Towards improved detection, prevention and integrated control of scrub typhus in Central Vietnam

Inauguraldissertation

zur

Erlangung der Würde eines Doktors der Philosophie

vorgelegt der

Philosophisch-Naturwissenschaftlichen Fakultät

der Universität Basel

von

Thi Duc Hanh Tran

Basel, 2022

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät auf Antrag von

First supervisor

Professor. Daniel Henry Paris
Department of Medicine
Swiss Tropical and Public Health Institute,
Department of Clinical Research
University of Basel
Basel, Switzerland

Second supervisor

Professor. Jakob Zinsstag
Department of Epidemiology and Public Health
Swiss Tropical and Public Health Institute
University of Basel
Basel, Switzerland

External reviewer

Professor. Nicholas Philip John Day
Centre for Tropical Medicine and Global Health
University of Oxford,
Oxford, United Kingdom
Faculty of Tropical Medicine
Mahidol University
Bangkok, Thailand

Basel, 22 June 2021

	The Dean of Faculty of Science
Professor Dr Daniel Henry Paris	Professor Dr Marcel Mayor

CONTENT

List of Tables

List of Supplement Tables

List of Figures

List of Abbreviations

PCR: Polymerase Chain Reaction; IFA: Indirect immunofluorescence assay EDTA: Ethylenediaminetetraacetic acid; ELISA: Enzyme-Linked Immunosorbent Assay; COVID-19: Coronavirus disease 2019; IgM: Immunoglobulin M; PPE: Personal protective equipment; ID: Identification number; HH: household; AIC: Akaike information criterion. ARDS: Acute respiratory distress syndrome; AUF: Acute undifferentiated fever.

Acknowledgements

First and foremost, I would like to say that no words can express my deepest gratitude and thanks to my beloved sisters and my brother, Thao Tran, Huong Dao and Hai Ha Hoang, who have looked after my parents during my four-year PhD study, especially in the most difficult period of my father's bad health. I could not go back home to take care of them as flights to Vietnam were limited due to the Covid pandemic.

I would like to express my heartfelt thanks to my initial PhD supervisor, Dr. Esther Schelling, who took my hand and supported me to start the journey with scrub typhus - the topic she never gives up!!!. She flow me over the sky with her knowledge and encouragement, however, she also kept my feet on the ground during my PhD study when she said that "Hanh, welcome to second base, Basel". Until the last moments of my PhD thesis submission, she was still here and told me that 'her thoughts be with me'!!!

I also would like to express my gratitude and appreciation to my first PhD supervisor, Professor Daniel Paris, who took my hands to an end – like a father walking his daughter to the aisle. He made all my dreams about scrub typhus come true. He helped me to pass strict and accurate academic gates, and guide me to practical study findings. Vietnam has almost nothing about scrub typhus in the primary health care until he came. He brought updated clinical practice on scrub typhus to my colleagues in hospitals. He helped to bring laboratory tests to Vietnam and organise everything about laboratory from knowledge to technical issues. Many thanks for his constructive critique, invaluable support throughout my living and studying in Switzerland. I also special thanks to his humour and encouragements, cause he has known how to push up an Asian student, experience from his love for another Asian country – Thailand, where he spent almost his youth and his PhD period. Many thanks to him, otherwise, I am not "I am" at present.

My deepest gratitude and acknowledgement also go to Professor Jakob Zinsstag, my second supervisor, my "grandpa" (he was the supervisor of my supervisor, Esther). His magical organizational and systematic thinking helps to keep me always on track, when I always have to report to him on every Monday morning unit meeting that: what I have done and what I am doing this week. Therefore, although no supervisor need to check me, I still by myself made all working plan and finished my work in time. That skills, including estimating workload, working planning, time planning, and deadline reaching, are all the crucial skills in my PhD program and casual life.

My sincere appreciation and thanks go to Dr. Christian Schiller, another "grandpa" of mine, who retired when I finished my first year. His devotion, patience and excitement with the

new topic helped me to pass ‘the easily-broken bridge– my second year’, when I moved from data to practical story in real life – “differentiating scrub typhus from dengue fever”.

Gratitude and acknowledgement also go to Dr Jan Hattendorf, for all his support and time taken to guide me everything in statistics and epidemiology approach during two years, when Esther could not be based in Basel.

My honor acknowledgement also go to Dr Do Manh Hung, from Nha Trang Pasteur Institute, the retired man with great passion in science. He is the first man help to make my dream come true in the field in Khanh Hoa, Vietnam, because scrub typhus was an ‘almost no one knows, no one hears about’ topic and seemed to be my solo one in Vietnam. Actually, I found Khanh Hoa as my ‘second academic home’ when many people there can talk about scrub typhus, from local people, to my colleagues in health care facilities to the Director of Provincial Department of Health. These are the great encouragements for myself and contribute to the whole project success.

Sincere appreciation and thanks also go to Dr. Pham Thanh Thuy, the head of department of infectious disease of the Bach Mai national hospital, the first person in Vietnam conducting the PhD in scrub typhus in 2002. Her working spirit and her patience in caring patients encouraged me to take the first steps in my PhD with scrub typhus. We all did not get ‘why’, felt so unreasonable and sad, when a patient with scrub typhus, an easy treatable disease - with a $0.3 doses of doxycycline (!!!), could be died or got severe complications unnecessarily, if accuracy diagnosis was early made !!! It is the purpose I made this PhD.

My special thanks go to the encouragements, ‘from head to toe’, on the academic and daily issues, from Lam Ngoc Hoa, Lan Lan, Lan Vu, Christine Mensch, and Nneka Onyejepu. If none of you had been there, no small steps on this PhD ways would have been completed.

Finally, @Dear Daniel @ step by step - schritt für schritt – ‘từng bước một’, are always in my mind and in my heart!!

My kind regards,

Hanh

SUMMARY

Background

Scrub typhus is a zoonotic infectious disease caused by obligate intracellular bacteria *Orientia* spp, transmitted to humans by the bites of infected larval-stage trombiculid mites, which are found mainly on rodents of forests and rice fields across the Asia-Pacific region. Scrub typhus is a severe public health problem, with one billion people at risk globally, causes illness in an estimated one million people every year, and became a leading cause of treatable non-malarial febrile illness. Case fatality reports vary widely around a median mortality of 6.0% (range 0-70%) for untreated and 1.4% (range 0-33%) for treated scrub typhus patients.

In Vietnam, after 40 years of neglect, scrub typhus (ST) is re-emerging as evidenced by expanding geographical distribution and increase in new cases. Unfortunately, both the clinical and laboratory diagnoses of ST remain challenging, even at the national hospital. Due to late diagnosis and late treatment effects, a severe case complication rate of 17% and a mortality rate of 1.2% among 251 infected and diagnosed patients was reported at the national hospital in 2003. Epidemiological and ecological information on scrub typhus is very scant in Vietnam, and there is no updated evidence on practical preventive measures and fostered case-detection.

Dengue fever (DF) has made a substantial impact over the two past decades in Vietnam and is unequivocally the leading cause of febrile illness throughout the country. Dengue incidence per 100,000 population has steadily increased from 32.5 in the year 2000 (24,434 cases) to 149.9 in 2018 (141,927 cases), at the third rank among the 28 most common communicable diseases. Because of non-specific symptoms such as high fever, headache, skin rash or myalgia are common to both ST and DF, differential diagnosis is required to decide on the treatment strategies.

The overall aim of the present PhD research was to contribute to a better understanding and improving case detection and practical prevention of scrub typhus in Vietnam. Specifically, the work aimed to i) differentiate scrub typhus from dengue fever using admission clinical manifestations and routine blood tests; ii) investigate behavioural and environmental related risk factors of scrub typhus; iii) evaluate temporal dynamics of DNA and serology-based assays and its efficacy in early diagnosis of scrub typhus in Vietnam.

Methods

First, a study including 221 and 387 confirmed acute cases of ST and DF, respectively, and use of multivariable logistic regression and classification and regression trees (CART), identified clinical and laboratory parameters differentiating ST from DF. Then in 2018/2020, a clinical hospital-based active surveillance study, and a retrospective residence-enrolment

date-age-matched case-control study were conducted to investigate the risk factors of ST in Khanh Hoa, Vietnam. Finally, were used data of two hospital active surveillances of suspected ST patients that were done in Khanh Hoa in the periods of 2013-2014 and 2018-2020. The PCR, IgM ELISA, IgM rapid test (RDT) results and days of fever on admission of these patients were used to evaluate temporal dynamics of DNA and serology-based assays and their efficacies in early diagnosis of scrub typhus in Vietnam.

Results

The main variables to distinguish scrub typhus from dengue included i) the eschar; ii) regional lymphadenopathy; iii) an occupation in nature; iv) increased days of fever on admission; v) increased neutrophil count; vi) decreased ratio of neutrophils/lymphocytes; vii) increased platelet count; and viii) the higher age of patients. Sensitivity and specificity of predictions for scrub typhus based on these seven factors reached 93.7% and 99.5%, respectively, in multi. When excluding the “eschar” variable, the values dropped to 76.3% and 92.3%, respectively. Using the CART model, the corresponding values for the alternative decision tree model were 95.0% and 96.9% when including the variable “eschar” and 77.4% and 90.7% without eschar.

Several factors were significantly associated with acquisition of scrub typhus, including sitting/laying directly on the household floor (adjusted OR=4.9, 95%CI:1.6–15.1), household with poor sanitation/conditions (aOR=7.9, 95%CI:1.9–32.9), workplace environment with risk (aOR=3.0, 95%CI:1.2–7.6), observation of mice around the home always (aOR=3.7, 95%CI:1.4–9.9), and use of personal protective equipment in the field (aOR=0.4, 95%CI:0.1–1.1).

PCR buffy coat performed best from day 1 to day 6, compared to ELISA and RDT, with an overall positivity rate of 73% during this early phase. ELISA IgM and RDTs performed better after day 7 of fever, with positivity rates of 90% and 81%, respectively, in the later phase – but contributed to diagnosis from day 3 of fever. The combination of PCR buffy coat with an RDT detected 93% to 100% of all positive cases during the first 14 days of fever.

Conclusions

This work provides evidence for better understanding on fostered case-detection and practical preventive measures of scrub typhus in Vietnam. A combined package with clinical training, risk factors training, and RDTs should be implemented at primary health care level to promote accurate diagnostic for scrub typhus in the hotspot area. The findings from this study are useful for training courses at the community level, support the establishment of preventive measures, create better awareness among the public and inform regional surveillance, and promote much-needed effective public health responses against scrub typhus - this after many decades of neglect in Vietnam.

CHAPTER 1. INTRODUCTION

1.1 GENERAL RESEARCH AIMS OF THIS PhD

Research objectives

The overall goal of this PhD is to contribute to a better understanding and improving case detection and practical prevention of scrub typhus in Viet Nam,

To the specific objectives were:

1) To differentiate scrub typhus from dengue fever using admission clinical manifestations and routine blood tests
2) To investigate behavioural and environmental risk factors of scrub typhus
3) To evaluate temporal dynamics of DNA and serology based assays and its efficacy in early diagnosis of scrub typhus in Vietnam

1.2 SCRUB TYPHUS INTRODUCTION

Scrub typhus is a chigger-borne zoonosis. The disease is also known as Japanese river fever, or “tsutsugamushi fever”, described in Japanese folklore, was associated with the jungle mite or chigger, therefore it was named ‘dangerous bug’ (tsutsuga=dangerous, and mushi=bug) [1]. The term “tsutsugamushi disease” was first mentioned in 1810 to describe mite-associated fevers in Niigata prefecture in Japan [1]. *Orientia tsutsugamushi* is the causative organism of scrub typhus and has its natural reservoir mainly in infected chiggers and possibly to a much lesser extent in rodents (“chiggers” are the larvae of the mites of the genus *Leptotrombidium*, a genus of the family *Trombiculidae*) [2]. Based on the current genetic classification evidence, the genus *Orientia* is considered as a single genus, out-group with genetic groups of *Rickettsia* in the family *Rickettsiaceae* [3]. The genus *Orientia* comprises the species, *Orientia tsutsugamushi* and *Orientia chuto* (firstly originated from Dubai, UEA in 2010) [4], which both cause scrub typhus in humans.

1.3 THE SITUATION IN VIETNAM

1.3.1 History of Fever of unknown origin/undifferentiated fever during the Vietnam war

The earliest reports of scrub typhus cases in Vietnam, referred to as “unknown etiology fever” (i.e.pseudo-typhus), were on two individuals in Saigon in 1915. The disease (pseudo typhoid) was similar to that described in Deli, Sumatra, that was later determined to be scrub typhus [5]. In the 1960s and 1970s, most of the patients recorded were military personnel, especially American servicemen in South Vietnam [6]. In fact, scrub typhus was reported as a leading cause of fevers of unknown origin (FUO) among US troops in Vietnam from

1960s to 1970s [7]. The first case observed in American personnel was in 1962 [8]. Between 1967 and 1970, about 225,000 man-days per year were lost to FUOs, whereas ~200,000 were lost to malaria [9]. In 1969, scrub typhus was reported as the primary cause of FUOs (18%), followed by amebiasis (17%) and murine typhus (15%). Specifically, 2000 cases of scrub typhus were estimated in that year [8]. In 1972, it was reported that 20%–30% of the FUOs were scrub typhus (when malaria and other identifiable diseases were excluded) [10]. In fact, no American death due to scrub typhus was reported during the Vietnam conflict [3].

1.3.2 Epidemiology of scrub typhus and dengue in Vietnam

1.3.2.1 Scrub typhus

1.3.2.1.1 Incidence among suspected cases and seroprevalence

The disease had been neglected in Vietnam until the end of the 20th century and the beginning of 21st century, resulting in a gap in publications till then. Differing from past studies, studies and case reports of scrub typhus since 2000s in Vietnam have confirmed the re-emerging presence of scrub typhus in 32 among 63 provinces throughout Vietnam, with main foci in the northern part of the country [11-14]. Up to present, scrub typhus has also been confirmed as one of the most acute undifferentiated fever (AUF) in Vietnam. A few recent studies of scrub typhus in the national hospitals in 2002 and 2012 in North Vietnam demonstrated that the seroprevalence of scrub typhus was about 1.1% (10/908) among the general population and its cumulative incidence were about 3.5% (251/7226) among total admission to the hospitals (2002) [15, 16]. A proportion of 40.9% (237/579) of patients was confirmed with scrub typhus among patients with suspected rickettsial infection, after excluding patients with malaria, dengue fever, and typhoid fever in the national hospitals during the 2001-2002 period [17]. The scrub typhus incidence was 2.9% (33/1127) among the enrolled patients reported in national hospital in 2012 [18]. The incidence of scrub typhus among the patients with acute undifferentiated fever and clinically suspected rickettsiosis were 34.1% (103/302) in the national hospitals and 32/66 (48%) in some hospitals in Hanoi city, during the 2015-2017 period [19].

Studies have confirmed the occurrence of scrub typhus in the central part of Vietnam. Scrub typhus confirmed by PCR among fever patients with negative malaria or dengue fever results was 47.9% in Khanh Hoa, 23.0% and 8.7% in Quang Ngai and Quang Nam, respectively (2010) [20]. The detection in Khanh Hoa in 2013-2014 was 62,6% (200/321) [21]. This illness contributed for more than half of the cases (21/41 patients; 51.2%) among the rickettsial agents detected in AUFs in Quang Nam hospital (2016) [22].

It is reported that the peak season for the incidence of scrub typhus is summer although there are cases all the year round. In the south of Vietnam, the transmission pattern of scrub

typhus seems to be different as it is more obvious in a temperate climate [16]. There was no significant difference between urban and rural areas [15].

1.3.2.1.2 Complications

The proportion of complications among cases was about 16% at both provincial and national levels. Complications are related to a delay in presentation at hospital. There were 15/88 patients (17%) who had one of 4 life-threatening complications (including respiratory failure, septic shock, encephalitis-meningitis and acute kidney damage) in Hanoi [23]. Complications included altered mental status (45/251; 17.9%), jaundice or hyper bilirubinaemia in (42/251; 16.7%) and pulmonary pathology in 39/251 (15.5%), among scrub typhus patients who hospitalised the Bach Mai national hospital in Hanoi from 2002 to 2003. The median time from symptom onset to treatment was 10 days (interquartile range 8–12) [16].

1.3.2.1.3 Case-fatality

Case-fatality rates ranged from 0.4%-6% among treated scrub typhus patients in Vietnam hospitals [16, 17, 23]. Most fatal cases presented to the hospital 10 days after the symptom onset and had acute respiratory distress syndrome [17] or, in older individuals, multi organ failure [16].

1.3.2.2 Dengue

Dengue has made a substantial impact in Vietnam over the two past decades and has unequivocally been the leading cause of febrile illness throughout the country [24-27]. Dengue morbidity per 100,000 population increased from 32.5 in the 2000s (24,434 cases) to 120.0 in 2009 (105,370 cases), to 78.0 in 2011 (69,680 cases) [28], and to 149.9 (141,927 cases). At national level it ranked third among the 28 most common communicable diseases in 2018 [29]. Provinces in the southern and central parts of the country had higher reported incidences compared to those from northern regions [24, 25]. The rate of mortality was appr. 0.1% during the 2007-2016 period [26]. Dengue has been extensively studied and its economical impact has been assessed. Recent studies have estimated that it is responsible for 39,884 disability-adjusted life years (DALYs) annually, leading to an economic burden of US$ 94.87 million per year (2016) [26].

1.3.3 Challenges in the management of scrub typhus and dengue in Vietnam

1.3.3.1 Diagnostic challenges

Capacity of diagnosis (rapid and confirmed) tests for scrub typhus at the provincial and district hospitals in Vietnam is limited. The standard confirmation tests for scrub typhus antigen is polymerase chain reaction (PCR). For serologic diagnosis of scrub typhus, it is the indirect immunofluorescence assay (IFA). Both PCR and IFA are expensive and require

considerable staff training and more sophisticated laboratory equipment. In Viet Nam, only PCR is available and only performs at central microbiology laboratories, but not in provincial or district hospital facilities. Early diagnosis is rarely achieved using serology alone [30]. All above causes delay the diagnosis of scrub typhus in the vast majority of clinics and hospitals in Viet Nam, leading to a mortality rate of estimated 1.2% among infected and diagnosed patients in central hospital of Viet Nam [16]. This is not only true for Viet Nam, but is a global phenomenon, particularly in low and middle income countries, due to limited laboratory diagnostic facilities.

According to the literature above, scrub typhus impacts become more serious in the context of Vietnam, which is a dengue hyper-endemic country but with limited dengue tests at primary health care lever [31, 32]. Because non-specific symptoms such as high fever, headache, skin rash or myalgia are common to both scrub typhus and dengue fever, these two acute febrile illnesses may mimic each other [33-36]. However, they require different treatment strategies. In the dual hotspot regions of scrub typhus and dengue fever, it is likely that this leads to misdiagnosis and inappropriate treatment.

1.3.3.2 Prevention and control

Scrub typhus is not transmitted directly from person to person; it is only transmitted by the bites of vectors. Therefore, at the community, the living environments, individual behaviours, agricultural activities and protective measures during outdoor activities are important factors to acquire the disease in communities in disease-endemic areas of several countries [37-39]. However, up to date, ecological and behavioural risk factor information on scrub typhus is very scant in Vietnam, and no updated evidence on practical preventive measures and fostered case-detection exist.

Given that little attention has been given to risk factors of scrub typhus, no national prevention and control strategy is available in Vietnam, which may have contributed to its emergence in newly identified foci and outbreaks.

1.4 SCRUB TYPHUS - *Orientia tsutsugamushi*

1.4.1 Global epidemiology

1.4.1.1 Incidence

1.4.1.1.1 Epidemiology inside the "tsutsugamushi triangle"

According to the literature, the majority of scrub typhus cases were found in the "tsutsugamushi triangle" in the Asia-Pacific region [40] (Figure 1.1. Global distribution of *Orientia* species in & outside tsutsugamushi triangle [40]). Some cases were reported in the neighbouring areas to the Asia Pacific, which were the Central Asia and the Middle East

[41]. Countries like China, Japan, South Korea, Thailand, Nepal and Taiwan have listed scrub typhus as a nationally notifiable disease [42-44].

