAM)

Results of the spatiotemporal analyses for scrub typhus cases, temperature and rainfall and the predictive variables from the best GLM model (excluding open forested habitat due to high VIF) contributed to the development of the initial GAM model using a negative binomial function (theta estimated as above). Using Akaike Information Criteria (AIC), the best GAM model was selected, details of which are shown in Table 5 and which did not include habitat fragmentation and grassland open cover. The model explained 50.4% of the deviance (R^2= 0.37) and performed better than the best GLM model above. The best GAM model showed the influence of sub-district location, rainfall, temperature, population, habitat complexity, mosaic land cover, rain-fed land cover and flooded land cover on number of scrub typhus cases (Figure 37).

Table 5. Results of GAM explaining the number of cases of scrub typhus per sub-district in Chiangrai province using a negative binomial link function (theta = 2.12), with approximate significance of smooth terms. For the best selected model, the deviance explained = 50.4%, R^2 = 0.37, maximum likelihood = 8868.2, AIC = 17689.8.

Explanatory variables	Estimated degrees of freedom	Reference degrees of freedom	Chi square	P value
Longitude and latitude	16.71	29	117.1	<0.0001
Total monthly rainfall	11.36	24	155.5	<0.0001
Average monthly temperature	0.97	20	1.9	0.10
Population size	2.89	9	87.2	<0.0001
Habitat complexity	1.14	9	6.6	0.002
Rain-fed land cover (%)	1.76	9	8.8	0.001
Mosaic habitat cover (%)	0.86	4	3.3	0.027
Flooded-irrigated land cover (%)	0.84	9	3.3	0.024

Figure 37. Results of the best GAM model explaining the number of scrub typhus cases in Chiangrai province over 2003-2018 by sub-district and by month. The smoothed variables selected in the best GAM were (A) the geographical distribution of sub-district (longitude and latitude of the centroid), (B) total monthly rainfall (mm), (C) temperature (°C), (D) population size, (E) rain-fed land cover, (F) flooded land cover, (G) habitat complexity and (H) mosaic land cover.



3.4 Discussion

In this chapter, I have shown that the burden of scrub typhus in Thailand is high with an increasing trend over the last two decades. However, it is unclear which factors have contributed to the remarkable rise in disease burden but improved awareness, reporting compliance, availability of diagnostic tests and/or changing human behaviour and agricultural practices may have played roles. The distribution of disease is far from uniform with burden highest in the northern region; Chiangrai, Chiangmai, Tak, Nan and Mae Hong Son provinces being particularly affected. Within Chiangrai province, the highest burden was observed in the mountainous and less developed western part of the province and to a lesser extent, the eastern region of the province.

The increase in reported cases to the national surveillance system in Thailand mirrors the trend seen in published surveillance data from South Korea, China and Bhutan [86, 236, 251]. In contrast, the disease burdens in Japan (2006-2017) and Taiwan (2003-2018) have remained stable [85, 255]. However, the reporting criteria differ for each country (as described in the introduction) which must be taken into account when comparing these datasets. There were more scrub typhus cases in men than women in Thailand which was similar to the situation in Japan and Taiwan but different to China and South Korea where the situation was reversed. Disease burden was greater in adults of working age in Thailand which differed to the other five countries. This deviation was most marked for Japan and South Korea where ageing farming communities are commonplace [237]. The proportion of children with scrub typhus has fallen during the study period, reflecting the accelerated increase in disease burden in adults. These results confirm the ongoing risk of scrub typhus in rural agricultural workers in Thailand and elsewhere within the Tsutsugamushi triangle. However, it must be noted that there was not a complete absence of scrub typhus in suburban or urban areas. Cases of scrub typhus have been described in Seoul, South

Korea and Bangkok, Thailand, which could be explained by increasing human exposure to rural habitats as urban centres expand or the increase in habitats amenable to vectors and natural hosts of scrub typhus within municipal boundaries [253, 282].

Seasonal variation of scrub typhus was observed in Thailand with the disease pattern resembling data from southern China and Taiwan. However, the temporal pattern of disease was not uniform with seasonality most marked in the northern and northeastern regions, becoming less pronounced as latitude decreases. This geographical effect on disease pattern was more prominent than South Korea and could be explained by Thailand's closer proximity to the equator and greater latitudinal range [66]. In Thailand, the southern region experiences a narrower temperature range, higher rainfall, and two main seasons (dry and wet). The west coast of the peninsula typically experiences wetter weather earlier in the year (April to October) due to the southwest monsoon while the east coast tends to have higher rainfall towards the end of the year (September to December). In the northern and northeastern regions, three main seasons are seen (cool, hot and wet) with a greater contrast in temperature observed. The wider temperature range can affect the endemic population of *Leptotrombidium* mites and this was demonstrated in Japan where the monthly incidence of Tsutsugamushi disease was more evenly distributed moving southwards [88, 283]. In the tropical southern region where temperature variation is less, rainfall appears to be the more important climactic factor influencing chigger abundance and thus, disease risk in humans [284, 285].

In Chiangrai Province, we investigated the effects of geographical, meteorological and land cover/land use variables on disease incidence to the sub-district level. Modelling revealed the effect of elevated/mountainous geography, rainfall, temperature, population size, habitat complexity and diversity of land cover on scrub typhus incidence per sub-district. In the cross-correlation analysis, a lag time of 2 months for temperature and 1 month for rainfall on

disease incidence was observed. In Chiangrai, March and April are often the hottest months of the year with the rainy season beginning in June. The climate effect on scrub typhus cases could be explained by agricultural activity occurring in June/July (clearing of scrubland and new vegetation prior to planting) and/or by increased chigger activity with the onset of the rainy season. Areas of new or transitional vegetation may provide the ideal habitat for infected rodents and chigger mites which in turn, become important factors in the ecology of scrub typhus [57, 79]. These habitats can be diverse and include forests, gardens, fringe habitats, water-meadows, beachheads, rice paddies, bamboo patches and oil palm or rubber plantations, many of which are abundant in Chiangrai Province.

The relationship between population, geography and scrub typhus cases in Chiangrai Province warrants further exploration. In the GAM, population was positively correlated with scrub typhus cases up to a peak population of 30,000 after which, negative correlation was observed. More rural, mountainous regions to the west of the province also correlated positively with disease burden. This suggests the risk of scrub typhus in a population is highest in medium-sized sub-districts with rural mountainous topographical features, which would allow a significant proportion of the population to be exposed to habitats described above. Larger populations suggest well-developed areas of the province with significant suburban and urban populations who are at lower risk of exposure to infected rodents and mites. The association of elevation and scrub typhus incidence was also described in southern China [286]. Rural, mountainous regions are less accessible and are thus less likely to undergo deforestation and maintain a greater habitat complexity. Finally, the increase in average monthly temperature from 24.5°C to 25.5°C in Chiangrai province from 2003 to 2018 coinciding with the increase in scrub typhus cases is intriguing and is in agreement with data from southern China [286-288]. Taken together, the data is suggestive of the impact of climate change on scrub typhus disease burden.

A major limitation to the study is that the majority of scrub typhus cases reported were probable cases based on the clinical criteria of fever and eschar (+/- positive rapid diagnostic test – RDT – results). The combination of these clinical features has a sensitivity and specificity of 47% and 81% in adults and 60% and 92% in children for scrub typhus in Chiangrai [289, 290]. The diagnostic capabilities of the laboratory in district general hospitals in Thailand are limited. In fact, most provincial hospitals are not able to perform confirmatory assays, requiring the aid of reference laboratories to confirm diagnosis [291]. Antibody detection-based rapid diagnostic tests (RDTs) for scrub typhus have been introduced (e.g. since 2008 in Chiangrai province) but are sub-optimal [292]. Despite these weaknesses, the current Thai scrub typhus surveillance system based on clinical features of fever and eschar +/- positive RDT results can play a valuable role in determining disease burden, particularly in regions where scrub typhus is the dominant eschar-associated disease (the main differential being spotted fever group rickettsial infections).

The reporting data used was derived from a passive surveillance system, the quality of which will be reliant on the compliance of healthcare and public health staff on reporting and applying the reporting criteria. Healthcare seeking behaviour will also impact the dataset. The limitations outlined so far suggest the true burden of scrub typhus in Thailand is likely to be underestimated based on the reporting data used. Other factors that may have influenced the impressive rise in disease burden since 1980 including disease awareness, availability of diagnostic tests, agricultural activity, human behaviour (hunting, foraging), and chigger abundance and activity were not available and not included in the single province case study.

Although patient location data for Chiangrai province was collected, difficulties surrounding geo-location data verification limited the utility of this high resolution data in the analysis. However, I was able to highlight the villages and sub-districts with the highest burden of

scrub typhus, allowing for the targeting of public awareness and engagement programmes. Paradoxically, despite the high incidence of scrub typhus in these areas, awareness among community health workers remains low and is almost completely absent in villagers. An additional complexity particular to Chiangrai province is its highly multi-ethnic population. The population of 1.25 million consists of an ethnic Thai majority but 14-15% belong to a hill tribe or other minority ethnic group, each with their own distinct language and culture. In Chapter 8, I shall briefly summarise our efforts to enhance awareness through our public engagement programme.

In this chapter, I have estimated the burden of scrub typhus in Thailand from national surveillance data from 2003-2018. I have performed an in-depth study of the disease burden at the national, regional and provincial level and investigated the characteristics of the disease within the population. Detailed spatio-temporal analyses were performed including the single province case study which has shown how geography, rainfall, temperature and landscape complexity may contribute to disease burden. The high burden of disease in Thailand and other neighbouring countries suggest that scrub typhus is the most clinically important rickettsial disease in the Asia Pacific region and perhaps, globally. The results will contribute significantly to our understanding of disease epidemiology both locally and further afield.

4 Causes of acute undifferentiated fever in Chiangrai, northern Thailand

4.1 Introduction

We have seen, as described in chapter 3, that the burden of scrub typhus in Thailand is high and has continued to rise over the last three decades. This is particularly true for northern provinces such as Chiangrai. However, it is unclear as to the degree to which scrub typhus contributes to the overall febrile disease burden locally and whether the disease can be practically differentiated from other common causes of acute undifferentiated fever (AUF). What other infectious aetiologies are likely to be prevalent in the province? Results from past fever aetiology studies from Southeast Asia draw attention to a group of non-malarial infections (in addition to scrub typhus) including dengue, leptospirosis, murine typhus, influenza and enteric fever [137, 243, 247, 293]. Despite its historical significance and shared clinical features, the impact and incidence of malaria in this region is declining although artemisinin resistance remains a major concern [294, 295].

In prospective studies of AUF in rural Thailand, leptospirosis, scrub typhus, murine typhus, influenza and dengue were identified most frequently among febrile adults and children [243, 244]. A study of febrile pregnant women in a large refugee population on the Thai-Burmese border highlighted the impact of arthropod-borne diseases, with malaria as the leading cause of fever followed by rickettsial infections, dengue, and leptospirosis [245]. Women with scrub typhus or murine typhus during pregnancy had severe disease with high proportions of miscarriage (17%) and adverse neonatal outcomes (42%) [133].

In Laos, a prospective multicentre study investigating the causes of non-malarial fever revealed dengue, scrub typhus, Japanese encephalitis and leptospirosis as the major aetiologies in hospitalised adults and children when influenza was excluded [246]. Disease

epidemiology differed significantly between the two regions studied (northwest and southern) and exhibited seasonal variability, emphasizing the importance of local epidemiological data in informing time and region-specific management [246]. Non-malarial febrile episodes in Lao pregnant women were most commonly attributed to dengue fever, followed by pyelonephritis, scrub typhus, murine typhus and typhoid. Severe outcomes, including maternal death, miscarriage, stillbirth, low birth weight and preterm birth, were found among 78% of mothers with dengue fever, rickettsial infection and typhoid [296].

In a prospective fever study of hospitalised Cambodian children, dengue, scrub typhus, bacteraemia (*Salmonella enterica* serovar Typhi was the commonest pathogen) and Japanese encephalitis were the major microbiological diagnoses [247]. The blood culture findings concurred with results from a systematic review on community-acquired bacteraemia in South and Southeast Asia where *Salmonella* Typhi was reported as the commonest pathogen isolated [297]. During the same period, another prospective cross-sectional fever study was performed in rural western and eastern Cambodia [298]. Unlike other fever studies from the region, laboratory diagnosis was heavily reliant on molecular (PCR-based) assays with serological assays excluded. In febrile adults and older children, malaria, leptospirosis, influenza, dengue, scrub typhus and other rickettsial infections were the commonest pathogens detected. However, a non-febrile control group was also included in the study with malaria parasites and *Leptospira* spp. being detected by PCR in 40.4% of participants, suggesting that there was asymptomatic carriage of these pathogens in the studied population.

A recently published prospective case-control study from Vietnam performed in 2016 on the causes of fever revealed that influenza, rickettsial infections (mainly scrub and murine typhus), dengue, leptospirosis, adenovirus and enterovirus infections were most frequently seen in the case cohort [299]. However, dengue would have been underestimated as the

study purposefully excluded patients with positive dengue rapid diagnostic tests (RDT) and malaria. Looking further back, a fever study conducted in the 1970s in rural Malaysia revealed that the most frequent diagnosis was scrub typhus, contributing to 19% of fevers in febrile adults and children, followed by enteric fever, flavivirus infection, and leptospirosis, contributing 7% each, while malaria accounted for only 6% of the study cohort [242].

These studies are limited by their heterogeneity, selection bias and suboptimal diagnostic tools. Despite this, the results provide a useful range of infectious diseases prevalent in Southeast Asia, information which is valuable to both local clinicians and epidemiologists. High quality fever studies are costly, difficult and challenging to perform. Two key aims are an accurate diagnostic panel that reflects the local epidemiology and representative geographical coverage [300].

The infectious diseases highlighted in the studies above present similarly, often with overlapping symptomatology. Compounding this issue is the lack of accurate, affordable diagnostic tests for many of these infections that can be used by frontline healthcare workers in the rural tropics, negatively impacting patient care [301]. There has been some progress: disease-specific RDTs for malaria and dengue have been developed and are widely used. For malaria, antigen screening for *Plasmodium* histidine-rich protein (HRP-2), lactate dehydrogenase or aldolase in blood using an immunochromatographic strip can either be species-specific, pan-specific or both [302]. Similarly, the combined NS-1 antigen/IgM RDT has allowed early accurate dengue diagnosis, improving on the previous situation in which false-positive results were commonly seen in RDTs seeking to detect dengue specific antibodies only [303]. However, a recent modelling study suggests that, when considering direct health benefits, testing for viral infections in this context is unlikely to be as cost-effective as diagnosing specific bacterial infections as this would differentiate patients who require antibiotic treatment and guide antibiotic choice [304].

In high income settings, biomarkers such as C-reactive protein (CRP) and procalcitonin are being used in patients with respiratory tract infections to help guide appropriate use of antibiotics [305]. In the absence of cheap and accurate disease-specific RDTs, studies on the utility of CRP and procalcitonin in differentiating bacterial and viral infections have been performed in the tropics. Using blood samples collected from febrile adults and children from well characterised fever studies from Cambodia, Laos and Thailand, it was shown that CRP was highly sensitive and moderately specific at discriminating bacterial from viral infections [306]. More recently, CRP-testing for adults and children with acute respiratory tract infections in primary care settings in Vietnam reduced overall antibiotic use [307]. In resource-poor tropical settings, common treatable infections are being missed while inappropriate antibiotic use is widespread. Therefore, it is crucial that causes of acute undifferentiated fever are characterised to improve patient care and allow targeted development of diagnostic assays. In settings where diagnostic capabilities are limited, simple biomarker tests could help differentiate common viral from bacterial infections.

In this study, I investigated the causes of acute undifferentiated fever in adults admitted to the provincial hospital in Chiangrai, northern Thailand. By doing so, I was able to determine the contribution of scrub typhus to the febrile disease burden. Additionally, I studied the clinical characteristics of the infectious diseases identified to determine whether specific variables could be used to differentiate them apart. Finally, I evaluated the use of CRP and procalcitonin tests in delineating patients who require antibiotics from ones who do not.

4.2 Method

4.2.1 Ethics statement and study site

The study took place at Chiangrai Prachanukroh Hospital, the provincial hospital for Chiangrai province, Thailand. It is situated in the far north of Thailand and borders Bokeo Province, Lao PDR, and Shan State, Myanmar. The three countries converge at the Mekong River in an area known as the "'Golden Triangle" where opium smuggling was once rife. The province population of 1.2 million consists mainly of ethnic Thais with 14-15% belonging to hill tribes and other minority ethnic groups.

Ethical approval was obtained from the Chiangrai Prachanukroh Hospital Ethical Committee, Ministry of Public Health, Thailand, and the Faculty of Tropical Medicine Ethics Committee, Mahidol University Bangkok (MUTM 2006-035). All study participants provided written informed consent and parents or guardians provided informed consent on behalf of participants aged <18 years old.

4.2.2 Patient data and samples

Patient screening, consent process, recruitment, data collection and collection of study samples were performed by local healthcare workers under the supervision of Dr Pacharee Kantipong and Dr Achara Laongnualpanich, Chiangrai Prachanukroh Hospital. Study design was by Dr Wirongrong Chierakul, Prof Daniel Paris and Prof Nicholas Day. The study was originally carried out from August 2006 to October 2008 with patient-level data and samples collected and stored.

Between August 2006 and October 2008, a total of 231 patients age ≥15 years old were prospectively recruited. Inclusion criteria were admission to hospital with a fever >37.5°C or a history of fever within the past 21 days, no evidence of a primary focus of infection (e.g. consolidation on chest X-ray, clinical features of a urinary tract infection, cellulitis), negative malaria screen on blood film and written informed consent. Demographic, clinical and laboratory data related to the admission were collected individually on study case-record forms (CRFs) from patient notes and hospital records. Demographic data included age, sex, and occupation. A rural/agricultural occupation was defined as those working as farmers, gardeners, agricultural/plantation workers, or fish and animal farm workers. Clinical data included symptoms, examination findings and vital signs on admission along with details of the current illness, prior antibiotic use, antibiotic treatment during admission and illness outcome (e.g. fever days, death). Laboratory data included haematology (complete blood count), biochemistry (renal and liver blood tests) and microbiological results from admission samples. Chest x-ray findings were also recorded if performed.

Routine blood tests were requested by the treating physician as per normal patient care. An additional study blood sample was collected by local study staff on enrolment (10ml EDTA whole blood and 10ml clotted blood for serum). Blood and other routine cultures were performed if requested by the local clinician and processed using conventional techniques at

the hospital microbiology laboratory. HIV testing was performed as part of routine hospital work using RDTs. Follow-up was carried out by study staff 7 to 14 days after enrolment and involved a clinical review and collection of a convalescent blood sample (10ml clotted blood for serum).

There were 19 patients with incomplete CRFs/datasets and 12 patients with incomplete sample collections. These 31 patients were excluded from further analysis resulting in a total of 200 patients in the study cohort. Of these, 171/200 (85.5%) provided paired blood samples obtained on admission and follow-up from days 7 to 14, and 29/200 (14.5%) patients had a confirmatory diagnosis made from admission samples alone. Both admission and follow-up samples were used for the diagnostic assays outlined below. Inflammatory biomarkers – CRP and procalcitonin – were tested on acute samples only. Clotted blood samples were processed for serum, aliquoted, stored locally at -30°C, and batch transported on dry-ice to the central laboratory of Mahidol-Oxford Tropical Medicine Research Unit (MORU) in Bangkok to be stored at -80°C. EDTA whole blood samples were transported at ambient temperature on the day of collection to Bangkok for further analysis. Some whole blood samples were processed immediately for culture for leptospirosis and scrub typhus (see below) with the remainder stored as aliquots of whole blood, plasma and buffy coat at -80°C. Specialist diagnostic assays were subsequently performed at MORU in appropriate biosafety level laboratories (mainly BSL-2 and BSL-3).

In addition, meteorological data comprising average monthly temperatures and total monthly rainfalls were retrospectively collected for the study period from the local Thai Meteorological Department office of Mueang district in Chiangrai province. The data was collected from a representative weather station for the district near the airport. Chiangrai Prachanukroh Hospital is located within this central district and receives patients from the area along with more seriously ill patients from other district hospitals within the province.

4.2.3 Diagnostic assays

The diagnostic panel was selected based on a number of factors including epidemiological data from previous fever studies, access and availability of assays and the expertise to use them, and study budget constraints. The study diagnostic assays (serology, molecular, cultures and *in vitro* isolation) were performed by Tippawan Anantatat, Dr Pruksa Nawtaisong, Suthatip Jintaworn, Ampai Tanganuchitcharnchai, Aunchalee Thanwisai and Kemajittra Jenjaroen at MORU. CRP and procalcitonin assays were performed by Dr Thomas Althaus at MORU.

The panel included assays to test for dengue, scrub typhus, murine typhus, leptospirosis and Japanese encephalitis. Dengue diagnosis was determined by using paired sera and the following serological ELISA tests: PanBio Dengue Early NS1 (Alere), PanBio Dengue IgM capture (Alere), PanBio Dengue IgG capture (Alere), and PanBio Japanese Encephalitis/Dengue IgM combo (Alere). An admission titer ≥10 U of NS1 PanBio units and/or ≥4-fold increase of IgM antibodies in the convalescent sample was considered diagnostic of acute primary dengue virus infection. Patients with anti-JEV IgM levels of >40 U were classified as having acute JEV infections only if anti-dengue IgM levels were <40 U using the combination ELISA test [308, 309]

Leptospirosis culture was performed at MORU within 24-48 hours by injecting 100µL of whole blood and 200µL of plasma sediment (the bottom fraction obtained from centrifuging 500µL of heparinized plasma) into 3 mL of Ellinghausen, McCullough, Johnson, and Harris (EMJH) medium, supplemented with 3% rabbit serum and 0.1% agarose. Both culture tubes were incubated aerobically at 25°C–30°C and examined every week for 3 months for evidence of growth [310]. The leptospirosis Standard Diagnostics Bioline RDTs were used for detecting anti-leptospira IgM and IgG [311].

Scrub typhus and murine typhus were diagnosed using the indirect immunofluorescence assay (IFA) to detect IgM antibody titers in paired sera (or in admission samples only if convalescent samples unavailable) against *Orientia tsutsugamushi* antigens (Karp, Kato and Gilliam strains for scrub typhus) and *Rickettsia typhi* antigens (Wilmington strain for murine typhus), respectively. IFA cut-off titer of ≥1:3,200 in an admission sample or ≥4-fold rise to ≥1:3,200 in a convalescent-phase sample was used, based on recent Bayesian latent class modelling to determine the optimal diagnostic cut-off points [143]. For scrub typhus, culture and polymerase chain reaction (PCR) assays were also performed as previously described [141]. Briefly, the PCR assays included conventional PCR assay to detect the 56kDa TSA gene and real-time PCR assays to detect the 47kDa *htra* and *groEL* genes. To fulfil the PCR criteria for diagnosis, a consensus of two out of three PCR assays was required.

The inflammatory biomarker procalcitonin was measured by the ELISA-based VIDAS PCT kit with a detection range of 0.05-195ng/ml (BioMérieux, France) and CRP serum levels were measured with the NycoCard Reader II (Axis Shield, Norway), with a detection range of 5-150mg/L in serum [312, 313]. Testing was performed on admission samples and two independent operators, blinded to the microbiological diagnoses, performed the procalcitonin and CRP assays in duplicate. Control reagents were provided with each test kit and calibration performed as per manufacturers' instructions. The following thresholds were evaluated for their usefulness in predicting bacterial causes of fever: 0.25ng/mL and 0.5ng/mL for procalcitonin and 20mg/L and 40mg/L for CRP [314-316].

4.2.4 Attribution of final diagnosis

The diagnostic results were considered in relation to each other and a final diagnosis was attributed to each case by the strength of evidence supporting each diagnosis, as previously described: (I) PCR/antigen/culture positivity > (II) dynamic serology (4-fold rise) > (III) single titer and/or unverified serological cut-off titer [317]. Prof Stuart Blacksell, Prof Daniel Paris and I discussed and derived a consensus.

Blood, urine, sputum and stool culture results from admission were collected from the hospital reporting system if performed. A final conservative diagnosis of culture-attributed infection (CAI) was made on the balance of clinical information, haematological and biochemical results, and results of our diagnostic panel.

4.2.5 Statistical analysis

Proportions, percentages and averages (median and interquartile range [IQR] or mean and standard deviation [SD]) were calculated. Seasonality was assessed by calculating proportions of patients (and 95% confidence intervals) admitted during discrete time-periods and assessing for overlap as well as performing two-sample tests of proportions. Univariate and multivariate logistic regression analysis were performed to determine predictor variables independently associated with the outcomes (e.g. viral/bacterial/unknown aetiologies or specific diagnoses such as scrub typhus or dengue). Categorical data were analysed using Pearson's Chi-squared test or Fisher's exact test where specified. Comparisons of receiver operating characteristic (ROC) curves evaluated the sensitivity, specificity and likelihood ratios for procalcitonin and CRP in differentiating bacterial from viral aetiologies. Classification and regression trees were generated for scrub typhus and dengue using Salford Predictive Modeler Software Suite v8.2 (Salford Systems, San Diego, CA, USA). Other analyses were performed using STATA 14 software (College Station, Texas, USA). All analyses were performed by me with support from Prof Mavuto Mukaka.

4.3 Results

4.3.1 Diagnostic findings

A diagnosis was determined in 103/200 (51.5%) patients: 77 (38.5%) had a bacterial infection, 24 (12%) had a viral aetiology and 2 (1%) had an invasive fungal infection. Scrub typhus was the leading cause of fever (45, 22.5%) followed by dengue (23, 11.5%), leptospirosis (15, 7.5%, murine typhus (7, 3.5%) and Japanese encephalitis virus infection (1, 0.5%). 12 (6%) patients had culture-attributed infections (CAI) as described below.

Twelve (6%) of the patients had multiple positive tests that required further scrutiny (11 dual, 1 triple). In 3 cases, anti-JEV IgM positive results were superseded by positive scrub typhus PCR results +/- dynamic serology. In 4 cases, positive JEV serology was offset by positive dengue NS1 antigen assays +/- dynamic serology. In 2 cases with positive leptospirosis RDT (detecting IgM and IgG), diagnosis was determined as scrub typhus in one patient (positive PCR) and murine typhus in the other (dynamic serology). One case had borderline positive PCR results and negative serology for scrub typhus (only 1/3 targets) and was superseded by positive dengue NS1 and IgM results. One case with dynamic rise in anti-dengue IgM but negative NS1 antigen was assigned a diagnosis of scrub typhus on the basis of PCR-positivity and dynamic serology. Finally, one case with positive leptospirosis RDT and anti-dengue IgM dynamic serology with negative NS1 antigen was diagnosed with scrub typhus on the basis of positive PCR assays.

142 patients (71%) had blood cultures performed of which, 126 (63%) were reported as no growth, 9 (4.5%) had microbiologically non-significant growth (likely skin contaminants such as coagulase-negative staphylococci, aerobic spore bearers) and 7 had microbiologically significant growth (3.5%). There were 2 *Talaromyces marneffei*, 1 *Haemophilus influenzae*, 1 *Staphylococcus aureus*, 1 *Burkholderia pseudomallei*, 1 *Escherischia coli*, and 1

Enterococcus faecium bacteraemia cases. The 2 patients with talaromycosis (previously penicilliosis) and the one with *Haemophilus influenza* bacteraemia tested positive for HIV. Additionally, there were 2 significant urine cultures (heavy growth of *E.coli*), 2 significant sputum cultures (*Klebsiella pneumoniae* in patients with severe respiratory syndromes) and 1 significant stool culture (*Salmonella* spp.). The culture-attributed infections group (CAI) comprised 12 patients, 10 with a bacterial infection and 2 due to invasive fungal infections.

4.3.2 Demographic, clinical and laboratory characteristics

Of the 200 febrile adults included in the study analysis, 114/194 (58.8%) were male, the median age was 41 (IQR 29-52) and the main reported occupation was agricultural worker at 64/136 (47.1%). 34/200 patients (17%) stated that they had received antibiotics prior to admission to the study hospital and the median days from onset of fever to admission was 4 (IQR 3-7).

The characteristics of patients in the viral, bacterial and unknown diagnosis groups are summarised in Table 6. Lower age (OR 0.966, 95%CI 0.937-0.996, p=0.026), lower CRP (OR 0.967, 95% CI 0.953-0.981, p=0.000), lower white blood count (OR 0.713, 95%CI 0.615-0.828, p=0.000), lower neutrophil count (OR 0.694, 95%CI 0.586-0.822, p=0.000) or higher haemoglobin (OR 1.259, 95%CI 1.023-1.549, p=0.029) were significantly more likely to be diagnosed with a viral aetiology on univariate logistic regression analyses. Only low CRP (aOR 0.972, 95%CI 0.957-0.987, p=0.000) and low white blood count (aOR 0.573, 95%CI 0.331-0.992, p=0.047) remained as significant predictors for viral infection on multivariate logistic regression analysis. Of note, characteristics were similar between the unknown and bacterial aetiology groups, suggesting possible bacterial diagnoses in the unknown aetiology group. 61/97 patients in the unknown aetiology group had a blood culture performed which showed no or non-significant growth.

Significant predictor variables for bacterial infection on univariate analyses included the presence of an eschar (OR 11.74., 95%CI 3.849-35.807, p=0.000) and a higher lymphocyte count (OR 1.366, 95%CI 1.027-1.816, p=0.032) but only the eschar remained a significant predictor on multivariate analysis (aOR 11.590, 95%CI 3.754-35.784, p=0.000). The finding of an eschar within the bacterial aetiology group was almost exclusively seen in patients diagnosed with scrub typhus (21/22, 95.5%), the exception being one patient with *Staphylococcus aureus* bacteraemia (1/22, 4.5%). Significant predictor variables for the

unknown aetiology group are shown in Table 6 but are clinically less useful. Details of univariate and multivariate analyses for diagnostic groups including odds ratio (OR), adjusted odds ratio (aOR) and 95% confidence interval (CI) can be found in Table 7.

When comparing the viral and bacterial aetiology groups directly (excluding unknown group), eschar, CRP, Hb, WBC, neutrophil count and lymphocyte count were significant variables on univariate analyses. A lower CRP (aOR 0.969 95%CI 0.951-0.987, p=0.001) was an important predictor for viral infection while presence of an eschar (completely absent in the viral group) and a higher CRP (aOR 1.032 95%CI 1.014-1.052, p=0.001) remained as significant predictor variables for bacterial infection on multivariate analysis.

Table 8 outlines the demographic, clinical, chest x-ray and laboratory results for patients diagnosed with scrub typhus, dengue, leptospirosis and murine typhus. It is important to highlight that although there were disease-specific features outlined below, it remained difficult to differentiate these infections in the majority of patients based on symptoms and signs alone. Features such as headache, abdominal pain, conjunctivitis and jaundice were proportionately present. On univariate analyses, higher number of days with fever before admission, presence of eschar, presence of cough or dyspnoea, presence of hepatomegaly, raised lymphocyte count and raised hepatic enzymes (aspartate aminotransferase – AST, alanine aminotransferase – ALT and alkaline phosphatase – ALP) were significant predictors for a scrub typhus diagnosis. On multivariate logistic regression analysis, presence of an eschar, high lymphocyte count and elevated AST and ALP remained significantly predictive for scrub typhus. If performed, chest x-rays were often normal and pulmonary infiltrates were the main abnormality reported (also proportionately seen in leptospirosis). Data shown in Table 9.

For dengue, female sex, younger age, presence of nausea or vomiting, absence of myalgia, higher haemoglobin and lower WBC, neutrophils, lymphocytes, platelets, ALP and CRP

were significant predictors of a dengue diagnosis. Lower CRP was the only consistently significant predictor variable on multivariate analysis. Elevated creatinine was significantly associated with leptospirosis on univariate analysis only. Details of the analysis can be found in Table 9.

Table 6. Clinical, demographic, and laboratory characteristics of patients by diagnostic group

	Viral aetiology (n=24)	*Bacterial aetiology (n=77)*	*Unknown aetiology (n=97)*
Demographics and History			
Male, n (%)	10/24 (41.7)	42/77 (54.5)	61/91 (67.0)[a]
Age, median (IQR)	32 (24-45)[a]	43 (28-50)	42 (30-54)
Rural occupation, n (%)	4/15 (26.7)	26/47 (55.3)	33/65 (50.8)
Pre-admission antibiotic, n (%)	2/8 (25.0)	14/42 (33.3)	18/54 (33.3)
Days with fever before admission, median (IQR)	5 (2.8-6)	5 (4-7)	4 (3-6)
Days of hospitalisation, median (IQR)	5.5 (5-7)	5 (4-7)	5 (4-7)
Clinical presentation*			
Fever, n (%)	23/24 (95.8)	75/77 (97.4)	92/97 (94.8)
Neurological findings n (%)	17/24 (70.8)	44/73 (60.3)	44/89 (49.4)
Respiratory findings n (%)	5/24 (20.8)	20/74 (27.0)	24/91 (26.4)
Gastrointestinal findings n (%)	16/24 (66.7)	37/73 (50.7)	47/90 (52.2)
Eschar, n (%)	0/24 (0.0)	22/74 (29.7)[a,b]	4/89 (4.5)[a,b]
Clinical severity, n (%)	6/24 (25)	14/74 (18.9)	20/91 (22.0)
Laboratory**			
CRP (mg/L), median (IQR)	12.5 (6.0-26.0)[a,b]	139.5 (67.5-150)	144 (56.0-150)[a]
Procalcitonin (ng/mL), median (IQR)	0.3 (0.1-1.3)	2.6 (0.9-7.3)	2.1 (0.4-24.0)[a]
Haemoglobin (g/dL), median (IQR)	13.2 (11.9-14.8)[a]	12 (10.6-13.0)	12.5 (11.0-14.0))
WBC (10^3/mm^3), median (IQR)	3 (2.1-5.6)[a,b]	10.6 (6.7-13.8)	9.8 (7.3-12.2)
Neutrophils (10^3/mm^3), median (IQR)	1.8 (1.0-3.4)[a]	7.7 (4.6-11.2)	7.3 (5.6-10.6)[a]
Lymphocytes (10^3/mm^3), median (IQR)	0.6 (0.4-1.2)	0.9 (0.5-1.8)[a]	0.8 (0.5-1.5)

NB – 2 fungal infections not included

[a] Significant predictor variable on univariate logistic regression analysis

[b] Significant predictor variable on multivariate logistic regression analysis

* Clinical presentation:

Fever: tympanic temperature >37.5°C on or after admission

Neurological findings: at least one of meningism, headache, focal neurological deficits

Respiratory findings: at least one of respiratory rate >22/minute, lung crepitation, cough, dyspnoea

Gastrointestinal findings: at least one of abdominal pain, vomiting, nausea, jaundice, hepatomegaly, splenomegaly

Severity criteria used in this study – one or more of: intubation; respiratory rate >30/min; pulse >100/min; systolic blood pressure <90mmHg or >160mmHg, or diastolic blood pressure <60mmHg; haematemesis; haemoptysis, seizures, reduced GCS

**Laboratory reference range: CRP <10mg/L, PCT <0.1ng/mL, Hb 12-18g/dL, WBC 4.8-10.8x10^3/mm^3, Neutrophils 2.6-7.0x10^3/mm^3, Lymphocytes 1.2-3.8x10^3/mm^3

Table 7. Results of univariate (A) and multivariate (B) logistic regression analyses for viral, bacterial and unknown aetiology groups

A	***Univariate logistic regression analyses***								
	Viral aetiology			***Bacterial aetiology***			***Unknown aetiology***		
	OR	**95% CI OR**	**P value**	**OR**	**95% CI OR**	**P value**	**OR**	**95% CI OR**	**P value**
Demographics and History									
Sex (M=1, F=2)	2.206	0.926-5.256	0.074	1.333	0.744-2.389	0.334	0.521	0.291-0.934	0.029*
Age	0.966	0.937-0.996	0.026*	0.996	0.978-1.015	0.706	1.016	0.998-1.035	0.084
Rural occupation	3.113	0.935-10.362	0.064	0.714	0.347-1.469	0.360	0.939	0.470-1.879	0.860
Pre-admission antibiotic	0.688	0.131-3.598	0.657	1.100	0.479-2.526	0.822	1.125	0.497-2.546	0.777
Fever days before admission	0.892	0.736-1.082	0.246	1.114	0.998-1.242	0.053	0.943	0.848-1.050	0.286
Days of hospitalisation	1.023	0.911-1.150	0.698	0.982	0.893-1.081	0.717	1.012	0.924-1.108	0.800
Clinical presentation									
Fever	0.953	0.112-8.098	0.965	1.923	0.378-9.779	0.431	0.552	0.128-2.375	0.425
Neurological findings	0.542	0.222-1.322	0.178	1.686	0.929-3.059	0.086	0.797	0.448-1.416	0.439
Respiratory findings	0.736	0.259-2.089	0.564	1.124	0.579-2.181	0.730	1.075	0.561-2.058	0.828
Gastrointestinal findings	1.837	0.745-4.528	0.186	0.806	0.448-1.451	0.473	0.874	0.493-1.551	0.646
Eschar	-	-	-	11.740	3.849-35.807	0.000*	0.167	0.055-0.506	0.002*
Clinical severity	1.257	0.464-3.404	0.653	0.778	0.377-1.603	0.496	1.060	0.531-2.115	0.869

Laboratory									
CRP	0.967	0.953-0.981	0.000*	1.005	1.000-1.011	0.060	1.005	1.000-1.011	0.042*
PCT	0.945	0.887-1.007	0.081	0.992	0.979-1.005	0.213	1.014	1.001-1.028	0.033*
Hb	1.259	1.023-1.549	0.029*	0.881	0.769-1.009	0.066	1.035	0.910-1.177	0.600
WBC	0.713	0.615-0.828	0.000*	1.040	0.986-1.096	0.153	1.054	0.999-1.113	0.056
Neutrophil count	0.694	0.586-0.822	0.000*	1.032	0.974-1.094	0.286	1.067	1.004-1.134	0.037*
Lymphocyte count	0.537	0.277-1.041	0.066	1.366	1.027-1.816	0.032*	0.912	0.694-1.199	0.510

**Significant predictor variable on univariate logistic regression analysis*

NB – Unless specified, for binary categorical variables, 0=no or absent, 1=yes or present

B	***Significant predictor variables associated with each aetiology group on multivariate logistic regression analysis***		
	aOR	**95% CI OR**	**P value**
Viral aetiology			
- WBC	0.573	0.331-0.992	0.047
- CRP	0.972	0.957-0.987	0.000
Bacterial aetiology			
- Eschar	11.590	3.754-35.784	0.000
Unknown aetiology			
- Eschar	0.148	0.041-0.535	0.004

NB – Unless specified, for binary categorical variables, 0=no or absent, 1=yes or present

Table 8. Demographic, clinical, imaging, and laboratory findings associated with scrub typhus, dengue, leptospirosis and murine typhus

	Diagnosis			
	Scrub typhus	***Dengue***	***Leptospirosis***	***Murine typhus***
No. of patients	45	23	15	7
Demographics				
Male, n (%)	24/45 (53.3)	9/23 (39.1)[a]	11/15 (73.3)	5/7 (71.4)
Age, median (IQR)	41 (28-47)	29 (24-45)[a]	33 (22-50)	49 (41-54)
Rural occupation, n (%)	16/27 (59.3)	4/14 (28.6)	6/11 (54.5)	1/2 (50.0)
Pre-admission antibiotic, n (%)	7/23 (30.4)	2/8 (25.0)	2/9 (22.2)	2/4 (50.0)
Days with fever before admission, median (IQR)	6 (4-8)[a]	5 (3-6)	5 (4-7)	5 (3-7)
Days of hospitalization, median (IQR)	5 (4-7)	5 (5-7)	5 (3.5-7)	5 (4-7)
Symptoms and signs				
Eschar	21 (46.7)[a,b]	0 (0)	0 (0)	0 (0)
Rash	1 (2.2)	3 (13.0)	0 (0)	0 (0)
Cough/dyspnoea	15 (33.3)[a]	3 (13.0)	2 (13.3)	0 (0)
Lung crepitation	5 (11.1)	1 (4.3)	0 (0)	0 (0)
Epistaxis	0 (0)	0 (0)	1 (6.7)	0 (0)
Haemoptysis	0 (0)	0 (0)	0 (0)	0 (0)
Nausea/vomiting	6 (13.3)	9 (39.1)[a,b]	3 (20.0)	1 (14.3)
Abdominal pain	12 (26.7)	8 (34.8)	5 (33.3)	3 (42.9)
Jaundice	8 (17.8)	2 (8.7)	3 (20.0)	1 (14.3)
Hepatomegaly	14 (31.1)[a]	7 (30.4)	1 (6.7)	0 (0)
Splenomegaly	2 (4.4)	0 (0)	0 (0)	0 (0)
Gum bleeding	0 (0)	1 (4.3)	0 (0)	0 (0)
Haematemesis	0 (0)	1 (4.3)	0 (0)	0 (0)
Headache	26 (57.8)	15 (65.2)	7 (46.7)	6 (85.7)
Conjunctivitis	11 (24.4)	3 (13.0)	5 (33.3)	1 (14.3)

Conjunctival haemorrhage	1 (2.2)	1 (4.3)	1 (6.7)	0 (0)
Tinnitus	2 (4.4)	0 (0)	0 (0)	0 (0)
Deafness	0 (0)	0 (0)	0 (0)	0 (0)
Neck stiffness	2 (4.4)	2 (8.7)	1 (6.7)	0 (0)
Myalgia	15 (33.3)	4 (17.4)[a]	6 (40.0)	3 (42.9)
Lymphadenopathy	1 (2.2)	0 (0)	0 (0)	0 (0)
Chest X-ray findings				
Performed, n (%)	32 (71.1)	14 (60.9)	11 (73.3)	4 (57.1)
- Normal or incidental findings, n (%)	24 (75.0)	13 (92.9)	9 (81.8)	4 (100.0)
- Pulmonary infiltrates	7 (21.9)	0 (0)	2 (18.2)	0 (0)
- Pulmonary oedema	1 (3.1)	0 (0)	0 (0)	0 (0)
- Pleural effusion	0 (0)	1 (7.1)	0 (0)	0 (0)
Laboratory findings				
Haemoglobin (g/dL), median (IQR)	11.9 (9.9-13.0)	13.3 (12.3-14.9)[a]	12.2 (11.0-13.8)	13.3 (12.9-15.0)
WBC (10^3/mm^3), median (IQR)	9.5 (6.4-12.3)	2.9 (1.9-5.1)[a]	10.9 (6.6-16.9)	9.9 (6.9-12.8)
Neutrophils (10^3/mm^3), median (IQR)	6.4 (4.3-10.5)	1.7 (0.8-3.3)[a]	8.6 (4.9-14.9)	8.1 (3.9-10.8)
Lymphocytes (10^3/mm^3), median (IQR)	1.2 (0.5-2.3)[a,b]	0.7 (0.4-1.3)[a]	0.9 (0.4-1.1)	0.8 (0.6-2.0)
Platelets (10^3/mm^3), median (IQR)	94 (61-170)	81 (25-101)[a]	96 (20-169)	72 (29-203)
BUN (mg/dL), median (IQR)	15.0 (12.0-28.0)	12.0 (7.3-14.8)[a]	30.0 (17.0-82.5)	21.0 (10.0-24.0)
Creatinine (mg/dL), median (IQR)	1.0 (0.9-1.6)	1.1 (0.8-2.3)	3.6 (1.2-6.3)[a]	1.3 (1.3-2.0)
Bilirubin total (mg/dL), median (IQR)	1.8 (0.9-3.1)	0.6 (0.5-1.8)	1.2 (0.6-7.2)	0.8 (0.6-2.3)
Bilirubin direct (mg/dL), median (IQR)	1.0 (0.3-1.8)	0.2 (0.1-0.8)	0.6 (0.1-5.1)	0.3 (0.1-1.5)
AST (IU/L), median (IQR)	148 (96-304)[a,b]	94 (57-240)	43 (27-68)	115 (63-303)

ALT (IU/L), median (IQR)	101 (54-189)[a]	58 (29-95)	41 (28-62)	69 (44-333)
ALP (IU/L), median (IQR)	271 (160-422)[a,b]	108 (78-134)[a]	127 (111-187)	132 (109-249)
Albumin (g/dL), median (IQR)	2.9 (2.5-3.2)	3.6 (3.1-3.8)	3.3 (2.7-3.7)	3.4 (1.8-4.2)
CRP (mg/L), median (IQR)	130.5 (67.5-150)	12.0 (5.5-30.3)[a,b]	150 (60.3-150)	103.0 (60.0-150)
PCT (ng/mL), median (IQR)	2.5 (1.0-6.6)	0.3 (0.1-1.4)	4.7 (2.7-25.2)	1.2 (0.2-3.7)

- [a] *Significant predictor variable on univariate logistic regression analysis*
- [b] *Significant predictor variable on multivariate logistic regression analysis*
- Rural occupation included farmers, gardeners, agricultural/plantation workers, fish and animal farm workers
- Incidental findings on CXR included hyperinflation, hiatus hernia, cardiomegaly without accompanying pulmonary signs, ground glass/fibrotic changes
- WBC, white blood cell count; BUN, blood urea nitrogen; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; CRP, C-reactive protein; PCT, procalcitonin
- Laboratory reference range:
  - Hb – 12-18g/dL
  - WBC – 4.8-10.8x10^3/mm^3
  - Neutrophils - 2.6-7.0x10^3/mm^3
  - Lymphocytes - 1.2-3.8x10^3/mm^3
  - Platelets – 130-400x10^3/mm^3
  - BUN – 8-20mg/dL
  - Creatinine – 0.51-0.95mg/dL
  - Bilirubin total – 0.3-1.2mg/dL
  - Bilirubin direct – 0-0.2mg/dL
  - AST – 0-35IU/L
  - ALT – 0-35IU/L
  - ALP – 30-120IU/L
  - Albumin – 3.5-5.2g/dL
  - CRP – <10mg/L
  - PCT – <0.1ng/mL

Table 9. Results of univariate (A) and multivariate (B) logistic regression analyses for scrub typhus, dengue, leptospirosis and murine typhus