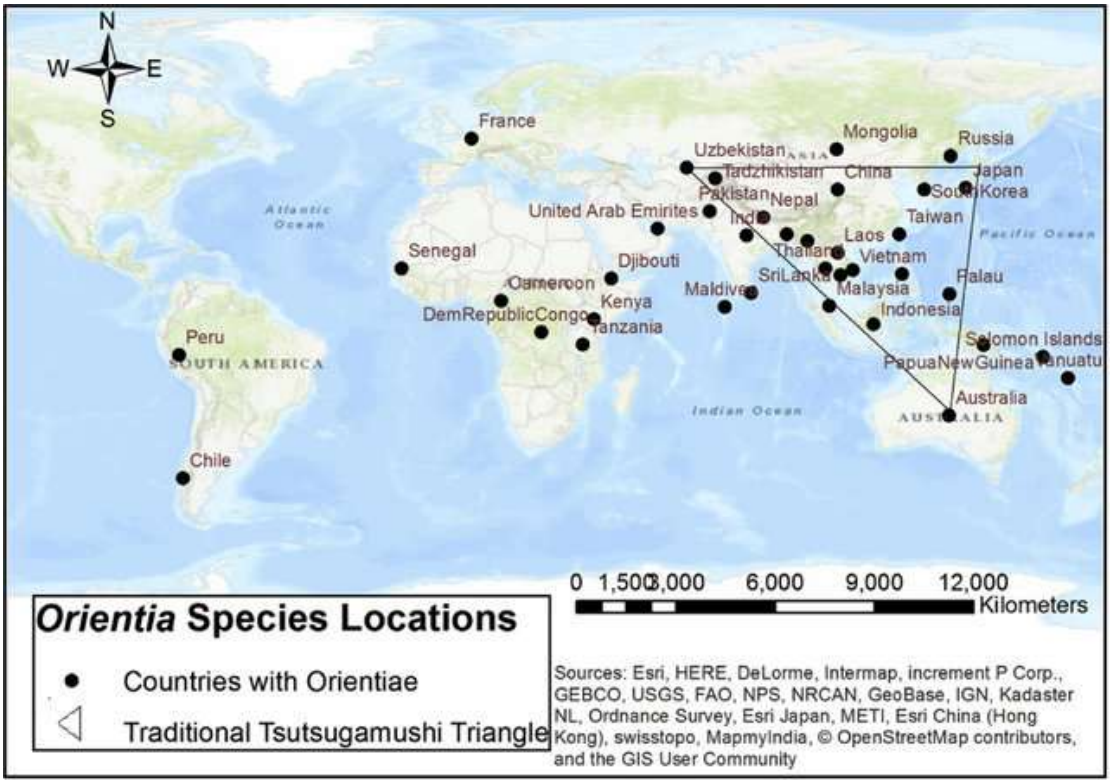


Figure 1.1. Global distribution of *Orientia* species in & outside tsutsugamushi triangle [40]

During the periods of 1952–1989 and 2006–2016, data collected in China showed that nearly 134,000 scrub typhus cases were reported [42]. For a decade of the 2006-2016 period, the country recorded 93,481 scrub typhus cases in 2527 hospitals, of which the proportion of confirmed cases was 4.7% among the suspected cases [43].

In contrast, Japan annually recorded a stable number of notified scrub typhus cases (median: 429, range: 320–505), with only little more than 4,000 cases of scrub typhus being notified during the period of 2007 to 2016 [44].

In South Korea, during 11 years, the annual lowest incidence of scrub typhus cases increased by three fold, i.e. from 5.7 to 17.7/100,000. It is also noted that the number of patients recorded in cities has expanded drastically. For instance, the annual lowest incidence in Ulsan Metropolitan City (South Korea) evolved from a mere 2.8/100,000 in 2003 to 59.7/100,000 in 2013 [41]. Further analysis discovered that outdoor activities in metropolitan areas were the most common risk factor [45, 46].

In India in 1917, the disease was treated as a typhus-like fever, and was endemic in many places in the country [47-49]. During World War II and the 1965 Indo-Pakistani war, it was

a major cause of fever in the army. Then, in 1990, scrub typhus re-emerged at the Pakistan border of India. Later, the national disease incidence declined, which was probably due to the extensive use of insecticides, empiric treatments of febrile illness and lifestyle changes. Despite these efforts, the disease is still under-diagnosed in India [6]. In 2014, only 66 out of 290 cases (22.8%) admitted to the hospital with acute undifferentiated fever (AUF) were diagnosed with scrub typhus in the Hadoti region of Rajasthan [50]. In Eastern India in 2017, scrub typhus was the most common cause of AUF (26.3%, 114/432), followed by dengue (19.2%, 83/432) [51].

In Thailand, the first case of human scrub typhus was in the central region in 1952. Within two years, scientists isolated the causative pathogen from rodents in the same geographic location. From 1980s to 2000s, an increase in the public awareness of scrub typhus and the invention of effective diagnostic tools partially led to a marked increase in the number of confirmed cases in the country [6]. The Bureau of Epidemiology noted a threefold increase in the annual incidence, i.e. from 6.0/100,000 in 2003 to 17.1/100,000 in 2013 [52].

In Central Nepal 2017 and 2018, 22.58% (358 patients attending different hospitals) were positive for IgM Antibodies to *Orientia tsutsugamushi* among patients with acute febrile illness suspected of scrub typhus infection [53].

In Taiwan, in the period of 2000 to 2004, 28.9% patients were confirmed by serological tests after the provisional diagnosis by clinicians. Cases found were not evenly distributed over the counties [54]. From 2004-2007, 10.2% (23/226) of cases were serologically confirmed as scrub typhus among those suspected of Q fever, scrub typhus, or murine typhus [55].

Aside from the above listed countries, some other countries in the tsutsugamushi triangle reported scrub typhus cases. In particular, the disease has been found on the islands of the southwest Pacific (Indonesia and the Philippines), South Asia (Sri Lanka) [56], and the continent of Australia [6].

1.4.1.1.2 Epidemiology outside the "tsutsugamushi triangle"

Nevertheless, scrub typhus does not limit itself within the traditional "tsutsugamushi triangle" in the Asia-Pacific area. Cases are sporadically reported from other countries such as the United Arab Emirates (UAE), Cameroon, Kenya, Congo, Djibouti, Tanzania in Africa, and Peru, Chile in South America [6, 40]. In 2015, Cosson and colleagues discovered the *Orientia* spp. DNA in rodent tissues in West Africa and in Europe [57].

1.4.1.2 Prevalence

Scrub typhus seroprevalences are available from Bangladesh, Indonesia, Laos, Malaysia, Papua New Guinea and Sri Lanka. The seropositivity ranging between 9.3% and 27.9% showed a high exposure level to *O. tsutsugamushi* in these countries [41]. Studies in Nepal showed that the seroprevalence of scrub typhus rose from 3.2% in 2001 to 52.4% in 2015 [58]. In Thailand, national sero-epidemiological studies also found a high prevalence of scrub typhus. In particular, the seroprevalences in Thailand varied widely from 13% - 31% in the suburban Bangkok to 59% - 77% in the northern and north-eastern regions [59].

1.4.1.3 Complications

Despite reported high prevalence of scrub typhus in many countries, its complications such as acute respiratory distress syndrome (ARDS), acute renal failure, ascites, pleural effusion, multiple organ failure, and encephalopathy have not been further studied in-depth. After an outbreak in Puducherry and Tamil Nadu (India) in 2013, people found that most complications included hepatic impairment (14.3%), meningeal involvement (9.6%), congestive cardiac failure and ARDS (4.8%) [49]. During the study period in 2014, 46 of 290 patients admitted with AUF developed respiratory dysfunction, including ARDS (73.9%), pleural effusion (65.2%) and pulmonary infiltrates (26.1%) [50]. Another study in Uttarakhand, India, in 2015 found that major complications were meningitis/ meningoencephalitis (12.5%), multiple organ failure and pneumonia (5.3 %) [47].

1.4.1.4 Case fatalities

Mortality rate of scrub typhus varies widely as countries with good healthcare systems have lower mortality rates in comparison with countries of limited access to healthcare. The case-fatality rate of 1.7% (14 deaths) was reported among a total of 831 cases reported from 47 districts out of 75 districts of Nepal in 2016 [60]. A review of showed that an estimated mortality of scrub typhus infection - if left untreated - was 6% (median, range 0-70%) [61]. On the other hand, the review of 39 studies and 91,692 patients found a median mortality of treated scrub typhus to be only 1.4% (range 0-33.3%) [41].

1.4.2 Transmission of scrub typhus

Scrub typhus is a zoonotic infectious disease caused by *Orientia tsutsugamushi* spp. bacteria. Human beings can be exposed to these bacteria through bites of infected *Trombiculidae* mites, which are found mainly in rodents in forests and rice fields across Asia Pacific regions. The mites are both vector and reservoir of *Orientia tsutsughamusi* spp. In Southeast Asia, *Leptotrombidium deliense* spp. mites and *Ascoschoengastia indica* spp.

mites (species of *Trombiculidae* mite family) are the key transmitters of *Orientia tsutsughamusi* spp in both indoor and outdoor settings [62, 63]. *O. tsutsugamushi* spp is maintained in the mites through transovarial and transstadial transmission during the mite metamorphosis life-cycle. Transmission of *O. tsutsugamushi* spp via the ovum is known as transovarial transmission and and through the various stages of the life-cycle as transstadial transmission. After being fertilized, infected female mites lay eggs for 3 weeks. For 5–7 days later, a infected 6-legged larva (chigger) emerges from the egg, and after 2 days, infected chiggers may start to display host-seeking behaviour by forming clusters on leaves, grasses and twigs above the soil surface. The chiggers can survive in outdoor environments for months without a vertebrate host. *Trombiculidae* mites have a large variety of hosts, including small mammals (rodents and shrews), birds, and larger mammals including humans (Figure 1.2. Chigger and scrub typhus life-cycle [64]). However, only monkeys, gerbils, hamsters and humans are thought to suffer clinically with scrub typhus [62-64].

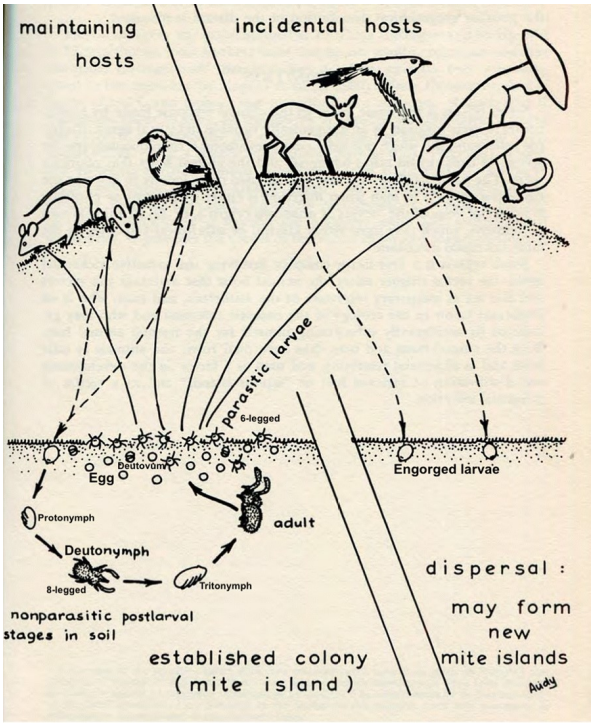


Figure 1.2. Chigger and scrub typhus life-cycle [64]

1.4.3 Risk factors of scrub typhus

Determinants of disease transmission of scrub typhus include i) socio-economic status, ii) behaviours related to use and contact to land/sand/soil, grass, bushes and their PPE, iii) species' habitat connections, vi) land use, and v) vector contact.

1.4.3.1 Socioeconomic status and occupation

In addition to the outdoor activity in urban areas, other common risk factors of contracting scrub typhus are socioeconomic status and occupation. Surveillance data in China, Japan, Korea and Taiwan suggested that the elderly population (60-69 years old) was at highest risk of scrub typhus, whereas in Thailand the younger group (45-54 years old) were the most commonly infected. In Japan and Thailand males were at higher risk of contracting the disease [41], whereas in were women in the other countries. Regarding occupation, South Korean, Chinese, Taiwanese and Thai farmers were at highest risk of having scrub typhus [41]. Interestingly, metropolitan residents in South Korea were found to have the highest risk. In 2009, the incidence of scrub typhus cases in the city dwellers increased from 20% (388 cases) in 2002 to 26.9% (1,345 cases) in 2009, whilst the one in farmers declined from 43.3% to 25%. Further analysis confirmed the finding that outdoor activity in metropolitan regions is the most common [46, 65]. Such variation in social risk factors is possibly owing to the difference in the nature of occupation, working behaviour in farms, genders, and age groups in the countries. Regarding education level, most scrub typhus patients in India are uneducated and live in rural areas [41].

1.4.3.2 Human behaviors

1.4.3.2.1 Work activities in the field and around the house

The literature review results showed that working in vegetable fields, hilly areas, harvesting in autumn were typically associated with scrub typhus infection [66-68]. In a case-control study in India, cases were more likely to be agricultural labourers (adjusted odds ratio (aOR) =1.8, 95% confidence interval (CI):1.0-3.2) [39]. Engaged in forest activities included visit of individual to forest for reasons like wood cutting, animal grazing (OR=4.2, 95% CI: 1.0-18.0) [69], working as labourers in vegetable fields (aOR =1.8, 95% CI=1.1-2.9) [70] were confirmed as risk factors of the illness. Work activities around the house such as cooking outside the house were also positively associated with scrub typhus infection (OR=5.6, 95% CI: 1.5-23.0) [71].

1.4.3.2.2 Personal Protective Equipment (PPE) and Hygiene

Personal Protective Equipment (PPE) and hygiene measures are considered to be useful in protecting people from bites of infected mites in the environment. Using PPE, bathing/changing clothes after work or changing clothes to sleep were protective factors in different studies. Wearing long-sleeved clothing when outside decreased the risk of scrub typhus in China, with aOR=0.3; 95% CI: 0.1-0.7 [72]. Changing clothes to sleep was also

protective against the illness in India, with OR=0.2 (95%CI: 0.1-0.5) [73]**.** The association between changing clothes to sleep and decreased odds of disease was stronger among those earning more than Rs1500 (US$30) (OR=0.05; 95% CI: 0.01-0.3) than among others (OR=0.4; 95% CI: 0.1-1.2). Washing/bathing after daily work also protected people from scrub typhus (analyses with hospital controls: aOR=0.3 (95%CI: 0.1-0.8), compared to community controls: aOR=0.4, 95%CI: 0.1-0.9). Not changing clothes to sleep, to wear gumboots at work and to wash after daily work accounted for 45%, 31% and 16% of cases in the population, respectively [73]. Wearing a long-sleeved shirt while working, keeping work clothes off the grass, and always using a mat to rest outdoors showed protective associations, with aORs and 95% CIs of 0.5 (0.3-0.9), 0.6 (0.4-0.9), and 0.7 (0.5-0.9), respectively [74]. This difference may be explained by the longer period of time available during the night for the mite to bite, while transfer of *Orientia tsutsugamushi* from an infected mite to humans takes more than six hours.

1.4.3.2.3 Outdoor exercise activities

Leisure activities such as camping, walking and resting on grassland were positively associated with infection risk and had an OR of 2.1 (95% CI: 1.0-4.2) [66]. Morning exercise in the park confirmed a positive association with scrub typhus in China (aOR=3.8, 95% CI=1.0-4.5) [70]. Another scrub typhus risk factor study during an outbreak in China showed that people who took their morning exercise or any activity in the park had significantly higher risk than those who did not (aOR=3.0, 95% CI: 1.1-8.2 and aOR=9.1, 95% CI: 3.3-25.5, respectively) [72]. Activities of participants of a Greenery pottery workshop had significantly higher risk of disease (aOR = 4.6, 95% CI: 1.7-12.5) [72]. One possible explanation is that due to the rapid development of the Chinese economy and urbanization, the habitat of scrub typhus no longer only included farmlands but also well-greened places such as grasslands in parks [72]. Of the 46 cases in Busan, Korea, 33 (71.7%) participated in government-run public work projects. The government-run public work projects included many occupations, such as maintenance of hiking trails, deforestation, and cutting grass. This occupation characteristic could result in high exposure to chigger mites [46].

1.4.3.2.4 Outdoor relaxing activities

Playing around the house in the two weeks before illness onset showed a positive association with scrub typhus infection, aOR=2.7, 95%CI: 1.1-6.3) [75]. Going to school by a vehicle (OR=3.1, 95%CI: 2.3-8.4) was associated with an increased risk among children [71].

1.4.3.2.5 Defecating/urinating in jungles or bushy areas

Practice of defecating/urinating in jungles or bushy areas was associated with the exposure to infected mites and increasing risk for scrub typhus in India and elsewhere [76]. In India, defecation in 2 weeks before illness in the field or open-air defecation has been identified as a risk factor for scrub typhus vs using toilets, OR=2.0, 95%CI: 1.2–3.4 [75], and aOR=1.6, 95%:1.1-2.3, respectively [77]. Defecation/urination in the jungle or bushy areas (OR 20, 95% CI: 2.3-174) was also determined a risk factor in an outbreak in India in 2007 [38], was well as in a scrub typhus peak in South Korea in 2009 (aOR=2.0, 95%CI: 1.4-2.9) [74].

1.4.3.3 Host - Vector - Animal contacts

1.4.3.3.1 Contact frequency with rats

Human contacts with rats and animals - scrub typhus vectors - showed positive association with scrub typhus infection. There were 65 % scrub typhus cases associated to a rat infestation in India [76]. Close contact with rats (aOR = 3.3, 95% CI: 1.2 -9.6), sitting near the rat holes (OR = 6.8, 95% CI: 1.2 - 38.1) were confirmed as risk factors of a scrub typhus outbreak in China [72].

1.4.3.3.2 Owning animals

Regarding the exposure to other animals than rats, some studies confirmed that keeping animal pets were significantly associated with exposure to *O. tsutsugamushi.* Peridomestic animals such as dogs and cats can serve as transport hosts as they harbour infected mites and may lead to exposure action to scrub typhus. Owning pets (OR=3.3, 95% CI: 1.2-9.1); p 0.031) was associated with scrub typhus infection among children under 15 years old in India [71].

1.4.3.3.3 Feeding animals

In addition, feeding animals or the leftover food to domestic animals attracts rodents, while households frequented by rodents can be more affected by scrub typhus, whereas a clean living-environment and control of rodents significantly decreased the incidence of scrub typhus among troops in China [78]. Handling of cattle fodder 2 weeks before illness or close contact with animals were determined as risk factors (OR=2.1, 95% CI: 1.1-3.7 [75] and OR=1.6, 95% CI: 1.1-2.3 [77], respectively).

1.4.3.3.4 Raising animals

The association between exposure to rodents at home and illness was stronger among those rearing animals in the yard (OR=5.6, 95% CI: 1.8-20.0) than among others (OR=2.1, 95% CI: 0.4-16.0) [73].

1.4.3.4 Species' habitat - Habitat connectivity

Apart from personal risk factors above, a recent study ascertained spatial connectivity between activities and habitats as risk factors, e.g. working/living near -water sources: lakes, ponds, streams, cannels, wells, irrigation systems surrounded by vegetation – Or being near: forests, stored wood or hay [69].

1.4.3.4.1 Household environment and household sanitation/condition with risk

Households (HH) near grassland, HH with length of grass higher than 2 feet, and presence of bushes within 5 metres of the house were confirmed as household environment risk factors of scrub typhus (aOR=3.3, 95% CI=1.9-5.6 [53], aOR=5.6, 95% CI: 1.2-25.5 [69], and aOR=2.8, 95% CI: 1.1-7.7, respectively [71]). Location of the house within or adjoining fields, and vegetation around household within 3 feet range were significantly associated with exposure to *O. tsutsugamushi*, OR=1.6, 95%CI: 1.02-2.4) [75], and OR=5.4, 95% CI: 1.1-25.8 [69], respectively. Presence of a water body within 100m of the house reported a higher risk of scrub typhus (OR=3.6, 95% CI: 1.4-9.8) [71], whereas using tap water was protective factors from scrub typhus vs using hand pump (aOR=0.5, 95%CI: 0.3-0.8) [77]. The risk of being infected were considerably higher in people who stored fuel (wood or dung) inside the house or veranda in 2007 (aOR=32.0, 95%CI: 4.0 – 265.0) [38], and in study 2017 in India (aOR=1.6, 95%CI: 1.1–2.5) [75]. Houses without a cement floor or HH with poor sanitary conditions (presence of rubbish, animal on ground) were further positively associated with scrub typhus illness, aOR=4.2, 95% CI: 1.0-17.0 [66] and aOR=1.7, 95% CI: 1.1–2.7 [67], respectively.

1.4.3.4.2 Workplace environment with risk

Workplace environment was confirmed as a risk factor for scrub typhus infection in Nepal and India. Working in the field, routine work involving contact with shrubs or activities around lakes (washing clothes, bathing/washing animals, feeding animals, etc.) were significantly associated with exposure to *O. tsutsugamushi,* aOR= 9.8, 95% CI=2.1-46.3 [53], aOR=3.7, 95% CI: 1.04-12.9 [69], and aOR=4.6 (95% CI: 1.25-16.77) [69], respectively.

1.4.3.5 Land cover (surface moisture for mite)

Natural transmission cycle of *Leptotrombidium* mites showed that natural moisture surface such as beach/sea, forest, brush, river branch, along streams, bean area or muddy area was a crucial micro-ecology factor favouring the presence of chiggers and increased risk of acquiring scrub typhus [64, 79, 80]. In details, the favouring surface for the presence of chiggers included i) woods, terrain, ground, forests for *L. deliense*; ii) grassy fields for *L. akamush*; iii) sandy beaches/sea coast for *L. arenicola*; iv) ground, trees, dwelling small mammals, rats close to man for *A. Indica* [64, 81, 82]. The chigger tends to aggregate closely in clusters on twigs and debris a few inches above the ground to await its host.