A	***Univariate logistic regression analyses***											
	Scrub typhus			***Dengue***			***Leptospirosis***			***Murine typhus***		
	OR	**95% CI OR**	**P value**	**OR**	**95% CI OR**	**P value**	**OR**	**95% CI OR**	**P value**	**OR**	**95% CI OR**	**P value**
Demographics												
Sex (M=1, F=2)	1.335	0.682-2.612	0.399	2.475	1.014-6.040	0.047*	0.493	0.151-1.607	0.241	0.559	0.106-2.956	0.494
Age	0.983	0.961-1.006	0.142	0.965	0.936-0.996	0.027*	0.976	0.941-1.012	0.181	1.040	0.993-1.090	0.099
Rural occupation	0.623	0.263-1.473	0.281	2.778	0.823-9.375	0.100	0.819	0.237-2.833	0.753	1.000	0.061-16.342	1.000
Pre-admission antibiotic	0.907	0.334-2.466	0.849	0.688	0.131-3.598	0.657	0.580	0.114-2.955	0.512	2.188	0.295-16.230	0.444
Fever days before admission	1.164	1.033-1.312	0.013*	0.905	0.746-1.098	0.310	1.066	0.905-1.255	0.443	0.968	0.746-1.256	0.809
Days of hospitalisation	1.036	0.935-1.148	0.497	1.017	0.901-1.147	0.790	0.960	0.793-1.162	0.675	0.952	0.724-1.253	0.727
Symptoms and signs												
Eschar	26.918	9.198-78.777	0.000*	-	-	-	-	-	-	-	-	-
Rash	0.299	0.038-2.388	0.255	2.600	0.649-10.408	0.177	-	-	-	-	-	-
Cough/dyspnoea	2.469	1.158-5.263	0.019*	0.488	0.138-1.731	0.267	0.515	0.112-2.380	0.396			
Lung crepitation	3.171	0.916-10.984	0.069	0.686	0.084-5.626	0.726	-	-	-	-	-	-
Epistaxis	-	-	-	-	-	-	5.929	0.506-69.507	0.156	-	-	-

Haemoptysis	-	-	-	-	-	-	-	-	-	-	-	-
Nausea/vomiting	0.556	0.215-1.433	0.224	2.829	1.120-7.144	0.028*	0.931	0.249-3.475	0.915	0.614	0.072-5.256	0.656
Abdominal pain	1.093	0.507-2.353	0.821	1.451	0.575-3.665	0.431	1.486	0.473-4.666	0.498	2.688	0.524-13.775	0.236
Jaundice	1.095	0.455-2.637	0.840	0.399	0.089-1.789	0.230	1.153	0.307-4.332	0.833	0.753	0.088-6.462	0.796
Hepatomegaly	3.182	1.395-7.256	0.006*	2.174	0.791-5.973	0.132	0.339	0.042-2.749	0.311	-	-	-
Splenomegaly	7.077	0.625-80.115	0.114	-	-	-	-	-	-	-	-	-
Gum bleeding	-	-	-	3.810	0.331-43.846	0.283	-	-	-	-	-	-
Haematemesis	-	-	-	7.667	0.462-127.198	0.155	-	-	-	-	-	-
Headache	1.347	0.664-2.731	0.409	2.098	0.774-5.683	0.145	0.654	0.227-1.887	0.432	4.875	0.575-41.351	0.146
Conjunctivitis	1.651	0.736-3.704	0.224	0.582	0.163-2.075	0.404	2.219	0.710-6.937	0.171	0.676	0.079-5.792	0.721
Conjunctival haemorrhage	0.690	0.078-6.078	0.738	1.495	0.167-13.425	0.719	2.343	0.256-21.470	0.451	-	-	-
Tinnitus	7.282	0.643-82.424	0.109	-	-	-	-	-	-	-	-	-
Deafness	-	-	-	-	-	-	-	-	-	-	-	-
Neck stiffness	1.198	0.232-6.185	0.829	2.365	0.448-12.491	0.311	1.881	0.213-16.572	0.569	-	-	-
Myalgia	0.738	0.361-1.505	0.403	0.278	0.090-0.857	0.026*	0.943	0.321-2.769	0.915	1.068	0.232-4.918	0.932
Lymphadenopathy	0.566	0.067-4.812	0.602	-	-	-	-	-	-	-	-	-
CXR												

Pulmonary infiltrates	2.062	0.723-5.882	0.176	-	-	-	1.347	0.266-6.814	0.719	-	-	-
Laboratory findings												
Hb	0.873	0.746-1.020	0.087	1.286	1.039-1.591	0.021*	1.002	0.786-1.278	0.985	1.354	0.943-1.944	0.101
WBC	1.001	0.940-1.065	0.987	0.688	0.586-0.807	0.000*	1.042	0.957-1.134	0.339	1.017	0.894-1.157	0.797
Neutrophils	0.984	0.917-1.056	0.650	0.673	0.562-0.807	0.000*	1.057	0.967-1.155	0.223	1.049	0.917-1.199	0.488
Lymphocytes	1.538	1.134-2.085	0.006*	0.470	0.225-0.981	0.044*	0.552	0.237-1.286	0.169	1.087	0.569-2.075	0.801
Platelets	1.000	1.000-1.000	0.501	1.000	1.000-1.000	0.027*	1.000	1.000-1.000	0.339	1.000	1.000-1.000	0.459
BUN	0.996	0.984-1.008	0.501	0.968	0.937-1.000	0.049*	1.013	1.000-1.027	0.055	0.981	0.943-1.021	0.349
Creatinine	0.920	0.799-1.059	0.245	0.964	0.817-1.138	0.666	1.132	1.001-1.279	0.048*	0.885	0.612-1.279	0.515
Bilirubin total	1.016	0.980-1.054	0.394	0.975	0.890-1.069	0.593	1.007	0.952-1.065	0.808	0.755	0.413-1.382	0.363
Bilirubin direct	1.021	0.925-1.126	0.684	0.925	0.746-1.146	0.476	1.063	0.943-1.200	0.317	0.750	0.353-1.597	0.456
AST	1.003	1.000-1.005	0.017*	1.002	0.999-1.004	0.144	0.988	0.975-1.001	0.063	1.001	0.997-1.005	0.655
ALT	1.004	1.001-1.007	0.007*	1.001	0.997-1.004	0.795	0.989	0.973-1.005	0.162	1.003	0.999-1.008	0.177
Alk phos	1.005	1.002-1.008	0.000*	0.991	0.983-0.999	0.020*	0.997	0.992-1.003	0.322	0.999	0.993-1.005	0.798
Albumin	1.024	0.979-1.072	0.292	0.989	0.902-1.084	0.814	0.973	0.787-1.205	0.805	0.714	0.210-2.431	0.590
CRP	1.003	0.997-1.010	0.313	0.968	0.954-0.982	0.000*	1.006	0.995-1.017	0.300	1.001	0.988-1.015	0.869

PCT	0.983	0.963-1.004	0.107	0.948	0.891-1.009	0.091	1.012	0.996-1.027	0.133	0.980	0.928-1.035	0.475

**Significant predictor variable on univariate logistic regression analysis*

NB – Unless specified, for binary categorical variables, 0=no or absent, 1=yes or present

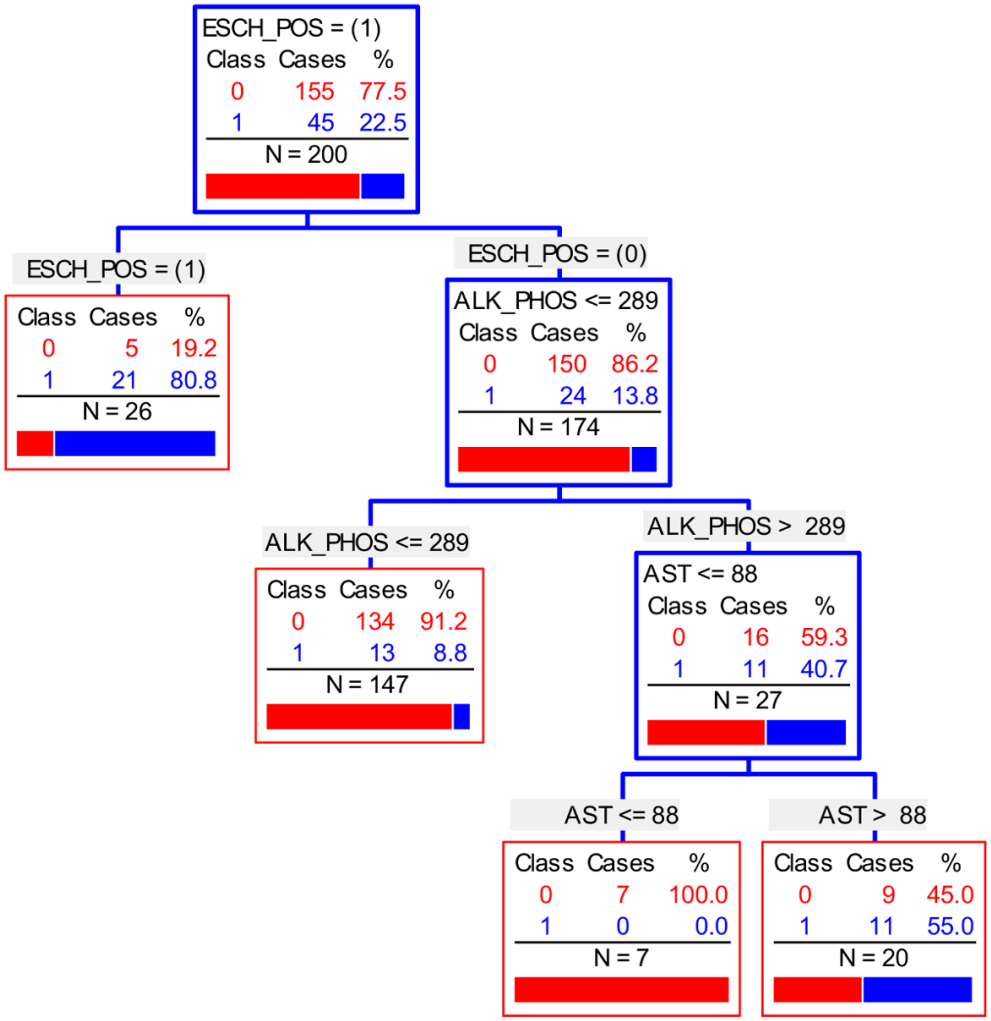
B	***Significant predictor variables associated with each diagnosis on multivariate logistic regression analysis***		
	aOR	**95% CI OR**	**P value**
Scrub typhus			
- Eschar	42.408	4.956-362.905	0.001
- Lymphocytes	2.063	1.146-3.713	0.016
- AST	1.014	1.004-1.023	0.004
- Alk phos	1.004	1.000-1.008	0.036
Dengue			
- CRP	0.956	0.927-0.986	0.005

NB – no variables were significantly associated with leptospirosis or murine typhus on multivariate logistic regression analysis in this study

– Unless specified, for binary categorical variables, 0=no or absent, 1=yes or present

In addition, classification and regression trees (CART) were generated for scrub typhus (Figure 38) and dengue (Figure 39) which revealed a similar set of significant variables when compared with multivariate logistic regression analyses above. The presence of an eschar, ALP>289IU/L and AST>88IU/L were used as decision nodes for scrub typhus while CRP≤37mg/L and WBC≤7.9x10^3/mm^3 were used for dengue virus infection. Area under ROC curves for both models were acceptable: 0.719 (95% CI 0.622-0.815) for scrub typhus and 0.784 (95% CI 0.661-0.907) for dengue.

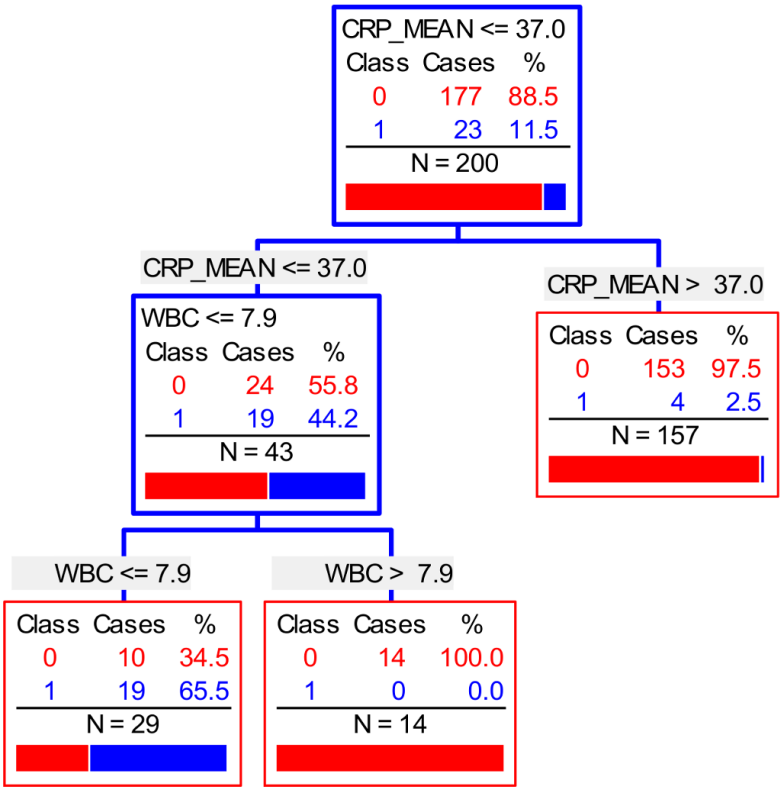
Figure 38. Classification and regression tree (CART) analysis for scrub typhus.



Classification and regression tree (CART) – Scrub typhus

Area under ROC curve (lower and upper confidence limits) = 0.71878 (0.62214-0.81542)

Figure 39. Classification and regression tree (CART) analysis for dengue.



Classification and regression tree (CART) – Dengue

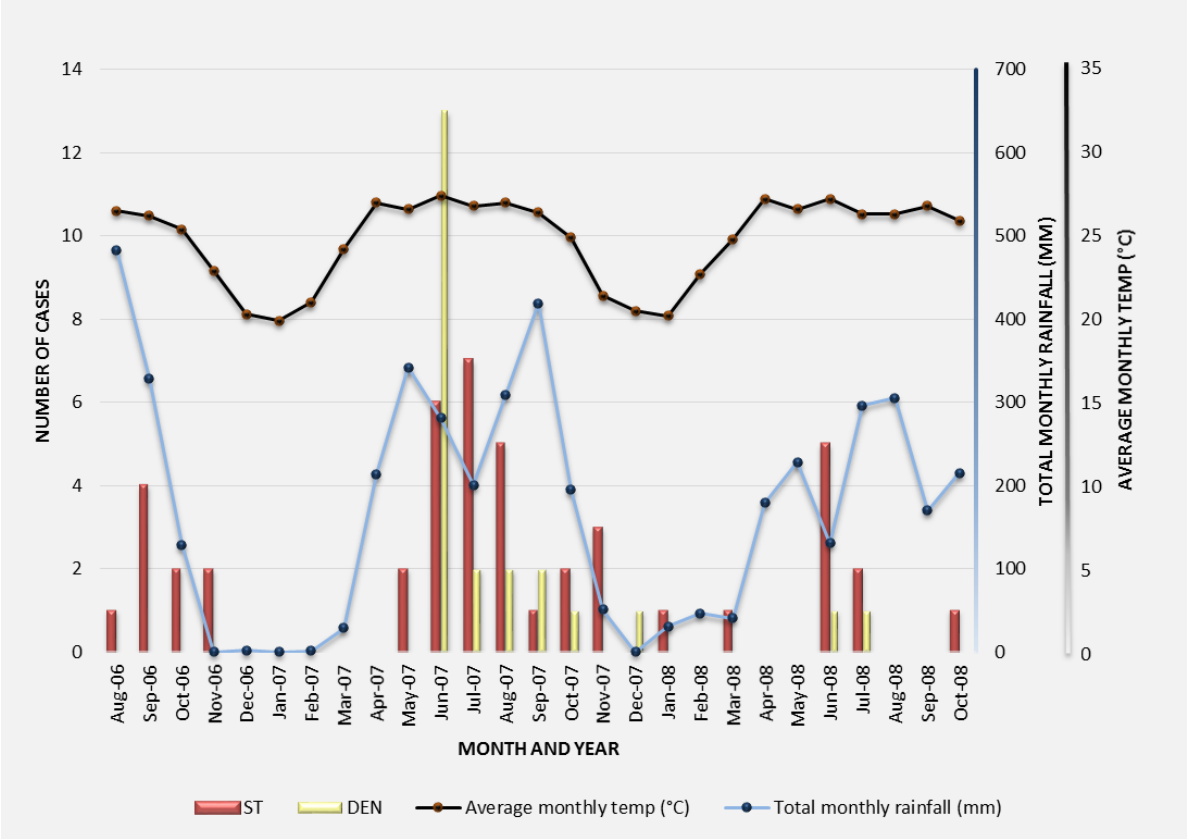
Area under ROC curve (lower and upper confidence limits) = 0.78384 (0.66055-0.90712)

4.3.3 Seasonality and deaths

Over 80% of the study cohort were admitted from June to November, coinciding with the rainy and beginning of the cool seasons. Proportions of patients admitted from June to November were compared to patients admitted from December to May for the study cohort, scrub typhus, dengue, leptospirosis, murine typhus, culture attributed infections and unknown groups: study cohort 0.82 (0.77-0.88):0.18 (0.12-0.23) p<0.001, scrub typhus 0.91 (0.83-0.99):0.09 (0.01-0.17) p<0.001, dengue 0.96 (0.87-1.00):0.04 (0.00-0.13) p<0.001, leptospirosis 0.80 (0.60-1.00):0.20 (0.00-0.40) p=0.001, murine typhus 0.57 (0.20-0.94):0.43 (0.06-0.80) p=0.595, CAI 0.83 (0.62-1.00):0.17 (0.00-0.38) p=0.04 and unknown 0.77 (0.68-0.86):0.23 (0.14-0.32) p<0.001. To illustrate further, cases of scrub typhus and dengue were plotted over time along with mean monthly temperature and total monthly rainfall in Figure 40.

Nine deaths were recorded: 3 patients had scrub typhus (3/45 scrub typhus cases, 6.7%), 2 had bloodstream infections (*Talaromyces marneffei* and *Haemophilus influenzae*, both HIV positive patients) and 4 deaths were in the unknown aetiology group. There were 5 men and 4 women, median age of 44 (IQR 40-51), 7 had an agricultural background and none were known to have received pre-admission antibiotics. 8 out of 9 patients were treated with antibiotics on admission. Patients were febrile on average 4 days (IQR 3-6) prior to admission and had comparatively shorter final admission duration (3 days, IQR 2-4). The common features in those who died included fever (8/9, 88.9%), neurological (7/8, 87.5%), respiratory (5/8, 62.5%) or gastrointestinal findings (5/8, 62.5%). 5/8 (62.5%) were classified as having severe disease on admission. The laboratory markers of infection reflected the severity of disease in this cohort (median, IQR): CRP 150 mg/L (149-150), PCT 37.2 ng/mL (2.0-59.7), WBC 10.2x$10^3/mm^3$ (8.3-14.8) and neutrophil count 9.3x$10^3/mm^3$ (6.9-12.2).

Figure 40. Temporal spread of scrub typhus and dengue cases and monthly meteorological data for Mueang district, Chiangrai.



4.3.4 Antibiotic use

A total of 84.5% (169/200) patients received antibiotics, including pre-admission and during admission to the study hospital (some patients were transferred from a district hospital). Thirty-one patients who did not apparently receive antibiotics had a diagnosis of dengue (7 patients), a bacterial infection (5 scrub typhus, 1 leptospirosis, and 1 bacteraemia) or the aetiology was unknown (18 patients). When a single antibiotic was used, intravenous ceftriaxone was the most frequently prescribed (131/169, 77.5%) followed by oral doxycycline (118/169, 69.8%) and intravenous chloramphenicol (26/169, 15.4%). Many patients received a combination of antibiotics during admission (105/168, 62.5%) while only 26.5% (9/34) received more than one antibiotic pre-admission. The most common combination was IV ceftriaxone and oral doxycycline (79/169, 46.7%).

The percentage of patients receiving antibiotics for the viral, bacterial and unknown infection groups were 75% (18/24), 93.3% (70/75) and 92.9% (79/85), respectively. Patients with scrub typhus or murine typhus (rickettsial infections) usually received appropriate antibiotics (e.g. doxycycline or chloramphenicol) – 42/51 (82.4%) patients – although more than 1 in 6 did not receive effective treatment. In contrast, almost all patients with leptospirosis received effective treatment with ceftriaxone +/- doxycycline – 14/15 (93.3%). Antibiotic use pre-provincial hospital and during admission is shown in Table 10. The combination of a third generation cephalosporin with an anti-rickettsial antibiotic was commonly used at the study hospital site. It is also interesting to note that patients with dengue were frequently treated with anti-rickettsial antibiotics, emphasising once again the difficulties faced by clinicians in differentiating dengue and rickettsial infections, mainly scrub typhus.

Table 10. Overview of antibiotic treatment before admission to the provincial hospital and during admission at the study site.

	3rd generation Cephalosporin + anti-rickettsial antibiotic(s)*	Ceftriaxone only	Doxycycline only	Chloramphenicol only	Other anti-rickettsial treatment#	Other antibiotic(s)	None
Scrub and murine typhus	3.7% (1/27)	18.5% (2/27)	0% (0/27)	7.4% (2/27)	0% (0/27)	3.7% (1/27)	66.0% (18/27)
	52.9% (27/51)	7.8% (4/51)	19.6% (10/51)	7.8% (4/51)	2.0% (1/51)	2.0% (1/51)	7.8% (4/51)
Leptospirosis	0% (0/8)	12.5% (1/8)	0% (0/8)	0% (0/8)	0% (0/8)	0% (0/8)	87.5% (7/8)
	73.3% (11/15)	20.0% (3/15)	0% (0/15)	0% (0/15)	0% (0/15)	0% (0/15)	6.7% (1/15)
Dengue and JEV	0% (0/8)	25% (2/8)	0% (0/8)	0% (0/8)	0% (0/8)	0% (0/8)	75% (6/8)
	29.2% (7/24)	4.2% (1/24)	33.3% (8/24)	4.2% (1/24)	4.2% (1/24)	0% (0/24)	25% (6/24)
CAI (bacterial)	0% (0/6)	33.3% (2/6)	0% (0/6)	0% (0/6)	16.7% (1/6)	0% (0/6)	50% (3/6)
	66.7% (6/9)	33.3% (3/9)	0% (0/9)	0% (0/9)	0% (0/9)	0% (0/9)	0% (0/9)
Unknown aetiology	5.7% (3/53)	17.0% (9/52)	3.8% (2/53)	0% (0/53)	1.9% (1/53)	3.8% (2/53)	67.9% (36/53)
	50.0% (42/84)	16.7% (14/84)	14.3% (12/84)	2.4% (2/84)	3.6% (3/84)	6.0% (5/84)	7.1% (6/84)

Unshaded line = pre-study hospital treatment, shaded line = study hospital treatment, CAI = culture-attributed infections

* Anti-rickettsial antibiotics = doxycycline, chloramphenicol, roxithromycin and ciprofloxacin (see discussion)

Other anti-rickettsial treatment = a combination of the antibiotics above (e.g. doxycycline and chloramphenicol) or a combination of 1 anti-rickettsial antibiotic with other antibiotics (e.g. amoxicillin and roxithromycin)

4.3.5 CRP and procalcitonin

Table 11 displays the biomarker results from this study. Low CRP was a significant predictor for the viral diagnostic group, mainly dengue. In cases with known diagnoses, high CRP was also a significant predictor for bacterial infections. 92% and 86% of bacterial cases had CRP levels above the pre-defined cut-offs of >20mg/L and >40mg/L, respectively. For the viral infections, 73% and 86% of cases had CRP levels below these cut-offs, respectively. The >20mg/L and >40mg/L CRP cut-offs correctly identified 87.2% and 86.2% of bacterial and viral cases, respectively. CRP plasma level of >36mg/L was determined to be the optimal cut-off to delineate bacterial from viral infections [sensitivity 88.9% (95%CI 79.3-95.1) and specificity 86.4% (95%CI 65.1-97.1)] with 88.3% of cases correctly identified.

Procalcitonin was generally less accurate than CRP at differentiating bacterial from viral infections, suffering significantly from lower specificity. The higher cut-off of 0.50ng/mL improved specificity when compared to 0.25ng/mL but was accompanied by a moderate fall in sensitivity and minor fall in correctly identified cases. If 0.70ng/mL was used as the cut-off, the accuracy would be sensitivity 79.2% (68.0 – 87.8), specificity 68.2% (45.1 – 86.1) and correctly identified cases 76.6%, which remains well below the performance of CRP.

Receiver operating characteristic (ROC) curves were generated for the use of CRP and PCT in differentiating bacterial versus viral infections and are shown in Figure 41 and 42. The areas under the ROC curve were 0.91 (0.85-0.96, 95% CI) and 0.80 (0.72-0.88, 95% CI) for CRP and PCT, respectively.

Table 11. Performance of CRP and procalcitonin at different cut-offs.

Value	Bacterial aetiology n (%)	Viral aetiology n (%)	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	Correctly identified (%)
CRP >20 mg/L*	66/72 (91.7)	6/22 (27.3)	91.7 (82.7-96.9)	72.7 (49.8-89.3)	91.7 (82.7-96.9)	72.7 (49.8-59.3)	87.2%
CRP >40 mg/L*	62/72 (86.1)	3/22 (13.6)	86.1 (75.9-93.1)	86.4 (65.1-97.1)	95.4 (87.1-99.0)	65.5 (45.5-82.1)	86.2%
PCT >0.25 ng/mL*	65/72 (90.3)	13/22 (59.1)	90.3 (81-96.0)	40.9 (20.7-63.6)	83.3 (73.2-90.8)	56.3 (29.9-80.2)	78.7%
PCT >0.50 ng/mL*	58/72 (80.6)	8/22 (36.4)	80.6 (69.5-88.9)	63.6 (40.7-82.8)	87.9 (77.5-94.6)	50.0 (30.6-69.4)	76.6%

* Significant difference ($p \leq 0.001$) between bacterial and viral groups at these cut-offs on direct comparison (Chi-squared test)

Figure 41. ROC curve for plasma CRP levels in differentiating bacterial from viral infections.

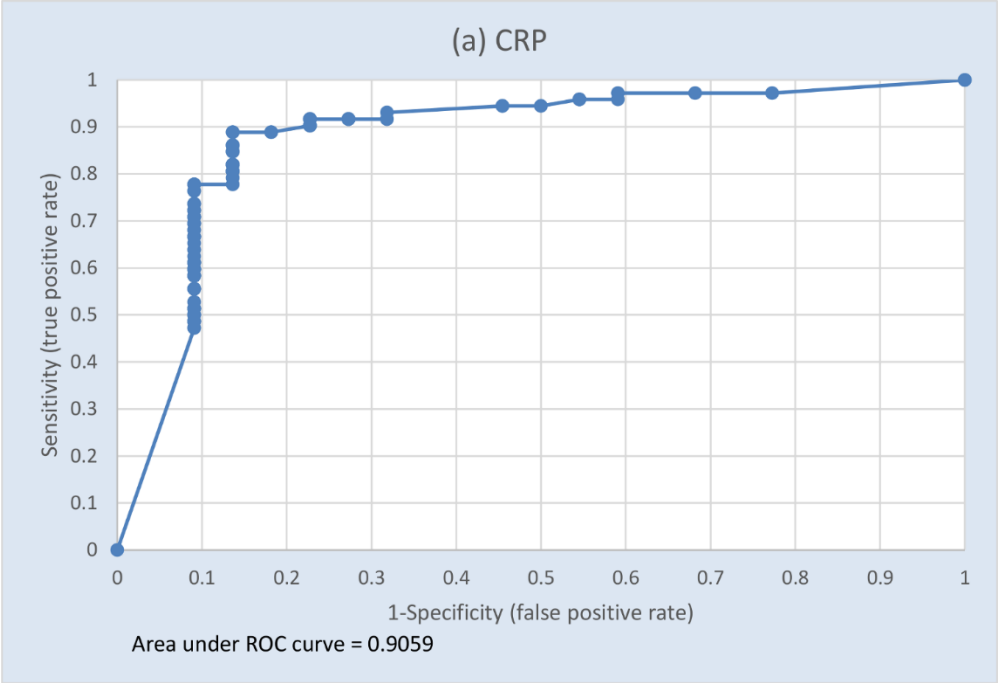
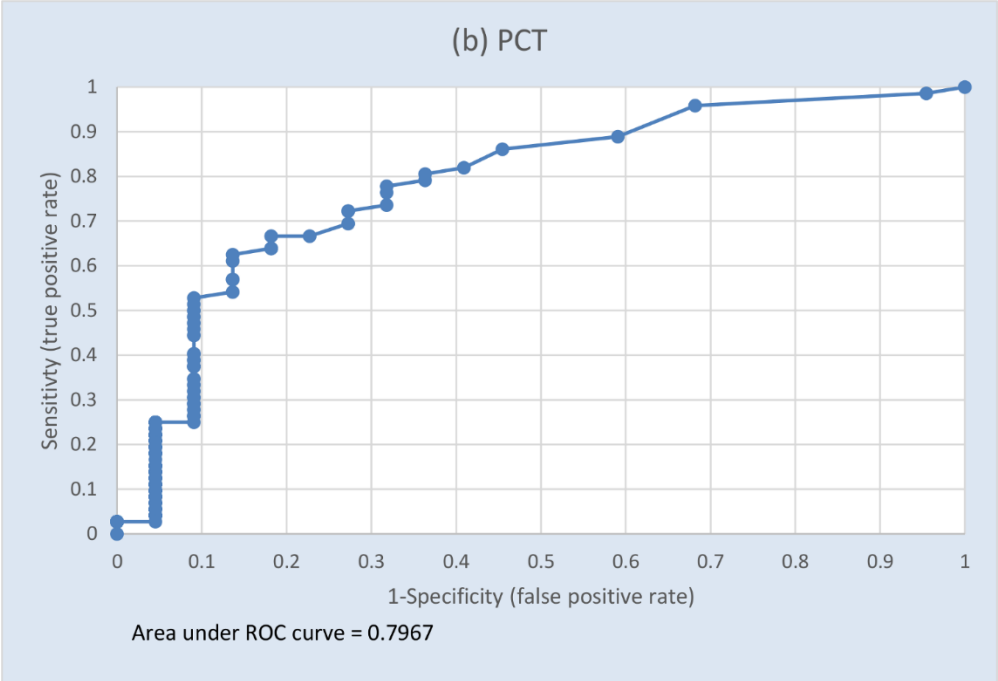


Figure 42. ROC curve for plasma procalcitonin levels in differentiating bacterial from viral infections.



Discussion

In this study, we were able determine the cause of acute undifferentiated fever in 51.5% of patients recruited. This low percentage is not unusual for cause of fever studies and is comparable to other similar studies from Southeast Asia in which diagnosis was determined in 41% to 73.3% of study patients [242, 243, 246, 247, 298, 299]. Scrub typhus contributes significantly to the febrile disease burden in Chiangrai and reflects surveillance data studied in chapter 3. By improving local diagnostic capacity to include testing for scrub typhus, dengue, leptospirosis and murine typhus and continuing conventional microbiological cultures, over half of acute undifferentiated fever would be expected to be determined.

Scrub typhus, the leading cause of acute undifferentiated fever

Scrub typhus was diagnosed in 22.5% of the study cohort and exhibited pronounced seasonality, being more common in the wet and early cool seasons. Presence of an eschar remains an important diagnostic clue and reflects previous findings [117, 244, 318]. However, eschars are not always present with past exposure to *Orientia tsutsugamushi* strains and degree of immunity determining whether an eschar forms at the site of the chigger feeding [109]. Eschar rates were observed to be variable between adults and children and in different parts of Thailand. Eschars were found in 70% of paediatric scrub typhus cases in northern Thailand while it was only detected in 7% of children in southern Thailand [200, 319, 320]. It was described in only 7% of adults in northeastern Thailand [228]. In our study cohort, 46.7% of febrile adults with scrub typhus had eschars. Conversely, five patients presented with eschars but tested negative for scrub typhus (1 with *Staphylococcus aureus* bacteraemia and 4 unknown aetiology). Interestingly, additional analysis of blood samples from one of these patients revealed positive 17kDa qPCR

suggestive of infection with *Rickettsia* sp. Therefore, although scrub typhus remains the most important eschar-associated febrile disease in the region, alterative causes such as spotted fever rickettsial infections (e.g. *Rickettsia conorii*, *Rickettsia honei*, and *Rickettsia felis*) should be considered. Clinically, it may be difficult to differentiate a true eschar from another skin lesion (including other insect bites) but eschars are completely painless whereas other causes may be associated with pain or pruritus.

Elevated ALP and AST were also found to be predictive of a scrub typhus diagnosis in febrile adults, which have been previously described in observational studies from northern Thailand and India [6, 136, 321]. Overall mortality was 4.5% and for scrub typhus, 6.7%. This was much higher than results from a recent systematic review where the median mortality was 1.4% for treated disease and 6.0% for untreated disease but lower than previously recorded mortality from northern Thailand of 13.1% from 2004-2010 [6, 7].

Antibiotic use and implications

The majority of patients received antibiotics once admitted to the study hospital (84.5%) and most patients with bacterial infections were treated appropriately. However, patients subsequently found to have scrub typhus or murine typhus were only treated correctly 82.4% of the time while for leptospirosis, this figure was 93.3%. Doxycycline and chloramphenicol were the main antibiotics used to treat rickettsial infections and the majority of patients with scrub typhus recovered despite previous reports of drug resistance [178]. Of the 3 scrub typhus patients who died, one did not receive appropriate antibiotics, one received chloramphenicol after a delay and one received treatment with oral doxycycline and IV chloramphenicol from admission. These observations suggest a high level of clinical

experience and awareness at the provincial hospital although additional diagnostic tools may help strengthen quality of care further.

Antibiotics such as doxycycline, chloramphenicol and rifampicin are effective for both scrub typhus and murine typhus [100]. Roxithromycin was used in one scrub typhus patient in combination with doxycycline. There are limited clinical studies on the effectiveness of roxithromycin in the treatment of scrub typhus and none reported for murine typhus [199, 200]. One case series from Chiangrai reported low efficacy of roxithromycin when compared to doxycycline or chloramphenicol in 20 children with scrub typhus [200]. *In vitro* susceptibility testing to roxithromycin has not been reported for *Orientia tsutsugamushi* while *Rickettsia typhi* appears susceptible [100]. Ciprofloxacin was used in two scrub typhus patients, one as the sole anti-rickettsial antibiotic and the other in combination with doxycycline and chloramphenicol. Fluoroquinolones have been shown to be moderately effective for murine typhus *in vitro* with limited efficacy data from clinical studies [100, 220, 322]. However, *Orientia tsutsugamushi* may be intrinsically resistant to fluoroquinolones which may explain the poor efficacy reported in clinical studies [201, 202, 208, 323].

Only 17% (34/200) of patients received antibiotics (or stated that they had) prior to admission to the provincial hospital. Of these 34 patients, only 5 received appropriate treatment for scrub typhus, a diagnosis which made up 22.5% of our febrile study cohort. Supporting this finding was a retrospective survey carried out between January 2015 and December 2016 on antibiotic use in primary care from the central Mueang District of Chiangrai province [324]. 39,242 patients received antibiotics and use of doxycycline was absent altogether. Even though roxithromycin and erythromycin were prescribed in 1.2% and 0.7%, respectively, there is little evidence to support their efficacy for scrub typhus infections [193, 325]. Tetracycline, although efficacious against scrub typhus, was used in 0.15% of patients for other indications such as acne or skin infections. These results

suggest that scrub typhus patients are not treated promptly due to limited access to appropriate antibiotics in the community, which may be contributing to prolonged illness duration and poorer outcomes.

Differentiating bacterial and viral infections

It remains difficult to differentiate common tropical infections based on symptoms and signs alone. It is also impractical and unlikely to be cost-effective for costly and expertise-reliant assays (e.g. IFAs, ELISAs and PCR) to be performed routinely in all hospitals [326]. Thus, the development of accurate and affordable disease-specific RDTs should be prioritised and guided by local, up-to-date, epidemiological data. In Chiangrai, the panel of RDTs should include those for scrub typhus, dengue, leptospirosis and murine typhus if possible. Indeed, RDTs for the first three diseases are now available locally although their accuracy in real-world settings, particularly for scrub typhus and leptospirosis, remains a work in progress.

Low CRP and WBC were shown to be significant predictors of a viral infection (mainly dengue) in this study. When directly comparing the viral and bacterial diagnostic groups, CRP was highly sensitive and specific in differentiating the two. We investigated two cut-offs in this study – 20mg/L and 40mg /L. The higher cut-off improves specificity by almost 14% with a small fall in sensitivity, suggesting the higher cut-off improves diagnostic accuracy. However, there are a couple of caveats. The reduction of incorrectly treated viral cases from 6/22 (27.3%) to 3/22(13.6%) is offset by “missing” 4/72 (5.6%) of potentially severe bacterial cases. In other words, reducing 3 cases with inappropriate antibiotic treatment comes at a cost of not treating 4 patients that could potentially lead to severe disease and death. Therefore, in this inpatient context, it is important to balance the risk between development of antibiotic resistance from unnecessary antibiotic use and adverse outcomes in

withholding antibiotics from a patient with an appropriate indication for treatment. The second caveat relates to the study setting. Patients admitted to hospitals are generally sicker with more severe disease than patients reviewed in the community. CRP levels will mirror this and thus, the pre-test probability of a patient having a raised CRP in the community is lower than the hospital setting, reducing its accuracy as a diagnostic tool. The CRP cut-offs studied here should not be translated directly to community settings (where lower thresholds are likely to be needed) and will require further investigation.

Procalcitonin performed less well in differentiating viral from bacterial infections and was limited by the low specificity. Elevated WBC was significantly associated with bacterial infections in Laos and elsewhere [246]. There have also been multiple reports from high-income settings of associations between neutrophilia, lymphopaenia and elevated neutrophil-to-lymphocyte ratio with bacteraemic medical emergencies in high-income settings [327-329]. Our results suggest that routine laboratory tests such as full blood count and CRP could be used to differentiate bacterial and viral infections in acutely febrile patients at the hospital level. A CRP-based point-of-care test is likely to be cost-effective in community settings in rural Southeast Asia although further validation of different cut-offs in this context are needed. As Thailand expands its community health care system to fulfil one of five core priorities in partnership with the World Health Organization – this information is relevant to the development and commissioning of laboratory and diagnostic services in community and rural hospital settings [330].

Limitations

A large proportion of the study cohort remained undiagnosed. However, elevated laboratory markers including CRP were comparable to the bacterial aetiology group, suggesting that a

significant number of patients with potentially antimicrobial treatable diseases remain undiagnosed. Cultures were performed locally in the hospital microbiology laboratory at the discretion of the treating physician. Therefore, not all patients had blood cultures collected and the local hospital laboratory quality assurance system may have been inadequate [331]. HIV tests were not uniformly performed and was limited to patients with a high suspicion for infection. Financial constraints restricted the diagnostic panel, which did not include testing for other common causes of fever such as influenza, other respiratory viruses and tuberculosis. There was an imbalance in the diagnostic assays performed due to costs, accessibility of accurate tests and availability of technical expertise which may have led to bias. For example, scrub typhus diagnosis used PCR, culture and serology while only culture and RDTs were used to diagnose leptospirosis. Finally, the sample size is relatively small and external validity limited although the data remains useful for meta-analysis and for comparing datasets from other published fever studies from the region.

Conclusion

In this chapter, I have highlighted the importance of scrub typhus and dengue in the aetiology of acute undifferentiated fever in Chiangrai. Scrub typhus contributes significantly to the febrile disease burden and reflects the national surveillance data studied in the previous chapter. I was able to outline the clinical characteristics of scrub typhus in adults and determine the features predictive of diagnosis. I have provided further evidence that antibiotics effective for scrub typhus should be included in empirical hospital treatment guidelines locally and be accessible to patients in the community. CRP testing was shown to be a useful tool for differentiating viral from bacterial infections and along with disease-specific RDTs, could significantly improve patient care while reducing antibiotic use.

5 Paediatric scrub typhus in northern Thailand

5.1 Introduction

The previous chapters have highlighted the burden of scrub typhus in northern Thailand and how this is reflected in the febrile disease burden locally at the provincial hospital in Chiangrai. In chapter 3, I described how the number of reported scrub typhus cases in children have remained relatively static over the last decade (around 1,000-2,000 cases per year) although the disproportionate increase in adult cases created the illusion that the burden of disease in children was falling. In previously reviewed studies, scrub typhus was found to be a major cause of acute non-malarial fever in children in Southeast Asia [242, 246, 247]. Historically, the burden of scrub typhus in children has been high. From 1932-1938, 74% of scrub typhus cases on the Pescadores Islands, Taiwan, were in children age <15 years old while in 1976, 52% of children were found to be seropositive for scrub typhus in central Thailand [332, 333]. Before 1987, around half of the cases in southern China occurred in children age <10 years old [334].

It remains a potentially severe but treatable disease in febrile adults with key clinical features described in chapter 4. These features included a prolonged period of fever prior to admission, headache, eschar, cough or dyspnoea, myalgia, hepatomegaly and abdominal pain. Pulmonary infiltrates were reported in over a fifth of patients who had chest x-rays and elevated hepatic enzymes and CRP were often observed. Of these features, presence of eschar and elevated AST and ALP were most predictive for scrub typhus while mortality in adults was 6.7%.

Are the clinical features observed in adults (aside from fever) similar to those in children? There is some evidence that they may differ. Studies from northern Thailand suggest that presence of eschar, lymphadenopathy, hepatomegaly and cough were more frequently

observed in children than in adults [200, 319]. Raised AST and ALT and interstitial infiltrates on chest x-ray were also more prevalent in children with scrub typhus than our findings in adults [200, 335]. In southern Thailand, hepatomegaly was found in 59% of paediatric scrub typhus patients [320]. In southern India, hepato-splenomegaly, eschar and oedema were seen in over half the children diagnosed with scrub typhus [336]. Presence of eschar and lymphadenopathy were often found in children with scrub typhus is South Korea and elevated CRP, erythrocyte sedimentation rate (ESR), AST and ALT were described in over 75% of the cohort [197]. In Taiwan, cough, anorexia, eschar, chills and lymphadenopathy were found in over 60% of children while hepatitis and pneumonitis were the main complications observed in over half the cohort [337]. Finally, in northern China children with scrub typhus presented commonly with headache, rash, eschar, lymphadenopathy and gastrointestinal symptoms in the majority of the study cohort [338].

Data from animal and human live vaccine studies allow us to approximate that children may be at greater risk of developing severe disease due to the higher likelihood of pre-existing immunity being absent [109, 110]. This could explain the higher prevalence of clinical symptoms and signs and greater complication rate observed. Despite this, are antibiotics as effective in children as in adults? In chapter 2, I described how adult scrub typhus infections poorly responsive to treatment with doxycycline or chloramphenicol, attributed to antibiotic resistance, were first reported from Chiangrai in 1996 [178]. However, two subsequent studies in children from northern Thailand revealed that doxycycline or chloramphenicol were uniformly effective in treating scrub typhus [200, 319]. More recently, a randomised controlled trial comparing the treatment of paediatric scrub typhus with doxycycline or chloramphenicol with azithromycin in Chiang Rai revealed no significant differences in efficacy [176]. However, treatment failure occurred in 17.5% of the patients studied [176]. It remains unclear which factors determine treatment outcome.

Studies of paediatric scrub typhus employing stringent diagnostic criteria are scarce. The lack of access to diagnostic tests, such as the gold standard indirect immunofluorescence assay (IFA), polymerase chain reaction (PCR) and *in vitro* culture, has contributed to this. In the reviewed studies above, many were retrospective, had limited or no follow-up period, lacked a control group and there was heterogeneity in the diagnostic criteria used. For example, the Weil-Felix assay and IgM ELISA were frequently used in India while the IFA and PCR assays were more commonly used elsewhere.

All of these assays have inherent weaknesses [139]. Of the serological tests, the Weil-Felix test lacks sensitivity and specificity while the perceived accuracy of other serological tests (e.g. enzyme-linked immunosorbant assay – ELISA, IFA) depend on the antigenic compliment of the assay with the infecting bacterial strains and the extent of residual antibodies in the population within endemic areas [151, 291]. PCR assays are specific but sensitivity can vary, depending on the quality of the samples, disease time-point, and bacterial load [151]. Moreover, the limited validation studies performed to date (e.g. IFA and ELISA diagnostic cut-offs in endemic and non-endemic regions) have focused on adult samples, generating a degree of uncertainty regarding the accuracy of these assays in children.

The aim of this study is to characterise paediatric scrub typhus and explore the determinants of treatment outcome in the location where suboptimal response was previously reported. To achieve these aims, a prospective observational study on paediatric scrub typhus was performed in Chiangrai, incorporating a healthy control group and a prolonged follow-up period. To aid our efforts, robust and validated diagnostic criteria not previously applied to paediatric populations were used to optimise diagnostic accuracy.

5.2 Method

5.2.1 Ethics statement and setting

Ethical approval for the study was obtained from the ethics committees of Chiang Rai Prachanukroh Hospital and Chiang Rai Provincial Public Health Office, Ministry of Public Health, Thailand; Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand; and University of Oxford (Oxford Tropical Research Ethics Committee), Oxford, UK. The study was registered at ClinicalTrials.gov: Identifier - NCT02398162.

Chiangrai Prachanukroh Hospital is located in Chiangrai province, the northernmost province in Thailand bordering Myanmar and Laos (**Latitude**: 19°90′07″ N **Longitude**: 99°82′70″ E **Elevation** above sea level: 401 m = 1315 feet). It is the main provincial hospital serving the main central Mueang district although it also accepts patients requiring escalation of care from district hospitals or private healthcare facilities and can directly admit severely ill patients from the entire province. Currently, the official capacity is 756 beds although recent additions and building work has increased this further.