1.4.4 Clinical definition of scrub typhus

Scrub typhus, also known as *Tsutsugamushi* disease, is caused by infection with *O. tsutsugamushi* 6–21 days after the bite of infected *Leptotrombidium* mites. The following acute febrile illness can be mild to fatal - depending on the virulence of the *Orientia* strain (although current knowledge of both virulence and host factor roles remains very limited). Illness typically presents with headache, myalgia, a local necrotic lesion at the site of the bite termed an eschar (not considered pathognomonic, but useful as a differential diagnostic guide – other illnesses can also present with eschar), maculopapular rash, lymphadenopathy and sometimes central nervous system involvement and jaundice [83]. There is typically a dramatic improvement with tetracycline or doxycycline therapy.

Accurate diagnosis is very important, because without appropriate antibiotic treatment a fatal course is possible, especially if complicated by disseminated intravascular coagulation [84, 85], central nervous system or pulmonary involvement [86, 87]. These complications include pneumonitis leading to ARDS, meningoencephalitis, renal failure and hepatic failure. Improved serologic and molecular diagnostic tests are now available. Although drug-resistant strains of *O. tsutsugamushi* have been reported, the infection usually responds to drugs not commonly used in the empirical therapy of undifferentiated fever such as doxycycline, azithromycin or chloramphenicol [83].

1.4.5 Immunity

Humoral [88, 89] and cell-mediated [90-92] immune responses are induced upon *Orientia* infection and both are required for protection in infected humans and animals.

The role of antibodies against the invading organism of *Orientia* spp. was studied by Hanson and Rikihisa [93, 94]. Serum containing antibodies can inhibit entry into target cells observed in chicken embryonic eggs. The inhibiting capacity of antibodies on binding and

invasion is strain-specific and measurable by strain-specific antibody titre rise [93]. Homologous immunity is when a strain-specific pre-existing immune response is raised against the same strain upon re-exposure – heterologous immunity is the cross-reactivity based immune response upon re-exposure to a different strain. Homologous immunity is supported by antibodies to the major surface protein, the 56-kDa TSA molecule that contains strain-specific epitopes. The *Orientia* phagocytosis capacity of macrophages and neutrophils is increased in the presence of the anti-*Orientia* antibodies [94-97].

The protective role of the cellular immune response was demonstrated in the studies of Shirai and Kobayashi [90, 98, 99]. When immune serum against Gilliam strain was given to mice infected with Karp strain, the mice remained infected; however the use of serum made from infected rat spleens resulted in complete protection against *Orientia* among these mice. Thus, cell-mediated immunity provides a T-lymphocyte-dependent broader cross protection against divergent strains in mice [90]. Rats without a thymus only survived after *Orientia* infection if T-lymphocytes were transfused [99]. The cellular immune response in mice starts to appear as early as 2 weeks after infection, and is related to the degree of stimulation of *Orientia*: early antibiotic treatment slows the development of the cellular immune response [98].

Bourgeois *et al.* studied the humoral immune response in infected cases and found two types of humoral immune responses [100]. In first infection patients, immunoglobulin (Ig) M and IgG appear in the blood/serum approximately 5-10 days after the onset of disease symptoms. However, IgM rapidly increases, thereafter IgG appears later and increases more slowly (Figure 1.3.) [100, 101]. Among secondary infection patients, the IgG response appears much earlier and more prominently around day 6 of illness; the IgM response is blunted, and only present in some patients around day 12, and no longer detected after day 63. In both first and secondary infections, the IgM anti-*O. tsutsugamushi*–specific humoral response is specific for the particular strain of *O. tsutsugamushi* being propagated [100]. The cellular immune response in the first infection is demonstrated by a decrease in the number of active lymphocytes (active T cells) in the early stages of the infection and an increase in the later stages of the recovery period. In re-infected patients, the number of active T cells did not decrease during the first week of illness, and increased in the second week.

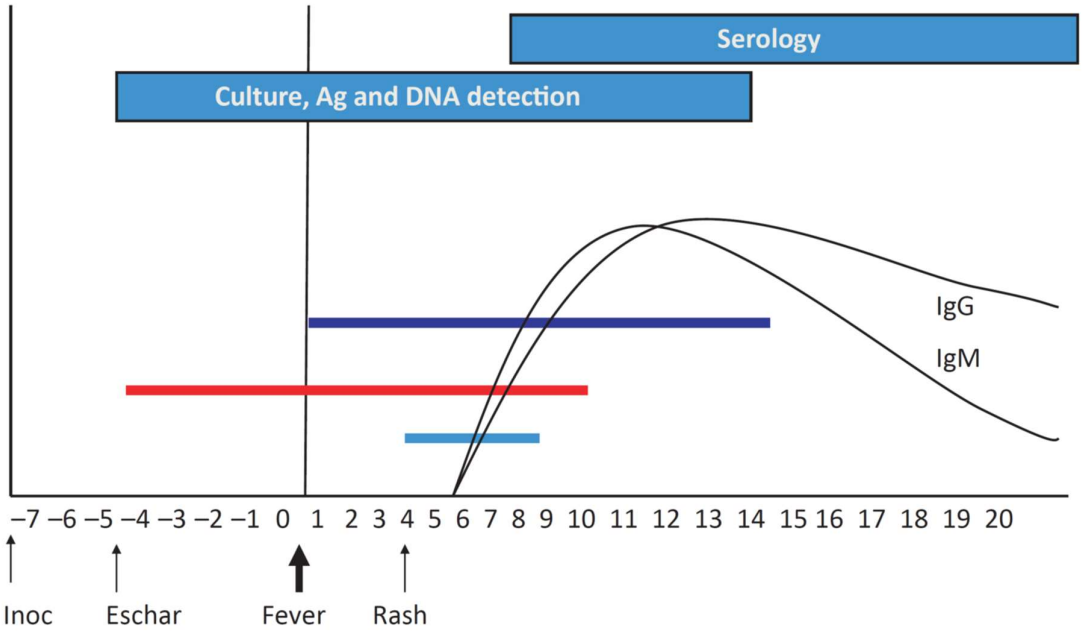


Figure 1.3. Scrub typhus progression: dynamics of pathogen detection, antibody dynamics and diagnosis.

'Inoc' refers to the time point that *Orientia* is inoculated into the host via the arthropod vector. 'Eschar' refers to the lesion at the bite site and the red bar to the time frame, when an erythematous and indurated plaque with a central necrotic crust is visible at the bite site. 'Fever' (royal blue bar) refers to the time period of fever and other symptoms. 'Rash' (light blue bar) refers to the presentation of a skin rash, approximately 3-5 days after fever onset. Immunoglobulin (Ig) M and IgG appear after 5-10 days since the onset of disease symptoms and can last for months to years, respectively. Pathogen, antigen (Ag) and DNA detection (culture, Ag, and DNA detection bar) can occur prior to clinical disease presentation and until approximately 10 days after disease onset. IgM, IgG, or both can reach in detectable levels about 5-10 days after disease onset (serology bar). Acute and convalescent samples should be taken about 7-10 days apart if possible, for detection of dynamic rise in antibody titer [102].

Saunders *et al.* studied the duration of antibodies in scrub typhus confirmed patients [103]. The authors found that the average time for conversion of the peak antibody titre to a negative reaction was 50.9 weeks. The rate of seroconversion to titres < 1:50 in 1 year was 61%. The prolonged existence of antibodies specific to typhus, together with the existence of many *Orientia* strains in endemic areas, are the factors explaining the high incidence of typhus in endemic areas [103].

1.5 DIAGNOSTICS: Methods to diagnose scrub typhus

1.5.1 Nucleic acid tests (molecular diagnosis)

1.5.1.1 Conventional PCR

Standardized conventional PCR (cPCR) use genes coding for the variable 56-kDa antigen of *O. tsutsugamushi* which are amplified through 35 cycles twice using 20-mer oligonucleotide primers and Taq polymerase. The Amplicon of extracted DNA is determined by visual detection of the ethidium bromide-stained bands following agarose gel electrophoresis of *5* pl of the PCR product. [104]. The cPCR based detection methods utilizes genetic markers such as *56 kDa, 47 kDa, GroEL, 16s RNA genes* and to detect specifically the target organism [105-107]

1.5.1.2 Nested PCR

Nested PCR utilizes repeated PCR rounds. The first round of N-PCR is for the amplification of the *47-kDa gene or 56-kDa gene* under the same conditions used for the C-PCRs. However, instead of using the same primers in the second PCR, a set of primers internal to the first set is used. Thus, the second round of N-PCR for the 47-kDa gene/56-kDa gene uses the first PCR product as the template DNA. This technique decreases the non-specificty of amplified DNA fragments. PCR products are stained with ethidium bromide and visualized by agarose gel electrophoresis [108].

1.5.1.3 Quantitative PCR

Real-time PCR-based detection methods targets genes such as 47-kDa HtrA outer membrane protein gene, 16S rRNA gene or the 60-kDa heat shock *GroEL* gene. The real-time qPCRs for the *O. tsutsugamushi* 47-kD assay requires a qPCR mixture consisting of DNA template, PCR buffer, platinum Taq DNA polymerase, primers and probes for the *O. tsutsugamushi* 47-kD assay. The qPCR undergoes 45−50 cycles of two-step amplification. Current PCR method uses the multiplex real-time PCR approach designed to target multiple genes using several probes labelled with different fluorochromes. The targeted multiple genes includes genes encoding the 47-kDa antigen and the GroEL protein with human interferon beta (IFN-β) as an internal control. Multiplex real-time PCR has improved sensitivity and specificity by its ability to monitor DNA amplification with a detection limit as low as ten copies per reaction.

1.5.1.4 Loop-isothermal Amplification PCR

LAMP detects acute scrub typhus infection by targeting the *groEL* gene, encoding the 60 kDa heat shock protein of *O. tsutsugamushi* [109]. LAMP technique utilizes isothermal amplification of DNA using polymerase and set of primer pairs that produce a hairpin DNA

template. The amplified product is determined via photometry for turbidity caused by the precipitation of magnesium pyrophosphate as a by product of the reaction. The reaction can also be quantified in real-time by measuring the turbidity or by fluorescence using intercalating dyes such as SYTO 9. LAMP is highly specific and sensitive with rapid response time as it can detect the DNA concentration of 1 mg/ml within 60–90 minutes.

1.5.2 Serology

1.5.2.1 Indirect immunofluorescence Assay IFA

The indirect immunofluorescence assay test (IFA) is considered as a the gold standard test for the detection of scrub typhus. It uses fluorescein linked anti-human reporter antibody to detect the presence of scrub typhus-specific antibodies. IFA test uses human or laboratory animal serum or plasma as a primary 'test' antibody, while Anti-IgM or anti-IgG antibodies (from the same species) are used as the secondary antibodies with conjugated fluorescein isothiocyanate (FITC). Spots on microscopic slides are prepared from cell culture with a known antigen or mixture of antigens, allowing for the detection of antibodies against all individual antigens simultaneously. These are serially diluted with doubling increments to allow semi-quantitative determination of antibody titres. IFA is advocated for seroepidemiological studies in endemic areas (with well established seroprevalence) due to specific selection of antibody isotypes and serovars in endemic regions.

1.5.2.2 ELISA IgM

ELISA IgM based methods target IgM antibodies in serum samples for the detection of *O. tsutsugamushi* at the early stages of disease process. IgM antibodies titers can be observed after the 1st week of *O. tsutsugamushi* infection, thereby differentiating nascent infection from past ones (IgG). Most advances in ELISA-based diagnosis were to improve specificity and sensitivity based on the use of recombinant antigens; 56-, 47- and 22-kDa proteins, and surface cell antigens (ScaA and ScaC) [110, 111].

1.5.2.3 Rapid diagnosis tests RDTs IgM

The rapid diagnosis test for IgM, is a point of care diagnostics for the detection of scrub typhus. RDT uses a recombinant mixture of 56-kDa outer-membrane proteins of *Karp, Kato Gilliam* strain and recently *Boryong,* and *Kangwon* strains as captured antigen for detection of IgG and IgM antibodies to *O. tsutsugamushi*. The aim is to improve the specificity of diagnosing scrub typhus in different endemic regions [112]. Its sensitivity and specificity are similar to the other standard methods used for the detection of scrub typhus. RDT can also be used in combination with another assay to produce more accurate results [113].

1.5.3 Limitations and difficulties

There is an urgent need for diagnostic methods for scrub typhus. The immuno-based methods such as IFA and ELISA are preferred for detection of scrub typhus due to their high sensitivity and specificity, but have limitations at early stages when no detectable IgM titers are yet developed. IFA and ELISA also need the convalescent sampling for verification of positive samples. On the other hand, DNA-based methods become preferred over serology based assays due to their possibility of early-stage diagnosis with higher specificity and sensitivity. However, PCR lacks its applicability in circumstances due to *Orientia tsutsugamushi* genetic diversity among its serotypes and across endemic regions [113]**.** All these methods require expensive equipment, require infrastructure, are sensitive to the sample type and timing and possible contamination [114]. RDT presents a lower sensitivity and specificity, but does not require specialized equipment, is rapid and simple. However, similar to the other immuno-based methods, RDT has limitations in case detection at early stages, needs convalescent sampling for verification, and has to be validated to local cut-off titres for each endemic region [30]. Recent approaches integrate nucleic acid amplification and recombinant protein-based serological tests for diagnosing scrub typhus. This approach has the potential for future improvement if advances in antibody-based immunochromatographic test (ICT) technology and simplification of DNA extraction methods are made available in the future [115]. In analogy, the combination of Dengue NS1 antigen and IgM antibody assays for the acute dengue infections diagnosis resulted in a dramatic increase in the sensitivity of admission diagnosis [116, 117]. Furthermore, biosensor, DNA-based diagnosis sensor with targeting multiple DNA markers, has been considered to become an ultimate choice for the detection of scrub typhus due to their higher specificity and sensitivity as well as its better possibility of target site detection and thus to deal with the problem of *Orientia tsutsugamushi* genetic diversity [113].

CHAPTER 2. SIMPLE CLINICAL AND LABORATORY PREDICTORS TO IMPROVE EMPIRICAL TREATMENT STRATEGIES IN AREAS OF HIGH SCRUB TYPHUS AND DENGUE ENDEMICITY, CENTRAL VIETNAM

Simple clinical and laboratory predictors to improve empirical treatment strategies in areas of high scrub typhus and dengue endemicity, central Vietnam

Hanh Thi Duc Tran [1,2,3,4], Christian Schindler [4], Thuy Thi Thanh Pham [5, 6], Mai Quang Vien [7], Hung Manh Do [8], Quyet Thi Ngo [9], Trieu Bao Nguyen [9], Hang Thi Hai Hoang [8], Lan Thi Hoang Vu [3], Esther Schelling [10], Daniel H. Paris* [1,2]

[1] Department of Epidemiology and Public Health, Swiss Tropical and Public Health Institute, Basel, Switzerland.
[2] Department of Clinical Research, University of Basel, Basel, Switzerland.
[3] Department of Epidemiology, Hanoi University of Public Health, Hanoi, Vietnam.
[4] University of Basel, Basel, Switzerland
[5] Department of Infectious Diseases, Bach Mai Hospital, Hanoi, Vietnam,
[6] The Partnership for Health Advancement in Vietnam (HAIVN), Hanoi, Vietnam,
[7] Nha Trang Pasteur Institute, Khanh Hoa, Vietnam,
[8] Department for Infectious Disease Control and Prevention, Nha Trang Pasteur Institute, Khanh Hoa, Vietnam.
[9] Department of Microbiology and Immunology, Nha Trang Pasteur Institute, Khanh Hoa, Vietnam.
[10] Vétérinaires Sans Frontières Suisse, Bern, Switzerland
[11] Department of Medicine, Swiss Tropical and Public Health Institute, Basel, Switzerland.
* Corresponding author: daniel.paris@swisstph.ch

This paper is accepted for publication in PLoS Neglected Tropical Diseases (ready for printing)

2.1 ABSTRACT

Background: Dengue fever is highly endemic in Vietnam, but scrub typhus - although recognized as an endemic disease - remains underappreciated. These diseases together are likely to account for more than half of the acute undifferentiated fever burden in Vietnam. Scrub typhus (ST) is a bacterial disease requiring antimicrobial treatment, while dengue fever (DF) is of viral etiology and does not. The access to adequate diagnostics and the current understanding of empirical treatment strategies for both illnesses remain limited. In this study we aimed to contribute to the clinical decision process in the management of these two important etiologies of febrile illness in Vietnam.

Methods: Using retrospective data from 221 PCR-confirmed scrub typhus cases and 387 NS1 protein positive dengue fever patients admitted to five hospitals in Khanh Hoa province (central Vietnam), we defined predictive characteristics for both diseases that support simple clinical decision making with potential to inform decision algorithms in future. We developed models to discriminate scrub typhus from dengue fever using multivariable logistic regression (M-LR) and classification and regression trees (CART). Regression trees were developed for the entire data set initially and pruned, based on cross-validation. Regression models were developed in a training data set involving 60% of the total sample and validated in the complementary subsample. Probability cut points for the distinction between scrub typhus and dengue fever were chosen to maximise the sum of sensitivity and specificity.

Results: Using M-LR, following seven predictors were identified, that reliably differentiate ST from DF; eschar, regional lymphadenopathy, an occupation in nature, increased days of fever on admission, increased neutrophil count, decreased ratio of neutrophils/lymphocytes, and age over 40. Sensitivity and specificity of predictions based on these seven factors reached 93.7% and 99.5%, respectively. When excluding the "eschar" variable, the values dropped to 76.3% and 92.3%, respectively. The CART model generated one further variable; increased days of fever on admission, when eschar was included, the sensitivity and specificity was 95% and 96.9%, respectively. The model without eschar involved the following six variables; regional lymphadenopathy, increased days of fever on admission, increased neutrophil count, increased lymphocyte count, platelet count ≥ 47 G/L and age over 28 years as predictors of ST and provided a sensitivity of 77.4% and a specificity of 90.7%.

Conclusions: The generated algorithms contribute to differentiating scrub typhus from dengue fever using basic clinical and laboratory parameters, supporting clinical decision making in areas where dengue and scrub typhus are co-endemic in Vietnam.

2.2 AUTHOR SUMMARY

Dengue fever is highly endemic in Vietnam, while scrub typhus is recognized as a re-emerging neglected disease. Both diseases are likely to account for more than half of the acute undifferentiated fever burden in Vietnam. However, scrub typhus is a bacterial disease requiring antimicrobial treatment, while dengue fever - of viral etiology - does not. Misdiagnosis and treatment delays cause potentially severe or fatal complications among scrub typhus patients, even though it is easily treatable. In this study, we used simple clinical and laboratory markers, which were identified upon admission of 221 PCR-confirmed scrub typhus cases and 387 NS1-positive dengue fever patients from Khanh Hoa province to identify the differences between scrub typhus and dengue. We found seven predictors that served to construct a simple clinical decision tree, holding great potential to distinguish scrub typhus from dengue using readily available clinical or laboratory findings. These predictors can strongly support medical staff in identifying scrub typhus cases from dengue, without using sophisticated diagnostic tests, and could improve the quality of diagnoses and appropriate treatment strategies at the primary health care level – especially in areas where scrub typhus and dengue fever are co-endemic in Vietnam and many parts of Asia and where diagnostic tests are not readily available.

2.3 INTRODUCTION

Scrub typhus and dengue fever are major under-diagnosed causes of febrile illness in many parts of Asia [118-125]. Scrub typhus and dengue fever together account for approx. 30-40% of the leading etiologies of acute undifferentiated fever in Thailand [126]. Sero-epidemiological data suggest that *Orientia tsutsugamushi* infection is common across Southeast Asia, with seroprevalences ranging from 9–28% [41, 127]. Case fatality rates from areas of reduced drug-susceptibility are reported at 12-14% for South India and northern Thailand, respectively [41]. High mortality rates were reported for complicated scrub typhus with central nervous system involvement (14%), multi-organ dysfunction (24%) and high pregnancy miscarriage rates with poor neonatal outcomes [128, 129].

After approximately half a century of neglect, scrub typhus is beginning to receive more attention as an important cause of non-malarial febrile illness in Vietnam. Recent reports highlight scrub typhus as a disease of high clinical relevance and expanding (documentation of) distribution due to a notable recent increase in the number of diagnosed and reported cases [15, 130]. In the 1960s, scrub typhus was considered a common disease among American veterans in Vietnam and an endemic disease in the midlands and mountainous forests of Vietnam, but after the discovery of Chloramphenicol the general interest in rickettsial diseases declined gradually with the availability of an effective antimicrobial [131, 132]. In Vietnam only a limited number of cases were registered after the 1970s, but the

increasing reports of scrub typhus in recent years suggest a re-emerging trend of this rickettsial illness with documented geographical expansion and distribution within the population of Vietnam [16, 125, 130]. Results from various causes-of-fever studies in Southeast Asia have confirmed the importance of this easily treatable rickettsial disease [16, 126, 130, 133]. Scrub typhus is a serious disease if untreated in elderly; the median mortality is 6% if untreated, and mortality increases with age (over 50 years old mortality >45%), while case fatality risks can reach 12-13% in South India or North Thailand [41, 134, 135].