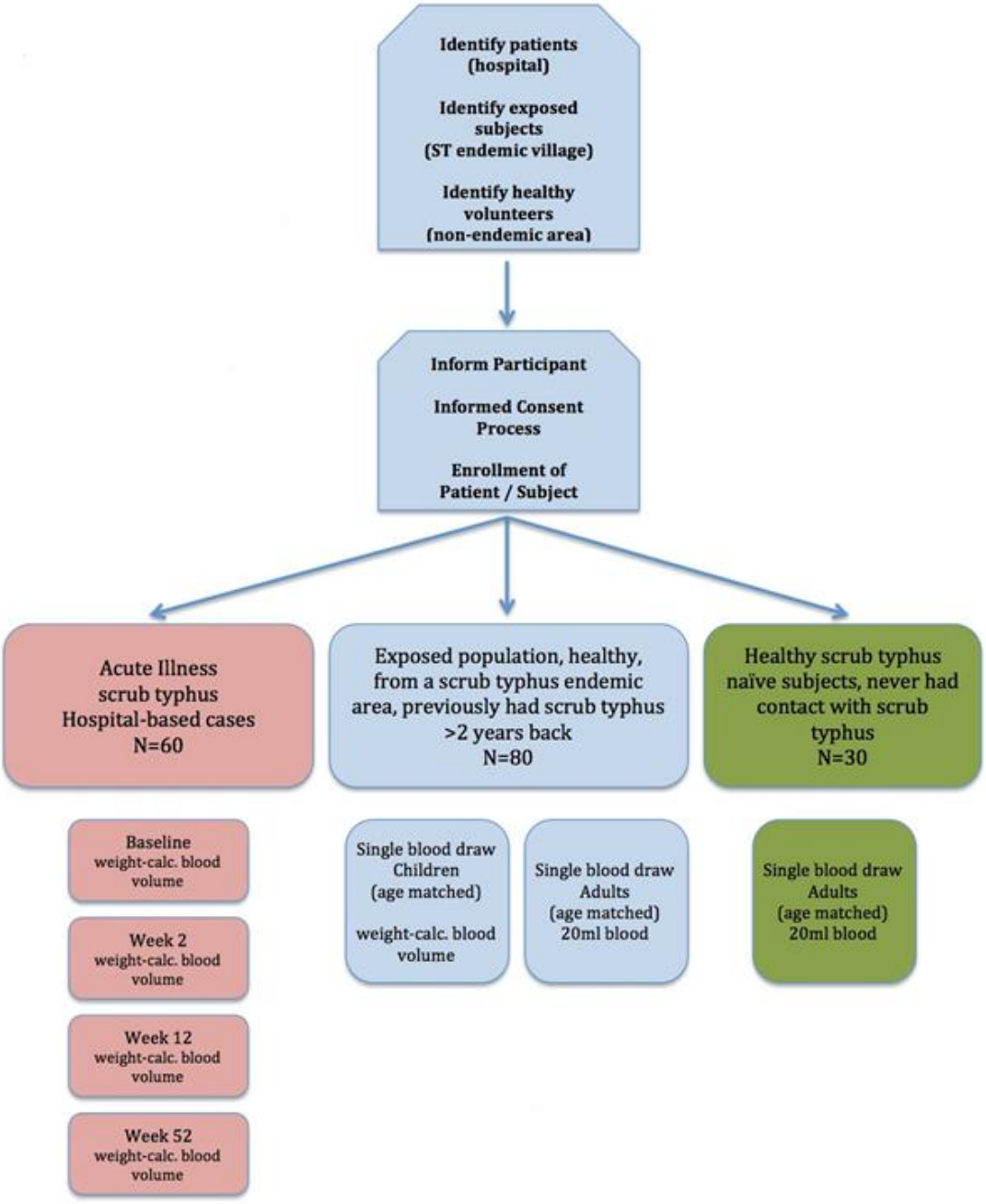
5.2.2 Study attributions and design

Study conception and design were performed by Prof Daniel Paris and Prof Nicholas Day with significant contributions from me. We are indebted to Dr Chulapong Chanta, Dr Supalert Nedsuwan and Dr Achara Laongnualpanich at Chiangrai Prachanukroh Hospital who were involved as local investigators and assisted with logistics. I was the acting Principal Investigator and responsible for obtaining EC approvals, designing the study case record form (CRF) and screening and sample logs, designing and testing the study database using the online data entry system MACRO (previously InferMed Ltd, London; now owned by Elsevier; version 4), data entry and verification, formulating and curating the laboratory protocols used during the study, organising and running the study initiation visit, initiating the study and coordinating the study until completion, organising study monitoring visits and performing patient follow-up and sample collection.

I was ably assisted by staff at Chiangrai Clinical Research Unit including Nidanuch Tasak, Piangnet Jaiboon, Suthathip Kaewta, Areerat Thaiprakong and Nattapon Pinthong. Dr Rachel Greer also assisted with data entry and validation. I was well supported in running the study by MORU's Clinical Trials Support Group (CTSG), particularly Pasathorn Sirithiranont (trial data manager), Noppawan Lunjarungsimalert (general support), Prayoon Yuentrakul (study monitor) and Zoe Doran (Head of CTSG).

The study flowchart is shown in Figure 43. The trial incorporated investigational aspects to study the natural immune response to scrub typhus and thus, also included the recruitment of healthy exposed children and adults (resident in an endemic region of Thailand i.e. Chiangrai) and healthy non-exposed adults (resident in Bangkok). For children, efforts were made to match age, sex and location of controls to patients. The immunological aspects along with other pathophysiological features (e.g. disease severity markers) are beyond the scope of this thesis and will be discussed in the Conclusions and future work chapter.

Figure 43. Paediatric scrub typhus study flowchart.



5.2.3 Patients, study schedule and data and sample collection

From July 2015 to August 2016, we prospectively recruited 60 children age <18 years old admitted to Chiang Rai Prachanukroh Hospital with fever (temperature >37.5°C) or history of fever within the preceding 14 days, positive scrub typhus IgM rapid diagnostic test (InBios, Seattle, USA), not diagnosed or being treated for tuberculosis, not immunocompromised and not pregnant. Written informed consent was obtained from the parent or guardian alongside assent if the child was aged ≥7 years old. Demographic, clinical and laboratory data were collected individually on study case-record forms (CRF, see Appendix 5). Chest x-ray (CXR) findings were recorded if performed. Fever clearance time (FCT) was defined as the time taken from initiation of appropriate antibiotic treatment (doxycycline, chloramphenicol, tetracycline, azithromycin or rifampicin) to defervescence (temperature ≤37.5°C) with the temperature remaining ≤37.5°C for 24 hours or more after this point. Data was collected on use of anti-pyretics and other anti-inflammatory drugs (e.g. paracetamol, ibuprofen, steroids) as they may affect FCT. Treatment failure was defined as FCT greater than 72 hours following initiation of appropriate antibiotic treatment. Relapse or reinfection was defined as return of fever and other symptoms compatible with scrub typhus with confirmatory laboratory diagnosis as described below.

Follow-up time-points were at 2, 12 and 52 weeks after admission. At each time-point, patients were clinically assessed with details of additional illnesses and treatment recorded. Blood was collected in EDTA and clotted blood tubes and processed to obtain aliquots of EDTA whole blood, plasma, buffy coat and serum. Additionally, blood was collected in heparin and citrate bottles and processed simultaneously for immunological assays (flow cytometry and ELISpot) and disease severity markers, respectively. If present, an eschar swab or crust was collected in 95% ethanol. Samples were stored at -80°C and transported to Bangkok for diagnostic processing on dry ice by overnight transport by road. A study

sample log with details on sample type, number of aliquots, location and storage conditions was maintained by study staff and updated regularly. No personal identifiers were included in the study sample log with only the patient study ID used. Recruitment and follow-up were completed by August 2017.

During the study period, we also recruited 40 children from the community to act as controls. These children were either siblings of patients or children living in areas where scrub typhus cases have occurred. All were healthy, none reported a past history of scrub typhus infection and the majority (32/40, 80%) had no knowledge of the disease. Written informed consent and assent were obtained. Demographic data and blood samples were collected for diagnostic, haematology and biochemistry tests. Data was recorded on dedicated CRF for controls (Appendix 5).

40 healthy adults from Chiangrai (living in scrub typhus endemic area) and 30 healthy adults living in Bangkok (not endemic for scrub typhus) were similarly recruited with identical blood sample types collected. However, their features and results from the immunological assays and disease severity markers will be analysed elsewhere.

5.2.4 Diagnostic assays and attribution of diagnosis

The study screening criteria included the use of the Scrub Typhus Detect™ IgM Rapid Test (InBios International Inc., Seattle, WA, USA), an immunochromatographic-based test utilising recombinant 56kDa type-specific antigen (TSA) of Karp, Kato, Gilliam and TA716 strains of *Orientia tsutsugamushi* [339]. The gold standard indirect immunofluorescence assay (IFA) was used to detect IgM antibody titres in paired plasma samples (or in admission samples only if convalescent samples were unavailable). The antibody targets were pooled *Orientia tsutsugamushi* antigens from Karp, Kato, Gilliam and TA716 strains as previously described [141]. Recently, validated diagnostic IFA cut-off titre of ≥1:3,200 in a single acute sample or ≥4-fold rise to ≥1:3,200 in a convalescent-phase sample was used to determine positivity [143].

Orientia tsutsugamushi specific PCR assays were performed as previously described [340-342]. Briefly, DNA extraction from whole blood, buffy coat or non-invasive eschar samples (if applicable) was performed. Real-time PCR assay to detect the 47kDa *htra* gene was performed in duplicate. If positive, the nested 56kDa TSA gene PCR assay was performed.

In controls, PCR was performed as above and serological testing included a screening ELISA at ≥0.5 net OD cut-off followed by IFA as appropriate [145]. The diagnosis of scrub typhus was made based on robust criteria as described previously: (I) PCR or culture positivity from either blood or eschar samples, (II) 4-fold rise in IgM to ≥1:3,200 in paired serum or plasma samples, or (III) single titre IgM ≥1:3,200 in an acute serum or plasma sample [143, 317]. Diagnostic PCR and serology assays were performed in the main laboratory facilities at Mahidol-Oxford Tropical Medicine Research Unit, Bangkok, Thailand. Serology was performed by Ampai Tanganuchitcharnchai and molecular assays by Manutsanun Sumonwiriya and Tippawan Anantatat. Attribution of scrub typhus diagnosis was performed by me with advice from Prof Stuart Blacksell.

5.2.5 Statistical analysis

Proportions, percentages and averages were calculated (median and interquartile range, IQR, unless specified). Comparison of demographic, exposure and laboratory variables between patient and control groups (STP and STE, respectively) were performed using Mann-Whitney U test, Pearson's Chi-squared test or Fisher's exact test as appropriate. Seasonality was assessed by calculating proportions, 95% confidence intervals and performing two-sample tests of proportions. Fever clearance time was plotted using Kaplan-Meier survival curve. Treatment failure (FCT > 72 hours) was analysed using logistic regression for variables significantly predictive of outcome. Analyses were performed using STATA 15 software (College Station, Texas, USA). I performed these analyses with support from Prof Mavuto Mukaka.

5.3 Results

5.3.1 Diagnostic, demographic and epidemiological findings

Only 35/60 (58%) patients recruited as positive by the scrub typhus IgM rapid diagnostic test were confirmed to have scrub typhus using our diagnostic criteria. This suggests that the performance of the InBios IgM RDT in children was poor with many false positive cases. Thirty-one patients were PCR positive while 34 had positive scrub typhus group IgM antibody titres using IFA above the diagnostic cut-offs. 30/31 (97%) of patients found to be PCR positive were also positive for scrub typhus group IgM, suggesting good concordance between the two diagnostic modalities. 39 healthy paediatric controls were negative for scrub typhus on the diagnostic assays with 1 patient having insufficient blood collected for testing.

Of the 25/60 (42%) children who fulfilled the screening criteria but failed the robust diagnostic criteria, 11 had weakly positive scrub typhus group IgM serology ranging from 1:200 to 1:1,600 titre while 14 had ≤1:100 titre suggesting negative or negligible positivity. 2/11 patients in the weakly positive IgM serology group had eschars detected with corresponding IgM titres of 1:400, suggesting that they may have had an alternative eschar-associated febrile illness such as spotted fever group rickettsia infection rather than true scrub typhus infection. PCR *Orientia tsutsugamushi* results were negative for all 25 children.

In Table 12, the demographic and exposure history data for STP (patients) and STE (controls) groups are shown. STE participants were generally older and more likely to identify as ethnically Thai while STP patients were younger, often belonged to a hill tribe, were more likely to be in contact with chickens and lived in a hilly/mountainous habitat. Although not reaching statistical significance, there was a suggestion that STE participants

had more exposure to ticks while STP patients had more exposure to rats and agricultural land/gardens. None of the patients or controls report to have had scrub typhus in the past.

STP patients belonging to a hill tribe (31/35, 89%) were mainly Akha (20/31, 65%), followed by Lahu (7/31, 23%). Karen, Hmong, Lisu, and Yao contributed 1/31 (3%) patient each. Hill-tribe distribution was less skewed in the controls: Karen 9/24 (38%), Akha 8/24 (33%), Yao or Mien 4/24 (17%), Lahu 3/24 13%), 1/24 unknown (4%) and 1/24 (4%) identifying as both Karen and Akha.

Figure 44 shows the geographical spread of 35 scrub typhus patients within the province by village of residence. Most of the cases were from the rural mountainous western region of the province. There were a few notable clusters which may be representative of multiple high transmission areas. As previously seen in adults, paediatric scrub typhus cases were almost exclusively admitted in the rainy and early cool seasons (Figure 45). Admissions from June to November far outweighed admissions from December to May: 33 versus 2 cases; 0.94 (0.87-1.02):0.06 (-0.02-0.13), $p<0.001$. Figure 47 portrays the temporal spread of cases by month.

Table 12. Demographic and exposure history among scrub typhus paediatric patients and healthy controls from the same endemic region.

	Patients (STP), n = 35	**Healthy controls (STE), n = 40**	***p* value**
Age (years; median, IQR)	6 (3-10)	11.5 (8.5-13.5)	*<0.001*
Sex:			
- Male, n (%)	24 (69%)	21 (53%)	0.156
- Female, n (%)	11 (31%)	19 (48%)	
Ethnicity:			
- Thai, n (%)	21 (60%)	38 (95%)	*<0.001*
- Hill tribe, n (%)	31 (89%)	24 (60%)	*0.008*
- Lao, n (%)	1 (3%)	0 (0%)	0.467
Student, n (%)	29 (83%)	38 (95%)	0.136
Arthropod exposure:			
- Flea, n (%)	8 (23%)	13 (33%)	0.353
- Mite, n (%)	3 (9%)	6 (15%)	0.489
- Tick, n (%)	3 (9%)	10 (25%)	0.073
- Lice, n (%)	1 (3%)	1 (3%)	1.000
Vertebrate exposure:			
- Rat, n (%)	25 (71%)	21 (53%)	0.093
- Cat, n (%)	27 (77%)	27 (68%)	0.353
- Dog, n (%)	25 (71%)	30 (75%)	0.727
- Pig, n (%)	14 (40%)	14 (35%)	0.655
- Cow, n (%)	2 (6%)	1 (3%)	0.596
- Horse, n (%)	1 (3%)	0 (0%)	0.467
- Rabbit, n (%)	1 (3%)	1 (3%)	1.000
- Chicken, n (%)	32 (91%)	26 (65%)	*0.011*
- Duck, n (%)	2 (6%)	0 (0%)	0.214

- Bird, n (%)	0 (0%)	4 (10%)	0.118
- Fish, n (%)	0 (0%)	1 (3%)	1.000
- Squirrel, n (%)	0 (0%)	2 (5%)	0.495
- Snake, n (%)	0 (0%)	1 (3%)	1.000
Visit place:			
- Rice field, n (%)	17 (49%)	22 (55%)	0.578
- Garden, n (%)	27 (77%)	23 (58%)	0.072
- Forest/jungle, n (%)	18 (51%)	17 (43%)	0.439
- Hill, n (%)	25 (71%)	13 (33%)	*0.001*
- Valley, n (%)	5 (14%)	6 (15%)	0.930
- Corn (maize) field, n (%)	2 (6%)	0 (0%)	0.214
- Tea plantation, n (%)	1 (3%)	0 (0%)	0.467
- Palm plantation, n (%)	0 (0%)	1 (3%)	1.000
Prior scrub typhus infection, n (%)	0 (0%)	0 (0%)	-

[STP – Scrub Typhus Patients, STE – Scrub Typhus Exposed healthy controls; exposure in the STP group was in the preceding 2 weeks prior to admission; subjects may have more than 1 ethnicity (e.g. Thai and hill tribe); analysis performed using Pearson's Chi-squared or Fisher's exact test as appropriate]

Figure 44. Location of scrub typhus patients by village of residence. National borders represented by pale yellow line, provincial border by pale grey line. Inset map shows location of Chiangrai town as a red dot and Chiangmai city as a black dot.

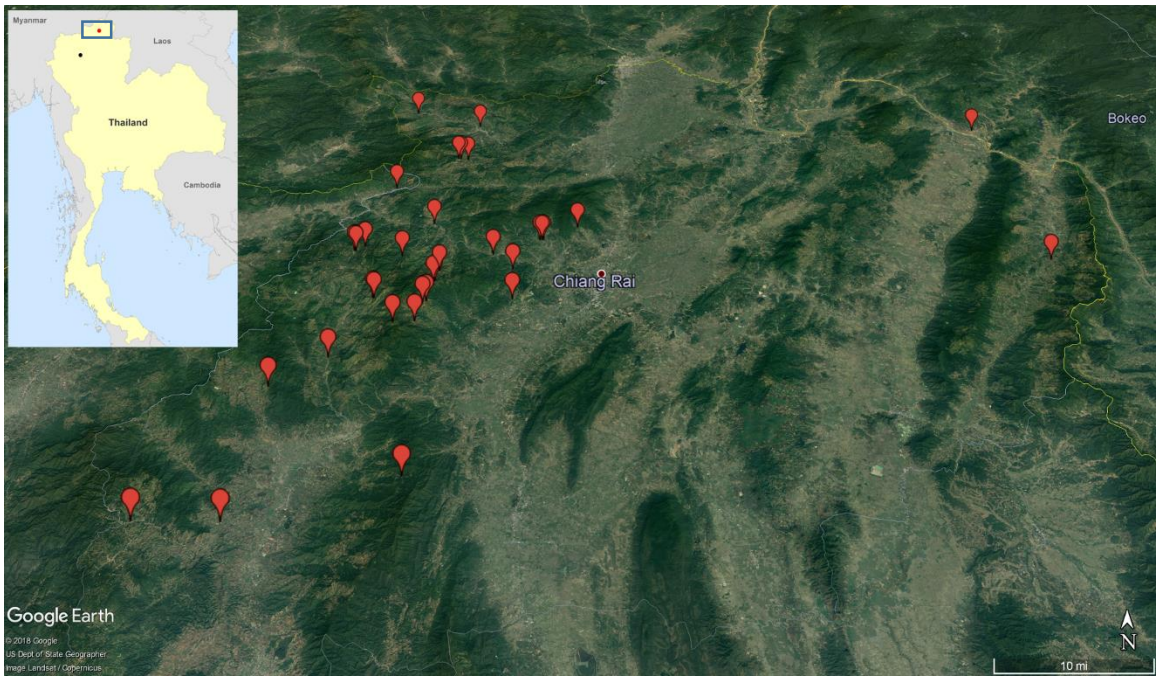
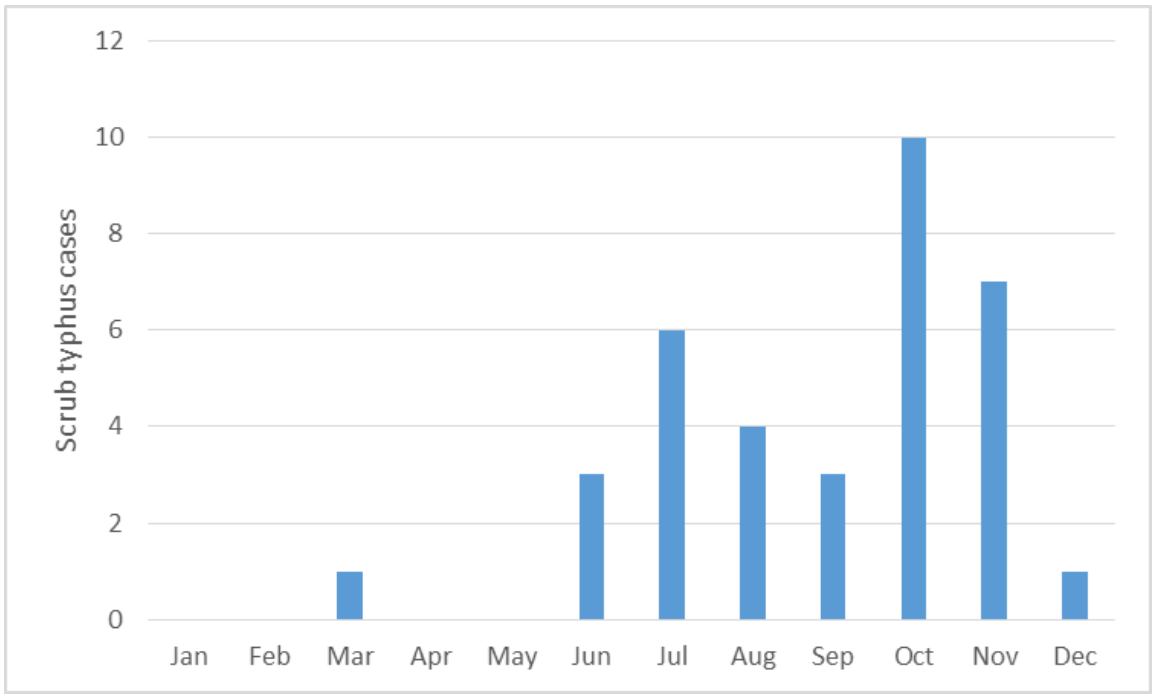


Figure 45. Temporal spread of paediatric scrub typhus cases by month.



5.3.2 Clinical, laboratory and radiological features

Clinical characteristics of scrub typhus patients on admission are shown in Table 13. All scrub typhus patients were febrile or had a history of fever prior to admission. Patients admitted directly to the provincial hospital had fewer fever days pre-admission than those transferred from another hospital [6 (IQR 3-9) days versus 10 (IQR 7-11) days, p=0.006]. The median duration of hospital admission was 5 days (IQR 4-7). A large proportion of patients were transferred from another hospital (40%), usually district general hospitals or private hospitals with absent or limited paediatric ICU capacity, respectively. Common clinical findings include presence of an eschar (60%), cough (60%), lymphadenopathy (43%), headache (40%), rash (34%), vomiting (31%), hepatomegaly (31%) and diarrhoea (29%). Eschars were most frequently observed on the external genitalia (exclusively in boys), head and inguinal regions. Temperature >37.5°C (31/35, 89%), tachypnoea (16/35, 46%) and tachycardia (11/35, 31%) were common vital sign features on admission.

In Table 14, the laboratory and CXR results for patients (STP) and available laboratory results for controls (STE) are shown. Scrub typhus patients had lower platelet, lymphocyte and eosinophil counts than healthy controls. Patients were also found to have lower haemoglobin and haematocrit than controls although these findings may be confounded by the difference in age between the groups. The main biochemical findings in patients included elevated AST, ALT and CRP as well as low albumin and ALP. In patients who had a CXR performed, normal appearances (12/21, 57%) and pulmonary infiltrates (9/21, 43%) were the main findings. Data on other routine microbiological tests performed locally as requested by the attending physician (blood, urine, stool, sputum and CSF cultures; malaria film; dengue and leptospirosis RDTs) were collected and were found to be negative or yielded non-significant results.

Table 13. Clinical features on admission of paediatric scrub typhus patients.

Clinical features	Scrub typhus patients (STP), n = 35
Days admitted, median (IQR)	5 (4-7)
Fever days before admission, median (IQR)	7 (5-10)
Transferred from another hospital	14 (40%)
Fever (%)	35 (100%)
Temperature (°C), median (IQR)	38.9 (38.0-39.5)
Rigors (%)	5 (14%)
Eschar (%)	21 (60%)
Eschar location (n=21):	
- External genitalia (%)	9 (43%)
- Head (%)	4 (19%)
- Inguinal region (%)	3 (14%)
- Chest (%)	2 (10%)
- Axillary region (%)	1 (5%)
- Neck (%)	1 (5%)
- Shoulder (%)	1 (5%)
- Buttock (%)	1 (5%)
- Waistline (%)	1 (5%)
Rash (%)	12 (34%)
Rash (n=12):	
- Petechial (%)	5 (42%)
- Maculo-papular (%)	5 (42%)
- Macular (%)	2 (17%)
Cough (%)	21 (60%)

Dyspnoea (%)	6 (17%)
Lung crepitations (%)	6 (17%)
Intubated on or within 24 hours of admission (%)	5 (14%)
Epistaxis (%)	1 (3%)
Haemoptysis (%)	0 (0%)
Nausea (%)	3 (9%)
Vomiting (%)	11 (31%)
Diarrhoea (%)	10 (29%)
Abdominal pain (%)	7 (20%)
Jaundice (%)	0 (0%)
Hepatomegaly (%)	11 (31%)
Splenomegaly (%)	3 (9%)
Gum bleeding (%)	0 (0%)
Haematemesis (%)	0 (0%)
Conjunctivitis (%)	8 (23%)
Sub-conjunctival haemorrhage (%)	2 (6%)
Retro-orbital pain (%)	1 (3%)
Tinnitus (%)	0 (0%)
Deafness (%)	0 (0%)
GCS, median (IQR)	15 (15-15)
Headache (%)	14 (40%)
Neck stiffness (%)	3 (9%)

Seizures (%)	3 (9%)
Confusion (%)	3 (9%)
Vertigo (%)	1 (3%)
Arthralgia (%)	0 (0%)
Myalgia (%)	4 (11%)
Lymphadenopathy (%)	15 (43%)

[Fever = temperature >37.5°C on or during admission]

Table 14. Laboratory and CXR results for paediatric scrub typhus patients and comparative laboratory results for the healthy control group.

Laboratory and CXR results	Patients (STP), n = 35	Healthy controls (STE), n = 40	*p* value
Haemoglobin (g/dl)	11.3 (10.3-11.9)	13.2 (12.5-14.1)	<0.001
Haematocrit (%)	34.4 (31.7-36.7)	40.8 (38.8-43.8)	<0.001
Platelets ($x10^3/mm^3$)	107 (58-178)	290 (256-355)	<0.001
White blood count ($x10^3/mm^3$)	8.1 (5.9-11.5)	8.0 (6.9-9.7)	0.733
Neutrophils, ($x10^3/mm^3$), median (IQR)	4.2 (3.1-7.2)	4.7 (3.2-5.5)	0.742
Lymphocytes, ($x10^3/mm^3$), median (IQR)	1.7 (1.3-3.4)	2.7 (2.1-3.1)	0.024
Monocytes, ($x10^3/mm^3$), median (IQR)	0.5 (0.2-0.7)	0.6 (0.4-0.7)	0.357
Eosinophils, ($x10^3/mm^3$), median (IQR)	0.0 (0.0-0.0)	0.2 (0.1-0.5)	<0.001
Blood urinary nitrogen (mg/dl)	9.8 (7.1-11.6)	10.0 (9.4-11.8)	0.345
Creatinine (mg/dl)	0.43 (0.37-0.55)	0.52 (0.42-0.59)	0.146
Sodium (mmol/l)	131 (129-134)	-	-
Potassium (mmol/l)	3.5 (3.3-4.0)	-	-
Chloride (mmol/l)	100 (97-105)	-	-
Globulin (g/dl)	3.1 (2.6-3.5)	3.2 (3.0-3.6)	0.120
Albumin (g/dl)	2.9 (2.6-3.3)	4.4 (4.2-4.5)	<0.001
Bilirubin direct (mg/dl)	0.2 (0.1-0.2)	0.2 (0.1-0.2)	0.508
Bilirubin total (mg/dl)	0.4 (0.3-0.5)	0.4 (0.3-0.6)	0.799

AST (IU/l)	140 (78-203)	23 (19-28)	<0.001
ALT (IU/l)	69 (44-111)	14 (11-18)	<0.001
ALP (IU/l)	162 (107-224)	229 (159-310)	0.032
CRP (µg/ml)	44 (22-88)	<5 (<5-<5)	<0.001
CXR performed (%)	21 (60%)	-	-
CXR findings (n=21):			
- Normal	12 (57%)	-	-
- Pulmonary infiltrates	7 (33%)	-	-
- Pulmonary infiltrates and oedema	2 (10%)	-	-

[AST – aspartate aminotransferase, ALT – alanine aminotransferase, ALP – alkaline phosphatase; median (IQR) used unless specified; analysis performed using Mann-Whitney U test]

5.3.3 Treatment, complications and outcomes

Unlike in adults with scrub typhus, the majority of patients with scrub typhus had received antibiotic treatment prior to admission to the provincial hospital (22/35, 63%). This is likely related to the proportion of patients being transferred from another healthcare facility (40%). Despite prompt antibiotic treatment for their febrile illness, only 4/22 (18%) paediatric scrub typhus patients received appropriate antibiotics (i.e. chloramphenicol, doxycycline, azithromycin or rifampicin). However, once admitted to Chiangrai Prachanukroh Hospital, all 35 children with scrub typhus received efficacious antibiotics, reflecting the expertise and experience of local paediatricians. Treatment is summarised in Table 15 below.

14/35 (40%) of patients developed complications of scrub typhus as summarised in Table 16. Patients often had more than one complication – 11/14 (79%) patients. Unsurprisingly, three patients with pneumonitis went on to develop acute respiratory distress syndrome while one patient with acute myocarditis progressed to circulatory shock. The most common complications seen were severe hepatitis [AST or ALT ≥5 times the upper limit of normal (ULN); 9/35 – 26%], severe thrombocytopenia (platelets $<50x10^3/mm^3$; 7/35 – 20%) and pneumonitis (5/35 – 14%). Despite the severity of disease observed in this study, almost all patients recovered. One child sadly died with multi-organ failure.

The main treatment outcome measure was fever clearance time (FCT) although data on headache clearance time were also collected. Treatment failure, defined as FCT >72 hours was another metric used to gauge outcome. The median FCT observed in the patient cohort was 36 (IQR 24-53) hours and median headache clearance time was 36 (IQR 24-48) hours. A Kaplan-Meier survival curve was plotted depicting the proportion of febrile patients over time and is shown in Figure 46. There were 8/35 patients who fulfilled the criteria for treatment failure: 7 patients with prolonged FCT beyond 72 hours and 1 patient who had persisting fever and complications despite appropriate treatment who passed away.

Mortality was 2.9%, potentially lower than the 6.7% mortality seen in adults with scrub typhus described in the preceding chapter. Study completion was high with 33/35 (94.3%) completing the study to the 52-week follow-up time-point. One patient died and one missed the final 52-week follow-up due to migration. Despite historical reports of disease relapse, there was no evidence (clinical or laboratory confirmed relapse) that this occurred throughout the entire 1 year follow-up period. Re-infection was also not observed in our paediatric scrub typhus cohort.

To analyse whether specific variables were significantly associated with treatment failure, univariate logistic regression analysis was performed (Table 17). Transfer from another hospital, hepatomegaly, severe hepatitis, high total bilirubin levels and chloramphenicol use during admission were significantly associated with treatment failure. It is important to highlight that at the study hospital, chloramphenicol was given exclusively via the intravenous route while doxycycline was only available orally. During the study, we observed that chloramphenicol (administered intravenously only) was used exclusively for severely ill children who were unable to take oral antibiotics due to being intubated. This would have selected for patients with more severe disease, thereby increasing the likelihood for treatment failure. In contrast, there was no evidence of treatment failure associated with doxycycline use during admission with the results highly suggestive that doxycycline was associated with treatment success (OR 0.1, 0.0-1.0, p=0.052) although patients receiving oral doxycycline generally had less severe disease.

Due to the small number of patients, I included transfer from another hospital, chloramphenicol use with severe hepatitis as the representative hepatic variable in the multivariate logistic regression model (Table 17). Severe hepatitis remained significantly predictive of treatment failure in this model.

Table 15. Treatment of scrub typhus patients.

Treatment	Scrub typhus patient group (STP), n = 35
Pre-admission antibiotics, n (%)	22 (63%)
Antibiotics (n=22):	
- Amoxicillin, n (%)	8 (36%)
- Ceftriaxone, n (%)	4 (18%)
- Ceftriaxone with scrub typhus active antibiotics, n (%)	4 (18%)
- Ceftriaxone with amoxicillin or ampicillin, n (%)	2 (9%)
- Unknown, n (%)	4 (18%)
Admission antibiotics, n (%)	35 (100)
Antibiotics (n=35):	
- 3rd generation cephalosporin and scrub typhus active antibiotics*, n (%)	16 (46%)
- Doxycycline alone, n (%)	15 (43%)
- Doxycycline and chloramphenicol, n (%)	2 (6%)
- Doxycycline and ampicillin, n (%)	1 (3%)
- Chloramphenicol and azithromycin, n (%)	1 (3%)
Steroids, n (%)	5 (14%)

* In this group (16 patients), the main antibiotic used to treat scrub typhus was chloramphenicol (used in 11/16 patients).

[Scrub typhus active antibiotics include doxycycline, chloramphenicol, azithromycin and rifampicin; 3rd generation cephalosporin mainly represented by ceftriaxone but also include cefotaxime and ceftazidime; steroids include dexamethasone and hydrocortisone]

Table 16. Complications observed in scrub typhus patients.

Complications	Number of patients (%)
Severe hepatitis	9 (26%)
Severe thrombocytopenia	7 (20%)
Pneumonitis	5 (14%)
Circulatory shock	4 (11%)
Acute respiratory distress syndrome (ARDS)	3 (9%)
Acute kidney injury	2 (6%)
Haematological abnormalities consistent with DIC	2 (6%)
Meningitis	2 (6%)
Meningoencephalitis	1 (3%)
Myocarditis	1 (3%)
Upper gastrointestinal haemorrhage	1 (3%)

[Severe hepatitis – AST or ALT ≥5 times the upper limit of normal (ULN);

Severe thrombocytopenia – platelets <50x10^3/mm^3;

Circulatory shock – severe circulatory failure requiring inotropic and vasopressive support;

ARDS – acute respiratory failure, hypoxia and bilateral infiltrates on CXR

Acute kidney injury – clinically denoted (reduced urine output), elevated creatinine in one case;

Haematological abnormalities consistent with disseminated intravascular coagulation (DIC) – tentative diagnosis based on thrombocytopenia, prolonged prothrombin time (PT) and prolonged activated partial thromboplastin time (aPTT);

Meningitis – clinically features (meningism), CSF leukocytosis in one case;

Meningoencephalitis – clinical features (confusion, disorientation and visual hallucination), CSF leucocytosis;

Myocarditis – reduced ejection fraction and biventricular enlargement on transthoracic echocardiogram, elevated troponin I]

Figure 46. Kaplan-Meier survival curve for fever clearance. [Dashed reference line indicates the 72 hours FCT treatment failure cut-off]

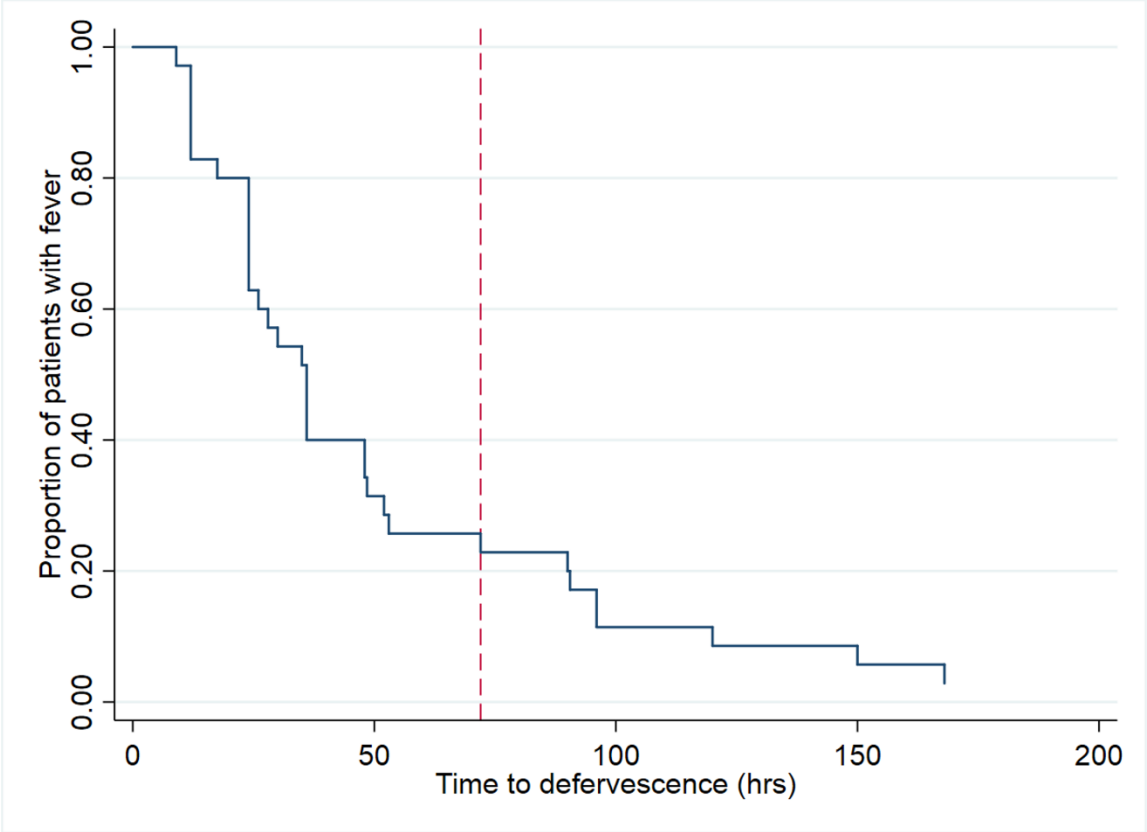


Table 17. Variables significantly associated with treatment failure on logistic regression analyses.

Variables	Odds ratio (OR)	95% Confidence interval (CI)	*p* value
Univariate logistic regression analysis			
Transferred from another hospital	7.1	1.2-43.1	*0.033*
Hepatomegaly	13.2	2.0-85.8	*0.007*
Severe hepatitis	24	3.2-177.4	*0.002*
Bilirubin total (mg/dl)	106.6	2.2-5191.9	*0.019*
Chloramphenicol use during admission	20	2.1-192.7	*0.010*
Multivariate logistic regression analysis			
Transferred from another hospital	2.7	0.2-29.1	0.420
Severe hepatitis	13.1	1.4-119.9	*0.023*
Chloramphenicol use during admission	7.6	0.6-102.7	0.128

[Severe hepatitis defined as AST or ALT ≥5 times the upper limits of normal (ULN);

Variables included in the multivariate logistic regression analysis limited to “transferred from another hospital”, “chloramphenicol use during admission” and “severe hepatitis” as the representative hepatic variable to minimise the risk of multi-collinearity]

5.4 Discussion

In this chapter, I have described the clinical characteristics of paediatric scrub typhus patients with diagnosis being determined using robust criteria. I was also able to include a prolonged follow-up period of a year and compare demographic, epidemiological and laboratory features between the patient cohort and healthy controls. Scrub typhus disproportionately affects hill-tribe populations (mainly Akha and Lahu people) and as they live in rural mountainous regions, it is unsurprising that the disease was significantly associated with this habitat. There was a significant association between disease and exposure to chickens which may reflect the village habitat most hill-tribe children live in. However, as reviewed in chapter 2, chickens have been shown to harbour chiggers although their role in disease transmission to humans is unknown [96]. The results were also suggestive that patients were more likely to have contact with rats, the natural host for chiggers, and agricultural habitats which is a risk factor for scrub typhus based on surveillance data studied in chapter 3. Disease seasonality in children was even more pronounced than in adults in Chiangrai province. Interestingly, the number of cases peaked in October but there was also a smaller but distinct peak in July. The large October peak could have coincided with long public school holidays with children helping with agricultural work and hunting and foraging in forests. Although July does not coincide with a long holiday, children may still be asked to help with agricultural activities and gathering food, especially at harvest time for the various crops frequently planted in this area.

Eschar was present in 60% of the patient cohort, a proportion comparable to previous studies from northern Thailand [176, 200, 319]. Studies in non-human primates suggest that eschar formation is dependent on the degree of host immunity to the infecting *Orientia tsutsugamushi* strain [109]. A recent seroprevalence study in Cambodian children showed peak seropositivity for scrub typhus IgG in 8-11 year olds, suggesting that primary infection

occurs during these years [343]. An older seroprevalence study on febrile patients aged 15 years and above from central Thailand suggested that around half of patients in the 15-24 year old age group had detectable antibodies to *Orientia tsutsugamushi*, a proportion which rose as age increased [241]. This could explain why eschar rates in children with scrub typhus in Chiangrai are higher than adults. Other common clinical findings such as cough, tachypnoea and lymphadenopathy also concurred with previous studies although hepatomegaly was not as frequently observed in our patient cohort [200, 319, 320]. When comparing clinical features of scrub typhus in children and adults in Chiangrai, aside from higher rates of eschar detection, there appears to be a preponderance of lymphadenopathy and rash in children. Thrombocytopenia, raised CRP, low albumin and elevated hepatic enzymes (particularly AST) were often seen with the latter previously suggested as a useful diagnostic tool in the absence of eschar in children [335].

The Scrub Typhus Detect™ IgM Rapid Test (InBios, WA, USA) RDT was selected for use in screening febrile children as it had previously been found to be accurate when validated using samples from 100 febrile adults from northeastern Thailand and Nepal [339]. In this study, included patients had previously been diagnosed with scrub typhus, murine typhus or dengue with around half the patients presenting with undifferentiated fever where the diagnosis was not determined. The RDT was sensitive and specific when the corresponding IFA IgM titre was high, suggesting that it would perform well in endemic areas where background antibody titre may be present in the population. Sensitivity and specificity depended on the choice of IFA IgM cut-off titre: at 1:400 over 1:1,600 to 1:3,200 would yield sensitivities (95% CI) of 52% (32-71), over 82% (57-96) to 92% (64-100), respectively, while retaining a specificity of ≥94% [339]. Unfortunately, the accuracy of the RDTs in children was disappointing with false positives accounting for around 40% of the patient cohort. What could account for the poor specificity seen in children in this trial? Some of the positive RDT

results could have been triggered by lower IgM titres which did not fulfil the strict diagnostic criteria – 5 patients with IFA IgM titres of 1:400-1:800. However, the majority of false positive results were seen in patients with undetectable IgM levels by IFA (<1:100), suggesting some form of cross reactivity. Samples from false positive RDT children have been shipped to InBios and work is underway to analyse the cause. It remains paramount that RDTs incorporating both antigen and antibody detection for scrub typhus are developed and validated for use in both adults and children.

Appropriate antibiotics were used once patients were admitted to Chiangrai Prachanukroh Hospital. A similar picture to adult scrub typhus patients was observed with regards to antibiotic treatment in the community and other healthcare facilities whereby antibiotics effective against scrub typhus were frequently excluded. This delay in treatment may, in part, explain why the complication and treatment failure rates in this study were high at 40% and 23%, respectively. The multitude of complications observed reflect the systemic vasculitic nature of severe scrub typhus infection [132]. Disease awareness remained negligible among patients and relatives while additional training of healthcare staff in district, community and private facilities could lead to significant improvement in the recognition and prompt treatment of scrub typhus.

Markers of hepatic dysfunction, previously shown to be associated with disease severity, were significantly associated with treatment failure in our study [335]. Specifically, severe hepatitis, as defined by markedly elevated AST or ALT, may be predictive of treatment failure in children. As discussed in Chapter 2, treatment failure, attributed to drug resistance in the past with limited objective evidence, may be due to a combination of factors such as delays in recognition and treatment, virulence of the infecting *Orientia tsutsugamushi* strain, pharmacological factors and degree of host immunity [2, 109, 110, 226, 229]. In patients

presenting with severe scrub typhus infection with evidence of organ failure on admission, there is a higher risk of poorer outcomes despite appropriate therapy being instigated.

There are several limitations to the study. Despite our efforts, there was a demographic mismatch between the scrub typhus patient group and healthy controls. The number studied was small although this was offset by the certainty of diagnosis and was comparable to previous studies on paediatric scrub typhus. Culture and susceptibility testing was not performed and the IFA IgM cut-off titres used were not previously validated using samples from the paediatric population. However, excellent concordance between IFA and PCR results suggest that the criteria was appropriate for this population and region.

In this chapter, I have described how we carried out the paediatric scrub typhus study in Chiangrai and was able to characterise scrub typhus in children. I have shown that paediatric scrub typhus in northern Thailand is often severe and potentially fatal, with disease severity on admission (particularly severe hepatitis) a useful marker of poor response to treatment. The strict diagnostic criteria used appears accurate in children although the development of accurate scrub typhus RDTs for both adults and children requires further innovation. Future studies on the immunological and microbiological aspects from this trial will provide further insight into treatment outcome and its determinants.

6 Scrub typhus antibiotic resistance trial

6.1 Introduction

In chapter 2, I reviewed the literature on the treatment of scrub typhus and concluded that, based on current evidence, doxycycline resistance is an illusion. These past studies, with recent improved understanding of key microbiological aspects of *Orientia tsutsugamushi* growth and pharmacokinetics of the antibiotics tested, are now thought to have used flawed methodologies. More recent work on antibiotic susceptibility of *Orientia tsutsugamushi* has shown that doxycycline MICs obtained for 2 reportedly resistant strains of *Orientia tsutsugamushi* (AFC-3 and AFSC-4) were found to be 0.125 mg/l and 0.250 mg/l, respectively [224]. These values fall within the expected wild type distribution of doxycycline MICs (median of 0.125 mg/l +/- one to two two-fold dilution steps) in 5 reference strains (including Karp and Gilliam, prototypical strains considered doxycycline susceptible) and 51 clinical isolates from Laos and Thailand, and remain well below the plasma concentrations achieved in humans at standard doses (100-200 mg/day, peak serum concentrations of 1.7-5.9 mg/l) [224, 344, 345]. The results strongly suggest that doxycycline resistance was not contributory to delayed defervescence in the original patients AFC-3 and AFSC-4 were isolated from. However, clinical details from 30 years ago are scant or were unreported and mutations in these strains may have occurred in the intervening years.

Scrub typhus treatment trials incorporating culture and antibiotic susceptibility testing, detailed pharmacokinetic analysis and correlates of immunity have not been performed. The additional information obtained will be crucial in studying the determinants of treatment outcome, especially in a region where uncertainties remain on efficacy of treatment. A recent large RCT comparing the efficacy of an intensive treatment regimen versus standard of care for TB meningitis in Vietnam illustrates this point well [346]. There was no survival

benefit with the intensive treatment regimen (higher rifampicin dose and addition of levofloxacin for first 8 weeks of treatment) and only through additional microbiological and pharmacological investigations could the results be objectively explained. In a subset of patients with drug resistance profiles available, multi-drug resistance TB was found to be a predictor of death while the intensified treatment regime improved survival in patients with isoniazid resistance and rifampicin sensitivity [347]. Similarly, in a subset of patients, although the intense treatment regime increased rifampicin exposure in patients' plasma and CSF, there was no relationship between rifampicin exposure and survival [348]. However, isoniazid exposure was associated with survival and low exposure, observed in fast metabolisers, was predictive of death [348]. These results suggest that screening for fast metabolisers should be performed and current isoniazid doses should be reviewed for TB meningitis, regardless of metaboliser status.

Doxycycline for 7 days is the preferred treatment for scrub typhus in adults [44, 167]. Shorter courses of doxycycline and azithromycin may be just as effective [170, 172-174]. Another completed but yet to be published RCT (ISRCTN47812566) comparing the treatment of scrub typhus in hospitalised Lao and Thai adults with doxycycline 7 days, doxycycline 3 days and azithromycin 3 days has been performed. However, the trial did not incorporate additional microbiological, pharmacological and immunological analyses and utilised smaller doses of azithromycin (500mg loading dose then 250mg a day) normally used for mild infections, the dosing regimen used in South Korea at the time.

In this final results chapter, I will explain how we are seeking to answer whether doxycycline resistance is present in northern Thailand by outlining my role in the Scrub Typhus Antibiotic Resistance Trial (START), which is currently underway. The formal title of the trial is "A randomised controlled trial comparing doxycycline and azithromycin for scrub typhus in adults in northern Thailand". We have embedded detailed analysis of potential determinants

of treatment outcome within the setting of a RCT. The trial protocol, methodology and patient information sheet/informed consent form are included in Appendix 6. The trial is summarised in the study synopsis below. The study case record form is included in Appendix 7 and miscellaneous study forms in Appendix 8. I will describe my contributions as the principal investigator along with challenges faced and limitations of the trial. Preparation for the interim and final analyses including code writing and formulating the trial report will be outlined. Finally, the blinded interim analysis results will be included.