Dengue has made a substantial impact in Vietnam over the two past decade and is unequivocally the leading cause of febrile illness throughout the country [24-27]. Dengue has been extensively studied and its economic impact assessed; recent studies have estimated that it is responsible for 39,884 disability-adjusted life years (DALYs) annually, representing an economic burden of US$94.87 million per year (2016) [26]. Vietnam is an endemic area for dengue fever, and the level of knowledge about the disease and its management in the population was promoted through broad publicity and knowledge dissemination (mainly TV and internet) [136, 137]. Dengue incidence per 100,000 population increased steadily from 32.5 in 2000, to 120.0 in 2009, and was 149.9 in 2018 in Vietnam [29, 138, 139]. The incidence distribution of dengue is higher and more consistent in the south than in the north of Vietnam.

The capacity for diagnosis (rapid and confirmatory tests) for scrub typhus in hospitals remains limited [30, 140]. The standard reference assays for scrub typhus antigen are polymerase chain reaction (PCR) and serological diagnosis (ELISA), which are expensive, require expertise and sophisticated laboratory equipment. Although testing for prevalent bacterial infections informs treatment and is cost-effective, access to useful tests is scarce [141]. For dengue, the NS1 antigen or combined NS1/IgM rapid diagnostic tests are highly appropriate for the early diagnosis of dengue infection as they are readily available, easy-to-use, inexpensive, accurate and cost effective compared to dengue ELISAs and PCR assays [142-144]. However, these tests are not readily available where needed most, especially at the primary health care level or in rural, tribal areas [31].

The similarities upon presentation of these two common causes of febrile illness complicate clinical management decisions at all health care levels of the country, from the primary health care centers to even the national tertiary hospital [18]. Non-specific symptoms such as high fever, headache, skin rash or myalgia are common to both scrub typhus and dengue, but different treatment strategies are required [33-36]. Frequent misclassification of undifferentiated febrile illnesses delay the diagnosis and treatment especially for scrub typhus [18, 125]. Approx. one million cases of scrub typhus occur each year, which - with an estimated 6% case fatality rate – account for a substantial mortality and economic burden for an easily-treatable disease. Improving access to diagnosis and appropriate antibiotic

treatment would have an important impact [145]. At the Vietnam national referral hospital a mortality rate mortality is estimated at 1.2% among confirmed patients, but numbers in district and community health care centers remain elusive [16].

Against this background, we conducted this study to improve differentiation between scrub typhus and dengue fever using admission clinical manifestations and routine blood tests, aiming to identify simple predictors based on their probability, when no diagnostic test is available.

2.4 METHODS

2.4.1 Study site

Khanh Hoa province lies in the coastal South Central region of Viet Nam. With a population of 1.2 mio (2019) in its 9 districts/townships, it covers 5.2 km^2 (2011) and includes 200 islands. Khanh Hoa has a tropical savannah climate and is a well-known tourism center in Viet Nam with over half a million visits per month; half of these are provincial residents. Nha Trang Bay in Khanh Hoa is an official member of the World's Most Beautiful Bay Club since 2003 [146].

Khanh Hoa is hyper endemic for dengue fever, and was a major hotspot among the 11 provinces in the central Vietnam with an average of 39,876 cases/100,000 population/year during 2011–2018 [24, 147]. Dengue incidence peaked at 40,204 cases /100,000 in 2016, and decreased to 920/100,000 in 2019. In the 2020 national report Khanh Hoa ranked 2nd among 63 provinces in Vietnam with 295.3 cases/100,000 population [148].

Khanh Hoa is recognized as endemic for scrub typhus since WWII. Scrub typhus was first reported in Khanh Hoa in a retrospective study of United States Air Force personnel at Cam Ranh Bay in 1969 [149]. From 2008 to 2010 there were 469 cases of scrub typhus reported in the province [150]. During 2013-2014 period, the Pasteur Institute in Nha Trang confirmed 201 of 321 suspected cases of scrub typhus in the 5 hospitals in Khanh Hoa [151].

2.4.2 Study design

This retrospective descriptive study included 608 patients consisting of 221 and 387 confirmed acute cases of scrub typhus and dengue fever respectively. Full medical records were accessed from the 5 major hospitals of Khanh Hoa (Provincial Hospital, Ninh Hoa branch provincial hospital, Dien Khanh district hospital, 87 Army hospital, Ninh Diem Hospital). The enrollment and selection procedures are presented in Figure 2.1.

The data of all patients hospitalised at the five hospitals with a diagnosis of “suspected scrub typhus” (Jan 2013 - Dec 2014 and Aug 2018 - Jul 2019) or “dengue fever” (2013-2017) were collected. We aimed to test following hypothesis: “There is no difference in

clinical manifestations and routine blood testing results between scrub typhus and dengue fever inpatients upon hospital admission".

All diagnostic assays and clinical assessments during admission and hospitalization in the five hospitals were made by trained local laboratory staff and physicians respectively, as part of routine clinical management, and following the scrub typhus "suspected case" definitions (criteria stated below) and the well-established "dengue fever" selection criteria. From Jan 2013 to Dec 2014, after excluding malaria, dengue and other diagnoses, 327 patients fulfilled the "suspected scrub typhus" definitions on admission and were enrolled to the study; 209 eschar samples were collected, and all provided admission blood samples. From Aug 2018 to Jul 2019, 31 patients with "suspected scrub typhus" on admission were enrolled and all provided admission blood samples. In total, 358 "suspected scrub typhus" patients were enrolled, of which 221 were confirmed to be scrub typhus.

As scrub typhus is by far the more neglected disease, we included all confirmed scrub typhus patients and randomly selected two controls from the dengue fever patient group as non-scrub typhus case controls for further analysis. These confirmed cases and random controls were assigned in a 1:2 ratio across the five hospitals. All dengue patients had a documented NS1 positive test result and presented without shock symptoms (n=378). Dengue patients with malaria co-infections (positive rapid diagnostic test (RDT) and Giemsa thin film) were excluded. Medical records were available for all dengue patients documented at the five hospitals during 2013-2017. In total, medical records of 608 scrub typhus and dengue cases were collected and included in analyses.

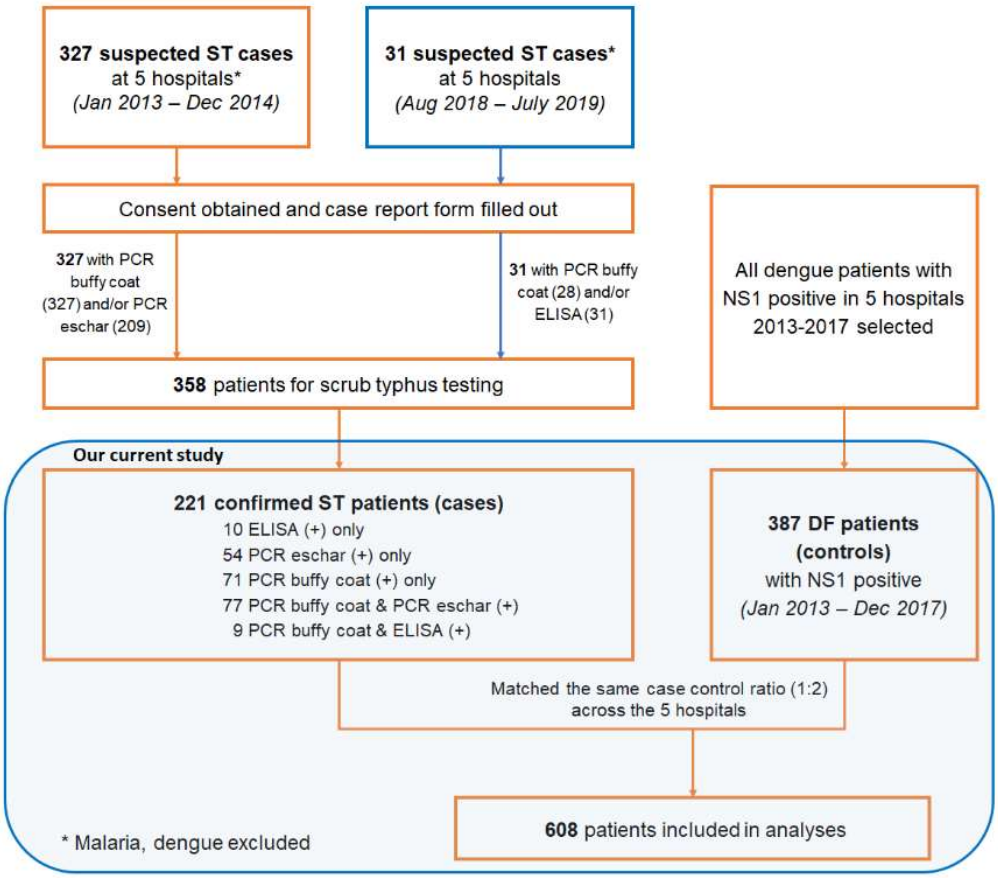


Figure 2.1. Investigational procedures and protocol for patients included in the study.

2.4.3 Diagnostic assays

Blood specimens from all enrolled patients were taken by trained laboratory technicians and if an eschar was present, swabs of the eschar area were collected at the respective hospitals upon admission, before transfer to the Nha Trang Pasteur Institute, Vietnam, for PCR and ELISA testing [152].

PCR assays: During 2013-2014, a quantitative SYBR green real-time PCR with primer designed from GroEL gene [153] was used to identify the presence of *O. tsutsugamushi* in 327 patients with 327 buffy coat samples and 209 eschar swab specimens. During 2018-2019, an in-house semi nested PCR [152], validated by the qualitative SYBR green real-time PCR [153], for detection of partial 56-Kda outer membrane protein gene was used to identify the presence of *O. tsutsugamushi* in 28 patients with 28 PBMCs samples (3 patients did not provide buffy coat samples). Primers used for the semi-nested PCR includes 2 forwards primers with the sequence of (F1): CAATGTCTGCRTTGTCRTTG; (F2): CCKTTTTCIGCTRGTGCGATAG and 1 reverse primer with sequence of (R): ATAGYAGGYTGAGGHGGYGTAAG. In total, there were 564 specimens available, from

358 suspected scrub typhus cases, including 355 buffy coat samples and 209 eschar swabs for PCR testing.

ELISA assays: The Scrub Typhus Detect IgM ELISA (part no. 500242, Lot no. XM5033; InBios International Inc., Seattle, WA, USA) was used for IgM detection all 31 serum samples of patients enrolled from 2018-2019. This ELISA uses recombinant p56kD type specific antigens of *Orientia tsutsugamushi* Karp, Kato, Gilliam, and TA716 strains. The manufacturer's methods were followed exactly. All sera were tested at a 1:100 dilution and absorbance was determined at 450 nm (OD@450 nm) using a microplate reader to give a final optical density (OD) result. The OD cut-off applied was 1.00 with a sensitivity of 91.5% and specificity of 90.9% for admission samples to confirm cases among suspected scrub typhus infection, as reported previously [154, 155].

2.4.4 Case definitions and selection criteria

"Suspected scrub typhus": Age not restricted with a febrile illness (axillary temperature ≥37.5^{O}C) and at least one of the following criteria needed to be fulfilled:

- Presence of an eschar
- Suspected dengue fever with a negative dengue NS1 test result
- Suspected malaria with a negative malaria test result (microscopy, RDT)
- Persisting or undifferentiated fever (≥10 days fever)

From August 2018 to July 2019, the same criteria were re-phrased to reflect more detail:

- Age ≥ 16 years old
- Patient with acute fever (axillary temperature ≥37.5^{O}C) and having had at least one of the following twelve secondary findings: eschar, nonspecific skin rash, headache, myalgia, retro-orbital pain, congestion of the conjunctival blood vessels, tinnitus, lymphadenopathy (regional/body), hepatomegaly, splenomegaly, dry cough, dyspnoea without upper respiratory tract discharge.
- Exclusion criteria: Patients diagnosed with malaria, dengue fever (confirmed by NS1), measles, influenza, bacterial pneumonia, urinary tract infections.

Confirmed acute cases

- Scrub typhus: Patients with a positive PCR result (buffy coat or eschar swab specimens) or positive IgM ELISA result (optical density [OD] of ≥ 1.0) for *O.tsutsugamushi* by the Institute Pasteur Nha Trang reference laboratory, in Vietnam.
- Dengue fever: Patients with a positive dengue NS1 antigen test (NS1) performed on site at each of the 5 hospitals. Patients with clinical symptoms of shock were excluded (due to the specific symptoms associated; *i.e.* circulatory failure,

pronounced tachycardia with weak and narrow pulse pressure, hypotension, cold, clammy skin, abnormal mental status, oliguria, metabolic acidosis, restlessness; or profound shock with undetectable blood pressure or pulse [156])

- Co-infections in scrub typhus and dengue fever cases with a positive malaria RDT and/or Giemsa staining method were excluded from the study.

2.4.5 Sample size considerations

A Monte Carlo simulation [157] showed that, 200 scrub typhus cases and 400 dengue fever controls would be sufficient to keep the estimation error of the area under the curve (AUC) associated with the prediction of scrub typhus within about 3.5% of the true value with 95% certainty, provided that the true value of AUC is ≥ 80% [158]. The precision increases with increasing AUC. The AUC was used because it represents overall performance of a prediction score. It allows to find a good threshold for the prediction score to distinguish between patients with and without the specific disease. The same ratio between cases and controls was applied across the five hospitals to avoid potential confounding by differences in the diagnosis capacities of physicians across hospitals.

2.4.6 Ethical approval

Ethical approval was provided by the Scientific and Ethical Committee in Biomedical Research, Hanoi University of Public Health (No. 382/2018/YTCC-HD3 and No.329/2019/ YTCC-HD3) and by the Ethics Committee of Northwestern and Central Switzerland (Ethikkommission Nordwest- und Zentralschweiz, EKNZ) (BASEC-Nr-2018-00974). All data retrieving procedures at the five sites were approved by Provincial Health Department of Khanh Hoa; the document No 2192/ SYT-NVY was signed by the Directors of the five study hospitals (16 August 2018). For the scrub typhus study in 2019 all participants provided written informed consent prior to study enrollment and sample collection.

2.4.7 Data sources and Data quality assurance

Complete medical records of all 608 scrub typhus (cases) and dengue patients (controls) were retrieved from the paper-based medical record filing cabinets stored at the storing units of the five hospitals in Khanh Hoa. The cases and controls (1:2 ratio) were associated with the same hospital, and the following data was extracted from the patients' medical record: clinical manifestations, routine blood testing results, method of diagnosis and management upon admission.

A structured data collection form was used to retrieve medical records; All skips, data format requirements, cross check, and data constraints were designed for quality assurance, prior

to building the form on Open Data Kit (ODK) [159], before uploading to the web-based server http://sg.smap.com.au/, from where it was downloaded onto Android devices (Samsung tablets). The Open Data Kit community produces free and open-source software for collecting, managing, and using data in resource-constrained environments [159]. The use of mobile data capture technology such as ODK and Android mobile devices have proven their efficiency and cost-effectiveness in cross-sectional surveys [160, 161] and are recommended by the WHO [162].

Four trained data collectors with experience in scrub typhus studies from the Department of Epidemiology, Institute Pasteur, Nha Trang used this e-form programmed on Samsung tablets to collect data. At the end of each day, the data supervisor checked the total numbers of forms and randomly 15% of the forms collected by each data collector, and any incomplete forms were completed. The ODK program checked for missing data, so that the form could only be closed and marked as "finished" when all information was provided (no information=99999). All completed forms were uploaded to the web-based server at http://sg.smap.com.au/ at the end of each working day. Copies were stored in the tablets, and all collected data was secured in the web-based server, and downloaded for subsequent analyses in STATA.

2.4.8 Statistical analyses

Identical variables were recorded for cases and controls, with the dependent variable chosen as scrub typhus (Yes/No). Primary independent variables were: fever, days of fever on admission, headache, hemorrhage, hepatomegaly, splenomegaly, lymphadenopathy (regional/body) and the basic blood laboratory results. A training data set was built by randomly selecting 60% of cases and 60% of controls. The remaining data was used for validation of the prediction models.

Descriptive statistics included counts, proportions and percentages for qualitative variables, and medians and interquartile ranges (IQR) for quantitative variables. Comparisons of demographic, social, and laboratory variables between patients and control groups were conducted using the Fisher's exact test and the Mann-Whitney U test, as indicated. Logistic Regression (LR) was applied to derive a prediction model for the dichotomous dependent variable (presence vs. absence of scrub typhus).

First, potential predictor variables for scrub typhus other than eschar were considered one by one in the training data set. The Bayes information criterion (BIC) was applied to determine the variables to be considered in the initial multivariable model. This initial model was then reduced using backward selection based on the BIC. A variable remained in the model if its removal increased BIC. The optimal cut points for the predicted probabilities of a patient having scrub typhus as opposed to dengue fever were determined by maximizing

the sum of sensitivity and specificity (i.e., the index of Youden). We derived 3 models, i.e. a model without laboratory variables, one only including laboratory variables, and one with both clinical and laboratory variables (no using eschar variable) (Table 2.2). In a further step, the variable eschar, which perfectly predicts scrub typhus, was added to the prediction model by setting the predicted probability of scrub typhus to one among patients with eschar. The resulting model was then applied to the validation data set and the receiver operating characteristic curves (ROC-curves) were generated to compare the performance of the model in both data sets based on the area under the curve (AUC). Finally, the model was fitted in the entire data set. This could be justified by the good performance of the training model in the validation data set.

An alternative approach for discriminating between scrub typhus and dengue fever consisted in deriving binary decision trees using the CART (classification and regression trees) method [133, 163-166]. The trees were developed for the entire dataset and were pruned based on the inbuilt cross-validation statistic of the CART program [167]. Each node of the tree represents a binary decision and the leaves of the tree are assigned to the diagnosis of either scrub typhus or dengue fever. Trees were pruned in order to avoid overfitting of the data. As for the regression-based prediction models, the model performance was assessed based on the sensitivity and specificity of predictions and on the index of Youden (i.e., the sum of sensitivity and specificity minus 1). The probability cut points used to assign final leaves to scrub typhus or dengue fever were chosen such as to maximise the index of Youden.

Descriptive and logistic regression analyses were conducted using STATA software version 14, while CART-analyses were conducted using R-software (Version 1.1.456 – © 2009-2018 RStudio, Inc.)

2.5 RESULTS

2.5.1 Socio-demographic and epidemiological findings

We included all 221 cases of scrub typhus and 387 cases of dengue in the analyses, reflecting approximately a 1:1,75 assignment. There were significant differences in age, occupation and number of days with fever before admission. The median (interquartile range - IQR) ages of the scrub typhus and dengue patients were 33 (22-45) and 20 (10-31) years, respectively ($p<0.001$). The proportion of occupation in nature was higher among patients with scrub typhus (38.5%) than among patients with dengue fever (15.0%) ($p<0.001$). There was also a significant difference in the days of fever on admission, between the patients with scrub typhus (median=5 days, IQR =3-7 days) and those with dengue fever (median=3 days, IQR = 2-4 days) ($p<0.001$).

The geographic distribution of the scrub typhus and dengue fever confirmed cases in this study is demonstrated in Figure 2.2. Scrub typhus cases occurred in all 8 districts in Khanh Hoa, and a similar distribution of dengue and scrub typhus confirmed cases was seen across the communes.

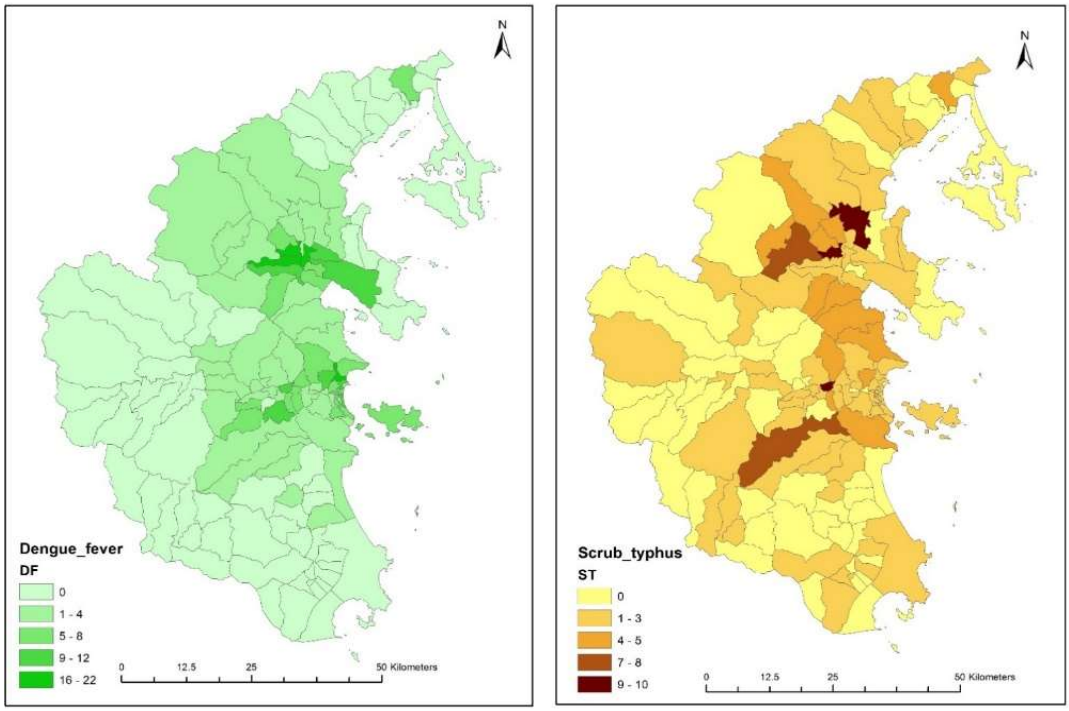


Figure 2.2. The geographic distribution in Khanh Hoa of all scrub typhus (n=221) and dengue fever (n=387) confirmed cases in this study is depicted in these maps.
(Note: The Figure 2.2 *was created using ArcGIS® software by Esri (www.esri.com). ArcGIS® is the intellectual property of Esri and is used herein under lisense. Source of administrative layer of Vietnam was obtained freely from the website (http://www.diva-gis.org/datadown#google_vignette) which is free for community user)*

2.5.2 Clinical and laboratory findings

Clinical manifestations and laboratory findings are summarised in Table 2.1. The strength of diagnostic factors for scrub typhus as opposed to dengue fever is expressed by the respective odds ratio. In the two scrub typhus cohorts, an eschar prevalence of 93.1% was seen among the 202 scrub typhus cases seen from 2013-2014, and 52.6% among the 19 scrub typhus cases seen in 2018-2019. The presence of an eschar is a vital diagnostic clue for scrub typhus; if the physician finds an eschar, it informs diagnosis and treatment of scrub typhus patients. But if there is no eschar, often scrub typhus is missed, and other differential diagnoses considered. Therefore, we conducted the analyses in datasets with and without the eschar variable for more feasible prediction approaches.