Study synopsis

Full Title	**The Scrub Typhus Antibiotic Resistance Trial (START)** *Comparing Doxycycline And Azithromycin Treatment Modalities In Areas Of Reported Antimicrobial Resistance For Scrub Typhus*
Short Title	Scrub Typhus Antibiotic Resistance Trial
Study Type	Randomized Controlled Treatment Trial
Conducted By	MORU (Mahidol Oxford Tropical Medicine Research Unit)
Co-PIs	Dr. Tri Wangrangsimakul, Dr. Carlo Perrone
Sample Size	Total sample size is n=177 patients, randomized into three treatment groups of each n=59 subjects. The study is powered at 90%, with alpha of 0.05, to detect an increase of 20% of the average FCT of 30 hours, with standard deviation of 10 hours, corresponding to an increase to 36 hours.
Study Population	Cases: Male and female patients with ≥15 years of age and acute scrub typhus infection
Study Duration	5 years
Study Design	Prospective, open-label, randomized-controlled treatment trial in patients ≥15 years old admitted to hospital with acute scrub typhus. Randomization into 3 oral treatment arms (each n=59 patients, total n=177): i) 7 days of doxycycline, ii) 3 days of doxycycline, and iii) 3 days of azithromycin
Primary Objective	To evaluate the clinical and microbiological responses in scrub typhus patients to three oral treatment regimens: 7 days of doxycycline, 3 days of doxycycline, and 3 days of azithromycin
Secondary Objectives	1. To perform pharmacokinetic/pharmacodynamics (PK/PD) characterization of the therapeutic responses for doxycycline and azithromycin, incl. serial bacterial load measurements. 2. To define clinical, bacterial, pathophysiological and pharmacological factors associated with disease severity, fever-clearance times (FCT), treatment failures, relapse/re-infection and latency. 3. To determine the minimum inhibitory concentrations (MIC) of clinical *Orientia tsutsugamushi* isolates to doxycycline, azithromycin and chloramphenicol, using *in vitro* growth-inhibition assays 4. To genotype all clinical isolates using whole genome sequencing for comparative genomics.

	5. To dissect the natural immune response in scrub typhus, using antigen-specific cellular immune and antibody studies, and cytokine profiling
Inclusion and Exclusion Criteria	Inclusion criteria: • Age ≥ 15 years old • Hospitalization with acute undifferentiated fever (temperature > 37.5°C, tympanic) ≤14 days or patients admitted to hospital with a history of fever ≤ 14 days who subsequently develop fever within 24 hours of admission • Clinically suspected scrub typhus: defined as acute undifferentiated fever with no clear focus of infection and negative malaria blood smear +/- negative malaria RDT • A positive scrub typhus RDT (e.g. Scrub Typhus IgM RDT, InBios International, Seattle, WA, USA or Scrub Typhus IgM/IgA/IgG SD Bioline, S.Korea) or positive PCR-based detection of *O. tsutsugamushi* DNA from the admission blood sample or presence of an eschar • Written informed consent and/or, written informed assent as required • Able to take oral medication Exclusion criteria: • Known hypersensitivity to tetracycline, doxycycline or azithromycin • Administration of doxycycline, azithromycin, chloramphenicol, rifampicin, or tetracycline during the preceding 7 days • Pregnancy or breast-feeding • Patients with myasthenia gravis or systemic lupus erythematosus • Patients with an alternative acute infection at the time of screening (diagnostic test required) e.g. acute malaria, dengue, leptospirosis, typhoid, Japanese encephalitis etc. • Current TB or TB treatment in ≤ 6 months (contain active antibiotics against Orientia tsutsugamushi) • Current HAART use for HIV, long term use of immunosuppressants (e.g. steroids, chemotherapy, TNF-inhibitors and related agents) • Patients with severe disease whom the clinical team feel their condition necessitates the need for additional scrub typhus treatment beyond the allocated antibiotic treatment assigned at randomization (e.g. IV chloramphenicol and/or PO/NG rifampicin)

Outcome measures	**The primary outcome measure** is fever clearance time (FCT); based on the time from first dose of antibiotic treatment to when the tympanic temperature first falls ≤37.5°C and remains ≤37.5°C for at least 24 hours (fever should be related to the presumptive diagnosis of scrub typhus). **The secondary outcome measures** are: ▪ Clinical treatment failure ▪ Microbiological treatment failure ▪ Relapse – possible and confirmed

6.2 Study initiation and running

6.2.1 Investigators

Investigators for the study included Dr Suwimon Khusuwan (Infectious diseases physician) and Dr Samroeng Seekaew (Deputy Director) from Chiangrai Prachanukroh Hospital, Dr Thundon Ngamprasertchai (Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand), Prof Rose McGready (Deputy Director, SMRU, Mae Sot, Thailand), Dr Tobias Brummaier (Research Physician, SMRU, Mae Sot, Thailand), Prof Daniel Paris (Clinical Director, Swiss Tropical and Public Health Institute, Basel, Switzerland) and Dr Carlo Perrone (Research Physician) and Prof Nicholas Day (Director) from MORU. The trial statistician will be Prof Mavuto Mukaka (Head of Statistics, CTSG, MORU). I am the principal investigator of the trial.

6.2.2 Study initiation

The trial began recruitment at the Chiangrai site in July 2017 and at Mae Sot site in September 2017. Prior to this, a study initiation visit was carried out at each site to review the study, the procedures, troubleshoot and identify any logistical or training needs required prior to starting. There was a focus on ensuring the trial was ICH/GCP compliant and meets other EC, sponsor and local regulatory requirements. The meeting was attended by study staff at each site along with CTSG staff from MORU, Bangkok.

Additional training for some study staff was required including data entry using MACRO and laboratory protocol training for SMRU site laboratory technicians (immunological assays). A study monitoring plan for the trial was also formulated and put in place. Monitoring is being performed by CTSG study monitors and the first visit took place after 5-10 patients were recruited at each site. At the first visit, all the CRFs and associated data entry along with study logs and documents were reviewed. The monitor assesses the quality of the study data, compliance with protocol and provide additional training and support as needed. A debrief and summary report (Corrective Action/Preventive Action, CAPA) is sent to the site investigators and study team. At subsequent monitoring visits (annually), 10% of randomly selected patient records and study documents/logs are reviewed along with all consent forms.

6.2.3 Study progress

Progress

Up until the end of January 2020, there were 88 patients recruited to the study and have completed follow-up, 64 from Chiangrai and 24 from Mae Sot. In Chiangrai, 1,889 patients were screened, while in Mae Sot this number was 597 patients. There were 5 patients who discontinued the trial early from Chiangrai (1 patient discontinuation, 3 deaths, 1 migrated) and 4 from Mae Sot (1 patient discontinuation, 3 migrated). There were 7 SAEs reported from Chiangrai and 1 from Mae Sot. Only 2 were possibly related to the study drug – both receiving doxycycline and they developed haematemesis (possible doxycycline-associated gastritis).

There were 13 protocol deviations in Chiangrai – 6 involving study drug administrative deviations by ward staff, 2 occurrences of a patient forgetting to take a dose of study drug, 1 case of 2 withheld study drug doses by the patient due to dizziness, 1 case with possible relapse but blood collection delayed due to admission to different health facility and 3 cases of laboratory errors (mislabeling and processing wrong sample). At SMRU, there were 10 protocol deviations - 3 related to insufficient PBMCs to perform ELISpot assays requiring additional blood collection, 3 related to no week 2 +/- week 8 follow-up due to migration, 1 related to patient discontinuation, 1 due to use of expired blood tube, 1 due to a patient being given 200mg doxycycline instead of 100mg and 1 due to delayed study drug taking by patient due to confusion with dosing time. There have been no protocol violations to this point.

As per study monitoring plan, there have been two monitoring visits at both sites up to January 2020 with a further monitoring visit planned for 2020. I regularly updated investigators, funders and DSMC for the trial through a quarterly report.

Challenges

The major difficulty faced during the study was recruitment rate being significantly slower than expected. Explanations include the normal variation in disease incidence (dependent on weather and agricultural activity of the population). Many patients, particularly in Chiangrai, were admitted late at night or during the weekend which coincided with study staff absence. This meant that a major exclusion criterion in Chiangrai was receiving treatment prior to screening (the other main exclusion criteria at this site were alternative diagnosis at the time of screening or severe disease/inability to take oral medications). In Mae Sot, the setting differed and healthcare staff carried out dual roles in clinical care and research. Also, more severe patients would be transferred to the local government hospital. Thus, the main exclusion criterion at SMRU was negative scrub typhus IgM RDT. Beyond the specific issues affecting recruitment, there were expected issues with staffing/investigator changes and ethics committee-related delays.

Measures to counter the slow recruitment rate were considered including recruitment from outpatient settings or additional district hospitals in Chiangrai. We also considered reducing target study numbers by reducing power to an acceptable 80%. However, these measures would have reduced the overall quality of the trial and particularly impacted the PK, bacterial load monitoring and immunological aspects of the trial. The relatively detailed PK analysis requires frequent sampling of blood by dedicated study staff at each additional site. The immunological assays also require intact white blood cells and therefore, require blood samples to be processed within 4 hours of collection. These assays themselves require laboratory expertise, equipment and materials beyond what is available at district hospitals. Recruitment at additional sites will require significant additional funding.

A major additional delay from 2020 onwards has been the COVID-19 pandemic which shut down non-essential research work throughout the MORU Tropical Health Network. Although study activities have restarted, valuable time during peak scrub typhus season has been lost and further rises in COVID-19 cases have subsequently led to additional lockdown measures, curtailing recruitment further. Funding from MIDRP has finished but additional funds from the Wellcome Trust (via MORU's institutional support) will allow the study to proceed.

Data safety and monitoring committee

The members of the DSMC are Prof Paul Newton (Prof of Tropical Medicine, Oxford University) as chair, Prof Elizabeth Ashley (Prof of Tropical Medicine and Director of the Lao-Oxford-Mahosot Hospital-Wellcome Trust Research Unit - LOMWRU), Assistant Prof Podjanee Jittmala (Mahidol University) and Dr Ronald Geskus (Head of Biostatistics Group, Oxford University Clinical Research Unit, Vietnam) as the DSMC statistician. DSMC will be charged with monitoring and evaluating the clinical data generated by this study, with a focus on safety, treatment efficacy and recruitment rate in an independent and objective manner (i.e. focus on SAEs, FCT, treatment failure, relapse, and recruitment progress). If required, the DSMC will make recommendations on the further conduct of the trial.

The following data will be sent to the DSMC for its review:

- SAEs – Final SAE reports will be sent to the DSMC as well as the ECs as they occur throughout the trial period
- SAEs that are of immediate concern to the Principle Investigator and Study Safety Team will be sent to the DSMC (initial reports)

- Efficacy data (including fever clearance time, treatment failure and relapse) when 90 patients have been recruited, and completed 8-week follow-up, and at the end of the study
- Quarterly trial progress reports to the study team (with additional treatment failure/relapse and expanded SAE data added for the DSMC version)
- Any additional data that is requested by the DSMC

The schedule for meetings will include an initial meeting after study initiation, after 90 patients recruited and completed the trial (interim analysis), at the end of the trial, and ad hoc meetings if required. Meetings will be by teleconference. The first teleconference will include the study PI. Thereafter, all meetings can be held without the study PI but the DSMC can ask the PI or other study staff to be present. Ad-hoc communications can be via email or teleconference, at the diction of the DSMC Chair. A minimum of three members should be present for the scheduled meetings including the Chair, another clinician and the DSMC statistician. To date, two START DSMC meetings have occurred, corresponding to the initial and interim analysis meetings as outlined above.

6.3 Data analysis plan and interim analysis

6.3.1 Data analysis

Demographic, clinical and laboratory variables will be described for the study cohort and each treatment arm using median and IQR or mean and SD as appropriate. The primary comparison is between doxycycline for 7 days vs 3 days of azithromycin. The main analysis will be intention-to-treat analysis (ITT) but per-protocol analysis will also be performed after completion of diagnostics work. The primary outcome of FCT will be analysed using using Kaplan-Meier survival analysis, Cox regression and Wilcoxon ranksum test. The categorical variables (Rx failure and relapse) will be analysed using Pearson's chi-squared test. Analysis will be performed by the principal investigator and trial statistician. STATA15 (StataCorp, College Station, Tx, USA) will be used for analysis.

Prof Mavuto Mukaka and I wrote the data cleaning and analysis code for the study and tested it on preliminary data prior to interim analysis. The exported study data (with treatment arms generically labelled) were sectioned into 34 separate files to match the different sections of the study CRF (e.g. demographics, medical history, SAE, outcomes, final status etc.). STATA codes were written for each file to first clean then analyse the results. The codes were tested on exported study data but with dummy randomisation of the treatment arms. Errors observed, which were mainly due to data variability within individual fields, were corrected. Examples of the codes written can be seen in Appendix 9 and include codes for outcome measures, final status, SAEs and demographics. The results will be presented in a report format which will include the CONSORT flowchart, patient characteristics table, SAE summary table and analysis results based on the main trial outcomes (report template is shown in Appendix 9).

Study population drug concentration-time curves will be formulated for each study arm using conventional non-compartmental analysis. The relationship of exposure parameters [max concentration (C_{max}), area under concentration/time curve (AUC), and half-life (t1/2)] to clinical outcomes and bacterial load will be investigated, and mechanistic PK/PD non-linear mixed-effects modeling will be performed.

6.3.2 Interim analysis plan

(Below is the plan as originally written – in the future tense. The planned interim analysis was carried out during thesis write-up, and the interim results are presented in the next section).

Interim analysis will take place after 90 patients have been recruited and completed follow-up. It will be preferable to have completed diagnostic data on the 90 patients. However, ITT analysis can be performed if there are delays in diagnostic processing of the specimens. The report will be in the same format as described above. Analysis will be performed by the investigator(s) and trial statistician while blinded to the treatment arms. Trial data and statistical script used for analysis will be sent for review and re-analysis by the DSMC statistician when the interim analysis report is sent to the DSMC.

The Haybittle-Peto rule ($p<0.001$ for interim analyses of treatment efficacy) will be used to guide the decision of the DSMC with regards to trial termination/discontinuation of treatment arm [188]. The DSMC may also stop the trial or discontinue a treatment arm if new evidence on scrub typhus treatment comes to light or the national treatment policy changes. It may stop the trial/discontinue a treatment arm on the basis of patient harm as evaluated by the DSMC members even if statistical significance is not reached – e.g. cases of fatal arrhythmias in the azithromycin arm.

The final recommendation on stopping or continuing the trial/treatment arms will rest on the judgement of the DSMC. The DSMC has the right to recommend to the sponsor, University of Oxford, and PI that the study be stopped if this is the considered view of the DSMC with clear justification outlined for such a recommendation. If during the course of the study, results of concern appear, these will be reported to the study DSMC.

6.4 Interim analysis results

Although START is designed as an open-label trial, the interim analysis results presented here have been blinded as a bias-reducing measure. The 3 treatment arms were labelled A, B and C with the investigators blinded to which treatment arm each letter corresponds to. Unblinding will only take place once the trial is completed and the final analyses for the main outcomes performed. The interim analysis shell report and data cleaning and analysis codes were prepared by me. Data extraction was performed by the study data manager at CTSG while the interim analysis using pre-prepared STATA codes was performed by Dr Carlo Perrone with supervision by Prof Mavuto Mukaka. Results were reviewed and collated by Dr Carlo Perrone, Prof Mavuto Mukaka and me. As per the data analysis plan, the interim analysis was performed when 90 patients had been recruited and completed the study. Diagnostic data were only partially available as only molecular testing had been performed for all 90 patients at the time of analysis. Serological testing will be performed as a batch at the end of the study and *Orientia tsutsugamushi* culture had been completed for only a small proportion of patients. Hence, only the results for the intention-to-treat interim analysis are depicted below.

The main study outcomes (also shown in Appendix 6) are:

- Fever clearance time (FCT) – time in hours from onset of 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 – persistence of fever >72 hours after initiation of antibiotic treatment OR failure of resolution of symptoms and/or complications attributable to scrub typhus within 5 days from the start of antibiotic treatment.

- Microbiological treatment failure - failure of resolution of bacteraemia (qPCR +/- culture) within 3 days from initiation of antibiotic treatment.
- Relapse – the recurrence of fever and/or associated symptoms, which the clinical and study teams have concluded as likely to be due to scrub typhus will be defined as “possible relapse”. The recurrence of bacteraemia as detected by qPCR +/- culture within the 8 week follow-up period after the patient had been treated and achieved clinical and bacteriological resolution will be defined as “confirmed relapse”. *Orientia tsutsugamushi* strain genotyping (if PCR +/- culture positive) will allow the differentiation of relapse from re-infection.

6.4.1 Baseline characteristics by treatment arm

Baseline characteristics are shown below in Table 18 with the variables being generally similar for the 3 treatment arms.

Table 18. Demographic, clinical, haematological and biochemical variables for START patients by treatment arm.

Demographics			
Treatment arm	**A n=30**	**B n=30**	**C n=30**
Age (years, median [IQR])	44 (33-58)	42.5 (26-56)	37 (24-49)
Sex (n [%]):			
- Male	19 (63.3)	17 (56.7)	18 (60.0)
- Female	11 (36.7)	13 (43.3)	12 (40.0)
Ethnicity (n [%])			
- Thai	19 (63.3)	17 (56.7)	19 (63.3)
- Karen	6 (20.0)	8 (26.7)	6 (20.0)
- Akha	3 (10.0)	2 (6.7)	4 (13.3)
- Lahu	5 (16.7)	9 (30.0)	5 (16.7)
- Hmong	3 (10)	1 (3.3)	2 (6.7)
- Other	6 (20.0)	5 (16.7)	6 (20.0)
Education (n [%]):			
- None	13 (43.3)	16 (53.3)	9 (30.0)
- Primary	8 (26.7)	9 (30.0)	14 (46.7)
- Secondary	6 (20)	2 (6.7)	2 (6.7)
- High school	1 (3.3)	1 (3.3)	3 (10.0)
- Vocational	0	0	1 (3.3)
- University	2 (6.7)	2 (6.7)	1 (3.3)
Occupation (n [%]):			
- None	5 (16.7)	3 (10)	4 (13.3)
- Agriculture	19 (63.3)	18 (60)	16 (53.3)
- Office	0	0	1 (3.3)
- Other	6 (20)	9 (30)	9 (30)
Clinical features			
Clinical features	**A n=30**	**B n=30**	**C n=30**
Fever days before admission - (median [IQR])	5.5 (4-7)	5.5 (3-7)	5 (3-7)
Fever (>37.5°C on or during admission) (n [%])	29 (96.7%)	30 (100%)	30 (100%)

Temperature on admission (°C) (median [IQR])	37.95 (36.9-39)	38.35 (37.5-38.9)	38.45 (37.7-39.3)
Respiratory rate (breaths/min) (median [IQR])	19 (18-24)	20 (18-24)	20 (18-24)
Pulse rate (breaths/min) (median [IQR])	90 (80-100)	94 (83-110)	93.5 (84-106)
Systolic BP (mmHg, median [IQR])	111.5 (100-120)	100 (100-117)	105 (100-120)
Diastolic BP (mmHg, median [IQR])	70.5 (60-80)	69 (60-77)	66 (60-80)
SpO2 (%, median [IQR])	97.5 (95-98)	98 (97-99)	98 (97-99)
Weight (kg, median [IQR])	54.5 (50-60)	50 (45-59)	55 (49-64)
Height (cm, median [IQR])	165 (154-168)	160 (150-165)	159.5 (155-165)
Eschar (n [%])	5 (16.7%)	3 (10%)	5 (16.7%)
Deafness (n [%])	0	0	0
Retro-orbital pain (n [%])	7 (23.3)	7 (23.3)	3 (10)
Arthralgia (n [%])	7 (23.3)	3 (10)	6 (20)
Jaundice (n [%])	0	0	0
Eye redness (n [%])	7 (23.3)	3 (10.0)	7 (23.3)
Abdominal pain (n [%])	14 (46.7)	10 (33.3)	8 (26.7)
Seizures (n [%])	1 (3.3)	1 (3.3)	0
Confusion (n [%])	1 (3.3)	2 (6.7)	2 (6.7)
Cough (n [%])	11 (36.7)	14 (46.7)	13 (43.4)
Epistaxis (n [%])	0	0	1 (3.3)
Bleeding gums (n [%])	2 (6.7)	0	0
Rigors/chills (n [%])	22 (73.3)	28 (93.3)	17 (56.7)
Tinnitus (n [%])	5 (16.7)	2 (6.7)	2 (6.7)
Headache (n [%])	28 (93.3)	24 (80.0)	28 (93.3)

Myalgia (n [%])	21 (70)	15 (50.0)	17 (56.7)
Skin rash (n [%])	5 (16.7)	4 (13.3)	3 (10.0)
Nausea (n [%])	13 (43.3)	11 (36.7)	13 (43.3)
Vomiting (n [%])	13 (43.3)	12 (40.0)	11 (36.7)
Diarrhoea (n [%])	6 (20.0)	11 (36.7)	7 (23.3)
Neck stiffness (n [%])	1 (3.3)	1 (3.3)	1 (3.3)
Vertigo (n [%])	6 (20.0)	5 (16.67)	4 (13.33)
Dyspnoea (n [%])	11 (36.7)	11 (36.7)	3 (10.0)
Haemoptysis (n [%])	0	0	1 (3.3)
Haematemesis (n [%])	0	0	0
Anaemic (n [%])	3 (10.0)	2 (6.7)	1 (3.3)
Lymphadenopathy (n [%])	8 (26.7)	8 (26.7)	5 (16.7)
Lung crepitation (n [%])	2 (6.7)	1 (3.3)	2 (6.7)
Hepatomegaly (n [%])	8 (26.7)	3 (10.0)	4 (13.3)
Splenomegaly (n [%])	0	0	0
Focal neurological deficits (n [%])	0	0	0
Haematology			
Treatment arm	**A n=30**	**B n=30**	**C n=30**
Haemoglobin, g/dl, median (IQR)	12.9 (12.1-14.4)	13.1 (11.6-13.9)	12.9 (12.1-13.9)
Haematocrit, %, median (IQR)	39.35 (36.5-44.3)	39.5 (35.9-41.2)	38.85 (36.5-43.4)
Platelets $x10^3/\mu L$, median (IQR)	135.5 (71-191)	142 (93-208)	125.5 (98-213)
White blood cells $x10^3/\mu L$, median (IQR)	8.35 (5.3-12.3)	7.6 (6.3-13.3)	6.85 (5.1-11.1)
Neutrophils $x10^3/\mu L$, median (IQR)	6.47 (4.33-9.60)	6.69 (4.69-11.54)	5.08 (3.85-8.48)

Lymphocytes $x10^3$/ µL, median (IQR)	1.39 (0.99-2.10)	100 (0.66-1.48)	1.05 (0.59-1.83)
Monocytes $x10^3$/ µL, median (IQR)	0.010 (0-0.050)	0.008 (0-0.36)	0.009 (0-0.222)
Eosinophils $x10^3$/ µL, median (IQR)	0.432 (0.310-0.886)	0.471 (0.285-0.602)	0.349 (0.185-0.434)
Biochemistry			
Treatment arm	**A**	**B**	**C**
Blood urinary nitrogen, mg/dl, median (IQR)	14 (11.8-25) n=29	14 (11-19.9) n=29	12 (10-20.7) n=28
Creatinine, mg/dl, median (IQR)	0.92 (0.78-1.12) n=29	1.005 (0.855-1.22) n=28	0.92 (0.77-0.98) n=29
Sodium, mmol/l, median (IQR)	134.5 (129.5-137) n=28	133.5 (131-136) n=28	133.5 (131-136.5) n=28
Potassium, mmol/l, median (IQR)	3.85 (3.45-4.15) n=28	3.8 (3.3-4.15) n=28	3.5 (3.4-4) n=28
Chloride, mmol/l, median (IQR)	100 (94-105) n=27	99 (95.5-104.5) n=28	100 (97-104) n=27
Globulin, g/dl, median (IQR)	3.45 (2.95-3.95) n=20	3.5 (3.1-3.85) n=20	3.3 (2.8-3.7) n=21
Bilirubin direct, mg/dl, median (IQR)	0.35 (0.25-0.45) n=28	0.4 (0.2-0.6) n=28	0.4 (0.2-0.5) n=29
Bilirubin total, mg/dl, median (IQR)	0.65 (0.45-0.9) n=28	1 (0.45-1.25) n=28	0.7 (0.4-1) n=29
AST, IU/l, median (IQR)	64 (37-119) n=29	63.5 (34.5-120.5) n=28	86 (40-183) n=29
ALT, IU/l, median (IQR)	62 (21-121) n=29	52 (27.5-85) n=28	94 (33-188) n=29
ALP, IU/l, median (IQR)	115 (89-208) n=27	104 (66-208) n=28	110 (84-167) n=29
CRP, µg/ml, median (IQR)	111 (41-156) n=30	98.5 (50-149) n=30	78.5 (39-140) n=30

6.4.2 Fever clearance time (FCT)

FCT for the 3 arms are shown in Table 19 and Kaplan-Meier survival curves were plotted and depicted in Figure 47. There were no statistically significant differences between the 3 treatment arms. For 4 subjects, the FCT could not be calculated and were not included in Table 19. Two subjects died before fever cleared – one subject during admission and the other subject after being transferred to another hospital who had persistent fever at the last follow-up (both in Arm B). One subject (Arm C) received rifampicin as TB treatment before fever cleared. The remaining subject (Arm A) was discharged soon after administration of the study drug so there were no temperature measurements and FCT could not be calculated. The subject had fever prior to study drug administration and reported no fever thereafter at follow-up.

Table 19. Fever clearance time by treatment arm.

FCT in hrs (median, IQR)		
Arm A (n=29)	**Arm B (n=28)**	**Arm C (n=29)**
24 (16-58)	29 (13-44)	31 (18-49)

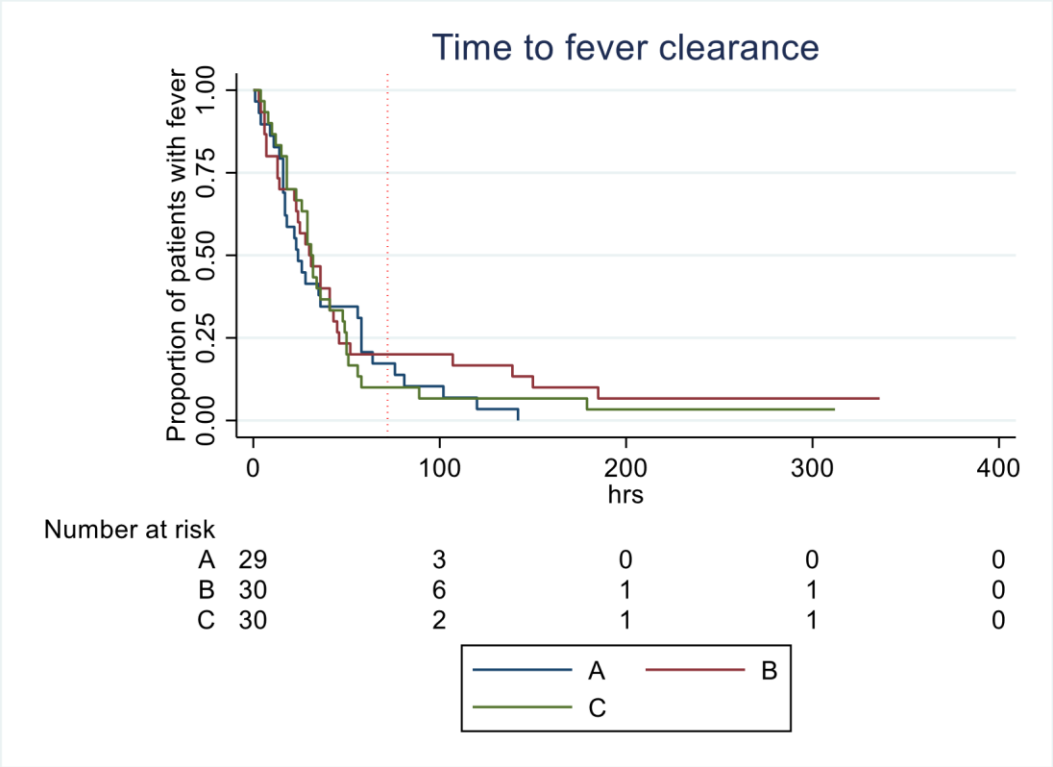
Kruskal-Wallis test – p = 0.908 (chi^2 0.194 with 2df)

Wilcoxon ranksum test for B vs A – p = 0.943

Wilcoxon ranksum test for C vs A – p = 0.828

Wilcoxon ranksum test for C vs B – p = 0.615

Figure 47. Kaplan-Meier survival curve for proportion of patients with fever by study arm.



NB – 1 subject (Arm A) had no recorded temperature post-drug administration so FCT could not be calculated. 3 subjects are included in survival time analysis as risk population but not recorded as failure events as FCT could not be calculated (one died without fever clearance, one had fever at last follow-up [day 7] and subsequently died following transfer to another hospital and one received non-study *Orientia tsutsugamushi* active medication).

Cox regression for survival analysis – Breslow method for ties

Treatment arm B vs A -p= 0.437 HR 1.23, 95% CI (0.73-2.10)

Treatment arm C vs A -p= 0.778, HR 1.08, 95% CI (0.64-1.82)

Treatment arm C vs B -p= 0.613, HR 0.87, 95% CI (0.52-1.48)

In addition, on request by the Data Safety and Monitoring Committee (DSMC), we also performed additional analysis to compare FCT between patients recruited at Chiangrai and Mae Sot. The results are shown in Table 20 and Figure 48. There were no significant differences in FCT between study sites.

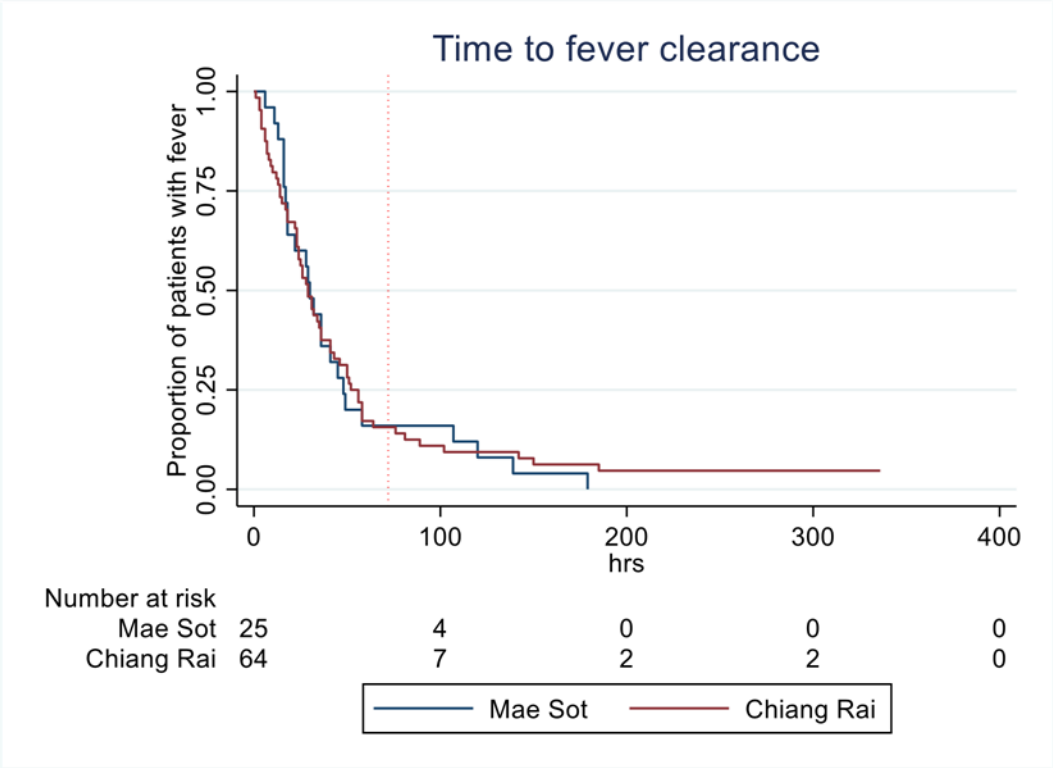
Table 20. Fever clearance time by study site.

FCT in hrs (median, IQR)			
Chiang rai site		**Mae Sot site**	
All n=61	PCR positive n=26	All n=25	PCR positive n=13
28 (14-50)	36 (18-58)	30 (17-48)	36 (18-49)

Kruskal-Wallis rank sum test: p=0.490 (all subjects)

Kruskal-Wallis rank sum test: p=0.823 (only PCR positive)

Figure 48. Kaplan-Meier survival curve for proportion of patients with fever by study site.



Cox regression -- Breslow method for ties

All subjects: p=0.819, HR 0.95, 95% CI (0.59-1.51)

6.4.3. Treatment failure

Results for treatment failure are shown in Table 21. All treatment failures included in the interim analysis are clinical treatment failures (mainly fever >72hrs) as PCR analysis of serial blood samples on individual study patients have not been completed. There were no significant differences between the 3 study arms.

Table 21. Treatment failure by treatment arm.

		Treatment Arm			Total
		A	B	C	
Treatment failure	Yes	5 (16.7%)	6 (20.0%)	3 (10%)	14 (15.6%)
	No	25 (83.3%)	24 (80.0%)	27 (90%)	76 (84.4%)
Total		30	30	30	90

Fisher's exact test all groups – p =0.553

Fisher's exact test B vs A – p = 1.000

Fisher's exact test C vs A – p = 0.706

Fisher's exact test C vs B – p = 0.472

Proportions (95% CI) for C= 2.1 to 26.5%

Proportions (95% CI) for B= 7.7 to 38.6%

Proportions (95% CI) for A= 5.6 to 34.7%

6.4.4 Disease relapse by treatment arm

There were only 2 patients with possible disease relapse during the 8 week follow-up period for the 90 patients included in the interim analysis. These were fever relapses without associated bacteraemia (negative PCR). There were no significant differences between the treatment arms. The results are shown in Table 22.

Table 22. Disease relapse by treatment arm.

		Treatment Arm			Total
		A	B	C	
Relapse	Yes	1	1 (3.3%)	0	2
	No	29 (96.7%)	29 (96.7%)	30	88
Total		30	30	30	90

Fisher's exact test all groups – p = 1

Fisher's exact test A vs B – p = 1

Fisher's exact test A vs C – p = 1

Fisher's exact test B vs C – p = 1

Proportions (95% CI) for A= 0.0 to 17.2%

Proportions (95% CI) for B= 0.0 to 17.2%

Proportions (95% CI) for C= 0.0 to 11.6%

6.5 Discussion

In this chapter, I have outlined how we are seeking to investigate the efficacy of scrub typhus treatment in northern Thailand and study the determinants of treatment outcome by conducting a RCT. I have outlined the trial protocol and described how I have been involved in the design, setting up, initiation and running of the trial up until and including the interim analysis stage. The data and interim analysis plans were formulated and codes written and tested for analysis. I coordinated the interim analysis and reporting to the DSMC along with formulating additional analyses based on the DSMC response.

The main difficulty with recruitment remains. In seeking to be the most definitive trial on scrub typhus treatment with inclusion of multiple embedded analyses, the recruitment rate has suffered. However, the potential outputs from this trial remain great and completion of the trial will improve our understanding of scrub typhus treatment and the determinants of outcome tremendously.

The interim analysis results have provided some insights into the potential outcomes of the trial. Despite blinding of the treatment arms, there do not appear to be any differences in FCT, clinical treatment failure and relapse between the 3 arms. If the results are confirmed at the completion of study, it will provide clinicians evidence to support the treatment of scrub typhus with shorter courses of doxycycline or azithromycin compared to the standard of care. However, it is to be noted that almost 16% of the recruited patients had treatment failure – mainly prolonged FCT – which occurred in all 3 study arms. Further analysis will be required to assess whether other factors (bacteriological, pharmacological or immunological) contribute to prolonged FCT.

It is intriguing to consider prolonged FCT in scrub typhus in the context of the COVID-19 pandemic and what has been learnt with regards to treatment. Most notably, the evidence

from the RECOVERY trial supporting the use of adjunctive and immunomodulatory treatments such as systemic corticosteroids and tocilizumab [349, 350]. In scrub typhus, a disease caused by an intracellular bacteria, it could be hypothesised that effective treatment may need to include both directed antibiotic therapy and adjunctive therapy targeting the immune sequelae of infection. Indeed, there is a suggestion that adjunctive corticosteroids may benefit scrub typhus treatment outcome in Chiangrai (see Chapter 2) although the lack of transparency regarding the protocol and methodology along with the absence of published results from the trial limit its utility.

Another highlight from the interim analysis results is the absence of differences in outcomes between the 2 study sites. This suggests that the original study which compared FCT between scrub typhus patients in Chiangrai and Mae Sot and concluded that the differences in FCT was due to doxycycline resistance was underpowered (only 19 patients were included across both sites) [178]. The patient selection methodology in this study was also unclear.

There are several limitations of START. The unblinded nature of the trial will leave the results open to bias although we are and will try to limit this by blinding the statistician and PI on analysis. Although screening is being performed with a validated RDT for this region, it is expected that many patients (estimated up to 25%) will be shown not to have scrub typhus based on the diagnostic criteria. The preliminary data suggest that 45% of the recruited patients so far are PCR positive with serological tests pending. However, it is encouraging that around two thirds of PCR positive patients (13/20 so far) are also culture positive which will allow further antibiotic susceptibility and genomic studies. The trial is focusing on admitted scrub typhus patients able to receive oral medications which, in reality, limits the patient cohort to moderate disease, limiting generalisability. Finally, we are not

including children or pregnant women in the trial which as previously explored, are particularly neglected populations.

Despite the historical reports of drug resistance in the treatment of scrub typhus from northern Thailand, most clinicians continue to use doxycycline to treat the disease. The START interim analysis results suggest this course of action remains sensible. However, prolonged fever occurs frequently and the reasons behind this slowed response to treatment remains uncharacterised. The results of this RCT should provide some clarification to the issue of scrub typhus and drug resistance along with supportive data for other determinants of outcome if drug resistance is proven to be an illusion.

7 Personal development through the thesis

In the last 4 years, I have developed both professionally, as a research physician, and have grown personally. During my studies, I have developed a greater understanding of scrub typhus and other rickettsial diseases. I have a better understanding of the history, epidemiology, ecology, microbiology, pathology, immunology, clinical features in adults and children, diagnosis, treatment and prevention of scrub typhus. For the majority of the DPhil period, I was based at Chiangrai Clinical Research Unit as head of the unit and led the staff including research physicians and nurses and laboratory technicians. I managed the unit and was actively involved in the finance, administrative, and personal development aspects of the staff. I was extremely keen to highlight our work to stakeholders and was closely involved in interactions with funders including hosting visitors from MORU, Wellcome, NMRC and elsewhere. I also participated in the future planning of the unit, both at CCRU and as part of the wider MORU Tropical Health Network. Prior to leaving the unit to return to Oxford, I was actively involved in succession planning and in the formal handover process of the unit to my successor (Dr Carlo Perrone, research physician).

I completed the International Funders' Award (Open University) during my studies which aims to enhance management and leadership skills in research professionals. I regularly undertook training in ICH/GCP and research ethics (both in English and Thai) and ensured that I was up to date with my GCP qualifications. I had further training in research statistics including the use of STATA, basic modelling (GLM and GAM) and mapping. I was able to develop data analytical skills which enabled me to perform most of the statistical analyses included in this thesis (e.g. comparative statistics, logistic regression and classification and regression tree analysis).

I learnt and gained experience in designing, preparing, initiating, running and coordinating clinical studies including multi-site studies. Inevitably, as principal investigator on multiple studies, this involved learning about interactions with ethics committees and a data and safety monitoring committee. I also had interactions with local stakeholders (health workers, community health volunteers, villagers and public health authorities) along with funders who supported the studies. I had additional contacts with funders through grant applications and they included Wellcome, MRC, NIH, NMRC and DTRA. I also successfully obtained funding for public engagement and awareness projects on scrub typhus in Chiangrai and was central to designing programmes aimed at improving awareness and the management of scrub typhus in high-risk communities. Through careful collaborations with local health workers, volunteers, villagers and public health staff, I was able to formulate materials to be used such as patient information leaflets, posters and audio-visual files, translated into multiple languages. Examples of these materials are depicted in Appendix 10. We are hopeful that through the efforts of the CCRU team, the local public health authorities will make doxycycline more widely available in at-risk areas of the province and that the burden of severe scrub typhus will decrease through early recognition and prompt treatment.

As part of my DPhil studies output, I have been involved in the writing and submission of papers to scientific journals for publication and also, formulating the replies to reviewers' and editorial comments. I was and continue to be actively involved in peer review. I have also regularly prepared and presented both posters and oral presentations at local, departmental, national, regional and international meetings. Near the end of my time in Chiangrai, I had the honour of organising, coordinating, preparing, hosting and running the 2nd Asia Pacific Rickettsia Conference (APRC2). This was attended by around 120 delegates from 21 countries from the Asia Pacific region and beyond (Figure 49). It is humbling to think that this was the last in-person major international conference on rickettsial diseases prior to the

COVID-19 pandemic. I have also been actively participating in the DTRA (Defense Threat Reduction Agency, US Department of Defense) funded Rickettsia Threat Reduction Network (TRN) which aims to bring together the relatively small but dedicated group of researchers involved in research into rickettsial diseases to brainstorm and highlight research gaps for focus. Both the conference and Rickettsia TRN have allowed me to broaden my research network which should prove beneficial in my future pursuit of scrub typhus research.

Figure 49. Delegates of the 2nd Asia Pacific Rickettsia Conference held at the Riverie Hotel, Chiangrai, Thailand; 3-6 November 2019.

8 Conclusions and future work

In this thesis I have focused on scrub typhus and its impact on the population of northern Thailand, which lies at the heart of the endemic region. In chapter 2, I highlighted how scrub typhus remains poorly studied and frequently neglected, a situation which contributes to unresolved issues and knowledge gaps. The fascinating historical context was explored along with the evolving epidemiology of scrub typhus. I carried out a comprehensive review of the global distribution of scrub typhus in humans and also included countries where the potential for human cases exist through detection of *Orientia tsutsugamushi* in other vertebrate and invertebrate hosts. I focused in detail on the chronology of scrub typhus discovery per country (Appendix 1). A similar exercise was carried out for Thailand which allowed the mapping of disease by province and a chronology of reported human cases of scrub typhus to be established (Appendix 2). A current overview of scrub typhus diagnostics outlined multiple issues and challenges yet to be met.

Scrub typhus treatment and the evidence for doxycycline resistance was extensively reviewed and a novel conclusion reached. This challenged the previously held orthodoxy that doxycycline resistance existed, and I provided an alternative scientific explanation for the findings previously reported. I believe that this will prove to be both clinically and academically significant and a key step towards our understanding of the determinants of treatment outcome in scrub typhus. Although a review on preventative measures for scrub typhus was limited by the lack of data, some practical measures could potentially be effective and these were later utilised in the preparation of materials for public awareness/engagement programmes (see below and Appendix 10).

In chapter 3, I was able to estimate the burden of scrub typhus in Thailand using national surveillance data which emphasised the elevated and increasing disease burden when

compared to published surveillance data from other endemic countries. The results suggest that scrub typhus is the most clinically important rickettsial disease in this region. Variability in disease epidemiology was observed with the highest burden in the northern and northeastern regions of the country. There was also marked seasonality in these regions with more cases reported in the wet and early cool seasons. Adults of working age and agricultural workers were most at risk while detailed analyses for Chiangrai province revealed how geographical and meteorological factors may contribute to disease burden. Human activities and behaviour (e.g. health-seeking behaviour) will undoubtedly impact disease epidemiology but data were unavailable and should be investigated in future studies. Detailed geo-location data has allowed high burden villages and sub-districts to be identified for public awareness/engagement programmes and will help inform local public health policies going forward.

Moving to the clinical interface, in chapter 4 I outlined how scrub typhus contributes to the febrile disease burden at a local provincial hospital in Chiangrai. Scrub typhus was diagnosed in 22.5% of patients recruited to the fever study which is one of the highest proportions reported from cause of fever studies. The disease characteristics in adults was reported with presence of eschar, elevated ALP and elevated AST found to be predictive for a scrub typhus diagnosis. This is particularly useful for clinicians without access to diagnostic tests within the endemic region. There was further evidence suggesting that scrub typhus patients were not being treated promptly in the community which may have a detrimental impact on morbidity and mortality. These data along with information on local disease burden will be used to inform local public health authorities of the need for scrub typhus treatment to be available at primary care units in high incidence areas. An additional output from this study was the finding that low CRP could be predictive of a viral infection (mainly dengue) in acutely unwell febrile patients admitted to hospital.

Scrub typhus in children has been particularly understudied, and in chapter 5 I was able to characterise paediatric disease in Chiangrai. Presence of eschar, cough, tachypnoea and lymphadenopathy were commonly observed clinical features. Thrombocytopenia, raised CRP, low albumin and raised hepatic enzymes were frequently seen in the patient cohort. Unlike previously published reports, diagnosis was confirmed using a robust diagnostic criteria. Similar to adults, children with scrub typhus did not receive appropriate treatment prior to hospital admission which may have contributed to the high complication and treatment failure rates. Severe hepatitis was significantly predictive of treatment failure regardless of whether appropriate antibiotics were instigated on admission.

In the final results chapter (chapter 6), the Scrub Typhus Antibiotic Resistance Trial (START) protocol was outlined. The trial was designed not only to assess the efficacy of doxycycline and azithromycin but to also study the host, microbiological and pharmacological factors determining treatment outcome. My role as principal investigator and trial progression up to the interim analysis stage was narrated along with challenges and limitations. The intriguing interim analysis results were also included. Once completed it will be one of the most definitive treatment trials on scrub typhus, with rich datasets allowing for additional studies on genomic, diagnostic, immunological, virulence, antibiotic susceptibility testing, pharmacokinetic/pharmacodynamic and epidemiological aspects of scrub typhus in adults.

Although the work described in this thesis has contributed significantly towards improving our understanding of scrub typhus, there remains much to be learnt. The burden of disease in Thailand is now clearer though further work on high resolution epidemiological data for the remainder of the country is still progressing. Estimating accurately the regional and global burden of disease remains a long term target, one which will require considerable advancements in diagnostic capability and coverage, and recognition of scrub typhus as a

significant disease by many more national public health authorities than currently. Researchers will continue to persevere and the hope is that through ongoing collaboration and international cooperation, rickettsial diseases, and scrub typhus in particular, will gain the prominence that reflects their public health importance.