Patients with scrub typhus were more likely to have regional lymphadenopathy (>1cm), with a high odds ratio (OR) of 98.9; 95% confidence interval (CI): 24.0 – 408. This was followed by rigors/chills OR=14.5 (95% CI: 5.04-42.0); lung crepitation OR=8.19 (95% CI: 1.75-38.3); documented dyspnoea OR=4.83 (95% CI: 1.27-18.4); retro-orbital pain OR=4.48 (95%CI:1.93-10.4); diarrhea (at least 3 days) OR= 3.02 (95%CI:1.08-8.44) and myalgia OR=1.63 (95%CI:1.15-2.29). On the other hand, patients with scrub typhus were significantly less likely to have pharyngo-laryngitis OR = 0.33 (95%CI: 0.17-0.65); respiratory rate >22 OR=0.42 (95%CI: 0.25-0.70); and hemorrhagic signs OR=0.14 (95%CI: 0.07-0.28), compared to dengue fever patients.

Significant hematological blood laboratory predictors for scrub typhus included higher white blood cell count OR=1.35 per unit increase in white blood cell (WBC) (95%CI 1.27-1.44, $p \leq 0.001$); neutrophil (NEU) count, OR=1.36 per unit increase in NEU (95%CI 1.25-1.47, $p \leq 0.001$); or lymphocyte count, OR=1.95 per unit increase (95%CI 1.65-12.3, $p \leq 0.001$), and aspartate aminotransferase (AST/GOT) or alanine aminotransferase (ALT/GPT) levels ≥45 U/L with an OR=2.55 (95%CI:1.08-6.04, $p \leq 0.034$).

Table 2.1. Demographic, clinical, diagnostic and laboratory characteristics of patients at admission

	Scrub typhus	Dengue fever	OR (95%CI)	P-value[a]
Demographics and History				
Male, n (%)	124/221 (56.1%)	204/387 (52.7%)	0.87 (0.63-1.22)	0.419
Age, median (IQR)	33 (22-45)	20 (10-31)	1.03 (1.02-1.04)	**<0.001**
Main occupation in nature, n (%)*	85/221 (38.5%)	58/387 (15.0%)	3.55 (2.40-5.23)	**<0.001**
Referral, n (%)	26/221 (11.8%)	37/ 357 (10.4%)	1.15 (0.68-1.96)	0.600
Days of fever on admission (>=37.5°C), median (IQR)**	5 (3-7)	3 (2-4)	1.68 (1.52-1.85)	**<0.001**
Clinical presentation at admission[#]				
Symptoms				
Headache, n (%)	145/220 (65.9%)	236/387 (61.0%)	1.24 (0.88-1.75)	0.228
Myalgia, n (%)	92/221 (41.6%)	118/387 (30.5%)	1.63 (1.15-2.29)	**0.006**
Retro-orbital pain, n (%)	19/220 (8.66%)	8/387 (2.07%)	4.48 (1.93-10.4)	**<0.001**
Rigors/chills, n (%)	29/220 (13.2%)	4/387 (1.0%)	14.5 (5.04-42.0)	**<0.001**
Dry cough, n (%)	36/220 (16.4%)	41/387 (10.6%)	1.65 (1.02-2.67)	**0.041**
Abdominal pain, n (%)	28/219 (12.8%)	60/387 (15.5%)	0.80 (0.49-1.29)	0.362
Diarrhea (at least 3 days), n (%)	10/220 (4.55%)	6/387 (1.55%)	3.02 (1.08-8.44)	0.035
Physical signs				
Body temperature > = 38 ° C, n (%)	155/216 (71.8%)	224/315 (71.1%)	1.03 (0.70-1.51)	0.871
Heart rate > 90/min, n (%)	112/221 (50.7%)	223/387 (57.6%)	0.76 (0.54-1.05)	0.098
Respiratory rate > 22/min, n (%)	21/203 (10.3%)	83/386 (21.5%)	0.42 (0.25-0.70)	**0.001**
Hypotension, n (%)	32/218 (14.7%)	54/348 (15.5%)	0.94 (0.58-1.51)	0.787
Eschar, n (%)	198/221 (89.6%)	0/387 (0.00%)	1.00 (1.00-1.00)	.
Rash, n (%)	13/221 (5.88%)	25/387 (6.46%)	0.88 (0.67-1.16)	0.777
Hemorrhagic signs (Petechial hemorrhage (epistaxis, bleeding gums, organs), skin hemorrhage, n (%)	10/221 (4.52%)	97/387 (25.1%)	0.14 (0.07-0.28)	**<0.001**
Regional lymphadenopathy (>1cm), n (%)	75/221 (33.9%)	2/387 (0.52%)	98.9 (24.0-408)	**<0.001**
Hepatomegaly and/or splenomegaly, n (%)	3/221 (1.36%)	5/387 (1.29%)	1.17 (0.19-7.05)	0.946
Pharyngo-laryngitis, n (%)	36/220 (16.4%)	41/387 (10.6%)	0.33 (0.17-0.65)	**0.041**
Documented dyspnoea, n(%)	8/220 (3.64%)	3/387 (0.78%)	4.83 (1.27-18.4)	**0.021**

	Scrub typhus	Dengue fever	OR (95%CI)	P-value[a]
Lung crepitation, n (%)	9/220 (4.09%)	2/386 (0.52%)	8.19 (1.75-38.3)	**0.007**
Fatigue, n (%)	90/221 (40.7%)	171/387 (44.2%)	0.87 (0.62-1.21)	0.407
Malaise, n (%)	2/219 (0.91%)	3/387 (0.78%)	1.18 (0.20-7.12)	0.857
Nausea, n (%)	15/219 (6.85%)	50/387 (12.9%)	0.50 (0.27-0.91)	**0.022**
Vomiting, n (%)	12/221 (5.43%)	35/387 (9.04%)	0.58 (0.29-1.14)	0.112
Lung crepitation and/or documented dyspnoea, n (%)	14/220 (6.36%)	4/386 (1.04%)	6.49 (2.11-20.0)	**0.001**
Gastrointestinal findings, n (%)	44/218 (20.2%)	109/387 (28.2%)	0.64 (0.43-0.96)	**0.031**
Clinical severity, n (%)	73/200 (36.5%)	122/347 (35.2%)	1.06 (0.74-1.52)	0.752
Laboratory findings[##]				
WBC (10^3/mm^3), median (IQR)	7.2 (5.1- 9.9)	4.1 (3.1- 6.4)	1.35 (1.27-1.44)	**<0.001**
NEU (10^3/mm^3), median (IQR)	4.5 (3.1-5.9)	2.5 (1.5-4.2)	1.36 (1.25-1.47)	**<0.001**
Lymphocytes (10^3/mm^3), median (IQR)	1.7 (1.1-2.7)	0.9 (0.6-1.3)	1.95 (1.65-2.30)	**<0.001**
N/L Ratio (neutrophils/lymphocytes)	2.5 (1.6- 3.8)	2.8 (1.4-5.0)	0.89 (0.83-0.95)	**<0.001**
HCT %, median (IQR)	38 (34.7-42.0)	37.7 (35.0-40.8)	1.00 (0.97-1.04)	0.827
RBC (10^{12}/L), median (IQR)	4.5 (4.2- 4.8)	4.5 (4.2-4.8)	0.89 (0.69-1.15)	0.377
PLT (~G/L), median (IQR)	121 (91-160)	127 (86- 177)	1.00 (1.00-1.00)	0.757
HGB (g/dL), median (IQR)	12.5 (11.3- 13.5)	12.4 (11.6-13.5)	0.96 (0.86-1.07)	0.497
Creatinine (umol/L), median (IQR)	84 (74-102)	81 (70-98)	1.00 (0.99-1.01)	0.975
AST (U/L), median (IQR)	97.5 (69-172)	81 (42.0- 118)	1.01 (1.00-1.01)	**0.001**
ALT (U/L), median (IQR)	108 (58-166)	62 (30.5- 99.5)	1.01 (1.00-1.01)	**0.001**
AST and/or ALT ≥45 U/L (n, %)	68/148 (45.9)	8/32 (25.0)	2.55 (1.08-6.04)	**0.034**

[a] Significant predictor variable on univariate logistic regression analysis (p<0.05) are indicated in bold.

** An occupation in nature: farmer, fisherman, working in forest.*

***Fever: tympanic temperature >37.5˚C measured by axillary method*

 # Clinical presentation

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

Clinical severity –at least one of these: intubation; respiratory rate >30/min; pulse >100/min; systolic blood pressure <90mmHg or >160mmHg, or diastolic blood pressure <60mmHg;

Laboratory reference range: WBC 4–10 G/L, NEU 2.6–7.0 G/L, L 1.2–3.8 G/L, HCT: 0.33-0.50L/L, PLT 150-450 G/L, HGB 12.0-16.5 g/dL (International Standard unit)

The significant predictors for scrub typhus in the training data set of the multivariable logistic regression model after backward selection are presented in Table 2.2. Besides eschar, we identified four significant clinical variables for scrub typhus and two significant routine hematological blood laboratory parameters. In the model combining these variables, the odds of having scrub typhus as opposed to dengue fever were positively associated with regional lymphadenopathy aOR=78.2 (95%CI: 9.20–665, p <0.001); followed by an occupation in nature, aOR= 3.87 (95%CI: 1.89-7.91, p <0.001); with age over 40, aOR=3.94 (95%CI:1.94-8.01, p <0.001); with increased days of fever on admission aOR=1.45 per additional day (95%CI: 1.22-1.66, p <0.001); and with neutrophil count, aOR=1.89 per unit increase (95%CI:1.54-2.32, p <0.001). On the other hand, the association with ratio of neutrophils/lymphocytes was negative with an aOR=0.68 per unit increase (95%CI: 0.57-0.81, p <0.001).

Table 2.2. Results from multivariate logistic regression with the most relevant predictors for the presence of scrub typhus (training part) [#]

	Clinical manifestations			**Routine hematological blood laboratory**			**Clinical manifestations & Routine hematological**		
	aOR	**95%CI**	**P-**	**aOR**	**95%CI**	**P-**	**aOR**	**95%CI**	**P-**
Eschar (no using)									
Regional	96.3	12.2-	**<0.001**				78.2	9.20-	**<0.001**
An occupation in	3.75	2.02-	**<0.001**				3.87	1.89-	**<0.001**
Age over 40	3.39	1.78-	**<0.001**				3.94	1.94-	**<0.001**
Days of fever on	1.49	1.30-	**<0.001**				1.42	1.22-	**<0.001**
Neutrophil count				2.09	1.75-	**<0.001**	1.89	1.54-	**<0.001**
Ratio of N/L				0.61	0.53-	**<0.001**	0.68	0.57-	**<0.001**
	AUC	**95%**		**AUC**	**95% CI**		**AUC**	**95%CI**	
ROC –analysis	0.862	0.823-		0.831	0.790-		0.912	0.878-	

[#] Footnote: *Results from multivariate logistic regression with the most important predictor variables in the training data set (n = 364). Initial clinical manifestation variables considered included days of fever on admission, myalgia, retro-orbital pain, rigor, hemorrhagic signs (epistaxis, bleeding gums, organs, or skin hemorrhage), regional lymphadenopathy (>1cm); at least one of: lung rales or documented dyspnoea, pharyngo-laryngitis, respiratory rate <22/min; an occupation in nature, 5-year age groups). The initial routine hematological blood laboratory variables included neutrophil count, lymphocyte count, ratio (Neutrophils/Lymphocytes), AST (GOT), ALT (GPT). The present models was obtained by backward selection guided by the Bayes information criterion (BIC).*

2.5.3 ROC curves

Besides eschar, we found the most relevant predictors of scrub typhus to be: increased days of fever on admission, regional lymphadenopathy, neutrophil count, ratio of N/L (neutrophils/lymphocytes), age over 40, and an occupation in nature (Table 2.2). ROC curves were generated to visualise the performance of these seven variables in

differentiating between scrub typhus and dengue fever, using mulvariable logistic regression (M-LR).

When adding eschar into the prediction model, i.e., by setting the probability of scrub typhus to 1 in patients with eschar, the areas under the ROC curve increased to 0.985 (95% CI:0.964-0.994), 0.993 (95% CI: 0.971–0.999) and 0.988 (95% CI: 0.976–0.995) in the training data set, validation data set and the whole data set, respectively (Figure 2.3: A1, A2, A3, respectively). When not using the eschar variable, the areas under the ROC curve for the 6 remaining variables were 0.912 (95% CI: 0.878-0.939), 0.888 (95% CI: 0.842–0.925) and 0.899 (95% CI: 0.873–0.922) in the training data set, the validation data set and the entire data set, respectively (Figure 2.3: B1, B2, B3, respectively).

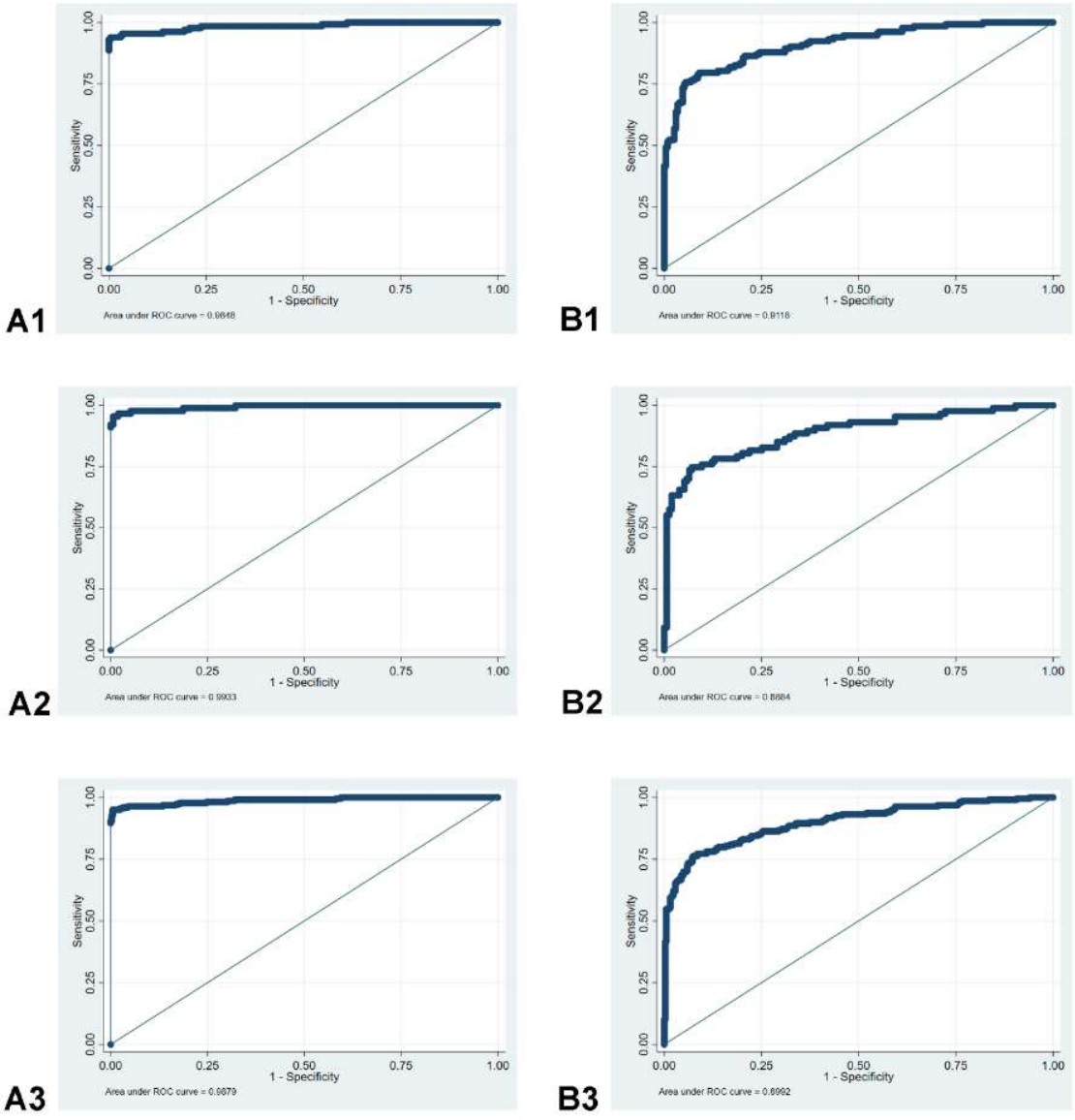


Figure 2.3 ROC curves-performance of prediction models for scrub typhus as opposed to dengue fever, using M-LR

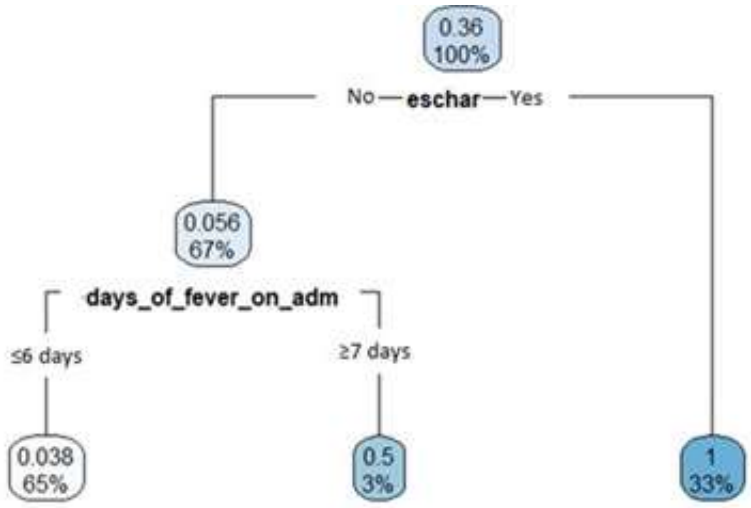
2.5.4 Decision tree analysis

In a second approach, CART analysis was applied to derive binary decision trees for distinguishing scrub typhus from dengue fever in the full data set (Figure 2.4) Each node of the tree represents a binary decision and the leaves of the tree are assigned to the diagnosis of either scrub typhus or dengue fever. In each node, 2 numbers are presented at the decision node level. The upper number indicates the positive predictive value associated with the respective node. The higher the probability of patients being scrub typhus, the darker the color of the node. The bottom number shows the percentage of patients at the respective node.

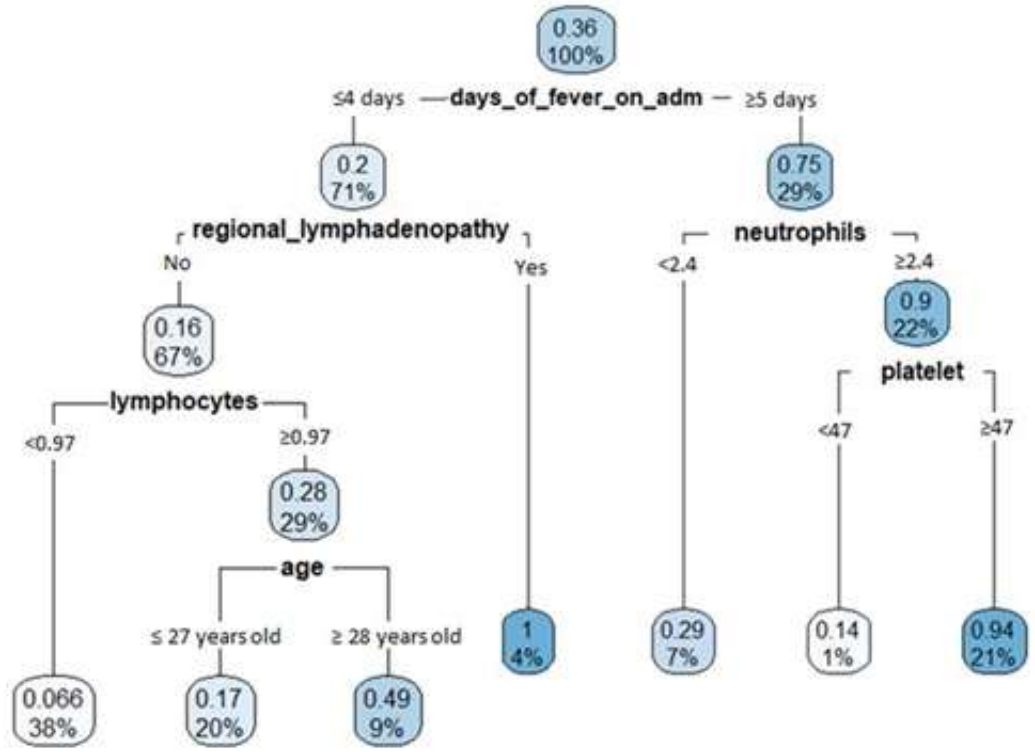
If the eschar variable was offered, the resulting tree involved the two variables “eschar” and “days with fever at admission” (Figure 2.4A). At the second decision node level, with “eschar” being positive, the probability of being scrub typhus is 1 (100%), and this node accounts for 33% of all patients. Among patients without “eschar” those with seven or more days of fever on admission had a probability of 50% of being diagnosed with scrub typhus. They accounted for 3% of the total sample.

When not offering “eschar” variable, the six variables days of fever on admission, regional lymphadenopathy, lymphocyte count, neutrophil count (without regional lymphadenopathy), platelet count and “age over 28 years old” were selected by the algorithm (Figure 2.4B). In detail, patients with ≥5 days of fever on admission had a probability of 75% of being scrub typhus – in a cohort of scrub typhus and dengue fever patients. This criterion was satisfied by 29% of the patients. Moreover, the positive predictive value of scrub typhus increased to 90% for patients who additionally had a neutrophil count ≥ 2.4 ($x10^3/mm^3$), and it further increased to 94% if patients additionally had a platelet count ≥ 47 (G/L).

Thus, we found slight differences between the two alternative approaches: while the CART tree included “platelet count”, the regression approach resulted in the inclusion of “an occupation in nature”. Moreover, the “age” cut point in the tree is at 28 years while we had found a cut-off of 40 years to discriminate well when deriving the regression model.



A with eschar variable



B without eschar variable

Figure 2.4. Regression tree for scrub typhus using the entire data set.

* Panel A: tree obtained when offering the variable “eschar”; panel B: tree obtained when not offering the variable “eschar”.

Table 2.3 presents the most relevant predictors of scrub typhus including eschar, regional lymphadenopathy, an occupation in nature, age, increased days of fever on admission, increased neutrophil count, decreased neutrophil/lymphocyte ratio, and increased platelet count, revealed by CART (using R) and by the multivariate logistic regression (LR) approach (using stata).

Table 2.3. The most relevant predictors of scrub typhus selected by CART (using R) and by the multivariate logistic regression (M-LR) approach (using STATA).

STRONG PREDICTORS OF SCRUB TYPHUS
1. Eschar
2. Regional lymphadenopathy
3. An occupation in nature*
4. Higher age**
5. Increased days of fever on admission (Nr)
6. Increased neutrophil count
7. Decreased Ratio (Neutrophils/Lymphocytes)▲
8. Platelet count ≥ 47 G/L #

** Fishing/agriculture/working in forest, only in M-LR*

*** Age over 40 in M-LR and age over 28 in CART*

▲ *Ratio (neutro/lymph) in M-LR and Lymphocytes in CART*

Only in CART

2.5.5 Model validation

The results from the M-LR model, using the set of 7 predictors: eschar, increased days of fever on admission, regional lymphadenopathy, an occupation in nature, increased neutrophil count, decreased ratio of N/L (neutrophils/lymphocytes), age over 40 was very sensitive and very specific for defining scrub typhus (using whole data, sensitivity=93.7%, specificity=99.5%, Youden=0.932), when directly comparing scrub typhus and dengue fever groups. The respective values were very similar in the training and the validation data set.

The regression tree generated for scrub typhus with the two predictors eschar and days of fever on admission had a slightly lower index of Youden (0.919) and a lower specificity (96.9%), while the sensitivity was slightly higher (95%) (Table 2.4).

Using data without "eschar" variable

The binary predictor derived from the M-LR model using the six variables increased days of fever on admission, regional lymphadenopathy, an occupation in nature, increased neutrophil count, decreased ratio of N/L (neutrophils/lymphocytes), and age over 40 had a moderate sensitivity (76.3%) but a high specificity (92.3%), providing an index of Youden of 0.686. The area under the ROC-curve defined by the underlying numerical prediction score was 0.899 (95% CI: 0.873–0.922). Again, the respective statistics were very similar in the training and the validation data set.

The decision tree algorithm in the entire dataset revealed six predictors: days of fever on admission, regional lymphadenopathy, neutrophil count, lymphocyte count, platelet count, age over 28. In the CART, the tree included platelet count and age over 28. In the full data set, the index of Youden of the decision tree model (0.681) was almost identical to the one of the regression-based model (Table 2.4).

Table 2.4. Model validation: Accuracy of Scrub typhus Prediction Models derived by Multivariate Logistic Regression vs. CART

Variables	**Multivariate**	**Multivariate**	**Multivariate**	**CART** [#]
N	N=364	N=244	N=608	N=608
Dataset	Training	Validation	Whole data	Whole
Using data with eschar variable				
Sensitivity	93.2%	94.4%	93.7%	95.0%
Specificity	99.6%	99.4%	99.5%	96.9%
Positive Predictive Value (PPV)	99.2%	98.8%	99.0%	94.6%
Negative Predictive Value (NPV)	96.3%	96.9%	96.5%	97.2%
Youden	0.928	0.937	0.932	0.919
Using data without eschar variable				
Sensitivity	77.3%	74.7%	76.3%	77.4%
Specificity	92.7%	91.6%	92.3%	90.7%
PPV	85.7%	83.3%	84.8%	82.6%
NPV	87.8%	86.6%	87.3%	87.5%
Youden	0.700	0.663	0.686	0.681

** after refitting the prediction model in the whole data set with the six variables: days of fever on admission, regional lymphadenopathy, an occupation in nature, neutrophil count, ratio (neutrophils/lymphocytes), and age over 40. For the derivation of the predicted probabilities using logistic regression, the model without the eschar variable was used as a basis, with the probability of scrub typhus then being changed to 1 among patients with an eschar.*

[#] Regression tree using R in entire dataset after pruning (Figure 2.4B).

2.5.6 Relevant findings from the models

2.5.6.1 The model of demographic characteristics, epidemiological information, clinical variables to predict scrub typhus

In the clinical model derived by M-LR without using the “eschar” variable, the most relevant clinical manifestation factors to predict scrub typhus were regional lymphadenopathy, days of fever on admission, an occupation in nature and age over 40. The clinical model worked well, and with these 4 factors, the area under ROC curve was 0.862 (95%CI: 0.823-0.896) in the training data without eschar variable (Table 2.2).

2.5.6.2 The model of the routine hematological blood laboratory variables to predict scrub typhus

In the M-LR model involving laboratory variables only, the most relevant routine complete blood count values to predict scrub typhus were neutrophil count and ratio of N/L (Neutrophils/Lymphocytes). The laboratory model had a higher predictive performance than the clinical model. The model with these 2 factors had an area under ROC curve of 0.831 (95%CI: 0.790-0.869) in the training data set without without eschar variable (Table 2.2).

2.5.6.3 The model combining demographic characteristics, epidemiological information, clinical and laboratory variables to predict scrub typhus

Combining the demographic characteristics, epidemiological information, clinical and laboratory variables, using M-LR, the seven most significant predictors for scrub typhus were; eschar, regional lymphadenopathy, days of fever on admission, an occupation in nature, increased neutrophil count, decreased ratio of N/L (Neutrophils/Lymphocytes), and age >40 years. The model with inclusion of all mentioned variables worked better than the models including the clinical or routine hematological blood laboratory variables only. With all of these factors, the area under ROC curve reached 0.988 (95%CI: 0.976-0.995) in the whole data set when including the eschar variable (Figure 2.3 - A3), and 0.899 (95% CI: 0.873–0.922) when excluding it (Figure 2.3 - B3).

The decision tree algorithm revealed the following seven most important predictors; eschar, regional lymphadenopathy, ≥5 days of fever on admission, increased neutrophil count, increased lymphocyte count, platelet count ≥ 47 G/L, and age >28 years. The tree demonstrated almost the same accuracy with the multivariate logistic regression analyses (index of Youden: 0.681 vs. 0.686), when not using the “eschar” variable (Figure 2.4).

2.6 DISCUSSION

Dengue fever is highly endemic in Vietnam, but scrub typhus - although recognized as an endemic disease - remains underappreciated. Scrub typhus is probably the most prevalent under-recognized treatable cause of undifferentiated febrile illness in Vietnam [16, 18, 125,

168]. One of the few clinical studies conducted in the national hospital in northern Vietnam suggested that up to 40.9% (273/579) of acute undifferentiated fever (AUF) patients had scrub typhus, after excluding patients with malaria, dengue fever, and typhoid fever, although this is likely an over estimation due to serological diagnostics and selection criteria of AUF patients considered as suspected rickettsial infections [125]. Dengue was responsible for one third (234/2108; 33.6%) of all acute undifferentiated fevers at the primary health care level in a year [31]. Hence, scrub typhus and dengue together are likely to contribute to more than half of undifferentiated febrile illnesses either at national referral hospital or at primary health care centers. In this study we identified simple predictors to assist in differentiating scrub typhus from dengue fever using basic clinical and laboratory parameters in Vietnam, to improve the quality of diagnoses and appropriate treatment strategies at primary health care level.

Following considerations regarding these two acute fevers need to be taken into account in Vietnam; Firstly, medical staff are not well aware of the potential causes of AUF [22, 31, 130], largely because robust “causes of fever” studies remain limited. Secondly, there is a strong general awareness of dengue due to the high case numbers and its impact between 2011 and 2018, the involvement of media campaigns, and with the broad availability of accurate RDTs leading almost to a perception bias towards dengue for any febrile illness in Vietnam [31]. Thirdly, the diagnostic capacity for scrub typhus (point-of-care and confirmatory assays) remains difficult and limited, even at the national referral hospital level [18]. PCR and serology are expensive, require considerable expertise and sophisticated laboratory equipment and simple RDTs are lacking [30]. Until this is improved, there is an absolute need for better predictors to inform empirical treatment or management for doctors.

2.6.1 Predictors to distinguish scrub typhus from dengue fever

In a large collection of characterized patients, this study identified predictors for scrub typhus to be; i) the eschar; ii) regional lymphadenopathy; iii) an occupation in nature; iv) ≥5 days of fever on admission; v) increased neutrophil count; vi) low ratio of neutrophils/lymphocytes; vii) platelet count ≥ 47 G/L; and viii) higher age (Table 2.4). In a Vietnamese cohort of dengue and scrub typhus patients, these predictors can identify scrub typhus with sensitivity of 93.7%, specificity of 99.5%, with a diagnosis accuracy (ROC curve) of 0.988 (95% CI: 0.976–0.995), if an eschar is present in scrub typhus cases. In the case of no eschar, the sensitivity and specificity of this approach drops to 76.3% and of 92.3% respectively, with a diagnosis accuracy (ROC curve) of 0.888 (95% CI: 0.878-0.939). This means that applying these predictors without using any diagnostic test, would strongly support medical staff in identifying scrub typhus cases (up to 99% if an eschar is found, and

89% if not). This also highlights the importance of a thorough clinical examination, especially as eschars are often hidden in skin folds or the genital areas [169].

2.6.2 Diagnostic considerations for the role of eschars

An underappreciated problem regarding the presence of eschars as a vital diagnostic clue is that their occurrence can vary broadly across different regions, and that pre-existing immunity can suppress eschar formation at the mite bite inoculation site [131, 170]. Several studies in Vietnam revealed eschar prevalence across communities from 18.2% to 46.6% [18, 125], while reports from other areas in Asia suggest eschar prevalence from 7%–97% among scrub typhus patients, depending on study site endemicity and study design [6, 145]. It is important to realise that although the presence of an eschar is helpful, many scrub typhus patients may not have an eschar. Clearly, eschars are not helpful in a setting where eschars are found in as few as 7% among children, like in southern Thailand [171] or where the occurrence of eschars is at 18.2% among patients aged 13 years or older in Vietnam [18]. The prevalence of eschars also depends on the selection criteria of studies – if a study is centered around eschar presence as an inclusion criterion, a high eschar rate is likely to be found. In this retrospective study a high presence of eschars in scrub typhus patients with 93.1% in the 2013-2014 cohort was seen, but only in 52.6% in the 2018-2019 cohort. It is likely that in the first phase, doctors diagnosed scrub typhus based on eschars leading to a high eschar rate, while in 2018-2019, after our training of the medical staff, the awareness about scrub typhus cases without eschar was raised and additional improved diagnostics were introduced (ELISA assays diagnostics improved, thus leading to a lower eschar prevalence than before. It is important to consider that patients with spotted fever group rickettsioses may also present with eschars, and although extremely rare, a local lesion has been described in murine typhus [18, 125, 171-173], so the “pathognomonic” role of eschar in scrub typhus diagnosis should be considered carefully. Eschars usually present within 30 cm below the umbilicus (including the perineal, inguinal, and buttock areas), under the breasts in female patients and in the axillae/under upper skinfold of umbilicus of children [6, 173, 174]. Patients are often not willing to reveal these body parts to doctors, if they are not specifically asked about this - often resulting in missed eschars due to incomplete examinations [174].

With all above reasons, the importance of the eschar in scrub typhus diagnosis should be critically considered and if no eschar is found – despite thorough examination – the remaining 6 predictors or the CART decision tree “without eschar” should be considered as predictive indicators.

2.6.3 Decision-supporting predictors based on multivariable logistic regression vs. CART

The CART analyses including “eschar” (i.e. an eschar was found), revealed after pruning that developing the tree necessitated two predictors only: “eschar” and “days of fever on admission”, leading to an index of Youden of 0.911. The application of the regression model to the entire data set revealed an index of Youden of 0.932 and involved the predictors: i) increased days of fever on admission, ii) regional lymphadenopathy, iii) an occupation in nature, iv) increased neutrophil count, v) decreased ratio of N/L (neutrophils/lymphocytes), and vi) age over 40, in addition to “eschar”. When not using the “eschar” variable, i.e. when no eschar was found – the regression model involved “an occupation in nature” and “age over 40”, while the CART tree involved “platelet count ≥ 47 G/L” and “age over 28” – in addition to the same predictors: days of fever on admission, regional lymphadenopathy, neutrophil, lymphocytes. However, both models resulted in similar accuracy for identification of scrub typhus (index of Youden: 0.681 vs. 0.686, respectively).

The documentation of “increased days of fever on admission” plays an important role in predicting scrub typhus. Due to self-treatment or misdiagnosis in community health centers, scrub typhus patients were likely to visit hospitals later than dengue patients. This can have an effect on diagnostics due to disease dynamics being characterized by an early bacteremia (7-10 days) followed by the antibody response – necessitating PCR to be coupled with a serological test for complete coverage of the diagnostic window [30]. If “an occupation in nature”, having “regional lymphadenopathy”, increased “days of fever on admission”, and “age >40 years” were combined, the area under the curve (ROC) was 86.2%. If neutrophils and lymphocytes were added, the AUC increased to 91.2 % (Table 2.2). After further inclusion of “eschar”, the AUC reached 98.5 % (Figure 2.3 A1), meaning that correct application of these predictors in this cohort can contribute substantially to a presumptive diagnosis without a diagnostic test.

In this study, we applied two different statistical approaches for deriving a model to discriminate between scrub typhus and dengue fever; i) multivariable logistic regression (M-LR) and ii) CART. As results from M-LR, a set of given seven predictors produced a slightly improved predictive performance when compared with CART analyses (the index of Youden from M-LR and CART were 0.932 vs 0.911, respectively). The higher index of Youden of the M-LR approach might be due to starting the model without “eschar” and correcting the predictions of this model to an ST-probability of 1 in patients with eschar. This gives other variables a chance to also enter the model while “eschar” overshadows all other variables in the development of the regression tree. However, when excluding the “eschar” variable, both models performed similarly (the index of Youden being 0.686 for M-LR vs

0.681 for CART). The decision tree approach has several advantages over the approach using logistic regression. The first and most important advantage of a decision tree is that the derived rules and subgroups of the tree are easy to understand and sequentially lead the clinician along the branches of the tree to the presumptive diagnosis proposed by the algorithm [165]. For example, starting from the second level of flowchart in Figure 2.4B, if a patient has days of fever on admission ≥ 5 days, chances are 74% that he has scrub typhus. After that, if he has a neutrophil count ≥ 2.4, this increases the probability to 90%. This probability is increased to 94%, if a platelet count ≥47 G/L is present. If the regression-based algorithm is programmed it can also be applied swiftly if the input data are available. But of course one will then first have to enter all values, which takes longer than just following the tree visually and decisions can also be obtained very fast.

2.6.4 Translating the findings into the real-world setting

Given the high probabilities of these predictors to make a presumptive diagnosis, they hold potential to inform a preemptive treatment strategy aiming to reduce complications and mortality, while creating improved awareness of scrub typhus all along.

These findings will be useful for medical staff working in areas where dengue and scrub typhus are endemic diseases. A simple medical history, a clinical examination and routine blood tests are available at primary health care centers, and will contribute to discriminating a bacterial from a viral disease in the 684 district hospitals and 11.083 community health centers. Correct application could lead to improved cost-efficiency by reducing medical and non-medical costs for patients, less unnecessary patient referrals to hospitals. Scrub typhus is an easily treatable disease with doxycycline which is inexpensive, readily available at local pharmacy agents and has a favorable age profile [175, 176].

However, the importance of these findings lies in that an improved interpretation of readily available clinical-laboratory information could accelerate diagnosis and improve empirical treatment strategies at the primary health care level. Mis-diagnosis can contribute to antibiotic overuse, which is a substantial problem in Vietnam. Since scrub typhus does not respond to broadband antimicrobials such as betalactams (especially the common derivative cephalosporins, which are widely used for undifferentiated febrile illnesses), a decision algorithm in distinguishing scrub typhus from dengue would inform medical staff to choose more adequate or targeted treatment strategies to reduce antibiotic overuse (i.e. doxycycline or macrolides) - especially at the primary health care level [119].

Thus, application of these simple predictors in the correct way holds potential to i) reduce the delay to treatment initiation; ii) inform on the use of an adequate antimicrobial, iii) shorten the disease course to reduce complications and fatality rates, as well as iv) improve the management of uncomplicated fevers and create better awareness of scrub typhus.

2.6.5 Limitations of the study

The data generated in this study is based on a large cohort of scrub typhus and dengue fever patients, since these together represent the major current burden of undifferentiated febrile disease. The findings need to be re-evaluated with a cohort including other co-endemic febrile illnesses, once more systematic evidence on the causes of undifferentiated febrile illness (UFI) becomes available. Likely diseases could be leptospirosis, murine typhus, Q fever, spotted fever group rickettsiae (SFGR), and/or melioidosis. This study has some limitations. Firstly: this was a retrospective study and the collected data could hold inconsistencies; secondly: eschar was among the main criteria to define a scrub typhus suspected case in the 2013- 2014 period thus could contribute to introducing a selection bias. To counteract this, we enrolled all other undifferentiated fevers (patients with long-lasting fever over 10 days/undifferentiated fever/used medicine to reduce fever without effect; dengue/malaria suspected cases with negative dengue/malaria test results) to minimize losing potential cases and reduce the eschar-positive patient proportion; thirdly: all of the confirmed scrub typhus were tested for dengue fever and malaria, and co-infections were not included, however maybe this was not documented in all the cases, and a prospective study would provide more reliable results. Co-infections of scrub typhus and other UFI such as leptospirosis, murine typhus, Q fever, SFGR, melioidosis are expected to be rare, a small chance for co-infections remains. fourthly: Investigations were limited to dengue, scrub typhus and malaria, while other endemic diseases were not considered (i.e. chikungunya, zika, lyme, Q fever, spotted fever group rickettsia (SFGR), leptospirosis, murine typhus, and/or melioidosis). However, epidemiological reports suggest that at present Vietnam is considered a low-risk area for chikungunya, zika, lyme, Q fever, and SFGR [17, 22, 177-180]. Leptospirosis and murine typhus have been reported as causes of UFI in Vietnam. Although both diseases do not associate with eschars, they respond to doxycycline as empirical therapy [22, 125], and clinical mis-classification of these two diseases as ST has no major therapeutic consequences [181-183]. The presented algorithms might not reach the accuracy reported if applied to areas with different epidemiological characteristics (i.e. settings with different risk factor profiles as in more urban areas), and the algorithms may require adaptation if improvements in dengue/scrub typhus diagnostic procedures occur. Positive and negative predictive values of the models need updating if an incidence change of these diseases/the other UFI above occurs over time; and fifthly, increased liver enzymes (ALT, AST) were described in differentiating scrub typhus from dengue fever patients previously in Thailand [133]. Although 67% of scrub typhus cases (148/221) and <1% dengue fever cases (32/387) had elevated liver enzyme findings upon admission, they were not statistically associated with any predictive power for

disease differentiation upon admission in the multivariable logistic model of this case-control study, which included >3 times more patients than the previous report.