Samples and data from the paediatric scrub typhus study, eschar study and START will continue to be processed and analysed. The natural cellular immune response will be characterised using ELISpot and flow cytometry assays. The dynamics of the humoral immune response will also be investigated. Additionally, we plan to study disease severity markers including cytokine responses and activation of the coagulation cascade. I continue to be involved in the eschar study which aims to study the dissemination dynamics of *Orientia tsutsugamushi*. We will also study the prevalence of non-scrub typhus eschar-associated disease (e.g. spotted fever rickettsia infection) in Chiangrai. Dr Jeanne Salje and I have applied for a NIH R01 grant to study *Orientia tsutsugamushi* persistence and quiescence which will be aided by our cohort of confirmed bacteraemic scrub typhus patients in Chiangrai.

The sample and data rich clinical studies carried out in Chiangrai will continue to benefit diagnostic advancement of scrub typhus. I continue to be involved in trying to identify *Orientia tsutsugamushi* antigens which will hopefully aid in the development of accurate and cost-effective rapid diagnostic tests. We are taking two main approaches: the first is to screen for *Orientia tsutsugamushi* antigenic targets from studying the natural humoral responses from patient samples and the second is to look for unique proteomic signatures using mass spectrometry in acute urine samples from patients. Improving the ease of culture with cell-free culture methods is another aspiring area of study and our past and current studies can contribute to this in future. In conjunction with Prof Stuart Blacksell, we will

continue studying ways to improve the accuracy and yield of current molecular and serological assays for scrub typhus in use at MORU.

One of the limitations of START is that it only targets mild to moderate disease. However, the methodology and protocols used have contributed to another current, ongoing RCT in India (the INTREST study (Clinical Trials Registry India: CTRI/2018/08/015159)), investigating the treatment of severe scrub typhus with intravenous antibiotics. Future trials investigating the optimal treatment for scrub typhus in young children and pregnant women should also be prioritised while the use of alternative antibiotic agents both novel and re-purposed may bring additional benefits for patients. Adjunctive treatment with immunomodulatory drugs such as steroids should also be investigated in a transparent and definitive manner to allow greater generalisability and maximise any benefit derived from these trials. We will complete START despite the difficulties surrounding trial recruitment during the COVID-19 pandemic. This will allow us to understand more regarding the determinants of treatment outcome and thus, improve the care of scrub typhus patients.

Finally, future work on disease prevention should continue and expand. Public engagement and awareness programmes in Chiangrai will continue with the focus on high disease burden sub-districts. We will assess the impact of these programmes and continue to evaluate the availability of doxycycline in the community as the hoped for changes are realised. Ongoing immunology studies will hopefully reveal immune correlates of protection against infection and severe disease, guiding future adjunctive treatment and vaccine development efforts. The potential of a scrub typhus vaccine based on mRNA technology is both exciting and intriguing and our results should contribute to this. Prophylaxis remains an understudied area and more could be done to optimise the antibiotic option and regimen for at-risk groups.

In this thesis, I have outlined my work on scrub typhus in northern Thailand. For the first time, the estimated disease burden for the country has been described and the changes over the last decades evaluated. The impact of scrub typhus on the febrile disease burden on hospitals have been highlighted along with the disease characteristics in adults and children. I highlighted the potential determinants of treatment failure and reviewed the controversial subject of drug-resistant scrub typhus infections, concluding that drug resistance may have been misconceived. Finally, the preliminary results from the ongoing scrub typhus antibiotic resistance trial are supportive of my conclusions on scrub typhus and doxycycline resistance and future outputs from the trial will significantly impact our understanding of the determinants of treatment outcome.

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10 Appendix

Appendix 1

Chronology of the global distribution of scrub typhus

Location	Year	Notes	Reference no.
China	313	Probably the earliest clinical description of scrub typhus	[11]
	2015	Increased geographical distribution and disease incidence 2006-2014, surveillance data	[86]
Japan	1810	Hashimoto describes a “tsutsuga” or disease, using the term “flood fever”	[5]
	2017	Stable disease incidence 2007-2016, disease prevalent in all prefectures, surveillance data	[85]
Indonesia (excluding New Guinea)	1902	Earliest observations in 1902 in Sumatra, outbreak of “pseudo-typhoid” in the tobacco estates of Senembah Company, Deli, northern Sumatra in 1910 (L. deliense being named after the town)	[25, 351, 352]
		Also reported on Borneo, Java and Sulawesi (Celebes)	[4, 57, 351]
	1997	Ongoing exposure occurring in East Java, seroprevalence study, 1.3% seropositive for *Orientia tsutsugamushi*	[353]
	2003	9.4% seropositive for *Orientia tsutsugamushi* on Gag Island, west of Papua, seroprevalence study	[354]
Taiwan	1908	Observed and suspected in Japanese police officers in Hualien, eastern Taiwan	[355]

	1931	First description of tsutsugamushi disease on the Pescadores Islands (Penghu)	[356]
	2006	Increase in cases in Eastern Taiwan 2000-2004	[249]
	2018	Stable disease incidence 2004-2016, highest disease burden in Eastern region and Kinmen, Lienchiang and Penghu counties in Kao-Ping region, surveillance data	[87]
Philippines	1908	Two suspected cases of tsutsugamushi disease, clinical description	[357]
	1946	Laboratory confirmed cases in Allied and Japanese forces and one *Orientia tsutsugamushi* strain isolated	[55]
	1981	Seroprevalence study, positivity in Sorsogon (6%), Palawan (7%), Cebu (8%) and Capiz (16%)	[358]
Australia	1910	Reports of Mossman fever in settlers on Daintree river near Mossman, northern Queensland, disease known among indigenous Australians previously	[27]
	1993-2007	Laboratory confirmed (serology and PCR) cases of scrub typhus in northern Queensland, Torres Strait Islands, Litchfield Park in the Northern Territory and the Kimberley region of northern Western Australia	[359-362]
Malaysia	1915	Suspected case of Kedani river fever	[24]
	1979	Serotyping reveal that Karp and Karp-like *Orientia tsutsugamushi* strains dominate among febrile patients in rural Malaysia	[363]
	1984	Scrub typhus accounted for 19.3% of 1,025 cases of febrile patients	[242]

	2013	High exposure in indigenous Malay populations (17.9%), seroprevalence study	[364]
Vietnam	1915	2 cases of “pseudo-typhus” described	[365]
	2014	Scrub typhus accounting for 3.5% of febrile cases 2001-2003, Hanoi	[366]
Korea	1915	Clinical description of cases of “continued fever” resembling scrub typhus	[68]
	2015	Increasing incidence 2001-2013 (surveillance data), urban cases becoming more common, seroconversion in US military personnel still occurring	[236, 253, 367]
Melanesia (New Guinea and adjacent islands)	1930	Probable scrub typhus case based on clinical findings alone	[368]
	1935	Reported as endemic typhus despite clinical and serological features of scrub typhus	[369, 370]
India	1932	Cases diagnosed clinically and with Weil-Felix OXK reactions, reported from the Simla Hills and other regions of India	[371, 372]
	2015	*Orientia tsutsugamushi* Kato-like strains most common followed by Karp-like strains in Vellore (South), Shimla (North) and Shillong (Northeast) – 56kDa genotyping	[373]
	2017	Review showing high burden of disease throughout India, 2002-2015	[7]
Burma	1932	1 case of probable scrub typhus admitted to Rangoon General Hospital, positive Weil-Felix OXK reaction, history of touring in forests in Upper Burma	[374]
	1944-1945	Cases of probable scrub typhus (Weil-Felix OXK reaction positive) reported	[57, 351]

		during WWII throughout Burma in at least 17 districts	
	2009	Case of serologically diagnosed scrub typhus imported to Japan from Myanmar	[375]
Singapore	1934	16 cases of probable scrub typhus (Weil-Felix OXK reactions positive)	[376]
	1999	6 autochthonous cases seropositive for scrub typhus by indirect immunoperoxidase test	[377]
Cambodia	1937	Clinical cases of scrub typhus with Weil-Felix OXK positive reactions	[378]
	2013	Scrub typhus caused 7.8% of febrile children hospitalised, 2009-2010	[247]
	2014	3.9% of acute undifferentiated fever had detectable *Orientia tsutsugamushi* by PCR, 2008-2010	[298]
	2017	*Orientia tsutsugamushi* the cause of 4.7% of acute meningoencephalitis in children, 2010-2013	[379]
Sri Lanka	1937	6 cases of tsutsugamushi disease diagnosed using the Weil-Felix OXK test, 1 clinical case described in 1940	[380]
	2017	Ongoing case reports and series of scrub typhus, genotypic characterisation revealed *Orientia tsutsugamushi* of all three prototypic strains (Karp, Kato and Gilliam) infecting patients – mainly Karp-related	[381, 382]
Laos	1939	Fatal case of tropical typhus contracted in upper Laos, positive Weil-Felix OXK reaction	[383]
	1996	3.9% of military personnel seroconverted to *Orientia tsutsugamushi* (ELISA)	[211]

	2003	14.8% of acutely febrile adult patients with negative blood culture had scrub typhus (serology - IFA)	[384]
	2010	Scrub typhus caused 7% of non-malarial fever cases (IFA, PCR)	[246]
Maldives	1941	Gan Island 1941-1944, 1098 cases reported in mainly military personnel	[385]
	2002	Outbreak and re-emergence of scrub typhus, 168 cases in under 1 year, 6% case fatality rate	[386]
Hong Kong	1941	Many records lost during the Japanese occupation but several cases reported from military personnel and civilians with positive Weil-Felix OXK test	[387]
	2006	58 cases of scrub typhus reported 1995-2004, surveillance data	[388]
Diego Garcia (Chagos Archipelago)	1942	47 cases of scrub typhus over 7 months, mainly around Eclipse Point	[57]
Melanesia (Solomon Islands and Vanuatu)	1943-1945	Reports of scrub typhus cases and isolation of pathogen in mites and rats	[54, 389]
	1975	Isolation of *Orientia tsutsugamushi* in rats in Eastern Solomon Islands and Northern Vanuatu	[390]
	1981	Seroprevalence study, widespread exposure in Melanesia but not Polynesia or New Zealand,	[391]
	2014	9 serologically (indirect micro-immunofluorescence assay) confirmed cases of scrub typhus in the Western Province of the Solomon Islands	[392]

Thailand	1943-1944	Japanese records suggest cases of scrub typhus in the Kwe Noi Valley and Phuket during WWII	[57]
	1952	First serologically confirmed scrub typhus patient from central Thailand, first *Orientia tsutsugamushi* strains isolated from rodents in the same area in 1954	[257, 393]
	2004-2009	Scrub typhus responsible for 7.5-19.9% of children and adults with acute undifferentiated fever	[243, 244, 394]
Rwanda and Burundi (formerly Belgian Congo)	1951	Serological evidence (complement fixation, CF) of exposure to *Orientia tsutsugamushi* in 2 native Africans and 2 African-born individuals of Indian origin, Musha Hill	[3, 395]
Oman	1951	8 healthy volunteers born in Muscat but moved to Musha Hill area in Belgian Congo had positive antibodies to *Orientia tsutsugamushi* (CF)	[3, 395]
Yemen	1951	Weil-Felix OXK positive agglutination reactions in serum from healthy men in Ta'izz district but not San'a district, 18% at ≥1:40 dilution	[396]
Pakistan	1962	Outbreak of scrub typhus in Sialkot area, *Orientia tsutsugamushi* isolated from patients, rats and mites	[397]
	1967	*Orientia tsutsugamushi* isolated from rodents in unusual habitats including alpine terrain, mountain deserts, semi-deserts and plains	[82]
	1975	*Orientia tsutsugamushi* Karp strain the main serotype from isolates collected from humans, rodents and mites	[398]

Tajikistan	1962	*Orientia tsutsugamushi* isolates from rodents and mites serotyped as Gilliam strain	[399]
Russia (Primorye region, Eastern Siberia)	1963	Seroprevalence rate of 4% (compliment fixation) in 1,838 human serum samples, *Orientia tsutsugamushi* strains isolated from rodents, mite vectors and 1 human case, mainly Gilliam serotype	[399-401]
	1993-1994	7.1% of rodents trapped were seropositive for scrub typhus, no *Orientia tsutsugamushi* strains isolated from rodents or mites	[402]
Iran	1974	Evidence of exposure to scrub typhus in small mammals (serological study)	[403]
Nepal	1978-1979	Seroprevalence rate of 10% (188 healthy human subjects) using micro-immunofluorescent assay	[404]
	2001	Scrub typhus caused 3.2% of acutely febrile patients admitted to a hospital in Kathmandu (multi-pathogen IgM RDT)	[405]
	2015	Outbreak of scrub typhus post-earthquake	[406]
Afghanistan	1982	Serological evidence of exposure in Kunduz and Badakhshan provinces, no clinical cases	[407]
Republic of Congo	1989	Case of returning traveller to Japan, clinically and serologically diagnosed (indirect immunoperoxidase assay, IIP)	[408]
Micronesia (Palau)	1995	Seropositive serum from 1995 (IFA IgG and IgM), 15 human cases diagnosed (IFA IgM) 2001-2003 on the Southwest Islands (mainly Sonsorol)	[409, 410]

Cameroon	1997	Scrub typhus in returning missionary, clinical case with eschar and positive serology (indirect immunofluorescence assay, IFA)	[411]
Tanzania	1999	Scrub typhus in returning traveler to the Netherlands, clinical and serological diagnosis (IFA)	[412]
Bangladesh	2003	19 cases diagnosed clinically with positive Weil-Felix OXK reaction	[413]
	2010	23.7% seropositivity (ELISA detecting IgM) for *Orientia tsutsugamushi* in non-febrile patients, 6 major teaching hospitals	[414]
	2015	Scrub typhus diagnosed (IFA and PCR) in 16.8% of febrile patients, 56kDa genotyping of *Orientia tsutsugamushi* strains revealed Karp-related clade were common	[248]
Uzbekistan	2005	Serological evidence (commercial IgM ELISA kit) of scrub typhus in 6% of 108 acutely febrile patients	[415]
United Arab Emirates	2006	Case of scrub typhus diagnosed using serology (IFA), PCR (16S rRNA, 47 kDa, 56kDa genes) and in vitro cell culture; gene sequencing revealed divergence from known *Orientia tsutsugamushi* strains, novel species Candidatus Orientia chuto proposed	[98]
Chile	2006	Scrub typhus in a biologist following an ecological trip to Chiloé Island, diagnosed using serology on paired blood samples (IgG ELISA) and PCR of eschar biopsy, 16S rRNA sequencing revealed a	[416]

		divergent form/species of Orientia spp.-like bacteria	
	2015-2018	12 further clinical cases described (3 from Chiloé Island and 9 from the mainland), diagnosed using serology (IgM and IgG, ELISA and IFA) and PCR, 56 kDa gene sequencing in 1 case showed close homology with *Orientia tsutsugamushi*	[417, 418]
Bhutan	2008	Outbreaks of scrub typhus in Gedu, Chukha district, diagnosed retrospectively using serology	[251, 256]
	2015	Seroprevalence (micro-immunofluorescence assay) of 22.6%, healthy volunteers from 8 districts	[419]
	2015	Scrub typhus diagnosed in 6.7% (IFA and PCR) of 1,044 patients with acute undifferentiated fever	[420]
Djibouti	2010	IgG antibodies to scrub typhus group orientiae detected in 6% of abattoir workers (ELISA, western blot)	[421]
Kenya	2011-2015	5-5.8% of febrile adults and children had detectable IgG antibodies to scrub typhus group orientiae using acute samples (ELISA, western blot)	[422, 423]
	2017	Orientia spp. DNA detected (PCR) in chiggers collected from captured rodents, 47kDa gene and 16S rRNA sequencing suggest Candidatus Orientia chuto as the closest phylogenetic relative	[424]
South Africa	2012	Scrub typhus group orientiae genetic material detected in the blood of 1 dog (16S rRNA sequencing)	[425]

Peru (Iquitos)	2013	Scrub typhus group orientiae IgG antibodies detected in 5.3% of febrile patients (ELISA, micro-immunofluorescence), 1 case with recent active infection (seroconversion)	[426]
Senegal	2015	*Orientia tsutsugamushi* DNA detected by PCR in spleens of exotic house mouse	[427]
France (Ardennes region)	2015	*Orientia tsutsugamushi* DNA detected by PCR in spleens of rodents in close proximity to human dwellings	[427]
Sao Tome and Principe	2019	Seroprevalence study. *Orientia tsutsugamushi* IgG/IgM detected by ELISA and IFA, and confirmed by western blot in large sample set from healthy pregnant women.	[428]

Appendix 2

Chronology of reported scrub typhus in humans in Thailand

Year	Location by province	Notes	Reference number
1943-1944	Kanchanaburi, Phuket	Suspected cases among Japanese troops during WWII	[57]
1952	Nakhon Pathom	First clinical case with confirmatory serology described	[257]
1954	Nakhon Pathom	First Thai *Orientia tsutsugamushi* strains isolated from rodent tissue	[393]
1964	Nakhon Pathom, Ratchaburi, Chiangmai	*Orientia tsutsugamushi* strains isolated from rodents and patients	[429]
1976	Prachinburi	77% seroprevalence of antibodies to *Orientia tsutsugamushi* (IFA) in children and adults in one village	[333]
1984-1985	Surin	40 clinically diagnosed cases described, 14 with confirmatory IFA testing performed, all cases were in displaced Khmer refugees	[430]
1985-1987*	Songkhla	28 paediatric scrub typhus cases diagnosed serologically	[431]
1985-2002*	Songkhla	73 paediatric cases serologically diagnosed (IFA or IIP) described	[320]
1986	Prachinburi	2 febrile displaced Khmers with scrub typhus diagnosed by micro-immunofluorescence assay	[432]

1988	Trat	1 serologically diagnosed case on another displacement camp on the Thai-Cambodian border	[433]
1989	Prachinburi	2 febrile adult patients serologically diagnosed with scrub typhus (IIP) at a camp site for displaced Khmers	[433]
1989-1991	Uttaradit, Phitsanulok, Tak, Kanchanaburi, Prachinburi, Ranong, Suratthani, Songkhla, Yala, Narathiwat	Varying seroprevalence rates (IFA) between provinces from 1.6% in Songkhla to 24.8% in Kanchanaburi, Thai Rangers had higher seroprevalence rates than Royal Thai Army soldiers (18.6% vs 6.8%)	[434]
1991	Chiangrai	54 adult patients attending outpatient clinics diagnosed serologically with scrub typhus (IIP); dengue patients more likely to have haemorrhagic complications, low platelets and low white blood cell counts	[435]
1991-1992	Si Saket	Point prevalence of antibodies to *Orientia tsutsugamushi* (IFA) 0-4.1% in soldiers	[436]
1991-1993	10 hospitals in all 4 regions of Thailand (sites not specified)	Scrub typhus diagnosed in 7.5% of febrile patients age ≥2 years old attending outpatient clinics (serology – Weil-Felix, IFA or IIP used)	[394]
1994	Ratchaburi, Petchaburi, Kanchanaburi	High seroprevalence (59.5%, IFA) in patients attending 6 malaria clinics	[241]
1994	Bangkok, Samut Prakan, Nonthaburi	21% seroprevalence rate (IIP) in suburban population associated with	[437]

		increasing age, orchard and orchid farm exposure	
1995-1997	Chiangrai	Randomised-controlled trial comparing rifampicin with doxycycline, fever-clearance time (FCT) significantly shorter with rifampicin – n=78	[177]
1996	Chiangrai, Tak	Delayed response to antibiotic treatment in Chiangrai attributed to drug-resistance	[178]
1999	Chiangrai	Azithromycin used to treat 2 pregnant women with serologically confirmed scrub typhus (immunoblot dipstick test, IIP)	[184]
1999-2002	Chiangmai, Phisanulok, Udon Thani, Khon Khaen, Chonburi, Bangkok, Songkhla, Surat Thani	240 patients diagnosed serologically, 25.8% PCR positive	[438]
1999-2002	Kanchanaburi	3 patients with acute febrile illness diagnosed serologically (IFA, western blot) with scrub typhus	[439]
2000-2001	Chiangmai	30 paediatric scrub typhus cases described, diagnosed with IFA	[319]
2000-2001	Udon Thani	183 febrile adult patients diagnosed serologically (IFA) with scrub typhus, 44.8% were 16S rRNA PCR positive, 29.0% were 56kDa gene PCR positive, specificity for both PCR	[228, 440]

		assays >99%, higher bacterial load associated with disease severity	
2000-2003*	Nakhon Ratchasima, Loei, Buriram, Udon Thani, Chumphon, Bangkok	16.1% of patients with AUF diagnosed with scrub typhus by IFA or PCR	[244]
2001-2002*	Nakhon Ratchasima, Loei, Buriram, Chumphon, Ratchaburi,	Scrub typhus diagnosed serologically in 19.9% of febrile adult patients	[243]
2001-2002	Nakhon Ratchasima	Scrub typhus (diagnosed by IFA) the major cause of septic shock in 35.3% of adult cases, 3 died	[441]
2001-2003*, 2009-2010	Nakhon Ratchasima, Loei, Buriram, Chiangrai, Chumphon, Ratchaburi, Bangkok	430 patients diagnosed by serology (IFA) and PCR from all provinces, 56kDa genotyping revealed Karp genotype as the dominant clade	[442]
2002	Chonburi	Outbreak of scrub typhus in soldiers following field training, 9.8% serologically diagnosed (IFA)	[443]
2003-2004	Buriram, Chaiyapoom, Chumphon	195 febrile patients serologically diagnosed (Weil-Felix, IFA), 56kDa genotyping of 10 PCR positive samples revealed diversity and possible new genotypes	[444]
2003-2005	Udon Thani, Tak	23 strains of *Orientia tsutsugamushi* isolated from patients, 56kDa genotyping revealed most were in the Karp-related clade	[445]
2003	Songkhla	12 febrile adult and paediatric patients diagnosed with scrub typhus (IFA,	[446]

		PCR), single 200mg dose of doxycycline used, persistence of *Orientia tsutsugamushi* DNA up to 27 days after treatment initiation	
2003	Chiangrai	20 cases of paediatric scrub typhus presented (IFA, rapid diagnostic test/RDT), doxycycline and chloramphenicol for at least 7 days effective, roxithromycin was not	[200]
2003-2005	Udon Thani, Nakhon Ratchasima, Chaiyapoom, Chumphon	Randomised-controlled trial of doxycycline vs azithromycin (n=57), median FCTs were not significantly different, higher proportion of patients treated with doxycycline defervesced within 48hrs	[174]
2004-2005	Chiangrai	54 children with scrub typhus (IFA) reported, 96.3% had abnormal liver blood profiles (mainly raised AST and ALT)	[335]
2004-2006	Tak	Scrub typhus diagnosed in 5.4% of febrile pregnant women (IFA, PCR, culture), abortion and poor neonatal outcome commonly seen	[133, 245]
2004-2007	Bangkok, Nakhon Ratchasima, Si Saket, Sakonnakhon, Chiangmai, Tak	Scrub typhus commonly found to be the cause of fever of unknown origin in Chiangmai and Tak and less so in other provinces (serology, PCR)	[447]
2004-2010	Chiangmai, Chiangrai	526 patients with eschar or positive RDT diagnosed, 13.2% mortality	[6]

2005-2006	Chiangrai, Nan, Phetchaburi, Nakhon Ratchasima, Ubon Ratchathani, Kanchanaburi, Lopburi	5.6% of acute febrile adult patients attending provincial army hospitals diagnosed with scrub typhus (IFA)	[448]
2005-2007	Chiangmai	4 children with scrub typhus (IFA) developed acute myocarditis with cardiogenic shock and ventricular tachycardia, 2 died	[449]
2006-2007	Chiangmai	65 febrile children diagnosed with scrub typhus (IFA, PCR), 26 belonged to Hmong hill tribe and lived in the same village area	[450]
2006-2008	Chiangrai	Scrub typhus diagnosed in 22.5% of acute undifferentiated fever (AUF) cases	[289]
2010-2013	Chiangrai	Randomised-controlled trial (n=57) in children with scrub typhus (eschar, RDT) comparing doxycycline or chloramphenicol vs azithromycin, no significant differences in cure rate but overall treatment failure rate of 17.5%	[176]
2011-2012	Nakhon Ratchasima	29.5% of patients with AUF diagnosed with scrub typhus (IFA, PCR) with 6.2% mortality	[210]
2013	Chonburi	10 cases serologically confirmed in soldiers following field training, 11.1% of healthy soldiers and 9.1% of residents around the site were	[451]

	seropositive for antibodies to scrub typhus group orientiae

[*NB - possibility of reports using overlapping datasets]

Appendix 3

Systemic manifestations and complications of scrub typhus. [adapted from [132]]

Complications	Notes
Cardiovascular	
Arrhythmias	ECG changes common, include sinus tachycardia, relative bradycardia, ST changes, atrial flutter or fibrillation, atrial standstill, PR prolongation, prominent U waves, ventricular premature beats, QT prolongation
Myocarditis	Case reports/series in both adults and children, associated with reduced ejection fraction, hypotension and arrhythmias; recovery usually seen if not fatal
Pericarditis	Isolated case report only (S. Korea)
Acute coronary syndrome, myocardial infarction	2 cases reported from S. Korea and India, increased risk of ACS observed in Taiwan
Respiratory	
Chest x-ray (CXR) changes	Common, changes up to 72% of patients based on observational studies, bilateral reticulo-nodular shadowing the main abnormality seen, others include pleural effusion, ground-glass opacities, consolidation, pulmonary oedema, cardiomegaly, hilar lymphadenopathy
Pneumonitis	Common, prevalence as high as 51%, usually diffuse reticular infiltrates but occasionally focal consolidation seen
Pleural effusion	Relatively common (15-20%), both transudative or exudative effusions seen; more frequent with older age, in men, in patients with cardiac failure or low albumin

Acute respiratory distress syndrome (ARDS)	Less common but associated with more severe disease, multi-organ failure, death; may be related to older age, delay in treatment
Gastrointestinal	
Hepatic dysfunction	Raised liver transaminases extremely common (80-90%) in both adults and children, elevated bilirubin less common, liver failure rare
Diarrhoea	Quite common, may occur in up to 45% of patients
GI haemorrhage	Haematemesis or melaena relatively common; usually caused by mucosal haemorrhage, erosions or ulcers of the stomach; bleeding from the small or large intestine more rare
Peritonitis	Rare, case reports of gastric perforation
Pancreatitis	Less common but associated with multi-organ failure and a high mortality (43%)
Cholecystitis	Rare, both calculous and acalculous cholecystitis in patients diagnosed with scrub typhus reported
Splenic infarction and rupture	Rare, the latter resulting in haemoperitoneum in one report
Renal	
Acute kidney injury (AKI)	Common (up to 53%) and may predict mortality, usually resolves with treatment but occasionally caused chronic kidney disease requiring dialysis
Nephrotic syndrome	Rare, 1 case report associated with membranous glomerulonephritis
Haematological	

Thrombocytopenia	Extremely common in both adults and children, normally transient and improves with treatment, persistent thrombocytopaenia reported in a child
Splenomegaly	Common (up to 47%), usually detected by imaging
Disseminated intravascular coagulation (DIC)	Relatively rare but associated with severe disease, multi-organ failure and high mortality
Haemophagocytic lymphohistiocytosis syndrome (HLH)	Rare but serious complication in adults and children, outcome improved with prompt antibiotic treatment (unlike the outlook for idiopathic HLH)
Thrombosis	Rare, case report of sagittal sinus venous thrombosis leading to seizures and localising signs in a 48 year old man
Endocrine	
Adrenal insufficiency	Rare, isolated case reports only, required short course of cortisone therapy but recovery achieved with antibiotic treatment
Sub-acute thyroiditis	Rare, case reports in adult women, scrub typhus accompanied by neck pain and raised free T4, resolved with scrub typhus treatment, no long-term sequelae reported
Epididymo-orchitis	Rare, case reports in children, resolved with treatment
Neurological	
Meningo-encephalitis	Relatively common (up to 26%); symptoms include headache, neck stiffness, altered sensorium, seizures and focal neurological deficits; CSF lymphocytic pleocytosis usually seen; associated with multi-organ failure although recovery with treatment is expected

Acute disseminated encephalomyelitis (ADEM)	Rare, case reports in adults only, improvement with treatment but persisting neurological sequelae in both cases
Cranial nerve complications	Relatively rare although may be seen more frequently in certain settings; abducens and facial nerve palsies along with optic neuritis and trigeminal neuralgia previously reported, hearing loss a classical complication, resolution normally seen with treatment/recovery, sequelae occasionally reported (e.g. persistent otalgia)
Vascular complications	Rare; cerebral infarction, subdural haematoma, thalamic haemorrhage and haemorrhagic transformation of scrub typhus-related encephalitis previously reported
Peripheral nervous system complications	Rare; Guillian-Barré syndrome (including the Miller-Fisher variant), brachial plexus neuropathy, lumbosacral radiculopathy and mononeuritis multiplex previously described
Other complications	Rare; transverse myelitis, transient parkinsonism, myoclonus, opsoclonus, visual hallucinations and cerebellitis reported
Ocular	Papilloedema, retinal haemorrhage, soft exudates, uveitis, subconjunctival haemorrhage can occur with visual acuity usually unaffected; optic neuritis and retinal vein occlusion rare

Appendix 4

Summary of prospective scrub typhus treatment trials and studies

Author(s)	Year	Country	Age group	Diagnostic modality	Antibiotic treatment	Numbers	Fever response (median and range unless specified)	Clinical response
Tierney [158]	1946	India	Adults	Clinical, Weil-Felix OXK	PABA: [8g LD, then 3g 2hrly; until 1 week after defervescence]	18	FCT – 6 days (3-12) Fever days – 11 (6-19)	17 had mild-moderate disease, 3 relapsed, no deaths
					None	16	Fever days – 19 days (10-79)	14 had severe-fatal disease, 3 deaths
Smadel *et al* [161]	1949	Malaya	Adults	Weil-Felix OXK, bacterial isolation in mice	Chloramphenicol: [single dose to 6 days, mainly <24hrs; total dose 1.6g to 12g]	30 – naturally infected	FCT – 26 hrs (6-96) Fever days – 7 (4-12)	No complications, relapses or deaths
						39 – prophylaxis study	FCT – 32 hrs (16-65) Fever days – 6 (2-15)	21 relapses, no deaths
					None	19	Fever days – 16 (12-31)	Complicated disease in 2, 1 died
Giles and Symington [452]	1950	Malaya	Adults	Clinical, Weil-Felix OXK, bacterial isolation in mice	Chloramphenicol: [single dose; mainly 3g, one patient received 5.25g]	16	FCT – 18hrs (12-72) Fever days – 12 (5-22)	3 had complicated disease, 1 relapsed, no deaths
Bailey and Ley [159]	1952	Malaya	Adults	Clinical, Weil-Felix OXK,	Chloramphenicol:	94	FCT – 31hrs (mean)	No treatment failures,

				bacterial isolation in mice	[3g LD, then 3g divided doses over 12-24hrs]			sequelae or deaths
					Chlortetracycline: 3g LD, then 3g divided doses over 12-24hrs]	30	FCT – 25hrs (mean)	Some gastric irritation, no deaths
					Oxytetracycline: [1.5g 6hrly, total 6g]	46	FCT – 37hrs (mean)	Some gastric irritation, no deaths
					PABA: [dose not stated]	15	FCT – 89hrs (mean)	Toxic haematological and renal reactions common, no deaths
					None	19	FCT – 17 days	1 death
Prezyna *et al* [165]	1954	Pescadores Islands	Adults	Clinical, Weil-Felix OXK, bacterial isolation in mice	Chloramphenicol: [1 day, 3g LD then 2-3g after 12hrs]	25	FCT – 40hrs (mean, upper range 104hrs)	5 relapsed, no deaths
					Chlortetracycline: [1 day, 3g LD then 2-3g after 12hrs]	12	FCT – 48hrs (mean, upper range 92hrs)	2 relapsed, no deaths
					Oxytetracycline: [1 day, 3g LD then 2-3g after 12hrs]	10	FCT – 49hrs (mean, upper range 120hrs)	2 relapsed, no deaths
Sheehy *et al* [166]	1973	Vietnam	Adults	Clinical, Weil-Felix OXK	Chloramphenicol: [3g daily, 3-6 days]	30	FCT – 34.7hrs (mean, 16-94) Fever days – 4.7 (mean, 1-14)	5 relapsed (ill for ≤4 days at treatment initiation), no deaths
					Tetracycline: [2g daily, 3-9 days]	30	FCT – 27.58hrs (mean, 14-68)	2 relapsed (ill for ≤4 days at treatment

							Fever days – 3.9 (mean, 1-10)	initiation), no deaths
Brown *et al* [170]	1978	Malaysia	Adults	Clinical, Weil-Felix OXK, IFA, bacterial isolation in mice	Doxycycline: [single dose, 200mg]	31	28/31 (90%) afebrile in 48hrs (upper range 96hrs)	No relapses or deaths in either groups
					Tetracycline: [7 days, 500mg 6hrly]	24	19/24 (79%) afebrile in 48hrs (upper range 96hrs)	
Olson *et al* [171]	1981	Pescadores Islands	Adults	Clinical, IFA, culture (probably in mice but method unclear)	Doxycycline: [single dose, 200mg]	22	No FCT or fever days reported, initial responses stated as satisfactory, mean FCT stated to be the same for all groups	2 relapses requiring further treatment, 6 with mild self-limiting relapses
					[200mg at baseline then 200mg after 7 days]	21		1 relapse requiring further treatment, 5 with mild self-limiting relapses
					Oxytetracycline: [500mg 6hrly, 7 days]	23		1 relapse requiring further treatment, 2 with mild self-limiting relapses
Song *et al* [172]	1995	South Korea	Adults	Clinical, IFA	Doxycycline: [3 days, 100mg 12hrly]	66	FCT – 34.0hrs (mean, SD 26.5hrs, upper range 120hrs)	4/66 (6.1%) required extension of treatment to 5 days to control fever, no relapses or deaths
					Tetracycline: [7 days, 500mg 6hrly]	50	FCT – 37.0hrs (mean, SD 26.6hrs, upper range 72hrs)	No relapses or deaths
Watt *et al* [178]	1996	Chiangrai, Thailand	Adults	Clinical, IIP	Doxycycline:	12 – Chiangrai	FCT – 80hrs (15-190)	Slower cessation of symptoms and

					[200mg LD, 100mg 12hrly, 7 days]	7 – Mae Sot	FCT – 30hrs (4-58)	signs, no relapses or deaths No relapses or deaths
Watt *et al* [177]	2000	Chiangrai, Thailand	Adults	Clinical, RDT, IIP	Doxycycline: [200mg LD, 100mg 12hrly, 7 days]	28	FCT – 52hrs (4-108)	Slightly slower cessation of symptoms and signs, 2 relapses, no deaths
					Rifampicin: [7 days, 300mg 12hrly]	26	FCT – 27.5hrs (4-84)	No relapses or deaths
					Rifampicin: [7 days, 450mg 12hrly]	24	FCT – 22.5 (3-76)	No relapses or deaths
Kim *et al* [173]	2004	South Korea	Adults	Clinical, IFA	Doxycycline: [200mg daily for 7 days]	46	FCT – 29hrs (4-176)	3/43 with fever >5 days but resolved by day 7, no relapses or deaths
					Azithromycin: [single 500mg dose]	47	FCT – 21hrs (1-120)	No relapses or deaths
Chanta and Chanta [200]	2005	Chiangrai, Thailand	Children	Clinical, RDT, IFA	Chloramphenicol: [100mg/kg/day IV, 7-14 days]	14	14/14 afebrile within 72 hrs	No relapses or deaths
					Doxycycline: [4mg/kg/day PO, 7 days]	2	2/2 afebrile within 72hrs	No relapses or deaths
					Roxithromycin: [10mg/kg/day PO, 7 days]	4	Only 1/4 was afebrile at 72hrs	2/4 required switch to doxycycline, no relapses or deaths

Phimda *et al [174]*	2007	Northeast and South Thailand	Adults	Clinical, IFA	Doxycycline: [200mg LD then 100mg 12hrly, 7 days]	27	FCT – 48hrs (16-120)	No relapses or deaths
					Azithromycin: [1g LD then 500mg 24hrly, 3 days]	30	FCT – 60hrs (12-128)	No relapses or deaths
Kim *et al* [175]	2007	South Korea	Adults	Clinical, IFA	Doxycycline: [200mg daily, 5 days]	45	FCT – 18hrs (4-105)	1/44 cleared fever at day 6 and relapsed at 2 weeks, no deaths
					Telithromycin: [800mg daily, 5 days]	47	FCT – 18hrs (4-72)	No relapses or deaths
Chanta and Ploenchai-wanit [176]	2015	Chiangrai, Thailand	Children	Clinical, RDT	Chloramphenicol age <8: [at least 5 days, 100mg/kg/day divided 6hrly]	19	FCT (Chl and Dox combined) – 30hrs (IQR = 21,48), range 8-104hrs	4/28 (14.3%) had FCT>72hrs, no relapses or deaths
					Doxycycline age ≥8: [at least 5 days, 2.2mg/kg/dose 12hrly]	9		
					Azithromycin: [20mg/kg LD max 1g then 10mg/kg 24hrly max 500mg, 3 days]	29	FCT – 36hrs (IQR = 20,68), range 8-184hrs	6/29 (20.7%) had FCT>72hrs, 1 child with severe disease requiring ICU support, no relapses or deaths
Kim *et al [179]*	2018	South Korea	Adults	Clinical, IFA, PCR	Doxycycline: [100mg 12hrly, 5 days]	83	FCT – 22hrs (IQR 12-24, range 4-96hrs)	No relapses or deaths
					Rifampicin:	75	FCT – 18hrs (IQR 12-24, range 1-72hrs)	No relapses or deaths

		[600mg daily, 5 days]

[PABA – para-aminobenzoic acid, RDT – rapid diagnostic test, IFA – indirect immunofluorescence assay, IIP – indirect immunoperoxidase assay, PCR – polymerase chain reaction, LD – loading dose, FCT – fever clearance time, IQR – interquartile range, ICU – intensive care unit]

Appendix 5

Paediatric scrub typhus study – Scrub typhus patients case record form (CRF)

1. DATE OF VISIT |__|__|/ |__|__|/ 20|__|__| *(dd/mm/yyyy)*

INFORMED CONSENT				
2. Date of informed consent obtained *(dd/mm/yyyy)* |__|__|/ |__|__|/ 20|__|__|			
DEMOGRAPHICS				
3.1 Birth date |__|__|/ |__|__|/ |__|__|__|__| *(dd/mm/yyyy)* ***OR*** 3.2 Age |__|__| years |__|__| months				
4. Sex: ○ Male ○ Female				
5. Ethnic group: ☐ Thai ☐ Karen ☐ Hmong ☐ Other, specify _____				
6.1 Occupation: ○ None ○ Agriculture ○ Office ○ Other 6.2 specify detail: _____				
7. Date of hospital admission *(dd/mm/yyyy)* |__|__|/ |__|__|/ 20|__|__|			
INCLUSION/EXCLUSION CRITERIA				
8. INCLUSION		**YES**	**NO**	**NA**
IN 1	Age > 6 months to ≤ 18 years	○Y	○N	
IN 2	Scrub Typhus Rapid test positivity OR *Orientia tsutsugamushi* PCR-positivity	○Y	○N	
IN 3	Fever in ≤ 14 days of presentation to Hospital	○Y	○N	
IN 4	Willingness to participate in the study and written informed assent and written informed consent obtained.	○Y	○N	
9. EXCLUSION		**YES**	**NO**	**NA**
EX 1	Current TB or TB treatment in ≤ 6 months (contains Orientia-effective antimicrobial)	○Y	○N	
EX 2	Documented HIV infection, use of steroids, chemotherapy, other immunosuppressant therapy or herbal remedies containing steroids and/or pregnancy	○Y	○N	
10. ELIGIBILITY (EL)				
10.1 Is subject eligible? ○ Yes ○ No				

10.2 Enrolment date *(dd/mm/yyyy)* |__|__|/ |__|__|/ 20|__|__|
ENROLMENT CONFIRMED BY _____ _____ DATE |__|__|/ |__|__|/ 20|__|__| INVESTIGATOR (PRINT NAME) SIGNATURE

MEDICAL HISTORY					
11. Onset date of symptoms			|__|__|/ |__|__|/ 20|__|__| *(dd/mm/yyyy)*		
12. Max number of days ill (เป็นมากี่วันแล้ว ก่อนมาเข้าโรงพยาบาล)			|__|__| days		
1) Fever (ไข้)	○Yes ○No	|__|__| days	14) Rigors chills	○Yes ○No	|__|__| days
2) Deafness (หูหนวก)	○Yes ○No	|__|__| days	15) Tinnitus (หูอื้อ)	○Yes ○No	|__|__| days
3) Retro-orbital pain	○Yes ○No	|__|__| days	16) Headache (ปวดหัว)	○Yes ○No	|__|__| days
4) Arthralgia (ปวดข้อ)	○Yes ○No	|__|__| days	17) Myalgia (ปวดกล้ามเนื้อ)	○Yes ○No	|__|__| days
5) Eschar	○Yes ○No	|__|__| days	18) Skin rash (ผื่น)	○Yes ○No	|__|__| days
6) Jaundice (ดีซ่าน)	○Yes ○No	|__|__| days	19) Nausea (คลื่นไส้)	○Yes ○No	|__|__| days
7) Eye redness (ตาแดง)	○Yes ○No	|__|__| days	20) Vomiting (อาเจียน)	○Yes ○No	|__|__| days
8) Abdominal pain (ปวดท้อง)	○Yes ○No	|__|__| days	21) Diarrhoea (ท้องเสีย)	○Yes ○No	|__|__| days
9) Seizures (ชัก)	○Yes ○No	|__|__| days	22) Neck stiffness (คอแข็ง)	○Yes ○No	|__|__| days
10) Confusion (สับสน)	○Yes ○No	|__|__| days	23)Vertigo (เวียนหัว บ้านหมุน	○Yes ○No	|__|__| days
11) Cough (ไอ)	○Yes ○No	|__|__| days	24) Dyspnoea (หายใจลำบาก)	○Yes ○No	|__|__| days
12) Epistaxis (เลือดกำเดาไหล)	○Yes ○No	|__|__| days	25) Haemoptysis (ไอเป็นเลือด)	○Yes ○No	|__|__| days
13) Bleeding per gum (เลือดออกตามไรฟัน)	○Yes ○No	|__|__| days	26) Haematemesis (อาเจียนเป็นเลือด)	○Yes ○No	|__|__| days

13. IN THE LAST 2 WEEKS,

13.1 HAVE YOU HAD CONTACT WITH: ☐ None

☐ rat ☐ cat ☐ flea (หมัด) ☐ mite (ไร หรือไรอ่อน) ☐ tick (เห็บ) ☐ other, specify _____

13.2 HAVE YOU VISITED: ☐ None ☐ rice field ☐ garden ☐ forest

☐ up-high hill ☐ jungle ☐ valley ☐ palm plantation ☐ other, specify _____

ANIMALS: 13.3 LIVE AT/CLOSE TO <u>YOUR HOME:</u> ☐ None

☐ rats ☐ cats ☐ pigs ☐ dogs ☐ cows/buffalos ☐ chicken ☐ other, specify _____

13.4 LIVE AT/CLOSE TO YOUR WORK PLACE: ☐ None

☐ rats ☐ cats ☐ pigs ☐ dogs ☐ cows/buffalos ☐ chicken ☐ other, specify _____

SCRUB TYPHUS HISTORY

14. Have you had "scrub typhus" before? *(It is called "kai rai oon" ไข้ไรอ่อน)* ○ Don't know ○ No ○ Yes

*(If NO or DON'T KNOW, Go to question **15**. Have you had any of following disease before?')*

14.1 How many years or months ago? *(most recent episode)* |__|__| years |__|__| months

14.2 Did you receive any antibiotic? ○ Don't know ○ No ○ Yes, specify _____

14.3 How many days did it take for the fever to go away?

○ Don't know ○ < 3 days ○ 3 – 7 days ○ > 7 days

15. Have you had any of following disease before?

15.1 Murine Typhus ○ Don't know ○ No ○ Yes

15.2 Leptospirosis ○ Don't know ○ No ○ Yes

15.3 Dengue ○ Don't know ○ No ○ Yes

16. VITAL SIGNS

Temp	|__|__|.|__| ˚C	Pulse rate	|__|__|__| beats/min
Respiratory rate	|__|__|__| breaths/min	BP	|__|__|__|/|__|__|__| mmHg
Weight	|__|__|__|.|__| kg	Height	|__|__|__|.|__| cm

TREATMENT

17. BEFORE ADMISSION: 17.1 Paracetamol ○No ○Yes

17.2 Antibiotics ○No ○Yes, specify (drug/ dose/ duration)_____

18. DURING ADMISSION: 18.1 Aspirin ○No ○Yes

18.2 Antibiotics ○No ○Yes, specify (drug/ duration)_____

18.3 Cortisone ○No ○Yes, specify (drug/ duration)_____

18.4 NSAIDs ○No ○Yes, specify (drug/ duration)_____

19.PHYSICAL EXAMINATION

1) Anemic	○No ○Yes	If Yes, detail _____

2) Jaundice	○No ○Yes	If Yes, ○ Mild ○ Moderate ○ Severe
3) Eschar lesion	○No ○Yes	If Yes, detail _____ total number |__|__|, and select location ☐ *head* ☐ *neck* ☐ *breast* ☐ *chest* ☐ *axilla* ☐ *arm* ☐ *abdomen* ☐ *groin* ☐ *genitalia* ☐ *buttocks* ☐ *legs* ☐ *belt* ☐ *sarong* ☐ *bra* (*ขอบยางเสื้อชั้นใน*) ☐ *underwear* (*ขอบยางกางเกงใน*) ☐ other, specify _____
4) Lymphadenopathy *(> 1cm)*	○No ○Yes	If Yes, Location _____
5) Skin rash	○No ○Yes	If Yes, Type/Location _____ *(Type: 1=macular, 2=maculo-papular, 3=papular, 4=petechial, 5=pustular, 6=vesicular, 7=blister, 8=petechial bruising)*
6) Petechial haemorrhage	○No ○Yes	If Yes, Location: ☐ Enoral ☐ Conjunctival ☐ Skin specify _____
7) Meningism	○No ○Yes	If Yes, detail _____
8) Subconjunctival hemorrhage	○No ○Yes	If Yes, detail _____
9) Conjunctival injection	○No ○Yes	If Yes, detail _____
10) Dyspnoea	○No ○Yes	If Yes, Intubated? ○No ○Yes
11) Lung crepitation	○No ○Yes	If Yes, Location _____
12) Liver enlarged	○No ○Yes	If Yes, size |__|__| cm below RCM
13) Spleen enlarged	○No ○Yes	If Yes, enlarged size |__|__| cm
14) Focal neurological deficits	○No ○Yes	If Yes, detail _____
15) Gum petechiae	○No ○Yes	If Yes, ○ Mild ○ Moderate ○ Severe Please describe: _____
16) Deafness	○No ○Yes	If Yes, • **Weber** (sensorineural): ○ Left ○ Right ○ symmetric • **Rinne:**

		Air Conduction: ○ Left ○ Right ○ symmetric Bone conduction: ○ Left ○ Right ○ symmetric

20. GLASGOW COMA SCORE

Motor |__| /6 Verbal |__| /5 Eyes |__| /4 Total |__|__| /15

21. PREGNANCY TEST ○ Positive ○ Negative ○ NA ○ Not done

22. CHEST X-RAY:

_____

_____

_____

23. LAB SAMPLES

Was following samples collected?