2.6.6 Conclusion

Scrub typhus and dengue fever are common sympatric endemic diseases in Vietnam. Basic clinical findings and routine hematological blood laboratory tests were investigated to develop a predictor-based clinical decision algorithm. The provided information by this study supports medical staff in the often challenging clinical decision-process for differentiating bacterial scrub typhus from viral dengue infections. Application of these simple predictors (Table 2.1) holds potential to i) improve clinical suspicion of scrub typhus cases; ii) reduce the delay to treatment initiation; iii) inform on the use of an adequate antimicrobial, iv) shorten the disease course to reduce complications and fatality rates, as well as v) improve the management of uncomplicated fevers and create better awareness of scrub typhus.

2.7 ANNEX

2.7.1 Acknowledgments

We would like to express our deep gratitude to the directors, senior hospital managers, staffs working at the planing and general departments, medical doctors, nurses and patients at 5 study sites: Khanh Hoa Provincial Hospital, Ninh Hoa branch provincial hospital, Dien Khanh district hospital, 87 Army hospital, Ninh Diem Hospital for their generous support and interest in this project. We would like to express our special thanks to Dr Phung Tan Le, the Deputy Director, and Dr Quan Hong Do of the Khanh Hoa Department of Health, for their approval for study implemetation in Khanh Hoa province. We are grateful to Dr Mai Quang Vien for providing support and advice in setting up this study. Further, we gratefully thank Dr Hung Manh Do for the many excellent discussions leading to impact, while drinking vietnamese coffee.

2.7.2 Author Contributions

Conceptualization: Hanh Thi Duc Tran, Thuy Thi Thanh Pham, Lan Thi Hoang Vu, Christian Schindler, Daniel H. Paris.

Data curation: Hanh Thi Duc Tran, Christian Schindler, Quyet Thi Ngo, Hang Thi Hai Hoang.

Formal analysis: Hanh Thi Duc Tran, Christian Schindler, Daniel H. Paris.

Funding: Esther Schelling, Daniel H. Paris.

Investigation: Hanh Thi Duc Tran, Christian Schindler, Thuy Thi Thanh Pham, Mai Quang Vien, Hung Manh Do, Quyet Thi Ngo, Trieu Bao Nguyen, Hang Thi Hai Hoang, Daniel H. Paris

Methodology: Hanh Thi Duc Tran, Christian Schindler, Thuy Thi Thanh Pham, Daniel H. Paris.

Laboratory work: Quyet Thi Ngo, Trieu Bao Nguyen

Project administration: Hanh Thi Duc Tran, Hang Thi Hai Hoang.

Resources: Esther Schelling, Daniel H. Paris.

Supervision: Christian Schindler, Thuy Thi Thanh Pham, Mai Quang Vien, Hung Manh Do, Daniel H. Paris.

Writing – original draft: Hanh Thi Duc Tran, Christian Schindler, Daniel H. Paris.

Writing – review & editing: Hanh Thi Duc Tran, Christian Schindler, Thuy Thi Thanh Pham, Mai Quang Vien, Hung Manh Do, Quyet Thi Ngo, Trieu Bao Nguyen, Hang Thi Hai Hoang, Lan Thi Hoang Vu, Esther Schelling, Daniel H. Paris

2.7.3 Financial Disclosure Statement

Research reported in this publication is a part of Hanh Tran's PhD project "Scrub typhus in Khanh Hoa, Viet Nam: an eco-epidemiological approach". This ongoing PhD work is financially partly covered by the Swiss Government Excellence Scholarship Award from 9/2017 to 12/2020. The remaining salary is covered by Esther Schelling and Daniel H. Paris. Esther Schelling funded the data collection and field trips. Daniel H. Paris funded the laboratory assays and technical laboratory training course. This data collection in Vietnam was also supported by a Wolfermann-Nägeli-Stiftung Award for Project Tsutsugamushi-Fieber (scrub typhus) in Khanh Hoa, Viet Nam (Hanh Thi Duc Tran PhD Candidate). The content is solely the responsibility of the authors.

2.7.4 Competing interests

The authors declare that there are no competing interests.

2.7.5 What next?

There is a need to implement better diagnostics for rickettsial illnesses (scrub and murine typhus – possibly SFG rickettsiosis) – these are difficult because they are still often based on the indirect fluorescent antibody (IFA) test – and to support the important transition to rapid diagnostic tests (RDTs) and ELISAs [30].

Until more simple and accurate diagnostic tests are available and appropriately validated, the use of clinical and routine laboratory predictors in the decision process for empirical antibiotic treatment will be important – and also increasingly to train and improve medical staff awareness of the problem.

Here is the first paper of Hanh Thi Duc Tran's publication series on scrub typhus in Vietnam. A recent paper presented risk factors to help preventing scrub typhus at the community

health care level; next she will evaluate the diagnostic accuracy of RDTs and ELISAs for scrub typhus in Vietnam and follow up with an evaluation on the implemention of these elements in the endemic scrub typhus setting of Central Vietnam.

CHAPTER 3. ECOLOGICAL AND BEHAVIOURAL RELATED RISK FACTORS OF SCRUB TYPHUS IN CENTRAL VIETNAM: A CASE CONTROL STUDY

Ecological and behavioural related risk factors of scrub typhus in central vietnam: a case control study

Hanh Thi Duc Tran[1, 2, 3*], Jan Hattendorf [1, 3], Hung Manh Do [4], Thanh Tien Hoang [4], Hang Thi Hai Hoang [4], Hoa Ngoc Lam [5], Mai Kim Huynh [6], Lan Thi Hoang Vu [2], Jakob Zinsstag [1, 3], Daniel Henry Paris [7, 8], Esther Schelling [9]

[1] Department of Epidemiology and Public Health, Swiss Tropical and Public Health Institute, Basel, Switzerland, [2] Department of Epidemiology, Hanoi University of Public Health, Hanoi, Vietnam, [3] University of Basel, Basel, Switzerland, [4] Department for Infectious Disease Control and Prevention, Nha Trang Pasteur Institute, Khanh Hoa, Vietnam, [5] Institute of Gastroenterology and Hepatology, Vietnam, [6] Department of Microbiology and Immunology, Nha Trang Pasteur Institute, Khanh Hoa, Vietnam, [7] Faculty of Medicine, University of Basel, Basel, Switzerland, [8] Department of Medicine, Swiss Tropical and Public Health Institute, Basel, Switzerland, [9] Vétérinaires Sans Frontières Suisse, Bern, Switzerland.
* corresponding author: thiduchanh.tran@unibas.ch, hanhtranch@gmail.com

This paper is published in the Infectious Diseases of Poverty BMC, vol 10, number 110, August 2021 (https://doi.org/10.1186/s40249-021-00893-6).

3.1 ABSTRACT

Background: The risk factors for scrub typhus in Vietnam remain unknown. Scrub typhus caused by *Orientia tsutsugamushi* often presents as an undifferentiated febrile illness and remains under appreciated due to the limited availability of diagnostic tests. This tropical rickettsial illness is increasingly recognized as an important cause of non-malaria acute undifferentiated fever in Asia. This study aimed to investigate behavioural and ecological related risk factors of scrub typhus to prevent this potentially life-threatening disease in Vietnam.

Methods: We conducted a clinical hospital-based active surveillance study, and a retrospective residence-enrolment date-age-matched case-control study in Khanh Hoa province, Vietnam, from August 2018 to March 2020. Clinical examinations, PCR and ELISA IgM tests were applied to define cases and controls. All enrolled participants filled out a questionnaire including demographic socio-economic status, personal behaviors/protective equipment, habitat connections, land use, and possible exposure to the vector. Multivariable conditional logistic regression was used to define the scrub typhus associated risk factors.

Results: We identified 44 confirmed cases and matched them with 152 controls. Among cases and controls, the largest age group was the 41–50 years old and males accounted for 61.4% and 42.8%, respectively. There were similarities in demographic characteristics between the two groups, with the exception of occupation. Several factors were significantly associated with acquisition of scrub typhus, including sitting/laying directly on household floor [adjusted *OR* (a*OR*) = 4.9, 95% *CI*: 1.6–15.1, *P* = 0.006], household with poor sanitation/conditions (a*OR* = 7.9, 95% *CI*: 1.9–32.9, *P* = 0.005), workplace environment with risk (a*OR* = 3.0, 95% *CI*: 1.2–7.6, *P* = 0.020), always observing mice around home (a*OR* = 3.7, 95% *CI*: 1.4–9.9, *P* = 0.008), and use of personal protective equipment in the field (a*OR* = 0.4, 95% *CI*: 0.1–1.1, *P* = 0.076).

Conclusions: Ecological and household hygiene-related factors were more associated with scrub typhus infection, than individual-level exposure activities in the hyper-endemic area. These findings support local education and allow people to protect themselves from scrub typhus, especially in areas with limitations in diagnostic capacity.

Keywords: Ecological, Environmental, Behaviour, Risk factor, Scrub typhus, *Orientia tsutsugamushi*, Vietnam

3.2 BACKGROUND

Scrub typhus is a zoonotic infectious disease caused by *Orientia* spp. Humans can be exposed to this bacterium through bites of infected larval-stage *trombiculid* mites, which are found in rodents of forests and rice fields across the Asia-Pacific region [184-187]. *Trombiculid* mites have a metamorphosis life-cycle; Female mites lay fertilised eggs in soil, from which 5–7 days later, 6-legged larva (chigger) hatch. These chiggers display host-seeking behaviour by forming clusters on leaves, grasses and twigs above soil surface, and can survive in outdoor environments for weeks-months without a vertebrate host. Chiggers have a large variety of hosts, including maintenance hosts i.e. small mammals (rodents and shrews), ground-dwelling birds, and incidental hosts i.e. larger mammals including humans. Only monkeys, gerbils, hamsters and humans are known to suffer clinically from scrub typhus [62-64, 184]. In severe cases, the disease can progress to multi-organ failure, with pathologic lesions in lungs, kidneys, liver, and brain [188-190]. Absence of eschars, the scrub typhus-specific symptom, makes clinical diagnosis challenging. Currently, standard confirmation tests are for scrub typhus antigen polymerase chain reaction (PCR) and for serologic diagnosis the indirect immunofluorescence assay (IFA) [191]. However, laboratory diagnosis shortly after infection remains difficult, as antibodies do not reach detectable levels for 5–10 days after onset, and the level of *Orientia* bacteria in the blood for PCR, only reaches detectable levels during acute episodes and is inapparent after appropriate initial antibiotic treatment [192, 193]. Therefore, scrub typhus is often misdiagnosed, especially in low-middle income countries with limited laboratory capacity [145].

Scrub typhus is a severe public health problem, with one billion people at risk globally, causes an estimated one million cases every year, and has became a leading cause of treatable non-malarial febrile illness [41, 145]. Scrub typhus is a serious public health problem in the Asia-Pacific [194]. In Vietnam, scrub typhus is re-emerging after 40 years of neglect [17]. Only sporadic cases were registered after 1970. However, in 1995, 45 scrub typhus patients were reported in Quang Ninh province, and 449 patients from 2000 to 2002 [195]. Nadjm et al. (2014) reported 255 confirmed cases from 24 northern provinces referred to the National Hospital of Tropical Diseases between 2001 and 2003 [16]. Khanh Hoa province confirmed 125 scrub typhus cases per year in 2013/2014, resulting in an estimated incidence of 1.1 per 10 000 [196]. However, laboratory capacity for scrub typhus diagnosis in Vietnam is not yet designated to cope with increasing numbers. Due to late diagnosis and treatment, Bach Mai national hospital estimated a complication rate of 17% and a mortality rate of 1.2% among 251 confirmed patients in 2003 [16, 197].

Epidemiological and ecological information on scrub typhus is very scant in Vietnam. Given the transmission of the bacteria via chiggers, factors at the community level need to be considered to prevent scrub typhus: household sanitation, household/working

surroundings, agricultural activities and personal protective measures during outdoor activities [38, 198, 199]. As crop fields are an important reservoir for chiggers, farmers are considered a high-risk group for scrub typhus [200]. However, the study in Khanh Hoa in 2013 showed patients with different occupations, including farmers (32%), students (10%), private industry workers (7%), administrative staff (4%), and 1 to 5% other professions: gardeners, traders, housewives, manual laborers, retirees, tour guides and soldiers [201]. These diverse findings suggest that behavioral risk factors associated with living and working environments in endemic areas are more relevant than occupation. Up to date, no further analyses on behavioral factors were performed in Vietnam.

There is an urgent need to gain a better understanding of disease transmission among humans within their ecosystem to make recommendations on practical preventive measures and fostered case-detection - this study aimed to investigate behavioural and ecological related risk factors of scrub typhus.

3.3 METHODS

3.3.1 Study site

The study took place in Khanh Hoa from August 2018 to March 2020. Khanh Hoa (Latitude 12°N and Longitude 109°E) is in coastal South Central of Vietnam. The population was 1.2 million residents (2019) [202], in 9 districts/townships. The coastal line is 385 kilometers with many lagoons, bays, and islands.. Forests and hilly landscapes cover more than half of province. Khanh Hoa is one of the few provinces with a higher gross product from fishing than from agriculture. The tropical savanna climate allows perennial grasses to grow all year, leads to an open shrub layer [203], suitable environment for chigger abundance and scrub typhus transmission.

There are 12 hospitals in Khanh Hoa: 11 public hospitals [Khanh Hoa Provincial Hospital; Ninh Hoa Hospital (Provincial Hospital Branch), Khanh Hoa Hospital for Tropical Diseases; and 8 district hospitals and Military Hospital 87. Nha Trang Pasteur Institute (IPN) located in Khanh Hoa is one of two Pasteur Institutes in Vietnam, operating directly under authority of the Vietnamese Ministry of Health. Khanh Hoa is known to be endemic for scrub typhus for over 50 years. Scrub typhus was reported among United States Air Force personnel in Khanh Hoa in 1969 [149]. In 2013–2014, 125 confirmed cases per year occurred in 8 of the 9 districts [149, 196].

3.3.2 Study design

We investigated scrub typhus risk factors using a hospital-based clinical active surveillance, and a retrospective residence-enrolment date-age-matched case-control study to determine risk factors and protective measures associated to disease.

3.3.2.1 Hospital-based clinical surveillance

Active surveillance was done at the Military Hospital 87, and 10 of 11 public hospitals of Khanh Hoa, excluding Truong Sa Island district hospital due to accessibility.

All clinical diagnoses during hospitalization admission were made by trained local physicians following suspected acute scrub typhus case definition (Table S3.1). All patients satisfying criteria were asked for their informed consent to be enrolled. Demographic and clinical data was collected with standardized questionnaires by trained health staffs. Blood specimens were collected from all enrolled patients at admission and discharge [each 2 tubes, with and without Ethylenediaminetetraacetic acid (EDTA)]. Using EDTA blood samples, buffy coat and plasma centrifugation (616 × *g* during 15 minutes) was conducted within 24 hours by trained technicians and stored at 2–8°C in the hospital laboratory of [152], before transfer to IPN, for PCR and enzyme-linked immunosorbent assay (ELISA) [152]. All samples were labeled with the subject's unique identification number (ID) and preserved in -a -20℃ freezer at the Department of Microbiology and Immunology until further processing.

3.3.2.2 Case-control study and sample size

For each confirmed acute scrub typhus case (Table S3.1), residence-enrolment date-age-matched controls were enrolled upon their informed consent.

Using Stata software version 14.0 (StataCorp, Texas, USA), we initially calculated a sample size of 128 cases and 512 controls for a matched case-control study, to detect a true odds ratio of 0.5 with 90% power at the 5%-significance level. Taking a ratio of cases and controls of 1:4, a prevalence of exposure (wearing a long-sleeved shirt/trousers for outdoor activities) of 66% among controls and a correlation of exposure between cases and control (rho) of 0.2 were assumed. Facing difficulties with enrolment of cases and controls due to COVID-19 pandemic, we adjusted our calculation and aimed for a more common 80% power. We calculated that 50 cases and 200 controls would be sufficient to detect an odds ratio of 0.4 with 80% power using the same set of assumptions.

For each confirmed case, we enrolled four matched controls: two hospital and two community controls. The inclusion and exclusion criteria of cases and controls were according to the Vietnam national guideline on diagnosis and treatment of infectious diseases 2016 [204] (Table S3.1).

Hospital and community controls were defined as residents with no acute scrub typhus, without fever or with fever ≥ 4 days. The 4 day criteria accounted for sufficient time to

develop a detectable IgM titer increase [1]. Based on evidence of macro-level factor effects on scrub typhus exposure risk, i.e. provincial climate (dry/rainy seasons), the controls were enrolled no longer than 1 month since the date of the case confirmation.

To prevent overmatching, hospital controls were selected in the same district as the case, however from any other commune than the case's commune. Regarding community controls, for each confirmed case, a list of community controls was prepared by the commune health center, including all eligible persons living around the case household within 500 meters, but not next door [64]. Two persons randomly chosen from the list were invited to participate and asked for their informed consent. At time of enrolment, blood samples were taken with the same sampling/storage procedure as described for suspected cases.

Using a residence-enrolment date-age-matched case-control study to determine disease risk factors allowed to minimise effects of ecological confounders related to residence such as mice density, changes of humidity, rain, temperature affecting chigger abundance, its chance to stick on people, and therefore changing exposure probability of people. Enrolment within one month limited changes in households' (HH) context and conditions. Age-matched case-control design helped to control potential confounders related to age such as typical work activities, occupations, behavior routines among each age group. While persons in the same communes (case-community controls) were likely to share some same working behaviours and personal protective equipment (PPE) wearing routines, the case-control design for both hospital and community controls was thought to address disadvantages of case–community controls while testing risk factors based on varied behaviours and household contexts across different areas.

3.3.3 Laboratory diagnostic assays

Buffy coat and plasma specimens from cases and controls were tested at IPN, using semi-nested PCR for *Orientia* spp. and using the immunoglobulin M (IgM) enzyme linked immunosorbent assay (ELISA), (Scrub Typhus Detect™ IgM ELISA, InBios International Inc., Seattle, WA, USA) for detection of antibodies to *O. tsutsugamushi* antigens.

PCR assay: A novel in-house semi-nested PCR on buffy coat, developed by IPN, was used for detection of partial 56-kDa outer membrane protein gene of *O. tsutsugamushi* [152]. Primers included 2 forward primers with the sequence of (F1): CAATGTCTGCRTTGTCRTTG; (F2): CCKTTTTCIGCTRGTGCGATAG and one reverse primer with sequence of (R): ATAGYAGGYTGAGGHGGYGTAAG. PCR was done with specimens of 114 suspected cases with peripheral blood mononuclear cells (PBMCs) samples (10 suspected cases without PBMCs).

ELISA assays: The Scrub Typhus Detect IgM ELISA (part no. 500242, Lot no. XM5033; InBios International Inc., Seattle, WA, USA) was used for 395 serum samples of 114

suspected cases (95 paired sera hospital admission and discharge, and 19 single admission sera), 103 hospital controls, and 83 community controls. The ELISA used recombinant p56kD type specific antigens of *O. tsutsugamushi* Karp, Kato, Gilliam, and TA716 strains to detect scrub typhus IgM antibodies. The manufacturer's manual was followed exactly. All sera were tested at a 1:100 dilution and the absorbance measured at 450 nm using a microplate reader to give a final optical density (OD@450 nm) result. The test kit was validated for diagnosis of acute scrub typhus in Asian countries [154, 155]. The OD positivity cut-off titer of 0.8 was used – this had previously shown a sensitivity of 91.5%, specificity of 88.3% for admission samples, and SE 69.8% and SP 89.5% for convalescent samples in another study under comparable conditions [155].

3.3.4 Building the questionnaire

The individual questionnaire was built on framework of landscape determinants of disease transmission [205]. The same questionnaire was applied to cases and controls. Participants were asked about 5 main items composed of several specific topical questions: i) socio-economic status, ii) behaviors related to land/sand/soil/grass/bushes and their PPE, iii) species' habitat connections, vi) land use, and v) vector contact (Figure S3.1).
To develop knowledge around the 5 topics, 12 in-depth interviews with two local scrub typhus epidemiologists, four heads of community health centers (where scrub typhus cases occurred the past years) and six residents (farmers, forest workers, governmental officers) explored local cultivation routines, farming seasons, PPE for daily working use, outdoor activities, and local languages. Following this contextualized "daily-life" assessment, a structured questionnaire and an environment observation checklist were constructed.

3.3.5 Pre-testing and revision of the questionnaire

The questionnaire was pretested with 30 residents including former scrub typhus patients. The questionnaire was revised according to i) specificity of questions, ii) understandability and clearness, iii) order (reflecting a daily life cycle), iv) suitability with local context, v) jumping questions, vi) language, and vii) duration of interview. Few cross-check questions were designed information quality checks. Data from pre-testing was not included in our study. The final questionnaire included a total of 96 questions and took about 40 minutes to complete (30 minutes for questions and 10 minutes for the environment observation checklist).