23.1 Blood?	○ Yes	○ No	○ Not applicable
23.2 Eschar swab?	○ Yes	○ No	○ Not applicable
23.3 Eschar crust?	○ Yes	○ No	○ Not applicable

24. ADMISSION LAB: CBC

Hb	|__|__|.|__| g/dL	Neutrophil	|__|__|.|__| %
Hct	|__|__|.|__| %	Lymphocytes	|__|__|.|__| %
Platelet	|__|__|__|.|__| $10^3/mm^3$	Monocytes	|__|__|.|__| %
WBC	|__|__|__|.|__| $10^3/mm^3$	Eosinophils	|__|__|.|__| %

25. ADMISSION LAB: BLOOD CHEMISTRY

BUN	|__|__|__|.|__| mg/dL	Bilirubin direct	|__|__|.|__| mg/dL
Creatinine	|__|.|__|__| mg/dL	Bilirubin total	|__|__|.|__| mg/dL
Na	|__|__|__| mmol/L	AST	|__|__|__| IU/L
K	|__|__|.|__| mmol/L	ALT	|__|__|__| IU/L
Cl	|__|__|__| mmol/L	Alk. phosphatase	|__|__|__| IU/L
HCO_3	|__|__| mEq/L	Albumin	|__|__|.|__| g/dL
Globulin	|__|__|.|__| g/dL	CRP (C-reactive Protein)	|__|__|__|.|__| µg/mL
Glucose	|__|__|__| mg/dL	Lactate	|__|__|.|__| mmol/L

26. OTHER LABORATORY TESTS:

_____

_____

_____

27. HOSPITAL DISCHARGE: date |__|__|/ |__|__|/ 20|__|__| *(dd/mm/yyyy)*

Discharge status ○ Alive ○ Dead

28. COMMENTS:

_____

_____

_____

_____

29. 2 WEEKS FOLLOW UP

29.1 Follow up attended? ○ No ○ Yes, go to 29.2

If No, select reason ○ Migration ○ Withdraw ○ Death ○ Other, _____

29.2 Date of visit |__|__|/ |__|__|/ 20|__|__|

29.3 Sample taken? ○No ○Yes, Glucose |__|__|__| mg/dL, Lactate |__|__|.|__| mmol/L

29.4 Any illness/symptoms? ○No ○Yes (refer to symptom list from baseline)

If Yes, describe _____

_____

29.4 How did you treat the illness/symptoms?

○ None ○ See doctor ○ Self-medication ○ Other, detail

_____

CRF completed by _____ (initial) _____ (date)

30. 12 WEEKS FOLLOW UP

30.1 Follow up attended? ○ No ○ Yes, go to 30.2

If No, select reason ○ Migration ○ Withdraw ○ Death ○ Other, _____

30.2 Date of visit |__|__|/ |__|__|/ 20|__|__|

30.3 Sample taken? ○No ○Yes, Glucose |__|__|__| mg/dL, Lactate |__|__|.|__| mmol/L

30.4 Any illness/symptoms? ○No ○Yes (refer to symptom list from baseline)

If Yes, describe _____

_____

30.4 How did you treat the illness/symptoms?

○ None ○ See doctor ○ Self-medication ○ Other, detail

_____

CRF completed by _____ (initial) _____ (date)

31. 52 WEEKS FOLLOW UP

31.1 Follow up attended? ○ No ○ Yes, go to 29.2

If No, select reason ○ Migration ○ Withdraw ○ Death ○ Other, _____

31.2 Date of visit |__|__|/ |__|__|/ 20|__|__|

31.3 Sample taken? ○No ○Yes, Glucose |__|__|__| mg/dL, Lactate |__|__|.|__| mmol/L

31.4 Any illness/symptoms? ○No ○Yes (refer to symptom list from baseline)

If Yes, describe _____

_____

31.4 How did you treat the illness/symptoms?

○ None ○ See doctor ○ Self-medication ○ Other, detail

_____

CRF completed by _____ (initial) _____ (date)

FINAL STATUS

32. Did subject complete the study?

- ○ Yes, specify completed date |__|__|/ |__|__|/ 20|__|__| *(dd/mm/yyyy)*
- ○ No, specify the date of below event |__|__|/ |__|__|/ 20|__|__| *(dd/mm/yyyy)*

  Select the reason of not complete below:

  - ○ Migration
  - ○ Lost to follow -up
  - ○ Subject withdraws consent
  - ○ Death
  - ○ Other, specify _____

33. REMARKS

_____

_____

_____

_____

_____

Investigator's Statement

I have reviewed the data recorded in this CRF and confirm that the data are complete and accurate.

Investigator (print full name) _____

Signature _____ Signature date |__|__|/|__|__|/20|__|__| *(dd/mm/yyyy)*

Paediatric scrub typhus study – Scrub typhus healthy controls case record form (CRF)

1. DATE OF VISIT |__|__|/ |__|__|/ 20|__|__| *(dd/mm/yyyy)*

INFORMED CONSENT				
2. Date of informed consent obtained *(dd/mm/yyyy)* |__|__|/ |__|__|/ 20|__|__|			
DEMOGRAPHICS				
3.1 Birth date |__|__|/ |__|__|/ 20|__|__| *(dd/mm/yyyy)* ***OR*** 3.2 Age |__|__| years |__|__| months				
4. Sex: ○ Male ○ Female				
5. Ethnic group: ☐ Thai ☐ Karen ☐ Hmong ☐ Other, specify _____				
6.1 Occupation: ○ None ○ Agriculture ○ Office ○ Other 6.2 specify detail: _____				
INCLUSION/EXCLUSION CRITERIA				
7. INCLUSION		**YES**	**NO**	**NA**
IN 1	Age >6 months to ≤18 years and age>18 years **(STE)** Age >18 years **(STH)**	○ Y	○ N	
IN 2	Currently well and healthy **(STH, STE)**	○ Y	○ N	
IN 3	Willingness to participate in the study and written, informed consent previously obtained **(STH, STE)**	○ Y	○ N	
IN 4	Historical diagnosis of scrub typhus, as defined by rapid diagnostic test, serology and/or PCR positivity (more than 2 years ago), or living in an endemic area with high risk of previous exposure. **(STE)** Residing in Bangkok for at least the 2 past recent years **(STH)**	○ Y	○ N	
8. EXCLUSION		**YES**	**NO**	**NA**
EX 1	Previous history of scrub typhus **(STH)**	○ Y	○ N	○ NA
EX 2	Significant acute intercurrent illness at the time of blood draw including fever >37.5°C or infection (including TB) requiring antibiotics **(STE, STH)**	○ Y	○ N	
EX 3	Documented HIV infection, use of steroids, chemotherapy, other immunosuppressant therapy or herbal remedies containing steroids and/or pregnancy **(STE, STH)**	○ Y	○ N	
9. ELIGIBILITY (EL)				
9.1 Is subject eligible? ○ Yes ○ No				
9.2 Enrolment date *(dd/mm/yyyy)* |__|__|/ |__|__|/ 20|__|__| *(dd/mm/yyyy)*				

ENROLMENT CONFIRMED BY

_____ _____ DATE |__|__|/ |__|__|/ 20|__|__|

INVESTIGATOR (PRINT NAME) SIGNATURE

QUESTIONNAIRE

10. DO YOU HAVE REGULAR CONTACT WITH: ☐ None

☐ rat ☐ cat ☐ flea (หมัด) ☐ mite (ไร หรือไรอ่อน) ☐ tick (เห็บ) ☐ other, specify _____

11. DO YOU VISIT ANY OF THESE PLACE REGULARLY:

11.1 rice field ○ No ○ Yes, reason* |__| _____ How often?# |__| _____

11.2 garden ○ No ○ Yes, reason* |__| _____ How often? # |__| _____

11.3 forest ○ No ○ Yes, reason* |__| _____ How often? # |__| _____

11.4 up-high hill ○ No ○ Yes, reason* |__| _____ How often? # |__| _____

11.5 jungle ○ No ○ Yes, reason* |__| _____ How often? # |__| _____

11.6 valley ○ No ○ Yes, reason* |__| _____ How often? # |__| _____

11.7 palm plantation ○ No ○ Yes, reason* |__| _____ How often? # |__| _____

11.8 other, specify _____ , reason* |__| _____How often? # |__| _____

**reason: 1=work, 2=walk to work, 3=collect food, 4=hunting, 5=other*

#how often?: 1=daily, 2=weekly, 3=monthly, 4=other timeframe

ANIMALS: 12.1 LIVE AT/CLOSE TO YOUR HOME: ☐ None

☐ rats ☐ cats ☐ pigs ☐ dogs ☐ cows/buffalos ☐ chicken ☐ other, specify _____

12.2 LIVE AT/CLOSE TO YOUR WORK PLACE: ☐ None

☐ rats ☐ cats ☐ pigs ☐ dogs ☐ cows/buffalos ☐ chicken ☐ other, specify _____

13. Do you or did you rest/sleep on grass during the day? ○ No ○ Yes, where _____

14. Does your house have a grass or cement yard? ○ No ○ Yes

15. Do you keep piles of grass/weeds there? ○ No ○ Yes

SCRUB TYPHUS HISTORY:

16. Have you had "scrub typhus" before?*(It is called "kai rai oon" ไข้ไรอ่อน)* ○ Don't know ○ No ○ Yes

*(If NO or DON'T KNOW, Go to question **18**. Have you had any of following disease before?')*

16.1 How many years or months ago? *(most recent episode)* |__|__| years |__|__| months

16.2 Did you receive any antibiotic? ○ Don't know ○ No ○ Yes, specify _____

16.3 How many days did it take for the fever to go away?

○ Don't know ○ < 3 days ○ 3 – 7 days ○ > 7 days

16.4 Did you have any of the following symptoms?

- Fever ○ Don't know ○ No ○ Yes
- Headache ○ Don't know ○ No ○ Yes
- Deafness ○ Don't know ○ No ○ Yes
- Eschar ○ Don't know ○ No
  ○ Yes, specify how many |__|__|, where _____
- Jaundice ○ Don't know ○ No ○ Yes
- Abdominal pain ○ Don't know ○ No ○ Yes
- Dyspnoea ○ Don't know ○ No ○ Yes
- Cough ○ Don't know ○ No ○ Yes
- Bleeding ○ Don't know ○ No ○ Yes
- Rash ○ Don't know ○ No ○ Yes
- Myalgia/arthralgia ○ Don't know ○ No ○ Yes

16.5 Did you go to hospital? ○ No ○ Yes, **answer below**

- Outpatient? ○ No ○ Yes
- Hospitalization? ○ No ○ Yes, how many days? |__|__| days
- ICU required? ○ No ○ Yes, how many days? |__|__| days

Note: Please check with hospital medical record if possible.

17. HOW DO YOU THINK YOU ACQUIRED SCRUB TYPHUS?

17.1Do you remember being bitten by a mite (or a very small insect)? ○ Don't know ○ No ○ Yes

1) Where do you think you were bitten? _____

2) What were you doing when you were bitten? _____

17.3Did you have any symptoms like "kai rai oon" since then? ○ Don't know ○ No ○ Yes

17.4Do you think you had this disease again only with less severe symptoms? ○ Don't know ○ No ○ Yes

17.5Did you visit the same area where you think you acquired the fever? ○ Don't know ○ No ○ Yes

18. MITES: 18.1 Do you know about "rai" or "rai oon" (Thai for red mites)? ○ No ○ Yes

18.2 Do you know a place where there are a lot of "rai" or "rai oon"? ○ No ○ Yes

18.3 Are there cases of scrub typhus reported from that area? ○ Don't know ○ No ○ Yes

18.4 Are there a lot of rodents in that area? ○ Don't know ○ No ○ Yes

19. Do you have friends or know anybody who has had this disease? ○ No, skip to question20. ○ Yes

19.1Where do they live? _____

19.2 Would you agree if we try to contact them? ○ No ○ Yes

20. COMMENTS: _____

_____

_____

_____

21. LAB: CBC			
Hb	|__|__|.|__| g/dL	Neutrophil	|__|__|.|__| %
Hct	|__|__|.|__| %	Lymphocytes	|__|__|.|__| %
Platelet	|__|__|__|.|__| $10^3/mm^3$	Monocytes	|__|__|.|__| %
WBC	|__|__|__|.|__| $10^3/mm^3$	Eosinophils	|__|__|.|__| %
22. LAB: BLOOD CHEMISTRY			
BUN	|__|__|__|.|__| mg/dL	Bilirubin direct	|__|__|.|__| mg/dL
Creatinine	|__|.|__|__| mg/dL	Bilirubin total	|__|__|.|__| mg/dL
Na	|__|__|__| mmol/L	AST	|__|__|__| IU/L
K	|__|__|.|__| mmol/L	ALT	|__|__|__| IU/L
Cl	|__|__|__| mmol/L	Alk. phosphatase	|__|__|__| IU/L
HCO_3	|__|__| mEq/L	Albumin	|__|__|.|__| g/dL
Globulin	|__|__|.|__| g/dL	CRP (C-reactive Protein) µg/mL	|__|__|__|.|__|
Glucose	|__|__|__| mg/dL	Lactate	|__|__|.|__| mmol/L

23. OTHER LABORATORY TESTS:

_____

_____

Investigator's Statement

I have reviewed the data recorded in this CRF and confirm that the data are complete and accurate.

Investigator (print full name) _____

Signature _____ Signature date |__|__|/|__|__|/20|__|__| *(dd/mm/yyyy)*

Appendix 6

Scrub typhus antibiotic resistance trial (START) – Study protocol

(*The first draft of this protocol was written by the candidate*)

A6.1 Objectives and outcome measures

The overall aim is to determine the efficacy of two common scrub typhus treatments in two distinct endemic areas of Thailand, including areas where previous reports of antibiotic resistance have originated from.

Primary objectives:

- To evaluate the clinical and microbiological responses in adult scrub typhus patients to three oral treatment regimens: 7 days of doxycycline, 3 days of doxycycline, and 3 days of azithromycin.

Secondary objectives:

- To perform pharmacokinetic/pharmacodynamic (PK/PD) characterization of the therapeutic responses for doxycycline and azithromycin, including serial bacterial load measurements.
- To define clinical, bacterial, pathophysiological and pharmacological factors associated with disease severity, fever-clearance times (FCT), treatment failures and relapse or re-infection.
- To determine the minimum inhibitory concentrations (MIC) of clinical *Orientia tsutsugamushi* isolates to doxycycline, azithromycin and chloramphenicol, using in vitro growth-inhibition assays.
- To genotype all clinical isolates using whole genome sequencing for comparative genomics.

- To study the natural immune response in scrub typhus, using antigen-specific cellular immune and antibody studies, as well as cytokine profiling, and compare the responses in the different treatment groups.

The primary outcome measure is fever clearance time (FCT). This will be based on the time from first dose of antibiotic treatment to when the tympanic membrane temperature first falls ≤37.5°C and remains ≤37.5°C for at least 24 hours. Fever should be related to the presumptive diagnosis of scrub typhus. The secondary outcome measures are treatment failure (clinical or microbiological) and disease relapse. These outcomes are defined as:

- Fever clearance time (FCT) – time in hours from onset of 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 – persistence of fever >72 hours after initiation of antibiotic treatment OR failure of resolution of symptoms and/or complications attributable to scrub typhus within 5 days from the start of antibiotic treatment.
- Microbiological treatment failure - failure of resolution of bacteraemia (qPCR +/- culture) within 3 days from initiation of antibiotic treatment.
- Relapse – the recurrence of fever and/or associated symptoms, which the clinical and study teams have concluded as likely to be due to scrub typhus will be defined as “possible relapse”. The recurrence of bacteraemia as detected by qPCR +/- culture within the 8 week follow-up period after the patient had been treated and achieved clinical and bacteriological resolution will be defined as “confirmed relapse”. *Orientia tsutsugamushi* strain genotyping (if PCR +/- culture positive) will allow the differentiation of relapse from re-infection.

A6.2 Study design

The trial is a prospective, open-label, randomised-controlled treatment trial in hospitalised patients ≥ 15 years old with acute scrub typhus infection that will evaluate and compare three different oral antibiotic treatment regimens:

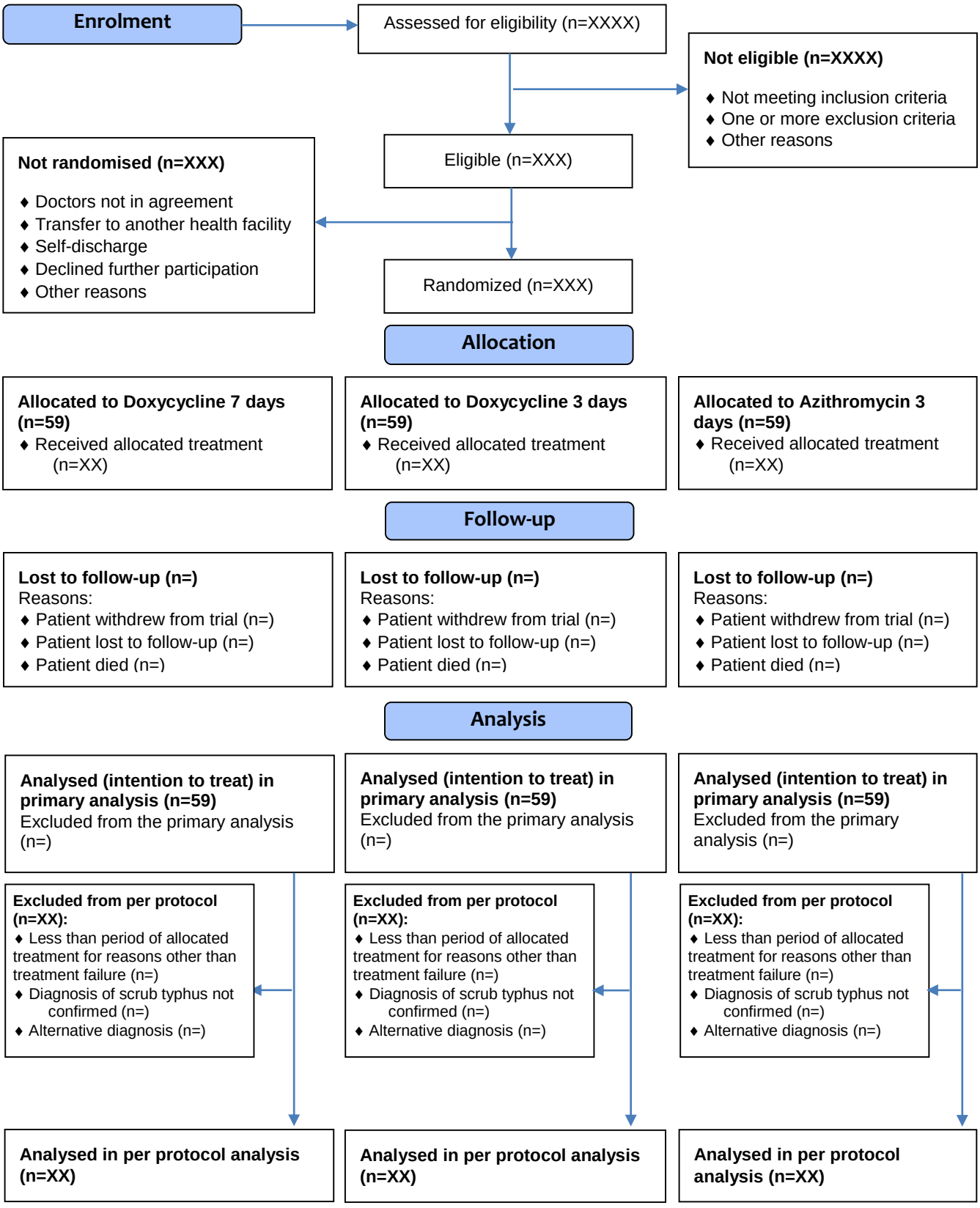
1. 7 days of doxycycline
2. 3 days of doxycycline
3. 3 days of azithromycin

Clinical and microbiological outcomes and responses to treatment are evaluated during admission and at the formal follow-up time-points at 2 weeks and 8 weeks. The trial CONSORT flowchart (Consolidated Standards of Reporting Trials) is shown in Figure A below. The study arms were similar to the trial from Laos with the exception of higher doses of azithromycin being used. Prof Nick Day, Prof Paul Newton, Prof Daniel Paris and I were involved in trial design.

A6.3 Study sites

Recruitment will be performed at Chiangrai Prachanukroh Hospital, Chiangrai, Thailand as the primary site and at Shoklo-Malaria Research Unit (SMRU), Mae Sot, Tak province in northwestern Thailand as the secondary site. SMRU is part of the MORU Tropical Health Network and provides health care for marginalized populations along the Thai-Myanmar border, focusing mainly on maternity-child health and infectious diseases. Health stations at Wang Pa and Mawker Thai will be the main areas of patient recruitment.

Figure A. START Consolidated Standards of Reporting Trials (CONSORT) Flowchart



A6.4 Inclusion and exclusion criteria

Inclusion criteria:

- Age ≥ 15 years old
- Hospitalization with acute undifferentiated fever (temperature > 37.5°C, tympanic) ≤14 days or patients admitted to hospital with a history of fever ≤ 14 days who subsequently develop fever within 24 hours of admission
- Clinically suspected scrub typhus: defined as acute undifferentiated fever with no clear focus of infection and negative malaria blood smear +/- negative malaria RDT
- A positive scrub typhus RDT (e.g. Scrub Typhus IgM RDT, InBios International, Seattle, WA, USA or Scrub Typhus IgM/IgA/IgG SD Bioline, S.Korea) **or** positive PCR-based detection of *O. tsutsugamushi* DNA from the admission blood sample **or** presence of an eschar
- Written informed consent and/or, written informed assent as required
- Able to take oral medication

Exclusion criteria:

- Known hypersensitivity to tetracycline, doxycycline or azithromycin
- Administration of doxycycline, azithromycin, chloramphenicol, rifampicin, or tetracycline during the preceding 7 days
- Pregnancy or breast-feeding
- Patients with myasthenia gravis or systemic lupus erythematosus
- Patients with an alternative acute infection at the time of screening (diagnostic test required) e.g. acute malaria, dengue, leptospirosis, typhoid, Japanese encephalitis etc.
- Current TB or TB treatment in ≤ 6 months (contain active antibiotics against Orientia tsutsugamushi)

- Current HAART use for HIV, long term use of immunosuppressants (e.g. steroids, chemotherapy, TNF-inhibitors and related agents)
- Patients with severe disease whom the clinical team feel their condition necessitates the need for additional scrub typhus treatment beyond the allocated antibiotic treatment assigned at randomization (e.g. IV chloramphenicol and/or PO/NG rifampicin)

A6.5 Informed consent

Written informed consent to participate in the study will be obtained prior to study inclusion:

- Febrile patients ≥ 18 years old: written informed consent sufficient.
- Febrile patients aged ≥ 15 to <18 years old, written parental/guardian consent with participant assent will be sought.

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 (Burmese or Karen for SMRU) forms 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 and Chinese). The witness to the informed consent procedure should be able to understand the language used.

In the event that the participant lacks capacity (e.g. acute delirium), the patient information sheet (PIS) and informed consent form (ICF) – shown below – 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.

A6.6 Treatment, allocation and randomisation

Patients who fulfill all the inclusion criteria and have none of the exclusion criteria will be randomized 1:1:1 to one of the three treatment arms according to a computer generated randomization schedule (permuted block randomisation in multiples of 3). Individual, sealed and sequentially numbered envelopes will be provided for the trial site with one envelope per patient, indicating the treatment allocation. Patients are randomized to either:

- Doxycycline 7 days, loading dose 200mg PO 1st dose, then 100mg PO every 12 hours for 7 days (14 doses in total inclusive of loading dose)
- Doxycycline 3 days, loading dose 200mg PO, then 100mg PO every 12 hours for 3 days (6 doses in total inclusive of loading dose)
- Azithromycin 3 days, loading dose 1,000mg PO on day 1, then 500mg PO every 24 hours on days 2 and 3 (3 doses in total inclusive of loading dose)

The code, treatment group allocation, and plasma drug level profiling group (pre-randomized to dense or sparse groups, ratio 1:3, computer generated) are kept in pre-prepared sealed envelopes. The envelopes are opened after patients are formally recruited. The study will not be blinded although investigators and trial statistician will be blinded to treatment arm at interim analysis.

Paracetamol may be given to the patient as required but the timing and doses will be recorded by the ward staff and study team. When calculating FCT, the timing and doses of paracetamol (and other anti-pyretic drugs) will be taken into account. Treatment of study patients with magnesium, aluminium and calcium salt-based antacids for symptoms of indigestion and acid reflux will be avoided as they may interfere with normal absorption of antibiotics. All antibiotic courses are provided by the study team free of charge and obtained from reputable pharmaceutical manufacturers working under good manufacturing practices

as stipulated by the clinical trial guidelines from the World Health Organization (WHO) and the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) [453, 454]. Batch testing of study antibiotics will be performed during the trial to ensure drug quality.

The ingestion of study drugs will be witnessed by the nursing or study staff. If the patient vomits the medication within the 1st hour, another antibiotic dose is re-administered and the time and dose recorded on the drug chart/log. Patients are withdrawn from the study if they do not tolerate the allocated drug treatment due to severe side effects or significant adverse drug reactions. All adverse drug reactions will be recorded.

Empirical treatment with another antibiotic in addition to the study antibiotic will be allowed if the additional antibiotic does not have any activity against *Orientia tsutsugamushi* e.g. beta-lactam antibiotics (such as penicillins, cephalosporins, carbapenems), aminoglycosides (such as gentamicin, amikacin, tobramycin), glycopeptides (such as vancomycin) etc.

If treatment failure occurs, the treatment regimen is reviewed by the medical team and study physician and alternative regimens are considered. Alternatives include a switch to the other allocated regimen (e.g. doxycycline to azithromycin), increasing the treatment duration or starting a combination of both doxycycline and azithromycin as no interactions are known that impede the efficacy of either drug. Alternatively, chloramphenicol +/- rifampicin may be used. Intravenous doxycycline and azithromycin are unavailable at the two study sites. The decision and reason for the treatment switch is recorded in the CRF (Appendix 7) and the trial monitor informed.

A6.7 Study conduct

Screening and enrolment

Staff at Chiangrai Prachanukroh Hospital or SMRU, will inform the study team if a patient presents with acute undifferentiated fever with a clinical suspicion of scrub typhus. The scrub typhus RDT will be performed (e.g. Scrub Typhus IgM RDT, InBios International, Seattle, WA, USA and/or Scrub Typhus IgM/IgA/IgG RDT, SD Bioline, S. Korea). Eligible patients (≥ 15 years old) who agree to participate in the study will take part in the informed consent procedure. If the patient has a fever and if the RDT is positive for scrub typhus or the patient has a fever and an eschar, the patient is formally enrolled into the study.

Every screened patient will receive a screening number and details are recorded on a screening log (Appendix 8). Reasons for rejection may include any exclusion criteria, non-conformity with the inclusion criteria or failure to obtain informed consent. Reasons for not enrolling must be recorded in the screening log.

Baseline

Upon study enrolment, the study team will open the sealed allocation envelope that contains the randomized pre-determined treatment arm, the PK group allocation (dense or sparse) and the study number of each participant. Pre-prepared antibiotic packages with the allocated treatment regimens will be available at each site and stored at 4°C. The first dose is administered and witnessed by study staff and/or clinical staff with the exact time and dose recorded on the drug chart/log. Patients are withdrawn from the study if they do not tolerate the allocated drug treatment after at least a couple of attempts and use of anti-emetics if required. All minor and significant adverse drug reactions are recorded.

Inpatient stay

Study patients will have their vital signs measured and recorded every 4-6 hours during hospitalisation. Details of all drugs administered including supportive treatment will be recorded. Any additional diagnostic test results (e.g. laboratory or imaging) requested by the medical team will also be collected and recorded on the CRF. Patients will be reviewed daily by study staff and clinical team. Patients will be discharged by the treating physician if there is clinical improvement and resolution of fever.

Follow-up

Scheduled follow-up visits occur at 2 weeks (+/- 2 days) and 8 weeks (+/- 2 weeks). Review and collection of samples are performed as previously at baseline, at both follow-up time points. Lost to follow-up will occur because of death, transfer to another hospital, migration from the area or withdrawal from the study (either patient or investigator instigated). If the patient cannot be followed-up, the reason will be recorded in the CRF.

If PK sampling time points fall outside the inpatient stay period (e.g. 7 days after the first dose of azithromycin and 4 days after the last dose of doxycycline) the study team will arrange additional follow-ups as required. We plan the sampling time-point schedule to be time-flexible and dependent on the availability of the patient to either attend the hospital or allow a visit at/near home by the study team.

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 8 weeks follow-up appointment). Clinical findings and samples are collected as previously at baseline to investigate the cause of clinical deterioration.

If the patient was discharged prior to completing the allocated treatment course, he/she will be asked to keep a diary of when the study drug was taken (date/time) and this information will be collected by the study team at the arranged follow-up time and recorded in the CRF. If the patient was discharged prior to cessation of fever due to hospital bed pressures, the patient will be given a tympanic membrane monitor and asked to measure their temperature 4 times a day and record the results in a diary until 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 FCT.

Study completion

The study will end when 177 scrub typhus patients (59 patients in each treatment arm) have been recruited and completed follow-up at 8 weeks. The initial proposed study period is 2 calendar years but is subject to change based on recruitment rates, local health system changes or other events beyond the control of the investigators.

A6.8 Study procedures

Samples and specimens

Sample receiving and specimen processing logs along with a summary of blood collection time-points for the treatment arms are included in Appendix 8.

Labelling: The prefix SAR followed by the site code (04 for SMRU, 29 for Chiangrai) and a 3 digit study number will serve as the patient study identifier. To avoid errors, Chiangrai study patients will start from SAR-29-001 while SMRU patients will start from SAR-04-501. An additional suffix for whole blood, serum, plasma (heparinized blood), plasma (citrated blood), plasma (EDTA blood), buffy coat, eschar swab, and eschar crust, will be added - e.g. SAR-CR-001-WB1, -S1, -PH1, -PC1, -EP1, -BU1, -ES1, -EC1, followed by a suffix for time of blood draw, e.g. SAR-CR-001-WB1-W0 for baseline and SAR-CR-001-WB1-W2 for week 2 etc.

Blood collection: Blood is collected in silica tubes (serum), sodium heparin tubes (plasma, peripheral blood mononuclear cells), EDTA tubes (plasma, buffy coat, whole blood), sodium citrate tubes (plasma) and Tempus™ Blood RNA tubes (Applied Biosystems, Foster City, CA, USA) for RNA transcription profiling.

Serial blood draws for PK and bacterial load can be collected through a venous cannula for the first 72 hours to ease collection. The first 0.5-1ml of blood will be discarded as this may contain flush fluid from previous use. After 72h blood is collected in the standard way.

Blood draw volume: The study requires approximately 15-20 ml of blood at each of the major time-points at baseline, 2 weeks and 8 weeks follow-ups. Minor/PK time-points will as standard require 2 ml of blood (EDTA tube) to be collected for PK and bacterial load analysis. In a small subset of patients a further 3 ml of blood (heparin tube) will be collected to allow comparison of intracellular and plasma drug levels.

In participants age ≥15 years to <18 years, to ensure blood volume collected is within acceptable parameters, guidelines from the Seattle Children's Hospital, 4800 Sand Point Way, NE Seattle, WA 98105, USA (http://www.seattlechildrens.org). Briefly, the total blood volume (TBV) is calculated according to the body weight. In a single blood draw, no more than 2.5% of the TBV is allowed. In a month, the maximum volume of blood draw recommended is max. 5% of the TBV.

Plasma drug profiling groups: Two groups are required for PK/PD analysis – a data "sparse" group (75% of all patients), with minor blood draws up to 7 time-points (baseline plus 6 additional minor blood draws), and the data "dense" group (25% of all patients) up to 13 time-points (baseline plus 12 additional minor blood draws).

Non-invasive eschar scrape samples: Patients with an eschar will have eschar samples collected – a sterilised dermal curette is used to scrape some dried crust fragments and collected in 95% ethanol with subsequent DNA extraction using DNAeasy Blood and Tissue kit (QIAGEN, Germantown, MD, USA) for PCR analysis, including genotyping.

Urine: A urine sample (10 ml) is collected from all study patients (if possible) at baseline, during admission (days 1 and 2) and major follow up visits for surrogate markers and metabolomics/proteomics analysis, supporting the development of urine-based rapid diagnostic tests for scrub typhus. Urine will be stored neat or centrifuged to obtain the supernatant (UU or US specimen code, respectively).

Cerebrospinal fluid: In a subset of study patients with central nervous system (CNS) involvement we will collect spare CSF (1-2 ml) for metabolomics and surrogate markers of disease severity only if a lumbar puncture is clinically indicated and performed by hospital doctors and spare CSF is available after adequate CSF volumes are apportioned for the tests requested by the clinical team.

Diagnostic assays

Scrub typhus RDTs: The Scrub Typhus *Detect*™ IgM Rapid Test (InBios International Inc., Seattle, WA, USA) will be used to screen patients and a trained study laboratory technician will perform the test on plasma, serum, or whole blood samples. In Chiangrai, the hospital laboratory uses an IgM/IgA/IgG immunochromatographic RDT (SD Bioline, South Korea) and we will also use the results from this test as part of the screening process if the test was requested by the clinicians in charge of the patient.

Serology: We will use a screening Scrub Typhus *Detect*™ IgM ELISA Kit and the gold standard IFA to detect scrub typhus IgM antibody titres as previously described in Chapter 5. The same robust and validated cut-off IFA IgM titres of the IFA assay of ≥1:3200 in a single acute sample or ≥4-fold rise to ≥1:3,200 in a convalescent sample will be used.

PCR: Real-time quantitative PCR assay to detect the 47kDa *htra* gene was performed in duplicate following DNA extraction from buffy coat, blood or eschar samples. If positive, the nested 56kDa TSA gene PCR assay was performed and DNA product sent for genotyping.

Culture: Blood, buffy coat or eschar specimens are propagated onto L929 mouse fibroblast cell monolayers in the BSL-3 laboratory, MORU, Bangkok. Cell culture media will be refreshed regularly and samples taken to assess for *Orientia tsutsugamushi* growth using qPCR. If detected, the clinical isolate is cultured further prior to purification and DNA extraction for genotyping and WGS.

Laboratory investigations

Specimens and target investigations include:

- Haematology tests are performed on EDTA blood samples.

- Biochemistry tests are performed on serum and plasma samples.
- Metabolomics and proteomics are performed on serum or plasma, urine or CSF samples.
- ELISA and IFA are performed on serum or plasma samples.
- Real-time quantitative PCR (qPCR) and nested conventional PCR use DNA extracted from whole blood, buffy coat or eschar samples.
- *Orientia tsutsugamushi* isolates are cultured from blood, buffy coat or eschar samples.
- Whole genome sequencing and genotyping will be performed on extracted DNA from blood, buffy coat, eschar or cultured clinical isolates.
- Disease severity surrogate markers and cytokines, coagulation factors and pathophysiology markers will be processed using serum and plasma samples.
- Fluorescence activated cell sorting (FACS) based flow cytometry are performed on purified white blood cells from anticoagulated blood (heparin) stimulated with *Orientia tsutsugamushi* antigens (e.g. whole cell, 56kDa, 47kDa and ScaC antigens with positive and negative controls).
- ELISpot assays will use purified PBMCs from heparinised blood which will be stimulated with *Orientia tsutsugamushi* antigens as above.
- Drug levels are measured from plasma samples (usually EDTA blood) using liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS).

Investigating treatment failure and relapse: If treatment failure occurs, the patient is managed as per standard of care by the attending clinical team in discussion with study staff

as per section A6.6 above. Details will be recorded in the patient CRF and a further blood sample (same tubes as baseline) is collected prior to any change in treatment. In the case of relapse after clinical cure and patient has been discharged, the study participant will inform study staff and will be reviewed by the study team (either at home, a healthcare facility close to home or at the hospital). A baseline blood sample panel will be collected and re-admission considered following discussions with the senior trial doctor at the respective study site.

PK/PD and serial bacterial load measurements: The number of sampling time-points will be defined by the randomised PK groups – "dense" or "sparse".

In the dense group, proposed plasma sampling time-points for doxycycline levels are - baseline and at 0.5, 1, 2, 3-5, 8-12, and 24 hours (plus 72hrs for doxycycline 7 days group) after the first dose and at 0, 3-8, 24, 48, and 96 hours after the last dose (up to 13 time points). For azithromycin, plasma sampling time-points are – baseline, at 0.5, 1, 2, 3-5, 8-12, 24 and 48 hours and at day 3, 4-5, 7 and week 2 (+/- 2 days) after the first dose (up to 12 time points).

In the sparse group, plasma sampling time-points for doxycycline levels are – baseline and at 3-5, 8-12 and 24 hours (plus 72hrs for doxycycline 7 days group) after the first dose and at 0 and 48-96 hours after the last dose (up to 7 time points). For azithromycin, sampling time-points are – baseline, at 3-5, 24, and 48 hours and at day 3 and week 2 (+/- 2 days) after the first dose (up to 6 time points).

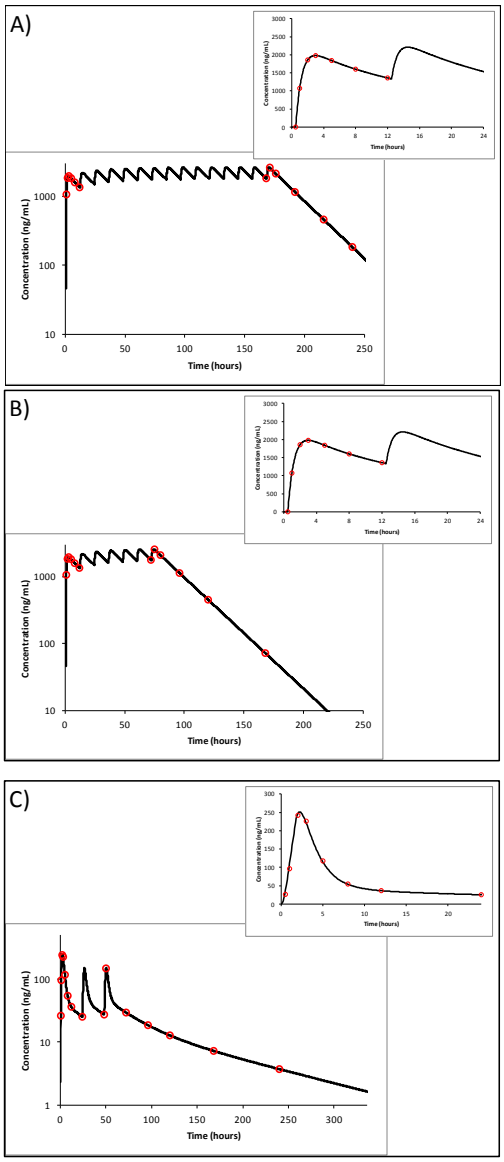
The time-points are not fixed and some flexibility will be allowed. We will avoid missing samples but if this occurs, the impact on the overall analysis will be minimised as individual concentration-time profiles will be plotted and results merged to give population

concentration-time profiles for each study arm via conventional non-compartmental analysis. Expected PK profiles are shown in Figure B below.

We will develop a bioanalytical assay to measure intracellular doxycycline and azithromycin in a subset of patients (up to 5 patients per study arm) for comparing drug levels within the cytoplasm of PBMCs to those in plasma. This will occur at day 1, day 3 and day 7 following initiation of treatment.

Cell culture and antibiotic susceptibility testing: We will determine the minimum inhibitory concentration (MICs) of *Orientia* spp. isolates for doxycycline, azithromycin and chloramphenicol in this study. All clinical *O. tsutsugamushi* isolates will be screened using a predetermined regional MIC cut off based on regional and reference strains [224]. If evidence for antimicrobial resistance is found, the isolate in question will be studied further. This will include genotyping (type specific genes, WGS) which will allow comparisons of susceptible and resistant isolates and further study of molecular markers of resistance.

Figure B. Expected pharmacokinetic profiles for A) 7 days doxycycline, B) 3 days doxycycline and C) 3 days azithromycin (inserts show the first 24 hours). Possible sampling time points are indicated by red circles. Simulated population mean profiles are based on Salman et al, 2010 and Beringer, 2012 [215, 455].



A6.9 Data and specimens management

Study data will be stored securely at all times and only designated study staff will have access. A data manager will assist the investigators in curating and maintaining the study database and export data for analysis when required. Data will also be audited regularly for accuracy. Data will be stored securely (including back-up facilities) for 10 years or sooner if analyses are completed.

Specimens will be stored at -80°C or in liquid nitrogen and kept for 10 years for analyses to achieve the study objectives. Residual specimens can be used for the study of scrub typhus and other infectious diseases with the exception of human immunodeficiency virus (HIV) or Creutzfeldt-Jakob disease (vCJD) due to implications for the patient. Study participants will be asked specifically to consent for the storage of leftover specimens for future research. No future analysis or study of legacy data/specimens will be performed without seeking further regulatory (EC/IRB) approval. Some laboratory analyses will need to be performed at other institutions outside of Thailand. In these situations, a Material Transfer Agreement will be made between MORU and other institutions as required with guidance sought from the relevant EC/IRB.

The overall responsibility of maintenance and storage of study data and specimens lies with the principal investigator. Maintenance and storage will comply with EC/IRB requirements and local regulations, whichever is strictest. Participants' data will not be released without the written permission of the subject except as necessary for study monitoring, regulatory authority inspection and site EC/IRB visit.

A6.10 Safety and risk management

Benefits

The participant will have access to additional diagnostic assays not currently available locally. Antibiotic treatment will be randomised and provided by the study team. Additional monitoring and follow-up will take place. As per local EC/IRB requirements, patients will receive THB300 remuneration for admission and every follow-up time-point.

Adverse and serious adverse events (AE/SAE)

Adverse events (AE)

An AE is any undesirable event or clinical deterioration that occurs to a study participant during the course of the study; that is, from the time of administration of study drugs until study completion, whether or not that event is considered related to the study drugs or to a concomitant drug or procedure. It includes:

- any unfavourable and unintended symptom or sign
- abnormal laboratory result
- an illness (e.g. after discharge)

Any new clinical deterioration that occurs between signing the consent form and the administration of study drugs is not an AE. This information will be recorded in the medical records as a pre-existing condition.

Serious adverse event (SAE)

A serious adverse event is an AE that:

- results in death

- is life-threatening i.e. the patient was at risk of death at the time of the AE
- requires inpatient hospitalisation or prolongation of existing hospitalisation
- results in persistent or significant disability/incapacity
- results in a congenital anomaly/birth defect
- results in any other significant medical condition

More than one of the above criteria can be applicable to one event. Important medical events that may not be immediately life-threatening or result in death or hospitalisation may be considered an SAE when, based upon appropriate medical judgment, they may jeopardize the patient or require medical or surgical intervention to prevent one of the outcomes listed in the definition above. We will use the Division of AIDS Table for Grading the Severity of Adult and Pediatric Adverse Events (v2.0, 2014) grading table as guidance on determining the severity of AEs (https://rsc.tech-res.com/docs/default-source/safety/daids_ae_grading_table_v2_nov2014.pdf). Grading the severity of the AE will also be based on clinical judgement of site investigators who will have the final say prior to submission of SAE reports. If the SAE is at least "possibly related" to the study drug, the site investigator will consider treatment change in conjunction with the attending physician.

All SAEs must be reported to the site lead investigator within one day of the awareness of the SAE, via email and phone. The site lead investigators will immediately inform the study PI and further reports should be submitted, if required, until the SAE is resolved. The site investigator must also report the SAEs to the local ethics committee in accordance with local requirements. The PI will be responsible for informing the data safety and monitoring committee (DSMC) regarding the SAE and any related updates.

Risk management

The risk of venepuncture will be minimised by the procedure being performed by experienced study staff using an aseptic technique. In-dwelling venous cannula used for collecting PK blood samples will be monitored daily for evidence of localised thrombophlebitis and removed if present.

Doxycycline side effects commonly seen (1-10%) include diarrhoea, indigestion, nausea, vomiting, pruritus or photosensitivity rash. Other symptoms reported infrequently include black/tarry/clay-colored stools, bloating, cough, dark urine or decreased appetite.

Azithromycin common side-effects (1-10%) include diarrhea, nausea and vomiting. Less common side-effects include rash and abdominal pain. Rarely, the drug can cause QTc prolongation and predispose the patient to ventricular tachyarrhythmias. Patients with a history of prolonged QTc, ventricular tachyarrhythmias or taking concomitant medications known to prolong the QTc interval will not be recruited.

Adverse events experienced as a consequence of study participation requiring additional medical care will be covered by the study sponsor.

A6.11 Clinical monitoring

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

We will perform an interim analysis after an adequate number of patients (around 50% of target number) have been enrolled to allow the DSMC to monitor the safety of the trial, treatment efficacy, recruitment progress and perform sample size recalculation if indicated. Further details of the interim analysis and stopping rules are discussed in the main text of chapter 6.

A6.12 Statistical considerations

Statistical analysis

Data analysis and interim analysis plan with results are presented in section 6.4 of the main body of the thesis.

Sample size calculation

Formal sample size calculation was based on previously published data on FCT and treatment failure. A median FCT of 30 (IQR 4-58) hours was observed in patients in Mae Sot, Tak Province, northwestern Thailand [178]. The study is powered at 90%, with alpha of 0.05, to detect an increase of 20% of the average FCT of 30 hours, with standard deviation of 10 hours, corresponding to an increase to 36 hours.

Based on this formal sample size calculation, the estimated sample size per arm is n=59 and a total of 177 scrub typhus patients. The primary comparison is between doxycycline 7 days as standard of care and azithromycin 3 days. We expect to recruit these patients within 24-48 months at the proposed study sites.

A6.13 Ethical considerations

The study is conducted in compliance with the conditions stipulated by the sponsor and local EC/IRB, applicable regulations and ICH/GCP requirements. Where requirements differ, those which afford greater protection to the safety of the subjects will be followed.

The study protocol has been submitted to the following ethics committees:

- Ethics committee of Chiangrai Prachanukroh Hospital, Chiangrai, Thailand
- Faculty of Tropical Medicine Ethics Committee (FTMEC), Mahidol University, Bangkok, Thailand
- Oxford Tropical Medicine Research Ethics Committee (OxTREC), University of Oxford, Oxford, UK

Study completion and participant withdrawal

Patients are deemed to have completed the study if they have been followed up to 8 weeks with data and samples collected as per study protocol. Participants may voluntarily withdraw from the study and investigators may also withdraw the participant from participating in the study at any time (e.g. poor compliance with protocol).

The reason for discontinuation or withdrawal will be recorded. If the study drug or participation in the study is discontinued due to an adverse event, the investigator will arrange for follow-up visits at least until the adverse event has resolved or stabilised.

The study may be stopped early for reasons including inability to recruit sufficient patients, DSMC recommendation based on trial data or trial termination by sponsor, EC/IRB or regulatory authority.

Funding

The study was funded by the Military Infectious Diseases Research Programme (MIDRP, N62645-16-C-4020), United States Department of Defense, USA and the Wellcome Trust, United Kingdom, as part of the MORU Tropical Health Network institutional funding support.

Study indemnity

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

Publication policy

Results of the study may be published on study completion in peer-reviewed scientific journals. The investigators will ensure that patient identifiers are not revealed.

The Scrub Typhus Antibiotic Resistance Trial (START)

PARTICIPANT INFORMATION SHEET

For Scrub Typhus Antibiotic Resistance Trial (START) Patient aged 15 years or more

This document may contain statements which you do not understand. If this is the case, please ask the study staff to explain until you fully comprehend. Once you decide to join the study, you may keep this document. You may also keep this document if you require some time to discuss further with friends, relatives, or your personal doctor before taking part in this study.

Principal Investigator	Dr Tri Wangrangsimakul
Study Site	Chiangrai Prachanukroh Hospital, Chiangrai, Chiangrai province, northern Thailand Shoklo-Malaria Research Unit (SMRU), Mae Sot, Tak province, north-western Thailand
Ethics committee reference	TMEC 16-055, OxTREC 4-17

Why we are doing this trial?

Scrub typhus is a common and potentially fatal cause of febrile illness in humans in the north and north-western regions of Thailand. It is caused by a bacteria which is transmitted through the bite of the immature stage of an infected mite. The disease mainly affects people in rural areas and agricultural workers.

Usually, patients with scrub typhus improve after 2 days of treatment with an appropriate antibiotic. However, a study on scrub typhus patients from Chiangrai 20 years ago demonstrated that some patients failed to respond appropriately to the standard antibiotic treatment, doxycycline. Further studies have shown that azithromycin is an effective alternative antibiotic that can be used to treat scrub typhus. Despite this, current treatment failure rates in Chiangrai remain high and the reasons for this have not been fully investigated.

We will perform this trial to evaluate the effectiveness of 3 days of doxycycline, 3 days of azithromycin, and the standard treatment course of 7 days of doxycycline, in regions of Thailand where drug-susceptible and potentially drug-resistant strains have previously been reported. In addition, we aim to investigate the reasons for treatment failure. We will enrol 59

patients in each treatment arm and will recruit patients with scrub typhus from Chiangrai, northern Thailand and Mae Sot, north-western Thailand.

What will happen to you if you voluntarily take part in the study?

- The study details will be explained to you by the study staff. You will be allowed adequate time to consider the information and the opportunity to ask questions. The researcher or study nurse will ask you to sign a consent form if you agree to participate in this study.
- If you agree to participate, we will test your blood for scrub typhus using the sample collected on admission to hospital. If the test is positive or if you have a fever and a small skin lesion called an eschar, we will enrol you into the study.
- On enrolment, you will be randomly allocated to receive either 7 days of doxycycline, 3 days of doxycycline, or 3 days of azithromycin as treatment for scrub typhus.
- A trained member of staff will aseptically insert an intravenous cannula into a vein in your hand or arm. This will be used by the study team to collect blood samples during your stay in hospital. We will collect a small amount of blood from you at baseline prior to the first dose of the allocated antibiotic. The amount of blood depends on your age/weight but will be approximately 3-4 teaspoons (15-20ml). We will also collect clinical information, details of tests performed as part of routine care, and other treatment started by your doctors.
- In some patients with scrub typhus, an eschar (a small dark crust of dead tissue) forms at the site of the mite bite. If present, we will collect an eschar swab specimen by using a wet cotton bud to rub on the eschar to collect a little bit of the dark crust. If the eschar crust is present, we will scrape a part of it off and collect it for testing. This is completely painless.
- During your stay in hospital, the treatment antibiotic will be administered orally by the ward nurses or study staff with a record made of the date and exact time of administration.
- If you do not tolerate the allocated antibiotic or develop an adverse reaction to the study drug, we will withdraw you from the study and an alternative treatment will be given by the doctors in charge of your care.
- You will be regularly reviewed by the ward nurses, the hospital doctors, and the study staff and your vital signs and clinical progress recorded. If you do not improve or become sicker despite the allocated treatment, the hospital doctors in charge of your care and the study doctor may alter your treatment regimen by extending the treatment course, switching antibiotics or adding a second antibiotic to your allocated treatment.
- You will have smaller blood samples (2ml) collected during and after your antibiotic treatment course to measure the drug levels and presence of bacteria in your blood. These

smaller blood collections will take place between enrolment and week 2 of the study. The number of time-points will be 6-12 and is dependent on which treatment group and which drug level measurement group you were randomised to. Study staff will keep you informed of the blood collection schedule for the duration of the study.

- You will be discharged by the hospital team after you are better and your fever has cleared. We will arrange to collect follow-up blood samples (3-4 teaspoons, 15-20ml) and further clinical information at weeks 2 and 8. If you cannot come to hospital, please let us know and we can arrange follow-up at a location closer to your home. You may also have additional follow-up(s) in the first week of study participation to complete drug level monitoring.
- If you become unwell after discharge from hospital, you will be asked to inform the study team, attend hospital to be reviewed and have further blood samples collected (3-4 teaspoons, 15-20ml). If you have deteriorated significantly, you will be readmitted for further care.

Expected length of time for the study procedure: We expect the study procedures to take around 45 minutes on the day of study enrolment. Subsequent daily reviews by the study team will take around 20 minutes each day until discharge from hospital. Scheduled follow-up at 2 and 8 weeks will take 20 minutes. Any additional follow-up required during the first week of the study (for drug level measurement) will take up to 10 minutes.

Possible risks are:

- Collecting blood may sometimes result in local pain, bleeding, bruising or a feeling light-headedness, which usually resolves without treatment.
- Blood for drug level measurements will be drawn through a small plastic cannula placed in a vein in your hand or arm. There is a small risk of localised skin/soft tissue infection which we will minimise by performing the cannula insertion under aseptic conditions, monitoring the cannula site daily, and replacing the cannula every 3 days if the inpatient stay exceeds this period.
- If you receive doxycycline for the treatment of scrub typhus, the common side-effects (1-10%) are diarrhoea, indigestions, nausea, vomiting, and itching and/or redness of the skin. Other less common side-effects include black-tarry or clay-colored stools, bloating, cough, dark urine, and decreased appetite.

- If you receive azithromycin for the treatment of scrub typhus, the common side-effects (1-10%) are diarrhea and loose stools. Less common side-effects include blistering/crusting/irritation/itching/reddening of the skin, cracked/dry/scaly skin, and abdominal pain.
- In case of any severe direct injury, adverse event, or harm as a result of participation in this study, the medical research team and study sponsor will be liable and cover any expenses for your treatment and care relating to the injury/complication.
- There are no extra costs for you if you decide to take part in this study.

We will ensure these procedures are carried out with minimal pain and risk as possible.

Compensation

By participating in this study, you will receive the antibiotic treatment course as per allocated study arm free of charge. You/your parent will receive 300 Baht for the additional time required by participating in the study during your inpatient stay. You/your parent will receive 300 Baht per visit by attending the scheduled follow-up visits at weeks 2 and 8. If the study team asks you to attend additional follow-up(s) during the first 2 weeks of the study, you/your parent will receive 300 Baht compensation per visit. Any travel costs incurred while travelling to attend the follow-up appointments at weeks 2 and 8 and additional follow-up time-points for drug level monitoring will be covered by the study team.

Contacting the research team

If you have any questions relating to this research project, you can contact:

- Nidanuch Tasak (Study Nurse) – 095-4534409
- Dr. Tri Wangrangsimakul – 052-029842 (business hrs) or 083-0745931
- Dr. Suwimon Khusuwan – 053-711300 (business hrs)

Should there be additional information

We will inform you if we have any new information about this study which might be important for you to know related to your participation in the study.

Confidentiality

Your identifiable information will be known only to the researchers performing this study or to specific groups for auditing purposes. These groups include research sponsors, government institutions, or organisations authorised to conduct audits such as the ethics committee. Your

information will be kept confidential by these groups. Individual participant identifiers are removed from all samples and replaced by codes to ensure that samples can only be linked to the participants by members of the research team. You have the right to withdraw from participating in this research project at any time without prior notice. Declining to participate or withdrawing from participation will not have any effects on services and medical care to which you are entitled. The clinical and laboratory data that are stored in our database may be shared with other investigators in the future. However, data will be anonymised and the investigators will not know your identity.

Future Study

Some of your samples will be stored for 10 years in a freezer for further tests in the future. One of the tests we will do is “genotyping”, where we compare parts of your genetic make-up with how you respond to infection. It is possible in the future that we will also test responses to other infections other than scrub typhus (e.g. dengue and leptospirosis). Most of the tests that will be done on your samples will be done in Thailand. However, for some tests that cannot be done in Thailand, part of the samples will be sent to laboratories overseas. At any stage you can instruct us to destroy stored samples. If the research physician wants to conduct further analysis, they will ask the relevant ethics committee for approval.

This research has been approved by the Faculty of Tropical Medicine Ethics Committee, Mahidol University (Bangkok, Thailand), OxTREC (an ethics committee in Oxford, UK) and the ethics committee at Chiangrai Prachanukroh Hospital, Chiangrai.

If you have not been treated as specified in this information sheet, or you wish to know more about participants’ rights, please contact:

Ethics Committee, Chiangrai Prachanukroh Hospital, Chiang Rai, Tel. 053-711300 ext. 2145

Ethics Committee, Faculty of Tropical Medicine, Mahidol University, Tel.02-3549100-4 ext. 1349

INFORMED CONSENT FORM/ASSENT FORM

For Scrub Typhus Antibiotic Resistance Trial (START) Patient aged 15 years or more

Date……Month……Year……

Participant aged 15 years old or more

First name (Girl/Boy/Ms/Mr/Mrs) …….

Family name…… age…….

Address……

Parent or guardian of participant aged 15 years to less than 18 years old

First name (Ms/Mr/Mrs) …….

Family name…… age…….

Address……

Parent/guardian of (Boy/Girl) ……

…… *(Name of study staff who obtained consent)* has informed me in detail about:

- Objectives of the study and time required to take part in the study
- Procedures required to be performed
- Expected benefits from the research
- Potential risks of taking part in the research
- Compensation and costs of relevance to me

I have understood all that has been read and had my questions answered satisfactorily.

Please tick the boxes below where relevant:

I □ agree to take part in this research and

□ Allow the study staff to use my blood, urine and cerebrospinal fluid samples collected as part of my routine care (which may be stored in the laboratory or on the ward) to test for scrub typhus and other tests as part of the study

□ Allow the study staff to collect blood, urine and eschar swab/crust samples from me

I □ allow / □ do not allow my blood +/- other samples to be stored for 10 years for future research

I □ allow / □ do not allow my blood +/- other samples to be sent to a research institute outside Thailand

I □ allow / □ do not allow my blood +/- other samples to be used for genetic testing

I □ allow / □ do not allow my anonymised clinical and laboratory data to be shared with other researchers for future use

I understand that I can change my mind at any stage without affecting further services and hospital care to which I am entitled to in the future. To consent to take part in this study, I allow the investigators to use my personal information obtained from this research. My information will be presented as part of research results without revealing my name or identity.

Participant's signature: _____ **Date** _____
(dd-mmm-yy)

Participant's name: _____
(Please print name)

***Parent or guardian's signature:** _____ **Date** _____
(dd-mmm-yy)

***Parent or guardian's name:** _____
(Please print name)

(*If participant's age is 15 to less than 18 years)

Witness's signature: _____ **Date** _____
(dd-mmm-yy)

Witness's name: _____
(Please print name)

Witness's signature: _____ **Date** _____
(dd-mmm-yy)

Witness's name: _____
(Please print name)

I certify that I have followed the study protocol to obtain consent from the participant. She/he apparently understood the nature and the purpose of the study and consents to the voluntary participation in the study. The participant has been given opportunities to ask questions which have been answered satisfactorily.

Designee/investigator's signature: _____ **Date** _____
(dd-mmm-yy)

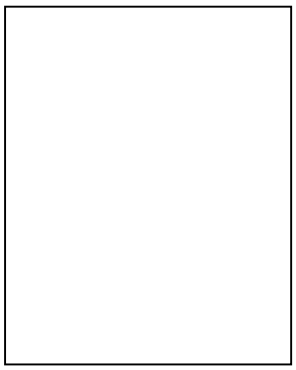
Designee/investigator's name: _____
(Please print name)

NB – A witness is a person who is independent from the trial or a member of staff who was not involved in gaining the consent/assent.

THE PARTICIPANT SHOULD NOW BE GIVEN A SIGNED COPY TO KEEP

****If the participant cannot read or write, use thumbprint instead:**

I cannot read, but the explainer has read all statements in the informed assent/consent form and explained the contents to me until I have understood. By imprinting my thumbprint on this form, I consent for my voluntary participation in the study.



Participant's thumbprint

Participant's name: _____ **Date** _____
(To be completed by person taking assent/consent) (dd-mmm-yy)

***Parent or guardian's signature:** _____ **Date** _____
(dd-mmm-yy)

***Parent or guardian's name:** _____
(Please print name)

(*If participant's age is 15 to less than 18 years)

Witness's signature: _____ **Date** _____
(dd-mmm-yy)

Witness's name: _____
(Please print name)

Witness's signature: _____ **Date** _____
(dd-mmm-yy)

Witness's name: _____
(Please print name)

I certify that I have followed the study protocol to obtain consent from the participant. She/he apparently understood the nature and the purpose of the study and consents to the voluntary participation in the study. The participant has been given opportunities to ask questions which have been answered satisfactorily.

Designee/investigator's signature: _____ **Date** _____
(dd-mmm-yy)

Designee/investigator's name: _____
(Please print name)

NB – A witness is a person who is independent from the trial or a member of staff who was not involved in gaining the consent/assent.

THE PARTICIPANT SHOULD NOW BE GIVEN A SIGNED COPY TO KEEP

Appendix 7

Scrub typhus antibiotic resistance trial (START) – Case record form

The following sections of the CRF are included:

- Screening
- Baseline/Day 0
- Day 1
- Follow-up
- Adverse event
- Serious adverse event
- Concomitant medications
- Supportive treatment
- Final status
- PK sample collections

Admission Day 2, Admission extra day and Unscheduled visit day are similar in content and format as Day 1 and have been excluded.

Scrub Typhus Antibiotic Resistance Trial (START)	**SCREENING**	SAR - |__|__| - |__|__|__| |__|__|__| Study site / Subject number / Subject initials

1. Screening date	|__|__|/ |__|__|/ 20|__|__| *(dd/mm/yyyy)*
2. Informed consent/assent date	|__|__|/ |__|__|/ 20|__|__| *(dd/mm/yyyy)*

INCLUSION/EXCLUSION CRITERIA

3. Inclusion criteria		**YES**	**NO**
IN 1	Age ≥15 years old	○	○
IN 2	Presentation to hospital with acute undifferentiated fever (temperature >37.5°C, tympanic) or history of fever ≤ 14 days	○	○
IN 3	Clinical suspicion of scrub typhus	○	○
IN 4	scrub typhus rapid test positive OR OT PCR positive OR presence of eschar	○	○
IN 5	Written informed consent and/or written informed assent obtained	○	○
IN 6	Able to take oral medications	○	○
4. Exclusion criteria		**YES**	**NO**
EX 1	Known hypersensitivity to tetracycline, doxycycline or azithromycin	○	○
EX 2	Administration of doxycycline, azithromycin, chloramphenicol, rifampicin, or tetracycline during the preceding 7 days	○	○
EX 3	Pregnancy or breast feeding	○	○
EX 4	Patients with myasthenia gravis or systemic lupus erythematosus	○	○
EX 5	Patients with an established infection (diagnostic test required), e.g. acute malaria, dengue, leptospirosis, typhoid, Japanese encephalitis etc.	○	○
EX 6	Current TB or TB treatment in ≤ 6 months (contains Orientia-effective antimicrobial)	○	○
EX 7	Current HAART use for HIV, long term use of immunosuppressants (e.g. steroids, chemotherapy, TNF-inhibitors and related agents)	○	○
EX 8	Patients with severe disease whom the clinical team feel their condition necessitates the need for additional scrub typhus treatment	○	○

5. ELIGIBILITY

5.1 Is subject eligible?	○ Yes ○ No
5.2 Enrolment date	|__|__|/ |__|__|/ 20|__|__| *(dd/mm/yyyy)*

Investigator's statement

I have reviewed all data contained on the screening form for this patient and have verified that the contents are consistent with observations and source records

Enrolment confirmed by:

_____ _____ DATE |__|__|/ |__|__|/ 20|__|__|

INVESTIGATOR (PRINT NAME) SIGNATURE *(dd/mm/yyyy)*

Scrub Typhus Antibiotic Resistance Trial (START)	**BASELINE/ ADMISSION DAY 0**	SAR - |__|__| - |__|__|__| |__|__|__| Study site / Subject number / Subject initials

DEMOGRAPHICS

1. Birth date |__|__|/ |__|__|/ |__|__|__|__| (dd/mm/yyyy) **OR** Age |__|__| years |__|__| months

2. Sex: ○ Male ○ Female

3. Ethnic group: ☐ Thai ☐ Karen ☐ Akha ☐ Lahu ☐ Hmong

☐ Other, specify _____

4. Highest educational attainment: ○ None ○ Primary school ○ Secondary school ○ High school ○ Vocational school/college ○ University

5. Occupation: ○ None ○ Agriculture ○ Office ○ Other

Specify detail: _____

TRANSFER INFORMATION

6. Was the patient transferred from another hospital/health facility/ward or clinic?

○ Yes, specify facility name _____

Date of admission |__|__|/ |__|__|/ 20|__|__| time |__|__|:|__|__|

Date of discharge |__|__|/ |__|__|/ 20|__|__|

Diagnosis _____

○ No, Go to Q10. '**MEDICAL HISTORY**' section

7. Observations and Vital signs

Temperature	|__|__|.|__| ˚C	Pulse rate	|__|__|__| beats/min
Respiratory rate	|__|__|__| breaths/min	BP	|__|__|__|/|__|__|__| mmHg
SpO_2	|__|__|__| %	Weight	|__|__|__|.|__| kg
FiO_2 (e.g. air = 21%)	|__|__|__| %	Height	|__|__|__|.|__| cm

8. Any diagnostic tests (disease specific) performed? ○ Yes ○ No

If ***Yes***, detail

_____

_____

9. MANAGEMENT AT PREVIOUS HEALTH FACILITY

Drug name	Dose and frequency	Route	Start date *(dd/mm/yyyy)* and time	Stop date *(dd/mm/yyyy)* and time	Duration (hrs/days)
Paracetamol ○ Use ○ Not use			|__|__|/ |__|__|/ 20|__|__| |__|__|:|__|__|	|__|__|/ |__|__|/ 20|__|__| |__|__|:|__|__|	
NSAIDs ○ Use, _____ ○ Not use			|__|__|/ |__|__|/ 20|__|__| |__|__|:|__|__|	|__|__|/ |__|__|/ 20|__|__| |__|__|:|__|__|	
Steroid ○ Use, _____ ○ Not use			|__|__|/ |__|__|/ 20|__|__| |__|__|:|__|__|	|__|__|/ |__|__|/ 20|__|__| |__|__|:|__|__|	
Antibiotics 1 ○ Use, _____ ○ Not use			|__|__|/ |__|__|/ 20|__|__| |__|__|:|__|__|	|__|__|/ |__|__|/ 20|__|__| |__|__|:|__|__|	
Antibiotics 2 ○ Use, _____ ○ Not use			|__|__|/ |__|__|/ 20|__|__| |__|__|:|__|__|	|__|__|/ |__|__|/ 20|__|__| |__|__|:|__|__|	
Antibiotics 3 ○ Use, _____ ○ Not use			|__|__|/ |__|__|/ 20|__|__| |__|__|:|__|__|	|__|__|/ |__|__|/ 20|__|__| |__|__|:|__|__|	
Antibiotics 4 ○ Use, _____ ○ Not use			|__|__|/ |__|__|/ 20|__|__| |__|__|:|__|__|	|__|__|/ |__|__|/ 20|__|__| |__|__|:|__|__|	

Scrub Typhus Antibiotic Resistance Trial (START)	**BASELINE/ ADMISSION DAY 0**	SAR - |__|__| - |__|__|__| |__|__|__| Study site / Subject number / Subject initials

10. MEDICAL HISTORY

10.1 Onset date of symptoms |__|__|/ |__|__|/ 20|__|__| *(dd/mm/yyyy)*

10.2 Max number of days ill เป็นมากี่วันแล้ว (ก่อนมาเข้าโรงพยาบาล) |__|__| days

a) Fever (ไข้)	○Yes ○No	|__|__| days	n) Rigors chills	○Yes ○No	|__|__| days
b) Deafness (หูหนวก)	○Yes ○No	|__|__| days	o) Tinnitus (หูอื้อ)	○Yes ○No	|__|__| days
c) Retro-orbital pain	○Yes ○No	|__|__| days	p) Headache (ปวดหัว)	○Yes ○No	|__|__| days
d) Arthralgia (ปวดข้อ)	○Yes ○No	|__|__| days	q) Myalgia (ปวดกล้ามเนื้อ)	○Yes ○No	|__|__| days
e) Eschar	○Yes ○No	|__|__| days	r) Skin rash (ผื่น)	○Yes ○No	|__|__| days
f) Jaundice (ซ่านดี)	○Yes ○No	|__|__| days	s) Nausea (คลื่นไส้)	○Yes ○No	|__|__| days
g) Eye redness (ตาแดง)	○Yes ○No	|__|__| days	t) Vomiting (อาเจียน)	○Yes ○No	|__|__| days
h) Abdominal pain (ปวดท้อง)	○Yes ○No	|__|__| days	u) Diarrhoea (ท้องเสีย)	○Yes ○No	|__|__| days
i) Seizures (ชัก)	○Yes ○No	|__|__| days	v) Neck stiffness (คอแข็ง)	○Yes ○No	|__|__| days
j) Confusion (สับสน)	○Yes ○No	|__|__| days	w)Vertigo (เวียนหัว บ้านหมุน)	○Yes ○No	|__|__| days
k) Cough (ไอ)	○Yes ○No	|__|__| days	x) Dyspnoea (จลำบากหายใ)	○Yes ○No	|__|__| days
l) Epistaxis (เลือดกำเดาไหล)	○Yes ○No	|__|__| days	y) Haemoptysis (ไอเป็นเลือด)	○Yes ○No	|__|__| days
m) Bleeding gums (เลือดออกตามไรฟัน)	○Yes ○No	|__|__| days	z) Haematemesis (อาเจียนเป็นเลือด)	○Yes ○No	|__|__| days

11. PAST MEDICAL HISTORY

a) Diabetes mellitus	○Yes ○No	g) Chronic lung disease	○Yes ○No
b) Hypertension	○Yes ○No	h) Chronic kidney disease	○Yes ○No
c) Dyslipidaemia	○Yes ○No	i) Chronic liver disease	○Yes ○No
d) Cerebrovascular disease	○Yes ○No	j) Heavy alcohol intake	○Yes ○No
e) Coronary artery disease	○Yes ○No	k) Current smoker	○Yes ○No
f) Deep vein thrombosis/pulmonary embolism	○Yes ○No	l) Other medical history? ○Yes ○No, specify:	

		_____

12. DRUG HISTORY

12.1 Do you have any known drug allergies? ○ Yes, specify _____ ○ No

12.2 Do you take any regular medications? ○ Yes ○ No

Drug name	Route	Dose	Frequency

SCRUB TYPHUS HISTORY

13. Have you had "scrub typhus" before? *(It is called "kai rai oon" ไข้ไรอ่อน)* ○ Don't know ○ No ○ Yes

*(If NO or DON'T KNOW, Go to question **14**. Have you had any of following diseases before?')*

13.1 How many years or months ago? *(most recent episode)* \|__\|__\| years \|__\|__\| months

13.2 Did you receive any antibiotic? ○ Don't know ○ No ○ Yes, specify _____

13.3 How many days did it take for the fever to go away?

○ Don't know ○ < 3 days ○ 3 – 7 days ○ > 7 days

14. Have you had any of following diseases before?

14.1 Murine Typhus ○ Don't know ○ No ○ Yes

14.2 Leptospirosis ○ Don't know ○ No ○ Yes

14.3 Dengue ○ Don't know ○ No ○ Yes

15. ANIMALS CONTACT

In the last 2 weeks,

15.1 Have you had contact with: ☐ None ☐ rat ☐ cat ☐ flea(หมัด) ☐ mite(ไร หรือไรอ่อน) ☐ tick(เห็บ) ☐ other, specify _____

15.2 Have you visited: ☐ None ☐ rice field ☐ garden ☐ forest ☐ up-high hill ☐ jungle ☐ valley ☐ palm plantation ☐ other, specify _____

15.3 Any animals live at/close to your home? ☐ None ☐ rats ☐ cats ☐ pigs ☐ dogs ☐ cows/buffalos ☐ chicken ☐ other, specify _____

15.4 Any animals live at/close to your work place? ☐ None ☐ rats ☐ cats ☐ pigs ☐ dogs ☐ cows/buffalos ☐ chicken ☐ other, specify _____

16. Date of admission |__|__|/ |__|__|/ 20|__|__| *(dd/mm/yyyy)*

17. OBSERVATIONS AND VITAL SIGNS

Temperature	|__|__|.|__| ˚C	Pulse rate	|__|__|__| beats/min_
Respiratory rate	|__|__|__| breaths/min	BP	|__|__|__|/|__|__|__| mmHg
SpO_2	|__|__|__| %	Weight	|__|__|__|.|__| kg
FiO_2 (e.g. air = 21%)	|__|__|__| %	Height	|__|__|__|.|__| cm

Scrub Typhus Antibiotic Resistance Trial (START)	**BASELINE/ ADMISSION DAY 0**	SAR - |__|__| - |__|__|__| |__|__|__| Study site / Subject number / Subject initials

18. PHYSICAL EXAMINATION		
a) Anaemic	○Yes ○No	If Yes, detail _____
b) Jaundice	○Yes ○No	If Yes, ○ Mild ○ Moderate ○ Severe
c) Eschar lesion	○Yes ○No	If Yes, detail _____ total number |__|__|, and select location ☐ *head* ☐ *neck* ☐ *breast* ☐ *chest* ☐ *axilla* ☐ *arm* ☐ *abdomen* ☐ *groin* ☐ *genitalia* ☐ *buttocks* ☐ *legs* ☐ *belt* ☐ *sarong* ☐ *bra* (*ขอบยางเสื้อชั้นใน*) ☐ *underwear* (*ขอบยางกางเกงใน*) ☐ other, specify _____
d) Lymphadenopathy *(> 1cm)*	○Yes ○No	If Yes, Location _____
e) Skin rash	○Yes ○No	If Yes, Type/Location _____ *(Type: 1=macular, 2=maculo-papular, 3=papular, 4=petechial, 5=pustular, 6=vesicular, 7=blister, 8=petechial bruising, 9=other)* Other details _____
f) Petechial haemorrhage	○Yes ○No	If Yes, Location: ☐ Enoral ☐ Conjunctival ☐ Skin specify _____
g) Meningism	○Yes ○No	If Yes, detail _____
h) Subconjunctival hemorrhage	○Yes ○No	If Yes, detail _____
i) Conjunctival injection	○Yes ○No	If Yes, detail _____
j) Increased respiratory effort	○Yes ○No	If Yes, Intubated? ○No ○Yes
k) Lung crepitation	○Yes ○No	If Yes, Location _____
l) Liver enlarged	○Yes ○No	If Yes, size |__|__| cm below RCM
m) Spleen enlarged	○Yes ○No	If Yes, enlarged size |__|__| cm
n) Focal neurological deficits	○Yes ○No	If Yes, detail _____
o) Gum petechiae	○Yes ○No	If Yes, ○ Mild ○ Moderate ○ Severe Describe: _____

p) Deafness	○Yes ○No	If Yes, • **Weber** (sensorineural): ○ Left ○ Right ○ symmetric • **Rinne:** Air Conduction: ○ Left ○ Right ○ symmetric Bone conduction: ○ Left ○ Right ○ symmetric

19. Glasgow Coma Score: Motor |__| /6 Verbal |__| /5 Eyes |__| /4 Total |__|__| /15

20. Is there a preliminary diagnosis (or diagnoses) in the admission record? ○Yes ○No

If ***Yes***, specify _____

LABORATORY TESTS

21. BLOOD: HAEMATOLOGY

Hb	|__|__|.|__| g/dL	Neutrophil	|__|__|.|__| %
Hct	|__|__|.|__| %	Lymphocytes	|__|__|.|__| %
Platelet	|__|__|__|.|__| $10^3/mm^3$	Monocytes	|__|__|.|__| %
WBC	|__|__|__|.|__| $10^3/mm^3$	Eosinophils	|__|__|.|__| %
PT	|__|__|__| sec	INR	|__|__|.|__|
PTT	|__|__|__| sec	ESR	|__|__|.|__| g/dL

22. BLOOD: BIOCHEMISTRY

BUN	|__|__|__|.|__| mg/dL	Bilirubin direct	|__|__|.|__| mg/dL
Creatinine	|__|.|__|__| mg/dL	Bilirubin total	|__|__|.|__| mg/dL
Na	|__|__|__| mmol/L	AST	|__|__|__| IU/L
K	|__|__|.|__| mmol/L	ALT	|__|__|__| IU/L
Cl	|__|__|__| mmol/L	Alk. phosphatase	|__|__|__| IU/L
HCO_3	|__|__| mmol/L	Albumin	|__|__|.|__| g/dL
Globulin	|__|__|.|__| g/dL	CRP (C-reactive Protein)	|__|__|__|.|__| µg/mL
Glucose	|__|__|__| mg/dL	Lactate	|__|__|.|__| mmol/L
Trop I	|__|__|.|__|__| ng/mL	CK-MB	|__|__|__| IU/L (ng/mL)

23. MICROBIOLOGY
23.1 Scrub typhus IgM/IgG/IgA RDT (SD Bioline): ○ Not done ○ Negative ○ Positive
23.2 Scrub typhus IgM RDT (InBios): ○ Not done ○ Negative ○ Positive
23.3 Scrub typhus PCR: ○ Not done ○ Done, time of PCR sample collection |__|__|:|__|__| Result: ○ Negative ○ Positive, copy number if positive _____
23.4 Blood culture: ○ Not done ○ Done, result _____
23.5 Urine culture: ○ Not done ○ Done, result _____
23.6 Urinalysis: ○ Not done ○ Done, result _____
23.7 CSF microscopy: ○ Not done ○ Done, result _____
23.8 CSF culture: ○ Not done ○ Done, result _____
24. OTHER LABORATORY TESTS
24.1 Pregnancy test: ○ Not done ○ Negative ○ Positive ○ Not applicable
24.2 CSF protein: ○ Not done ○ Done, result _____
24.3 CSF glucose: ○ Not done ○ Done, result _____
24.4 Arterial blood gas: ○ Not done ○ Done, result _____

24.5 Any other tests? ○Yes, details below ○No

Test performed	Result/Value	Units

25. STUDY SAMPLE COLLECTION

Were the following study samples collected:

25.1 Blood ○Yes, collection date \|__\|__\|/ \|__\|__\|/ 20\|__\|__\| ○No

25.2 Eschar swab ○Yes, collection date \|__\|__\|/ \|__\|__\|/ 20\|__\|__\| ○No ○Not applicable

25.3 Eschar scrape ○Yes, collection date \|__\|__\|/ \|__\|__\|/ 20\|__\|__\| ○No ○Not applicable

25.4 Urine ○Yes, collection date \|__\|__\|/ \|__\|__\|/ 20\|__\|__\| ○No ○Not applicable

25.5 CSF ○Yes, collection date \|__\|__\|/ \|__\|__\|/ 20\|__\|__\| ○No ○Not applicable

26. IMAGING AND OTHER NON-LABORATORY TESTS

26.1 Chest X-ray: ○ Not done ○ Done, result _____

26.2 Ultrasound: ○ Not done ○ Done, details _____

26.3 CT scan: ○ Not done ○ Done, details _____

26.4 Echocardiogram: ○ Not done ○ Done, result _____

26.5 Any other tests performed? ○Yes, specify result below ○No

_____

_____

Treatment arm allocation	○ Doxycycline 7 days	○ Doxycycline 3 days	○ Azithromycin 3 days	
DOXYCYCLINE (First dose/loading dose always 200mg followed by 100mg BD)				
Dose	**Date**	**Time**	**Patient vomit?**	**If vomit, re-dose time**
Dose 1	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|	○Yes ○No	|__|__|:|__|__|
Dose 2	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|	○Yes ○No	|__|__|:|__|__|
Dose 3	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|	○Yes ○No	|__|__|:|__|__|
Dose 4	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|	○Yes ○No	|__|__|:|__|__|
Dose 5	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|	○Yes ○No	|__|__|:|__|__|
Dose 6	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|	○Yes ○No	|__|__|:|__|__|
Dose 7	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|	○Yes ○No	|__|__|:|__|__|
Dose 8	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|	○Yes ○No	|__|__|:|__|__|
Dose 9	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|	○Yes ○No	|__|__|:|__|__|
Dose 10	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|	○Yes ○No	|__|__|:|__|__|
Dose 11	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|	○Yes ○No	|__|__|:|__|__|
Dose 12	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|	○Yes ○No	|__|__|:|__|__|
Dose 13	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|	○Yes ○No	|__|__|:|__|__|
Dose 14	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|	○Yes ○No	|__|__|:|__|__|
AZITHROMYCIN (First dose/loading dose always 1,000mg followed by 500 mg OD)				
Dose	**Date**	**Time**	**Patient vomit?**	**If vomit, re-dose time**
Dose 1	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|	○Yes ○No	|__|__|:|__|__|
Dose 2	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|	○Yes ○No	|__|__|:|__|__|
Dose 3	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|	○Yes ○No	|__|__|:|__|__|

(* file PK sample collection CRF after this page****)***

Scrub Typhus Antibiotic Resistance Trial (START)	**ADMISSION DAY 1**	SAR - |__|__| - |__|__|__| |__|__|__| Study site / Subject number / Subject initials

1. Date of review |__|__|/ |__|__|/ 20|__|__| *(dd/mm/yyyy)* **time** |__|__|:|__|__| *(hh:mm, 24hr)*

2. SYMPTOMS (present on day of review and number of days that they have been present)

a) Fever (ไข้)	○Yes ○No	|__|__| days	n) Rigors chills	○Yes ○No	|__|__| days
b) Deafness (หูหนวก)	○Yes ○No	|__|__| days	o) Tinnitus (หูอื้อ)	○Yes ○No	|__|__| days
c) Retro-orbital pain	○Yes ○No	|__|__| days	p) Headache (ปวดหัว)	○Yes ○No	|__|__| days
d) Arthralgia (ปวดข้อ)	○Yes ○No	|__|__| days	q) Myalgia (ปวดกล้ามเนื้อ)	○Yes ○No	|__|__| days
e) Eschar	○Yes ○No	|__|__| days	r) Skin rash(ผื่น)	○Yes ○No	|__|__| days
f) Jaundice (ดีซ่าน)	○Yes ○No	|__|__| days	s) Nausea (คลื่นไส้)	○Yes ○No	|__|__| days
g) Eye redness(ตาแดง)	○Yes ○No	|__|__| days	t) Vomiting(อาเจียน)	○Yes ○No	|__|__| days
h) Abdominal pain (ปวดท้อง)	○Yes ○No	|__|__| days	u) Diarrhoea(ท้องเสีย)	○Yes ○No	|__|__| days
i) Seizures (ชัก)	○Yes ○No	|__|__| days	v) Neck stiffness(คอแข็ง)	○Yes ○No	|__|__| days
j) Confusion (สับสน)	○Yes ○No	|__|__| days	w)Vertigo(เวียนหัว บ้านหมุน)	○Yes ○No	|__|__| days
k) Cough (ไอ)	○Yes ○No	|__|__| days	x) Dyspnoea (หายใจลำบาก)	○Yes ○No	|__|__| days
l) Epistaxis (เลือดกำเดาไหล)	○Yes ○No	|__|__| days	y) Haemoptysis (ไอเป็นเลือด)	○Yes ○No	|__|__| days
m) Bleeding gums (เลือดออกตามไรฟัน)	○Yes ○No	|__|__| days	z) Haematemesis (อาเจียนเป็นเลือด)	○Yes ○No	|__|__| days

Any other symptoms not listed above? ○Yes, specify symptoms below ○No

_____

_____

_____

_____

_____

_____

Scrub Typhus Antibiotic Resistance Trial (START)

ADMISSION DAY 1

SAR - |__|__| - |__|__|__| |__|__|__|
Study site Subject number Subject initials

3. OBSERVATIONS AND VITAL SIGNS

<table>
<tr><th>Time</th><th>Temp. (˚C)</th><th>Pulse (per min)</th><th>Resp. rate (per min)</th><th>BP (mmHg)</th><th>SpO₂ (%)</th><th>FiO₂ % (air = 21%)</th></tr>
<tr><td>|__|__|:|__|__|</td><td>|__|__|.|__|</td><td>|__|__|__|</td><td>|__|__|__|</td><td>|__|__|__|/|__|__|__|</td><td>|__|__|__|</td><td>|__|__|__|</td></tr>
<tr><td>|__|__|:|__|__|</td><td>|__|__|.|__|</td><td>|__|__|__|</td><td>|__|__|__|</td><td>|__|__|__|/|__|__|__|</td><td>|__|__|__|</td><td>|__|__|__|</td></tr>
<tr><td>|__|__|:|__|__|</td><td>|__|__|.|__|</td><td>|__|__|__|</td><td>|__|__|__|</td><td>|__|__|__|/|__|__|__|</td><td>|__|__|__|</td><td>|__|__|__|</td></tr>
<tr><td>|__|__|:|__|__|</td><td>|__|__|.|__|</td><td>|__|__|__|</td><td>|__|__|__|</td><td>|__|__|__|/|__|__|__|</td><td>|__|__|__|</td><td>|__|__|__|</td></tr>
<tr><td>|__|__|:|__|__|</td><td>|__|__|.|__|</td><td>|__|__|__|</td><td>|__|__|__|</td><td>|__|__|__|/|__|__|__|</td><td>|__|__|__|</td><td>|__|__|__|</td></tr>
<tr><td>|__|__|:|__|__|</td><td>|__|__|.|__|</td><td>|__|__|__|</td><td>|__|__|__|</td><td>|__|__|__|/|__|__|__|</td><td>|__|__|__|</td><td>|__|__|__|</td></tr>
</table>

4. PHYSICAL EXAMINATION

<table>
<tr><td>a) Anaemic</td><td>○Yes ○No</td><td>If Yes, details ______________________________</td></tr>
<tr><td>b) Jaundice</td><td>○Yes ○No</td><td>If Yes, ○ Mild ○ Moderate ○ Severe</td></tr>
<tr><td>c) Eschar lesion</td><td>○Yes ○No</td><td>If Yes, details ________________________________
total number |__|__|, and select location
☐ <i>head</i> ☐ <i>neck</i> ☐ <i>breast</i> ☐ <i>chest</i>
☐ <i>axilla</i> ☐ <i>arm</i> ☐ <i>abdomen</i> ☐ <i>groin</i>
☐ <i>genitalia</i> ☐ <i>buttocks</i> ☐ <i>legs</i> ☐ <i>belt</i>
☐ <i>sarong</i> ☐ <i>bra (ขอบยางเสื้อชั้นใน)</i> ☐ <i>underwear (ขอบยางกางเกงใน)</i>
☐ other, specify _________________________</td></tr>
<tr><td>d) Lymphadenopathy
<i>(> 1cm)</i></td><td>○Yes ○No</td><td>If Yes, location and details______________________
____</td></tr>
<tr><td>e) Skin rash</td><td>○Yes ○No</td><td>If Yes, type ___________ , location _____________
<i>(Type: 1=macular, 2=maculo-papular, 3=papular, 4=petechial, 5=pustular, 6=vesicular, 7=blister, 8=petechial bruising, 9=other)</i>
Other details ______________________________</td></tr>
<tr><td>f) Petechial haemorrhage</td><td>○Yes ○No</td><td>If Yes, location: ☐ Enoral ☐ Conjunctiva ☐ Skin
Specify details ______________________________</td></tr>
<tr><td>g) Meningism</td><td>○Yes ○No</td><td>If Yes, details ______________________________</td></tr>
<tr><td>h) Subconjunctival haemorrhage</td><td>○Yes ○No</td><td>If Yes, details ______________________________</td></tr>
<tr><td>i) Conjunctival injection</td><td>○Yes ○No</td><td>If Yes, details ________________________________</td></tr>
</table>

j) Increased respiratory effort	○Yes ○No	If Yes, Intubated? ○No ○Yes, details _____ _____
k) Lung crepitation	○Yes ○No	If Yes, location and details _____ _____
l) Liver enlarged	○Yes ○No	If Yes, size |__|__| cm below right costal margin, details _____
m) Spleen enlarged	○Yes ○No	If Yes, enlarged size |__|__| cm, details _____ _____
n) Focal neurological deficits	○Yes ○No	If Yes, details _____ _____
o) Gum petechiae	○Yes ○No	If Yes, ○ Mild ○ Moderate ○ Severe Describe: _____
p) Deafness	○Yes ○No	If Yes, • **Weber** (*sensorineural*): ○ Left ○ Right ○ symmetric • **Rinne:** Air Conduction: ○ Left ○ Right ○ symmetric Bone conduction: ○ Left ○ Right ○ symmetric
Glasgow Coma Score:	Motor |__| /6 Verbal |__| /5 Eyes |__| /4 Total |__|__| /15	
5. COMPLICATIONS (Have any complications developed?)		
5.1 Respiratory	○Yes ○No	If Yes, detail _____
5.2 Cardiovascular	○Yes ○No	If Yes, detail _____
5.3 Renal	○Yes ○No	If Yes, detail _____
5.4 Liver	○Yes ○No	If Yes, detail _____
5.5 Neurological	○Yes ○No	If Yes, detail _____
5.6 Haematological	○Yes ○No	If Yes, detail _____

5.7 Any other complications?	○Yes ○No	If Yes, detail _____

LABORATORY TESTS			
6. BLOOD: HAEMATOLOGY			
Hb	|__|__|.|__| g/dL	Neutrophil	|__|__|.|__| %
Hct	|__|__|.|__| %	Lymphocytes	|__|__|.|__| %
Platelet	|__|__|__|.|__| $10^3/mm^3$	Monocytes	|__|__|.|__| %
WBC	|__|__|__|.|__| $10^3/mm^3$	Eosinophils	|__|__|.|__| %
PT	|__|__|__| sec	INR	|__|__|.|__|
PTT	|__|__|__| sec	ESR	|__|__|.|__| g/dL
7. BLOOD: BIOCHEMISTRY			
BUN	|__|__|__|.|__| mg/dL	Bilirubin direct	|__|__|.|__| mg/dL
Creatinine	|__|.|__|__| mg/dL	Bilirubin total	|__|__|.|__| mg/dL
Na	|__|__|__| mmol/L	AST	|__|__|__| IU/L
K	|__|__|.|__| mmol/L	ALT	|__|__|__| IU/L
Cl	|__|__|__| mmol/L	Alk. phosphatase	|__|__|__| IU/L
HCO_3	|__|__| mmol/L	Albumin	|__|__|.|__| g/dL
Globulin	|__|__|.|__| g/dL	CRP	|__|__|__|.|__| µg/mL
Glucose	|__|__|__| mg/dL	Lactate	|__|__|.|__| mmol/L
Trop I	|__|__|.|__|__| ng/mL	CK-MB	|__|__|__| IU/L (ng/mL)

Scrub Typhus Antibiotic Resistance Trial (START)	**ADMISSION DAY 1**	SAR - |__|__| - |__|__|__| |__|__|__| Study site / Subject number / Subject initials

8. OTHER TESTS DONE (laboratory, imaging etc.)? ○Yes ○No

Test performed	Result/Value	Units

9. STUDY SAMPLE COLLECTION

Further study samples collected:

9.1 Blood for bacterial load/drug level: ○Yes, collection time |__|__|:|__|__| ○No

9.2 Urine ○Yes, collection time |__|__|:|__|__| ○No

9.3 CSF ○Yes, collection time |__|__|:|__|__| ○No

OUTCOME MEASURES

10. Body temperature (oral or tympanic):

10.1 Highest temperature in last 24hrs |__|__|.|__| °C

10.2 Lowest temperature in last 24hrs |__|__|.|__| °C

10.3 Has fever cleared (temp. ≤37.5°C and remained ≤37.5°C for ≥24hrs without paracetamol use)?

○Yes ○No

If ***Yes***, specify fever clearance time |__|__| days |__|__| hours

11. Clinical symptoms:

11.1 Have clinical symptoms resolved? ○Yes ○No

If ***Yes***, specify time taken for symptoms to resolve after initiation of study antibiotic

|__|__| days |__|__| hours

11.2 Has headache cleared? ○Yes ○No ○Not applicable

If ***Yes***, specify headache clearance time |__|__| days |__|__| hours

12. Has scrub typhus bacteraemia resolved? ○Yes ○No ○Not applicable

If ***Yes***, specify time taken for resolution of bacteraemia after initiation of study antibiotic

|__|__| days |__|__| hours

DISCHARGE

13. Is subject discharged? ○ Yes, fill details below ○ No

13.1 Hospital discharge date |__|__|/ |__|__|/20|__|__|

13.2 Discharge status ○ Alive ○ Dead

13.3 Discharge destination ○ Home ○ Another health facility ○ Other, specify _____

Scrub Typhus Antibiotic Resistance Trial (START)	**ADVERSE EVENT NO. |__|**	SAR - |__|__| - |__|__|__| |__|__|__| Study site / Subject number / Subject initials

☐ tick if this is the last page

AE DIAGNOSIS	**START DATE** |__|__|/ |__|__|/ 20|__|__| *(dd/mm/yyyy)*	**END DATE** |__|__|/ |__|__|/ 20|__|__| *(dd/mm/yyyy)* ☐ ONGOING

EVENT DESCRIPTION

SEVERITY ○ Mild ○ Moderate ○ Severe ○ Life-threatening

RELATIONSHIP TO STUDY DRUG

○ Not related, detail_____

○ Possibly related, detail_____

○ Probably related, detail_____

○ Definitely related

Alternative etiology (If *'Not related' or 'Possibly related' or 'Probably related'* was selected)

☐ Concurrent illness, specify_____

☐ Underlying disease

☐ Concomitant medication, specify _____

☐ Other, specify _____

Outcome	**Action taken**
○ Fatal	○ none
○ Not recovered/ not resolved	○ Discontinue study drug
○ Recovered/ resolved	○ Treatment - medication *(If ticked, fill ConMed form)*
○ Recovered/ resolved with sequelae	○ Other treatment, specify _____
○ Unknown	

Is this adverse event serious? ○ Yes, select below crieteria ○ No

☐ Results in death

☐ Is life-threatening

☐ Requires inpatient hospitalization or prolongation of existing hospitalization

☐ Results in persistent or significant disability/incapacity

☐ Is a congenital anomaly/birth defect

☐ other medically important condition

Scrub Typhus Antibiotic Resistance Trial (START)	**SERIOUS ADVERSE EVENT NO. |__|__|**	SAR - |__|__| - |__|__|__| |__|__|__| Study site / Subject number / Subject initials

Report Status ○ Initial report **Date of Report** |__|__|/|__|__|/20|__|__| *(dd/mm/yyyy)*

○ Follow-up report **Date of Report** |__|__|/|__|__|/20|__|__| *(dd/mm/yyyy)*

○ Final report **Date of Report** |__|__|/|__|__|/20|__|__| *(dd/mm/yyyy)*

SUBJECT INFORMATION

Date of Birth |__|__|/|__|__|/|__|__|__|__| *(dd/mm/yyyy)* **OR** **Age** |__|__| years |__|__| months

Sex: ○ Male ○ Female

If **Female**, was she pregnant at time of SAE? ○ Yes ○ No ○ Unknown

DESCRIBE THE SERIOUS ADVERSE EVENT (include all relavant laboratory results, treatment given to treat the current event)

CONCOMITANT MEDICATION (medication used in the days before and at the moment of the SAE)

○ Yes ○ No *(if yes, fill in below table)*

Medication *(Generic name)*	**Start date** *(dd/mm/yyyy)*	**End date** *(dd/mm/yyyy)*	**Dose**	**Dose unit**	**Frequency**	**Route**	**Indication**

Scrub Typhus Antibiotic Resistance Trial (START)	**SERIOUS ADVERSE EVENT NO. |__|__|**	SAR - |__|__| - |__|__|__| |__|__|__| Study site / Subject number / Subject initials

Final Diagnosis/Syndrome *(provitional if initial SAE report)*: _____

_____

Onset date: |__|__|/|__|__|/20|__|__| *(dd/mm/yyyy)*	**End date:** |__|__|/|__|__|/20|__|__| *(dd/mm/yyyy)* or ○ ongoing

Action taken in relation to study drug:

☐ No action taken

☐ Concomitant Medication is required

☐ Permanent discontinuation → Discontinue on \|__\|__\|/\|__\|__\|/20\|__\|__\| *(dd/mm/yyyy)*

☐ Temporary discontinuation → Discontinue on \|__\|__\|/\|__\|__\|/20\|__\|__\| Restart on \|__\|__\|/\|__\|__\|/20\|__\|__\|

☐ Continue study drug

Relationship to study drug:

○ Not related, detail_____

○ Possibly related, detail_____

○ Probably related, detail_____

○ Definitely related

*If relationship to study drug is selected as '**Not related**' or '**Possibly related**' or '**Probably related**' complete below:*

Alternative Etiology	☐ Concurrent Illness *(Specify)* _____ ☐ Concomitant Medication *(Specify)* _____ ☐ Other *(Specify)* _____ ☐ Unknown

SAE Criteria:*(Check all that apply)*

☐ Results in death

☐ Is Life-threatening

☐ Requires inpatient hospitalization/ prolongation of existing hospitalization

☐ Results in persistent or significant disability/incapacity

☐ Is a congenital anomaly/birth defect

☐ Any other significant medical condition

Severity: ○ Mild ○ Moderate ○ Severe ○ Life-threatening

Outcome:

○ Continuing/ongoing ○ Resolved ○ Resolved with sequelae ○ Permanent

○ Fatal, Date of death \|__\|__\|/\|__\|__\|/20\|__\|__\| *(dd/mm/yyyy)*

Scrub Typhus Antibiotic Resistance Trial (START)	**SERIOUS ADVERSE EVENT NO. |__|__|**	SAR - |__|__| - |__|__|__| |__|__|__| Study site / Subject number / Subject initials

Additional remarks

_____

_____

Investigator:_____Signature _____ Date: |__|__|/|__|__|/20|__|__|

***Please email this form to the Study safety team within 24 hours of awareness of SAE** *(dd/mm/yyyy)*

<table>
<tr><td>Scrub Typhus Antibiotic Resistance Trial (START)</td><td>CONCOMITANT MEDICATIONS
Number |__|</td><td>SAR - |__|__| - |__|__|__| |__|__|__|
Study site Subject number Subject initials</td></tr>
</table>

<table>
<tr><td colspan="8">1. Did subject receive any concomitant medication? ○ Yes, details below ○ No</td></tr>
<tr><th>MEDICATION NAME</th><th>START DATE
(dd/mm/yyyy)</th><th>END DATE
(dd/mm/yyyy)</th><th>DOSE</th><th>DOSE UNIT</th><th>FREQ</th><th>ROUTE</th><th>INDICATION</th></tr>
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☐ ONGOING</td><td></td><td></td><td></td><td></td><td></td></tr>
</table>

Scrub Typhus Antibiotic Resistance Trial (START)	**SUPPORTIVE TREATMENT** **Number** |__|	SAR - |__|__| - |__|__|__| |__|__|__| Study site / Subject number / Subject initials

1. Any supportive treatment? (e.g. IVIs, vasopressors, haemofiltration) ○ Yes, details below ○ No			
Treatment	**Start date** *(dd/mm/yyyy)* **and time**	**Stop date** *(dd/mm/yyyy)* **and time**	**Indication**
	|__|__|/|__|__|/20|__|__| |__|__|:|__|__|	|__|__|/|__|__|/20|__|__| |__|__|:|__|__|	
	|__|__|/|__|__|/20|__|__| |__|__|:|__|__|	|__|__|/|__|__|/20|__|__| |__|__|:|__|__|	
	|__|__|/|__|__|/20|__|__| |__|__|:|__|__|	|__|__|/|__|__|/20|__|__| |__|__|:|__|__|	
	|__|__|/|__|__|/20|__|__| |__|__|:|__|__|	|__|__|/|__|__|/20|__|__| |__|__|:|__|__|	
	|__|__|/|__|__|/20|__|__| |__|__|:|__|__|	|__|__|/|__|__|/20|__|__| |__|__|:|__|__|	
	|__|__|/|__|__|/20|__|__| |__|__|:|__|__|	|__|__|/|__|__|/20|__|__| |__|__|:|__|__|	
	|__|__|/|__|__|/20|__|__| |__|__|:|__|__|	|__|__|/|__|__|/20|__|__| |__|__|:|__|__|	
	|__|__|/|__|__|/20|__|__| |__|__|:|__|__|	|__|__|/|__|__|/20|__|__| |__|__|:|__|__|	

Scrub Typhus Antibiotic Resistance Trial (START)	**FINAL STATUS**	SAR - |__|__| - |__|__|__| |__|__|__| Study site / Subject number / Subject initials

1. Did subject complete the study as per protocol?

- ○ Yes, specify completed date |__|__|/ |__|__|/ 20|__|__| *(dd/mm/yyyy)*
- ○ No, specify the date of event below |__|__|/ |__|__|/ 20|__|__| *(dd/mm/yyyy)*

If ***No***, select the reason for not completing the study per protocol:

- ○ 1. Migration
- ○ 2. Early discontinuation by patient decision
- ○ 3. Early discontinuation by investigator/study team decision
- ○ 4. Withdrawn consent
- ○ 5. Death, date of death |__|__|/ |__|__|/ 20|__|__| *(dd/mm/yyyy)*
- ○ 6. Other, specify _____

If 2, 3, or 4 specify a reason if possible:

_____

_____

_____

2. Final study outcome:

- ○ Clinical cure
- ○ Clinical treatment failure
- ○ Microbiological treatment failure
- ○ Relapse/reinfection

Investigator's Statement

I have reviewed the data recorded in this CRF and confirm that the data are complete and accurate.

Investigator (print full name) _____

Signature _____ Date |__|__|/|__|__|/20|__|__| *(dd/mm/yyyy)*

Scrub Typhus Antibiotic Resistance Trial (START)	**PK SAMPLE COLLECTION**	SAR - |__|__| - |__|__|__| |__|__|__| Study site / Subject number / Subject initials

DOXYCYCLINE 7 days – data DENSE group				
	Time-point	**Sample collected?**	**Collection date**	**Collection time**
	Pre-dose	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|
After first dose	0.5 hours	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|
	1 hours	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|
	2 hours	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|
	3 - 5 hours	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|
	8-12 hours	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|
	24 hours/1 day	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|
	72 hours/3 days	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|
After last dose	0 hour	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|
	3 - 8 hours	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|
	24 hours/1 day	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|
	48 hours/2 days	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|
	96 hours/4 days	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|

Scrub Typhus Antibiotic Resistance Trial (START)	**PK SAMPLE COLLECTION**	SAR - |__|__| - |__|__|__| |__|__|__| Study site / Subject number / Subject initials

DOXYCYCLINE 7 days – data SPARSE group				
	Time-point	**Sample collected?**	**Collection date**	**Collection time**
	Pre-dose	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|
After first dose	3 - 5 hours	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|
	8-12 hours	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|
	24 hours/1 day	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|
	72 hours/3 days	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|
After last dose	0 hour	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|
	48 - 96 hours/2-4 days	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|

Scrub Typhus Antibiotic Resistance Trial (START)	**PK SAMPLE COLLECTION**	SAR - |__|__| - |__|__|__| |__|__|__| Study site / Subject number / Subject initials

DOXYCYCLINE 3 days – data <u>DENSE</u> group				
	Time-point	**Sample collected?**	**Collection date**	**Collection time**
	Pre-dose	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|
After first dose	0.5 hours	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|
	1 hours	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|
	2 hours	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|
	3 - 5 hours	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|
	8-12 hours	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|
	24 hours/1 day	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|
After last dose	0 hour	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|
	3 - 8 hours	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|
	24 hours/1 day	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|
	48 hours/2 days	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|
	96 hours/4 days	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|

Scrub Typhus Antibiotic Resistance Trial (START)	**PK SAMPLE COLLECTION**	SAR - |__|__| - |__|__|__| |__|__|__| Study site / Subject number / Subject initials

DOXYCYCLINE 3 days – data <u>SPARSE</u> group				
	Time-point	**Sample collected?**	**Collection date**	**Collection time**
	Pre-dose	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|
After first dose	3 - 5 hours	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|
	8-12 hours	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|
	24 hours/1 day	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|
After last dose	0 hour	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|
	48 - 96 hours/2-4 days	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|

Scrub Typhus Antibiotic Resistance Trial (START)	**PK SAMPLE COLLECTION**	SAR - |__|__| - |__|__|__| |__|__|__| Study site / Subject number / Subject initials

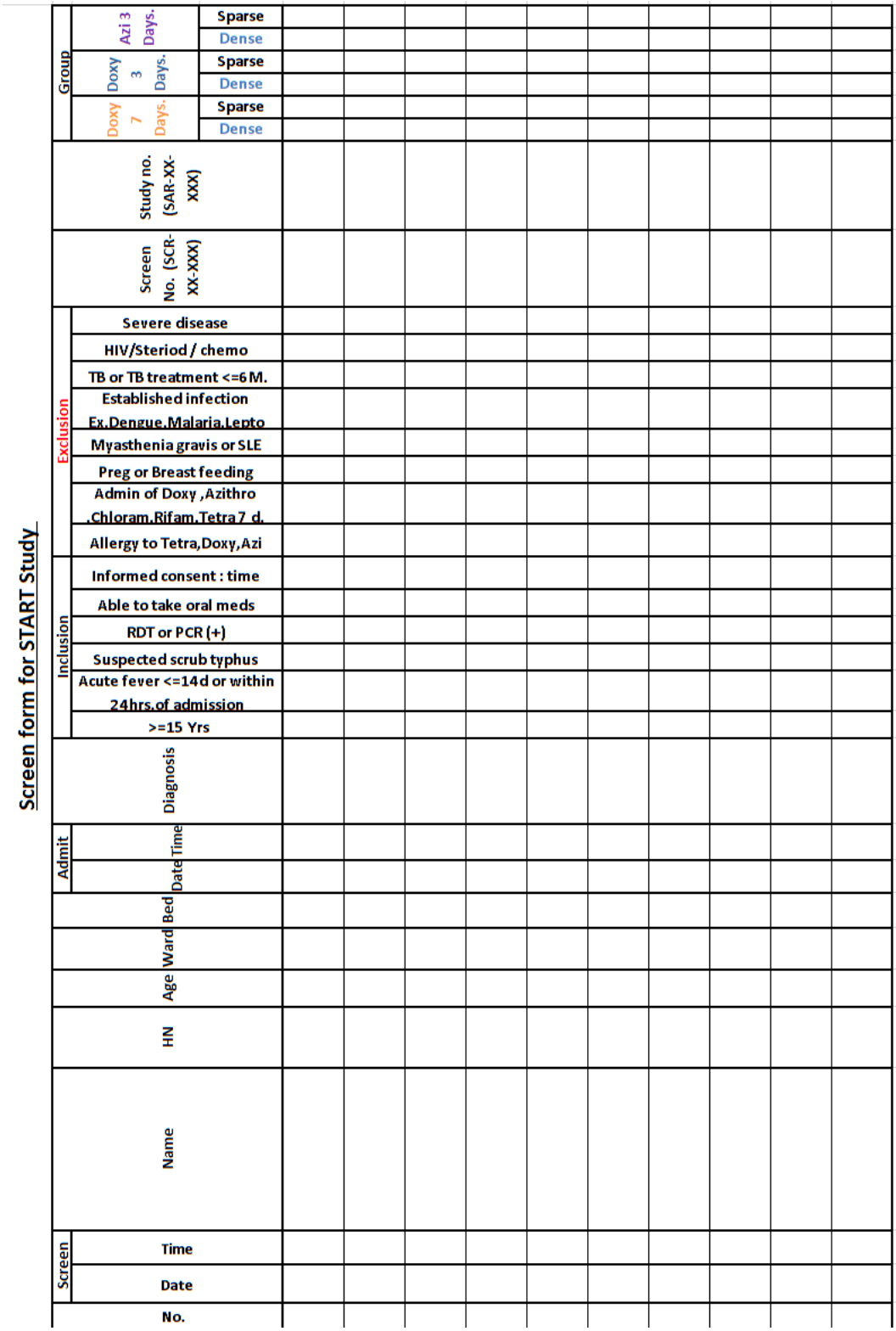
AZITHROMYCIN – data DENSE group				
	Time-point	**Sample collected?**	**Collection date**	**Collection time**
	Pre-dose	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|
After first dose	0.5 hours	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|
	1 hours	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|
	2 hours	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|
	3 - 5 hours	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|
	8 - 12 hours	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|
	24 hours/1 day	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|
	48 hours/2 days	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|
After last dose	24 hours/1 day	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|
	48-72 hours/2-3 days	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|
	120 hours/5 days	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|
	12-16 days	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|

Scrub Typhus Antibiotic Resistance Trial (START)	**PK SAMPLE COLLECTION**	SAR - |__|__| - |__|__|__| |__|__|__| Study site / Subject number / Subject initials

AZITHROMYCIN – data SPARSE group				
	Time-point	**Sample collected?**	**Collection date**	**Collection time**
	Pre-dose	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|
After first dose	3 - 5 hours	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|
	24 hours/1 day	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|
	48 hours/2 days	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|
After last dose	24 hours/1 day	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|
	10-14 days	○Yes ○No, specify_____	|__|__|/|__|__|/20|__|__|	|__|__|:|__|__|

Appendix 8

Scrub typhus antibiotic resistance trial (START) – Screening log



Screen form for START Study												
Group	Azi 3 Days.	Sparse										
		Dense										
	Doxy 3 Days.	Sparse										
		Dense										
	Doxy 7 Days.	Sparse										
		Dense										
Study no. (SAR-XX-XXX)												
Screen No. (SCR-XX-XXX)												
Exclusion	Severe disease											
	HIV/Steriod / chemo											
	TB or TB treatment <=6 M.											
	Established infection Ex.Dengue,Malaria,Lepto											
	Myasthenia gravis or SLE											
	Preg or Breast feeding											
	Admin of Doxy ,Azithro ,Chloram,Rifam,Tetra 7 d.											
	Allergy to Tetra,Doxy,Azi											
Inclusion	Informed consent : time											
	Able to take oral meds											
	RDT or PCR (+)											
	Suspected scrub typhus											
	Acute fever <=14d or within 24hrs.of admission											
	>=15 Yrs											
Diagnosis												
Admit	Time											
	Date											
Bed												
Ward												
Age												
HN												
Name												
Screen	Time											
	Date											
	No.											

Sample Receiving Log

Scrub Typhus Antibiotic Resistance Trial (START)

No.	Subject Number	Trial Group	Blood Collection (Venous) (Total vol/#tube)					Date & Time of Blood Collection	Eschar Swab (in 95% EtOH)	Eschar Crust (in 95% EtOH)	Eschar Crust (in SPG Medium)	Urine - vol + time collected	CSF – vol + time collected	Received by (initials)/ time
			Heparin	EDTA	Serum	Citrate	Tempus Tube							

Blood samples collected by (initials):_____

Eschar collected by (initials): _____

Date (dd/mm/yy)_____

Specimen Processing Log

Scrub Typhus Antibiotic Resistance Trial (START)

No.	Subject Number	Plasma			Serum (0.5ml/ tube)	PCR samples			Culture EDTA-BU (in SPG Medium)	WBA	PBMC		ELISpot plate number	Urine		CSF	Performed by (Initials)
		Heparin (0.5ml/ tube)	EDTA (0.5ml/ tube)	Citrate (0.3ml/ tube)		EDTA-WB (0.5ml /tube)	EDTA-Buffy coat	Citrate -Buffy coat		All Ag/ Partial	Total cell count by Sceptor (10^6)	Number of spare tube (5X10^5cells/tube)		Whole Urine	Supernatant		

QC (Initials)/Date (dd/mm/yy)_____

Page (___/___)

Specimen Processing Log for PK

Scrub Typhus Antibiotic Resistance Trial (START)

No.	Study No.	Group	EDTA Blood (number of aliquots)																													
			PK-T01 Time:____ Date:____		PK-T02 Time:____ Date:____		PK-T03 Time:____ Date:____		PK-T04 Time:____ Date:____		PK-T05 Time:____ Date:____		PK-T06 Time:____ Date:____		PK-T07 Time:____ Date:____		PK-T08 Time:____ Date:____		PK-T09 Time:____ Date:____		PK-T10 Time:____ Date:____		PK-T11 Time:____ Date:____		PK-T12 Time:____ Date:____		PK-T13 Time:____ Date:____	
			PE	BU	PE	BU	PE	BU	PE	BU	PE	BU	PE	BU	PE	BU	PE	BU	PE	BU	PE	BU	PE	BU	PE	BU	PE	BU	PE	BU	PE	BU

START: ADVERSE EVENTS AND SERIOUS ADVERSE EVENTS (SAE) TRACKING LOG

The Scrub Typhus Antibiotic Resistance Trial (START) Comparing Doxycycline And Azithromycin Treatment Modalities In Areas of Reported Antimicrobial Resistance For Scrub Typhus: START Study	
INVESTIGATOR NAME & ADDRESS: **Dr. Tri Wangrangsimakul / MORU, Faculty of Tropical Medicine, Mahidol University, 420/6 Rajvithi Road, Bangkok, Thailand**	**PROTOCOL NO: START Study** **SITE NUMBER: -**

Subject No.	AE/SAE	Onset Date	Date site was first aware of AE/SAE	Date AE/SAE was reported to Safety Team	Initial (I) or Follow-up (F) AE/SAE Report	IRB submission Date (Only for SAE)	Comments
					☐ I ☐ F		
					☐ I ☐ F		
					☐ I ☐ F		
					☐ I ☐ F		
					☐ I ☐ F		
					☐ I ☐ F		
					☐ I ☐ F		
					☐ I ☐ F		

Protocol Deviation and Violation Log

Protocol Title: *The Scrub Typhus Antibiotic Resistance Trial (START) Comparing Doxycycline And Azithromycin Treatment Modalities In Areas Of Reported Antimicrobial Resistance For Scrub Typhus* (START Study)	
Site Name:	**Principal Investigator:**

Item no.	Subject Number	Description of Violation	Corrective Action
		☐ Deviation ☐ Violation	
		☐ Deviation ☐ Violation	
		☐ Deviation ☐ Violation	

Principal Investigator's signature: _____ Date: _____/_____/_____ Page ___/___

Scrub typhus antibiotic resistance trial (START) – Protocol deviation/violation record form

START Study	Protocol Violation / Deviation NO. |__|	SAR - |__|__| - |__|__|__| |__|__|__| Subject number Initials

PROTOCOL VIOLATION / DEVIATION FORM

1. Description of Violation / Deviation (please be brief):

_____

_____

_____

_____

2. Reason(s) for Violation / Deviation, if any (please be brief):

_____

_____

_____

3. Action taken in response to the violation / deviation :

_____

_____

_____

4. Date the violation / deviation occurred

\|__\|__\|/ \|__\|__\|/ 20\|__\|__\| *(dd/mm/yyyy)*

_____ _____ DATE \|__\|__\|/ \|__\|__\|/ 20\|__\|__\|

INVESTIGATOR (PRINT NAME) SIGNATURE

Scrub typhus antibiotic resistance trial (START) – Summary of blood collection schedule

		Treatment Groups					
		Doxycycline 7 days		**Doxycycline 3 days**		**Azithromycin 3 days**	
	Blood collection time-points	PK Group Dense	PK Group Sparse	PK Group Dense	PK Group Sparse	PK Group Dense	PK Group Sparse
Major blood draws	Baseline D0 (15-20ml)	X	X	X	X	X	X
	Week 2 +/- 2 days (15-20ml)	X	X	X	X	X	X
	Week 8 +/- 2 weeks (15-20ml)	X	X	X	X	X	X
After first dose	0.5hr D0 (2ml)	X		X		X	
	1hr D0 (2ml)	X		X		X	
	2hrs D0 (2ml)	X		X		X	
	3-5hrs D0 (2ml)	X	X	X	X	X	X
	8-12hrs D0 (2ml)	X	X	X	X	X	
	24hrs D1 (2ml)	X	X	X	X	X	X
	48hrs D2 (2ml)					X	X
	72hrs D3 (2ml)	X	X				
After last dose	0hr (2ml)	X	X	X	X		
	3-8hrs (2ml)	X		X			
	24hrs (2ml)	X		X		X	X
	48hrs (2ml)	X		X			
	48-72hrs (2ml)					X	
	48-96hrs (2ml)		X		X		
	96hrs (2ml)	X		X			
	120hrs (2ml)					X	
	10-14 days (2ml)						X*
	12-16 days (2ml)					X*	

*Blood collection coincides with week 2 major blood draw, PK = Pharmacokinetic/drug level studies

Appendix 9

Scrub typhus antibiotic resistance trial (START) – STATA data cleaning and analysis codes for outcome measures, final status, SAEs and demographics

Outcome measures

```
cd "C:\Users\Tri\Documents\Thailand\Chiang Rai\START\Data\START_31May2019"
use dummy_randomization, clear
rename subjid label
sort label
save dummy, replace
insheet using STARTOM20190531.csv,clear
set more off
br
saveold Outcome_measures,version(12) replace
gen fevct=omfevcldy*24+omfevclhr
gen fevct1=omfevcldy1*24+omfevclhr1
gen fevct2=omfevcldy2*24+omfevclhr2
br label omfevcldy omfevclhr fevct omfevcldy1 omfevclhr1 fevct1 omfevcldy2 omfevclhr2 fevct2
br label fevct fevct1 fevct2

gen compfevct=fevct
replace compfevct=fevct1 if compfevct==. & fevct1!=.
replace compfevct=fevct2 if compfevct==. & fevct2!=.

gen id=real(substr(label,8,3))
gen ref=_n
*sort id ref
replace compfevct=0 if compfevct==.
drop if label[_n]==label[_n+1]
```

```
replace compfevct=. if compfevct==0

br label fevct fevct1 fevct2 compfevct
sort label
merge label using dummy
keep if _merge==3
saveold outcomemeasures_single_record, version(12) replace

tabstat compfevct, stats(n median p25 p75) by(group)
kwallis compfevct, by(group)
ranksum compfevct, by(group) ,if group=="A" | group=="C"
ranksum compfevct, by(group) ,if group=="B" | group=="C"
ranksum compfevct, by(group) ,if group=="A" | group=="B"

gen feverclear=.
replace feverclear=0 if compfevct==.
replace feverclear=1 if compfevct!=.
replace compfevct = 336 if label=="SAR-29-019" //no fct because fever not cleared
replace compfevct = 192 if label=="SAR-29-049" //no fct because fever not cleared
replace compfevct = 312 if label=="SAR-29-054" //no fct because fever not cleared
replace compfevct = 41 if label=="SAR-29-031" //fever cleared at home as per MACRO comment
replace compfevct = 58 if label=="SAR-29-037" //fever cleared at home as per MACRO comment

stset compfevct,fail(feverclear)
sts graph, by(group) graphregion(fcolor(white)) xtitle(hrs) xline(72,lcolor(red) lstyle(dot)) title(Time to fever clearance) ytitle(Proportion of patients with fever)
```

Final Status

```
cd "C:\Users\Tri\Documents\Thailand\Chiang Rai\START\Data\START_31May2019"
insheet using STARTFS20190531.csv,clear
set more off
br

gen fscomdat_str =fscomdat
replace fscomdat_str = subinstr(fscomdat_str,"-","",1)
replace fscomdat_str = subinstr(fscomdat_str,"-","20",1)
*br fscomdat fscomdat_str

gen fscomdat_mo = substr(fscomdat_str,3,3)
gen fscomdat_mo_num = month(date(fscomdat_mo,"M"))

capture label drop label_mon
label define label_mon 1 "Jan" 2 "Feb" 3 "Mar" 4  "Apr" 5 "May" 6 "Jun" 7 "Jul" 8 "Aug" 9 "Sep" 10 "Oct" 11 "Nov" 12 "Dec"

label values  fscomdat_mo_num label_mon

gen fscomdat_str1 = substr(fscomdat_str,1,2) +"/"+"0" + string(fscomdat_mo_num) +"/"+ substr(fscomdat_str,-4,4) if fscomdat_mo_num <=9

replace fscomdat_str1 = substr(fscomdat_str,1,2) +"/"+ string(fscomdat_mo_num) +"/"+ substr(fscomdat_str,-4,4) if fscomdat_mo_num >=10

gen fscomdat_num = date(fscomdat_str1,"DMY",2019)
format fscomdat_num %td

drop fscomdat_str1 fscomdat_mo fscomdat_mo_num *_str
br
```

```
gen fsnodat_str =fsnodat

replace fsnodat_str = subinstr(fsnodat_str,"-","",1)

replace fsnodat_str = subinstr(fsnodat_str,"-","20",1)

*br fsnodat fsnodat_str


gen fsnodat_mo = substr(fsnodat_str,3,3)

gen fsnodat_mo_num = month(date(fsnodat_mo,"M"))


capture label drop label_mon

label define label_mon 1 "Jan" 2 "Feb" 3 "Mar" 4  "Apr" 5 "May" 6 "Jun" 7 "Jul" 8 "Aug" 9 "Sep" 10 "Oct" 11 "Nov" 12 "Dec"


label values  fsnodat_mo_num label_mon


gen fsnodat_str1 = substr(fsnodat_str,1,2) +"/"+"0" + string(fsnodat_mo_num) +"/"+ substr(fsnodat_str,-4,4) if fsnodat_mo_num <=9


replace fsnodat_str1 = substr(fsnodat_str,1,2) +"/"+ string(fsnodat_mo_num) +"/"+ substr(fsnodat_str,-4,4) if fsnodat_mo_num >=10


gen fsnodat_num = date(fsnodat_str1,"DMY",2019)

format fsnodat_num %td


drop fsnodat_str1 fsnodat_mo fsnodat_mo_num *_str

br


sort label

merge label using dummy

keep if _merge==3


gen eventdat=fscomdat_num

replace eventdat=fsnodat_num if eventdat==.
```

```
format eventdat %td
replace finalst=1 if label=="SAR-29-013" //patient withdrew from study but after fever cleared, labelling as clinical cure
tab group finalst,row

*****************************************************************
*treatment failure
                                *
*****************************************************************
gen treatfail=.
replace treatfail=1 if finalst==2
replace treatfail=0 if finalst==1 | finalst==4
capture label drop treatfail
label define treatfail 1 "failure" 0 "cure"
label values treatfail treatfail
tab group treatfail,row
tab group treatfail,chi2 exact
tab group treatfail,chi2 exact, if group=="A" | group=="B"
tab group treatfail,chi2 exact, if group=="A" | group=="C"
tab group treatfail,chi2 exact, if group=="B" | group=="C"
cii proportions 25 5  //for group A
cii proportions 24 3  //for group B
cii proportions 23 4  //for group C

encode group, gen(groupnew)
logistic treatfail i.groupnew

*****************************************************************
*relapse
                                        *
*****************************************************************
gen relapse=.
```

```
replace relapse=1 if finalst==4
replace relapse=0 if finalst==1 | finalst==2
capture label drop relapse
label define relapse 1 "yes" 0 "no"
label values relapse relapse
tab group relapse,row
tab group relapse,chi2 exact
tab group relapse,chi2 exact, if group=="A" | group=="B"
tab group relapse,chi2 exact, if group=="A" | group=="C"
tab group relapse,chi2 exact, if group=="B" | group=="C"
cii proportions 25 1  //for group A
cii proportions 24 1  //for group B
cii proportions 23 0  //for group C
saveold Final_status,version(12) replace
```

SAEs

```
cd "C:\Users\Tri\Documents\Thailand\Chiang Rai\START\Data\START_31May2019_extracted1Oct2019"

insheet using STARTSAE_PI_CR_20190502.csv,clear

set more off

br

tab1 studynumber treatmentarm relationshiptostudydrug alternativeaetiology /*

*/ saecriteria saeseverity presumptivediagnosis

saveold SAE_CR,version(13) replace

cd "C:\Users\Tri\Documents\Thailand\Chiang Rai\START\Data\START_31May2019_extracted1Oct2019"

insheet using STARTSAE_PI_MS_20190502.csv,clear

set more off

br

tab1 studynumber treatmentarm relationshiptostudydrug alternativeaetiology /*

*/ saecriteria saeseverity presumptivediagnosis

recast str244 description, force

saveold SAE_MS,version(12) replace
```

Demographics

```
/*

Use variable only label and enrdat from file STARTSC to merge with STARTDM


Convert the dates enrdat and brthdat to date format( numeric)

Then generate age based on the formatted date variable.

Write a few data cleaning commands.

*/

cd "C:\Users\Tri\Documents\Thailand\Chiang Rai\START\Data\START_31May2019"


insheet using STARTDM20190531.csv, clear

br
```

```
count
save STARTDM20190531, replace
insheet using STARTSCR20190531.csv, clear
br
count
save STARTSCR20190531, replace

use STARTDM20190531, clear
merge 1:1 label using STARTSCR20190531 ,keepusing(enrdat) //Keep using enrolment date to calculate age from Screening
count
drop _merge

br trial site label personid brthdat ageyr agemo sex enrdat

//DOB
gen year=real(substr(brthdat,7,4))

gen brthdat_num = date(brthdat,"DM19Y") if year<=1999
replace brthdat_num = date(brthdat,"DM20Y") if year>1999 & year<=2019

format brthdat_num %td


//Enrollment date
gen enrdat_str =enrdat
replace enrdat_str = subinstr(enrdat_str,"-","",1)
replace enrdat_str = subinstr(enrdat_str,"-","20",1)


gen enrdat_mo = substr(enrdat_str,3,3)
gen enrdat_mo_num = month(date(enrdat_mo,"M"))
```

```
//label define label_mon 1 "Jan" 2 "Feb" 3 "Mar" 4  "Apr" 5 "May" 6 "Jun" 7 "Jul" 8 "Aug" 9 "Sep" 10 "Oct" 11 "Nov" 12 "Dec"


//label values  enrdat_mn_num label_mon


gen enrdat_str1 = substr(enrdat_str,1,2) +"/"+"0" + string(enrdat_mo_num) +"/"+ substr(enrdat_str,-4,4) if enrdat_mo_num <=9


replace enrdat_str1 = substr(enrdat_str,1,2) +"/"+ string(enrdat_mo_num) +"/"+ substr(enrdat_str,-4,4) if enrdat_mo_num >=10


gen enrdat_num = date(enrdat_str1,"DMY",2019)

format enrdat_num %td


gen agedy_DOB = (enrdat_num - brthdat_num)

gen ageyrd_DOB = (enrdat_num - brthdat_num)/365.25

gen ageyr_DOB = int((enrdat_num - brthdat_num)/365.25)



gen ageyr_dec_DOB = real("0." + substr(string(ageyrd_DOB),4,.))


gen agemo_DOB = int(ageyr_dec_DOB * 12) if brthdat_num != .


br label sex brthdat_num enrdat_num ageyr   ageyr_DOB  ageyr_DOB agemo_DOB


gen ageyr_new=ageyr


replace ageyr_new= ageyr_DOB if brthdat_num != .


br label enrdat_num sex ageyr_new brthdat_num ageyr_DOB agemo_DOB ageyr

drop enrdat_str1 ageyr_dec_DOB enrdat_mo enrdat_mo_num *_str
```

```
br

rename educa education
capture label drop education
//recode education 3 = 4
//label define education 1 "None" 2 "Primary" 4 "Secondary/High school" 5 "Vocational" 6 "University"
label define education 1 "None" 2 "Primary" 3 "Secondary" 4 "High school" 5 "Vocational" 6 "University"
label values education education

rename occup occupation
label define occupation 1 "None" 2 "Agriculture" 3 "Office" 4 "Other"
label values occupation occupation

sort label
merge label using dummy
keep if _merge==3

tab group occupoth if occupation==4

tab sex group, m
tabstat ageyr_n, stat(N min max mean sd median p25 p75) by(group)
tab group education, row
tab group occupation, row
drop year agedy_DOB ageyrd_DOB ageyr_DOB agemo_DOB
saveold Demographics, version(12) replace
```

Baseline characteristics

Variables	**A**	**B**	**C**
Age (years, median [IQR])			
Sex (n [%]): - Male - Female			
Ethnicity (n [%])			
Education (n [%]): - None - Primary - Secondary - Vocational - University			
Occupation (n [%]): - None - Agriculture - Office - Other			
Fever days before admission (median [IQR])			
Fever (>37.5°C on or during admission) (n [%])			
Temperature on admission (median [IQR]) (°C)			
Respiratory rate			
Pulse rate			
Systolic BP (mmHg, median [IQR])			
Diastolic BP (mmHg, median [IQR])			
SpO2 (%, median [IQR])			
Weight (kg, median [IQR])			

Height (cm, median [IQR])			
Eschar (n [%])			
Deafness (n [%])			
Retro-orbital pain (n [%])			
Arthralgia (n [%])			
Jaundice (n [%])			
Eye redness (n [%])			
Abdominal pain (n [%])			
Seizures (n [%])			
Confusion (n [%])			
Cough (n [%])			
Epistaxis (n [%])			
Bleeding gums (n [%])			
Rigors/chills (n [%])			
Tinnitus (n [%])			
Headache (n [%])			
Myalgia (n [%])			
Skin rash (n [%])			
Nausea (n [%])			
Vomiting (n [%])			
Diarrhoea (n [%])			
Neck stiffness (n [%])			
Vertigo (n [%])			
Dyspnoea (n [%])			
Haemoptysis (n [%])			
Haematemesis (n [%])			
Anaemic (n [%])			
Lymphadenopathy (n [%])			
Intubated (n [%])			
Lung crepitation (n [%])			
Hepatomegaly (n [%])			
Splenomegaly (n [%])			
Focal neurological deficits (n [%])			

Haemoglobin, g/dl, median (IQR)			
Haematocrit, %, median (IQR)			
Platelets, x103/mm3, median (IQR)			
White blood count, x103/mm3), median (IQR)			
Neutrophils, x103/mm3, median (IQR)			
Lymphocytes, x103/mm3, median (IQR)			
Monocytes, x103/mm3, median (IQR)			
Eosinophils, x103/mm3, median (IQR)			
Blood urinary nitrogen, mg/dl, median (IQR)			
Creatinine, mg/dl, median (IQR)			
Sodium, mmol/l, median (IQR)			
Potassium, mmol/l, median (IQR)			
Chloride, mmol/l, median (IQR)			
Globulin, g/dl, median (IQR)			
Albumin, g/dl, median (IQR)			
Bilirubin direct, mg/dl, median (IQR)			
Bilirubin total, mg/dl, median (IQR)			
AST, IU/l, median (IQR)			

ALT, IU/l, median (IQR)			
ALP, IU/l, median (IQR)			
CRP, µg/ml, median (IQR)			

Serious adverse events summary

SAE Criteria	SAE severity	SAE no.	Study number	Rx arm	Relationship to study drug	Description

95% CI and z test of proportion (compare between arms)

Data analysis

Fever clearance time

FCT in hrs (median, IQR)		
A	**B**	**C**

Kruskal-Wallis test – p =
Wilcoxon ranksum test for A vs B – p =
Wilcoxon ranksum test for A vs C – p =
Wilcoxon ranksum test for B vs C – p =
Hazard ratio (95%CI) = (p=)
Kaplan-Meier survival curve for proportion of patients with fever

Treatment failure

		Treatment Arm			Total
		A	B	C	
Treatment failure	Yes				
	No				
Total					

Fisher's exact test all groups – p =
Fisher's exact test A vs B – p =
Fisher's exact test A vs C – p =
Fisher's exact test B vs C – p =

Proportions (95% CI) for A
Proportions (95% CI) for B
Proportions (95% CI) for C

Relapse

		Treatment Arm			Total
		A	B	C	
Relapse	Yes				
	No				
Total					

Fisher's exact test all groups – p =
Fisher's exact test A vs B – p =
Fisher's exact test A vs C – p =
Fisher's exact test B vs C – p =

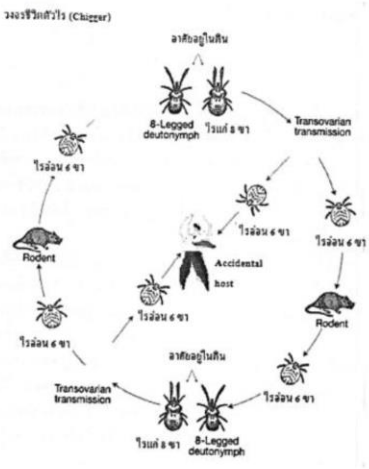
Proportions (95% CI) for A
Proportions (95% CI) for B
Proportions (95% CI) for C

Appendix 10

Scrub typhus public engagement and awareness – training material

Scrub typhus

Scrub typhus is a vector-borne disease and a major cause of fever in Thailand and throughout Asia. It is caused by *Orientia tsutsugamushi*, a bacteria, and is transmitted by the larval stage of the trombiculid mite or chiggers. These chiggers normally feed on rodents but humans can be infected when venturing into habitats where infected chiggers and rodents are present. These habitats include forested areas adjacent to agricultural land, mixed cropland, new vegetation on previously cleared land and areas close to waterfalls and rivers.

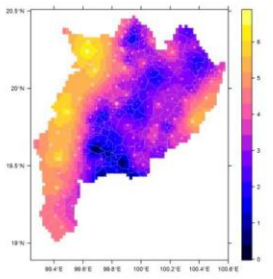


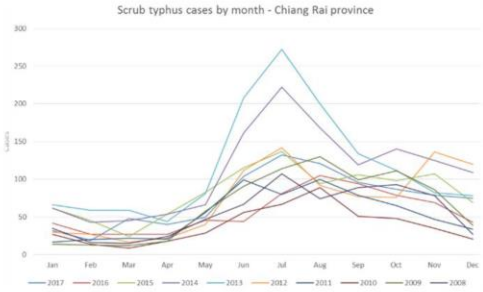
From 2003-2017, data from the national disease reporting system (R506) reveal that the northern region has the highest disease burden and Chiangrai province has the highest number of cases.

Chiangrai province 2008-2017 (R506), 8722 cases total:

- Scrub typhus cases highest in Mae Fah Luang, Mae Suai, Mae Chan, Mueang and Wiang Pa Pao districts (see table)
- Male to female ratio – 1:0.81
- 68% of cases occurred in the 15-64 age group
- Main occupational groups were agriculture (35%), labour (24%) and student (21%)
- 77% reported from DGH, 22% from Chiangrai provincial hospital
- 66% outpatients, 24% inpatients
- 72% of cases occurred from June to November

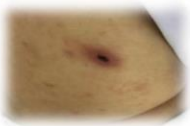
Research data from 2015 onwards from Chiangrai Prachanukroh Hospital suggest hill tribe populations are disproportionately affected (80-90% of scrub typhus cases).

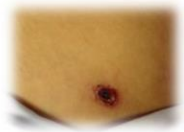




Clinical

- Incubation period of 6-21 days following infected chigger bite
- Main symptoms – fever, headache, nausea, vomiting, cough, rash, tinnitus, rigors, myalgia, conjunctival injection, lymphadenopathy, hepatosplenomegaly
- Eschar develops in 30-40% of adults and 60-70% of children – begins as a papule at the bite site, ulcerates, develops central necrosis, usually painless
- Eschar normally located in warm moist skinfold areas or areas covered by underwear – groin, axillae, external genitalia, waist, thigh, neck, abdomen, chest
- Complications can occur if presenting late – can affect the liver, lungs, blood, CNS, kidneys, heart





Diagnosis

- Clinical diagnosis based on the presence of fever, eschar and other related symptoms
- In Chiangrai, patients with fever and eschar >90% likely to have scrub typhus
- If eschar not present, difficult to differentiate from other causes of acute febrile illness such as dengue, leptospirosis, malaria, typhoid, Japanese encephalitis, Zika, Chikungunya

Tests rely on detecting antibodies or bacterial antigens or DNA (only available at reference or research laboratories).
Rapid diagnostic tests detecting scrub typhus antibodies available at DGHs and provincial hospital.

Treatment

- Mortality if untreated – 6% (range 0-70%)
- Mortality if treated – 1.4% (range 0-33%)

DDC guidance:

- Patients with mild/moderate disease can be treated at the PCU with specific antibiotics e.g. doxycycline, azithromycin, chloramphenicol, tetracycline
- Patients to be reviewed 48hrs after treatment started
- Patients with shortness of breath, deranged vital signs, bleeding, shock, confusion or reduced consciousness, jaundice, neck stiffness, abnormal urine output – refer to hospital

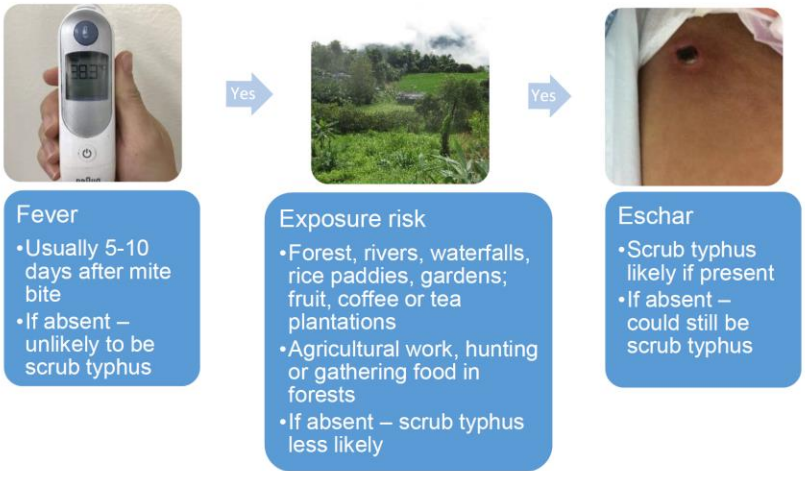
Prevention

- No effective vaccine available
- Immunity short-lived, repeat infection common
- Insect repellent (DEET) impractical for residents in endemic areas

Other preventative measures:

- Protective clothing – long sleeve tops, trousers, socks, scarf, hat, boots, gloves
- Avoid laying clothes on the ground
- Take off work clothes after finishing work, was straightaway or keep separate from home clothes
- Shower or bathe after finishing agricultural work, use soap
- When working – avoid bending close to the ground or kneeling; during rest time, avoid lying or sitting on the ground
- If possible, avoid habitats where mites and rodents are abundant e.g. hay/grass stacks, edge of forests, areas with new vegetation close to forests or waterways

Flowchart for PCU staff



MORU Tropical Health Network

Chiangrai districts with high burden of scrub typhus, 2008-2017

Districts, cases (% of total, province)	Sub-districts, cases (% of total, district)	Villages, cases (% of total, sub-district)
Mae Fah Luang – 1746 (20%)	Mae Salong Nai – 791 (45%) Thoet Thai – 471 (27%)	Hin Taek – 82 (10%) Huai Yuak Pa So – 79 (10%) Hua Mae Kham – 76 (10%) Huai Phung – 63 (8%) Pong Hai – 58 (7%) Huai Uen – 78 (17%) Thoet Thai – 74 (16%) Ah Gu Ah Hai – 40 (8%) Mae Mor – 34 (7%) Phaya Prai Lao Ma – 27 (6%) Thoet Thai 9 – 27 (6%)
Mae Suai – 1638 (19%)	Wawi – 522 (32%) Tha Ko – 409 (25%)	Doi Chang – 84 (16%) Pong Klang Nam – 51 (10%) Saen Jaroen – 36 (7%) Huai Nam Yen – 34 (7%) Doi Lan – 33 (6%) Huai Nam Khun – 52 (13%) Ba La – 51 (12%) Pa Giah – 41 (10%) Doi Ngam – 33 (8%) Panaseri – 32 (8%)
Mae Chan – 996 (11%)	Pa Tueng – 414 (42%) Mae Chan – 128 (13%)	Santisuk – 105 (25%) Huai Gang Pla – 62 (15%) Lao Foo – 50 (12%) Huai Tang – 44 (11%) Pang Sa – 30 (7%) Jor Pa Ka – 20 (16%) Den Pa Sak – 16 (13%) Sala – 16 (13%) San Ton Han, Pong Tong, Suan Sak – 15 (12%) Thammajarik – 14 (11%)
Mueang – 958 (11%)	Huai Chompoo – 240 (25%) Mae Yao – 216 (23%)	Mae Mon – 43 (18%) Huai Ma Liam – 38 (16%) Pang Khon – 38 (16%) Huai Sam – 25 (10%) Pa Lang – 25 (10%) Huai Mae Sai – 48 (22%) Panasawan – 32 (15%) Huai Khom – 25 (12%) Ruam Mitr – 22 (10%) Huai Khom Nai – 20 (9%)
Wiang Pa Pao – 718 (8%)	Wiang – 217 (30%) San Sali – 119 (17%)	Mae Poon Lang – 68 (31%) Mae Poon Luang – 52 (24%) Dong Lai Nah – 25 (12%) Hua Wiang – 25 (12%) Pong Nong – 14 (6%) Pong Nok – 31 (26%) Mae Poon Noi – 24 (20%) Loh – 13 (11%) Mae Tala – 13 (11%) Pong Nuea – 12 (10%)

Scrub typhus video clip in Thai

https://youtu.be/rOd9Cigvtnc