3.3.6 Data collection

Interviews with cases were conducted within 30 days after laboratory confirmation. In parallel, controls were enrolled, tested and interviewed. Data collectors worked at the Department of Epidemiology, IPN, and the team of four all had experiences from the study

in 2014 [206]. A trained laboratory technician joined the field data collection team. Every day, the data supervisor checked total numbers and content of all (paper-based) forms collected. Incomplete forms were completed by the data collector or after re-contacting the participants. This was the case in a total of 10% of questionnaires.

Coordinates of all participants' households were collected using a short form built in Open Data Kit (ODK) on Android devices (Samsung table) [159], before uploading at http://sg.smap.com.au/. GPS accuracy was set up ± 5 meters.

3.3.7 Data management and quality

The unique ID included a group ID. The composite code was cross checked. The completed questionnaires were double entered by 2 independent data entry clerks, using Epi data 3.1 (EpiData Association, Odense M, Denmark). After that the data was compared using Epi data 3.1 Mismatches were corrected case by case using Stata 15.0 (StataCorp, Texas, USA) to have a clean dataset.

3.3.8 Statistical analyses

3.3.8.1 *Generating new variables*

Five PPEs for field work (socks, boots, long/extra shirt, long/extra trousers, and gloves) were in 2 questions: one binary, and one on the frequency of use on a range from 1 to 10 that was further categorized to the binary variable "use of PPE in the field". "Use of PPE in the field" meant that the person used all 5 PPEs with a minimum frequency of 5/10 times when working in the field.

We used a pre-specified meaningful grouping algorithm to combine related exposure variables into a single binary or ordinal composite variable: a) "field work group", b) "work around house group", c) "household (HH) with poor sanitation/conditions", d) "HH surroundings with risk" and e) "workplace environment with risk". The key risk habitats for the presence of infected chiggers in Southeast Asia were (i) forests, bushes, shifting cultivation area, and (ii) water meadows including grassy edges of water bodies and seepages in drier areas [64, 79, 80, 207-209]. All participants/HH/workplace environment characteristics that related to at least to one of these key risk habitats and were defined as the highest risk group. The others were defined as lower risk groups.

In detail, "field work group" was generated as an ordinal composite variable with 5 sub-groups from highest to lowest risk, including: i) work in forest/hilly field and others, ii) work in vegetable garden and others (except forest/hilly areas), iii) work in sugar cane/crop/rice field and others (except forest/hilly areas/vegetable garden), iv) work in fruit/industrial tree gardens and others (except forest/hilly areas/vegetable garden/sugarcane/crop/rice field), and v) no work related to land/sand. By the same way, "work around house group" was

created as an ordinal composite variable with 5 sub-groups, containing: i) watering plants/bonsai or carpenting and others, ii) cleaning around house and others (except watering plants/bonsai or carpenting), iii) clearing bushes/barns and others (except watering plants/bonsai, carpenting, cleaning around house), and iv) no activities around house. “HH with poor sanitation/conditions”, the binary composite variable, was generated as HH with at least one of following characteristics: bushes within 5 meters, a mud yard, a mud house floor, or drainage on yard. Two other binary composite variables, “HH surroundings with risk” and “workplace environment with risk” were defined as HH or workplace surrounded by at least one of four natural characteristics: in/close to forest, in/close to hilly field, near water bodies within 100 meters or bushes within 10 meters.

These composite variables were very useful in examining effect of the full set of related exposure variables, while people or HH could have one or many related exposures of scrub typhus.

3.3.8.2 Statistical analyses

We have used composite (as described above) and single variables from the questionnaire. Descriptive statistics included variables to explore major differences between groups. Comparisons of demographic, social, and potential risk factor variables between cases and all controls were analysed using univariable conditional logistic regression. Strength of associated exposures for scrub typhus cases as opposed to controls was expressed by the matched odds ratio. In primary analyses, we pooled both control groups to ensure sufficient statistical power. We used conditional logistic regression to estimate odds ratios and corresponding 95% confidence intervals. In subsequent analyses, we analysed two control groups separately. Community controls were analysed with conditional logistic regression. For hospital controls we preferred adjusted logistic regression as recommended [210]. All analyses were conducted using Stata 15.0 (StataCorp, Texas, USA).

In initial analyses, all potential risk factors were selected by biological plausibility, professional knowledge via literature review, and prior analyses adjusted for field study experience before consideration in models. Subsequently, all potential explanatory variables with $p<0.1$ in matching univariable models were retained and considered in multivariable models using pooled sample data. The multivariable model was derived using manual backward selection, and considered for effects of retained explanatory variables, confounders and interaction terms. Comparing Akaike information criterion (AIC) of the models, we decided which model had the best fit to our data. In addition, we had the other two models, one comparing cases and hospital controls (subsample 1) and one for cases and community controls (subsample 2). Using the selected potential risk factor variables, the same data analyses procedure described above was applied to two subsamples. We present here results of the final model.

To build distribution maps, satellite imagery of Khanh Hoa captured from Google Earth was used to create a base-map. GIS software ArcGIS 10.6.1 (Esri, California, USA) was used for mapping.

3.4 RESULTS

The study flow chart is shown in Figure 3.1. A total of 114 suspected acute scrub typhus cases were initially enrolled to the study. Paired blood specimens (admission and discharge) were collected from 95 suspected cases and admission samples alone from 19 ones. Buffy coats were collected from 104 suspects. Forty-five of the 114 suspects were positive with PCR buffy coat and/or ELISA IgM. After exclusion of one positive due to living outside the study area, we included 44 confirmed cases in the case-control study. Eighty-three hospital controls were initially aligned to the matching-criteria to the 44 confirmed cases, however, 13 of these were excluded for other criteria. Finally, 70 hospital controls were included. We enrolled 82 eligible community controls. In summary, we included data of 196 participants, whereof, 44 confirmed scrub typhus cases and 152 controls.

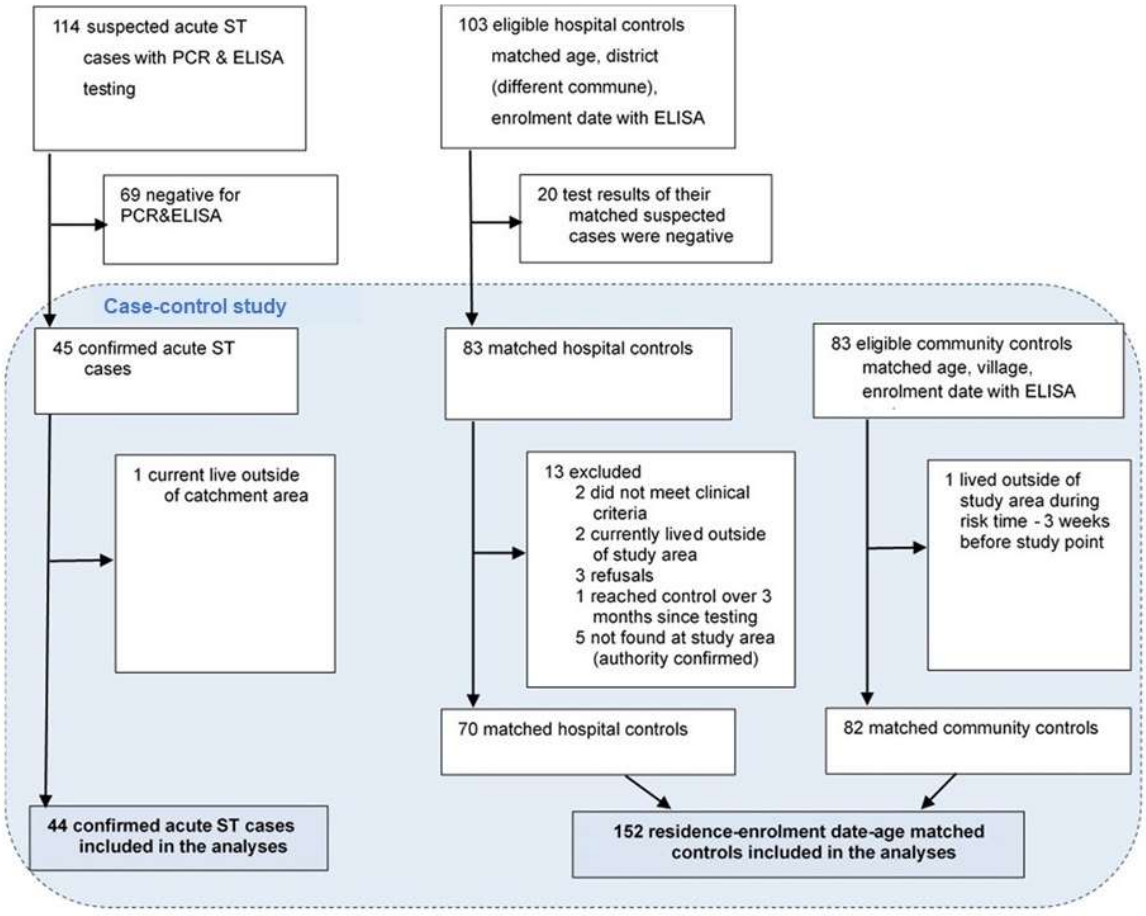


Figure 3.2. Study flow chart- Overview of enrolment of scrub typhus cases, hospital and community controls

3.4.1 Socio-demographic findings and geographic distributions

Among cases and controls 61.4% and 42.8% were males, respectively. The largest age group was 41–50 years old, and comparable between the case and control groups. The two groups were balanced with respect to ethnicity, education level, family size, excepting occupations (9.1% farmers among cases and 21.1% among controls) and working in forests/mountain fields (27.3% among cases and 11.2% among controls). The majority of cases and controls had no history of scrub typhus (90.9% and 98.7%, respectively) (Table 3.1)

Table 3.1. Socio-demographic comparison between scrub typhus cases and controls, Khanh Hoa, August 2018–March 2020

Variables	**Cases (*n* = 44) *n* (%)**	**Controls (*n* = 152) *n* (%)**	***P*-value**	**m*OR* (95%*CI*)***
Age group, year				
≤ 30	12 (27.3%)	45 (29.6%)	..	..
31–40	11 (25.0%)	27 (17.8%)	0.29	1.9 (0.6–6.4)
41–50	13 (29.6%)	47 (30.9%)	0.78	1.2 (0.3–5.3)
≥ 51	8 (18.2%)	33 (21.7%)	0.98	1.0 (0.2–6.5)
Sex				
Male	27 (61.4%)	65 (42.8%)	0.05	2.0 (1.0–4.1)
Female	17 (38.6%)	87 (57.2%)	..	..
Ethical group				
Kinh	38 (86.4%)	125 (82.8%)	0.35	1.8 (0.6–5.9)
Others	6 (13.6%)	26 (17.2%)	..	..
Education				
Illiteracy	2 (4.7%)	10 (6.6%)	..	..
Primary school	16 (37.2%)	35 (23.0%)	0.34	2.3 (0.4–12.4)
Secondary school	11 (25.6%)	60 (39.5%)	0.87	0.9 (0.2–4.7)
High school	11 (25.6%)	30 (19.7%)	0.56	1.6 (0.3–8.7)
University and higher	3 (7.0%)	17 (11.2%)	0.69	0.7 (0.1–5.0)
Occupation				
Not related field/sea/sand	16 (36.4%)	78 (51.3%)	..	..
Farmer	4 (9.1%)	32 (21.1%)	0.17	0.6 (0.4–2.1)
Gardening, growing vegetables	4 (9.1%)	6 (4.0%)	0.11	3.5 (0.8–16.0)
Working in forests/ mountain fields	12 (27.3%)	17 (11.2%)	0.006	**4.5 (1.5–12.9)**
Others	8 (18.8%)	19 (12.5%)	0.24	1.9 (0.7–5.4)
Number of people living in same household				
1–3 people/household	9 (20.5%)	40 (26.5%)	..	..
4–5 people/household	22 (50.0%)	75 (49.7%)	0.43	1.4 (0.6–3.5)
≥ 6 people/household	13 (29.6%)	36 (23.8%)	0.30	1.7 (0.6–4.8)
History of scrub typhus				
Participants 2 years prior to study				
Yes	4 (9.1%)	2 (1.3%)	0.02	7.3 (1.3–39.9)
No	40 (90.9%)	150 (98.7%)	..	..
Family members 2 years prior to study				
Yes	2 (4.7%)	5 (3.3%)	0.64	1.5 (0.3–8.5)
No	41 (95.4%)	147 (96.7%)	..	..
Number of years living in same house				
≤ 1 year	2 (4.6%)	2 (1.3%)	..	..
2–3 years	2 (4.6%)	6 (4.0%)	0.48	0.4 (0.04–4.6)
≥ 4 years	40 (90.9%)	144 (94.7%)	0.22	0.3 (0.04–2.1)

** mOR: matched odds ratio, using conditional logistic regression ** **bold**: P-value <0.05*

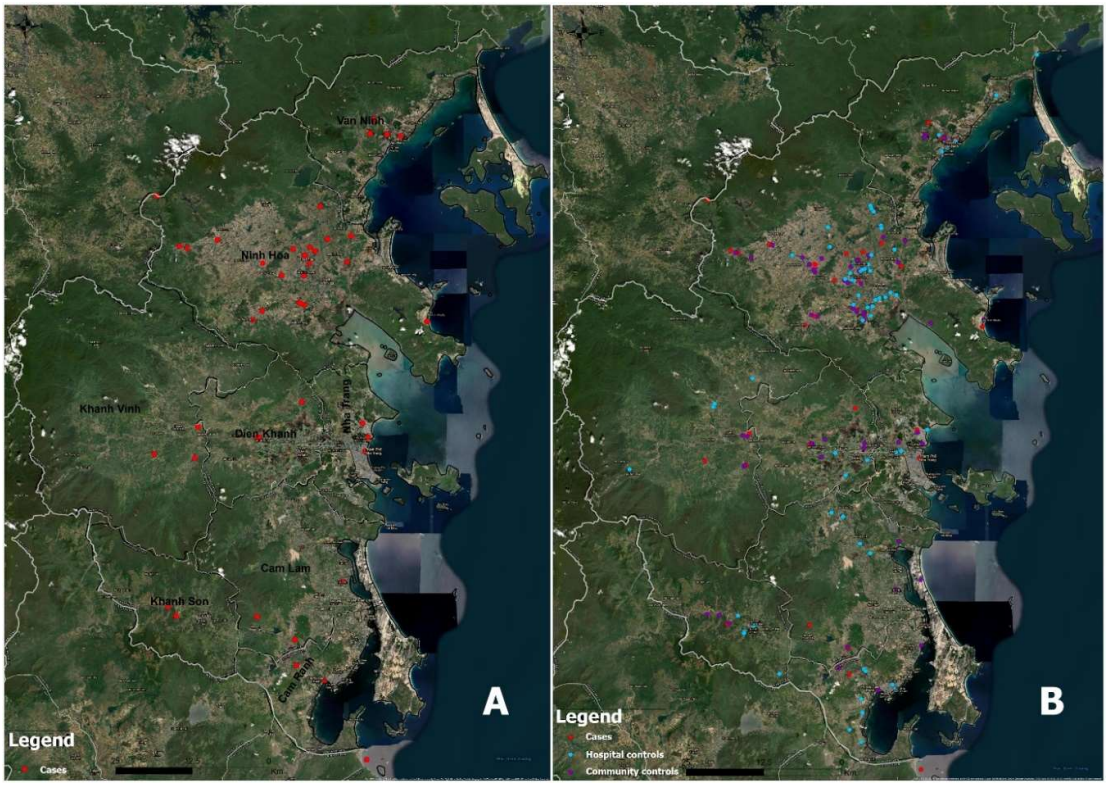


Figure 3.3 Geographic distributions of scrub typhus cases and controls, Khanh Hoa, August 2018 - March 2020

We plotted geographic distributions of scrub typhus confirmed cases (A) and controls (B) (Figure 3.3Figure 3.3). Scrub typhus cases occurred in all 8 districts. There were differences in numbers of confirmed cases across the districts. Most cases occurred in Ninh Hoa while a few were in other districts. Cases were observed in all zones, including flat areas, near mountains, near forests, or alongside beaches.

3.4.2 Risk factors

Exposures associated with scrub typhus in univariable conditional logistic regression are provided in Table 3.2. Cases were more likely than controls to get scrub typhus when they worked in forest/hilly areas (matched odds ratio (m*OR)* = 23.9, 95% *CI*: 5.8–98.2), worked in fruit/industrial tree gardens (m*OR* = 10.5, 95% *CI*: 1.6–69.4), urinated in the forest/near bushes/field (m*OR* = 5.6; 95% *CI*: 2.4–13.1), passed regularly at the river side (m*OR* = 2.8, 95% *CI*: 1.4–5.6), used the same work clothes the next day (m*OR* = 2.4, 95% *CI*: 1.0–5.6), sat/laid directly on the HH floor (m*OR* = 3.9, 95% *CI*: 1.7–8.7).

Always observing mice around home (m*OR* = 3.1, 95% *CI*: 1.5–6.6), HH surroundings with risk (m*OR* = 8.4; 95% *CI*: 2.4–29.1), and workplace environment with risk (m*OR* = 3.6, 95% *CI*: 1.7–7.7) were associated with increased acquisition risk. Lower risks for acquiring scrub

typhus were the use of PPE in the field and changing clothes when at home,(m*OR* = 0.3, 95% *CI*: 0.2–0.7 and m*OR* = 0.3, 95% *CI*: 0.1– 0.8, respectively).

Table 3.2. Exposures associated with scrub typhus in Khanh Hoa, Vietnam, August 2018 - March 2020

Variables	Cases (*n* = 44) *n* (%)	Controls (*n* = 152) *n* (%)	*P*-value	m*OR* (95% *CI*)[#]
Field work group				
Work in forest/ hilly field and others	26 (59.1%)	24 (15.8%)	< 0.001	**23.9 (5.8–**
Work in vegetable garden and	6 (13.6%)	15 (9.9%)	0.07	**3.9 (0.9–16.6)**
Work in sugarcane/crop/rice field	3 (6.8%)	37 (24.3%)	0.88	0.9 (0.1–5.2)
Work in fruit/industrial tree garden	3 (6.8%)	8 (5.3%)	0.02	**10.5 (1.6–**
No work related to land/sand	6 (13.6%)	68 (44.7%)	..	..
Use of Personal Protective Equipment in the field				
Yes	14 (31.8%)	86 (56.6%)	0.004	**0.3 (0.1–0.7)**
No	30 (68.2%)	66 (43.4%)	..	..
Urinating in the forest/near bushes/field				
Yes	25 (56.8%)	34 (22.4%)	< 0.001	**5.6 (2.4–13.1)**
No	19 (43.2%)	118 (77.6%)	..	..
Using the same work clothes the next day				
Yes	15 (34.1%)	30 (19.7%)	0.05	2.4 (1.0–5.6)
No	29 (65.9%)	122 (80.3%)	..	..
Changing clothes when at home				
Yes	34 (77.3%)	140 (92.1%)	0.01	**0.3 (0.1–0.8)**
No	10 (22.7%)	12 (2.9%)	..	..
Always observing mice around				
Yes	21 (47.73%)	37 (24.34%)	0.003	**3.1 (1.5–6.6)**
No	23 (52.27%)	115 (75.66%)	..	..
Work around house group				
Watering plants/bonsai or carpenting + others	19 (43.2%)	54 (35.5%)	0.48	1.3 (0.6–2.9)
Cleaning around the house +	6 (13.6%)	34 (22.4%)	0.46	0.7 (0.2–1.9)
Clearing bushes/barns + others	4 (9.1%)	9 (5.9%)	0.36	1.9 (0.4–7.2)
No activities around house	15 (34.1%)	55 (36.2%)	..	..
Passing regularly at the riverside				
Yes	23 (52.3%)	42 (27.6%)	0.004	**2.8 (1.3–5.6)**
No	21 (47.7%)	110 (72.4)		

Variables	Cases (*n* = 44) *n* (%)	Controls (*n* = 152) *n* (%)	*P*-value	m*OR* (95% *CI*)[#]
Sitting/laying directly on household floor				
Yes	23 (52.3%)	40 (26.7%)	0.001	**3.9 (1.7–8.7)**
No	21 (47.7%)	110 (73.3%)		
Household with poor sanitation/conditions				
Yes	41 (93.2%)	96 (63.2%)	<0.001	**8.4 (2.4-29.1)**
No	3 (6.8%)	56 (36.8%)		
Household surroundings with risk				
Yes	22 (50%)	45 (29.6%)	0.007	**3.1 (1.4-7.2)**
No	22 (50%)	107 (70.4%)		
Workplace enviroment with risk				
Yes	30 (68.18%)	58 (38.16%)	0.001	**3.6 (1.7–7.7)**
No	14 (31.82%)	94 (61.84%)	..	..

*[#] mOR: matched odds ratio, using univariable conditional logistic regression, ** **bold**: P-value <0.05*

The most relevant factors associated with scrub typhus in the multivariable conditional logistic regression model are presented in Table 3.3. We found four significant risk factors and one protective determinant for scrub typhus, including: sitting/laying directly on HH floor (adjusted odds ratio (a*OR)* = 4.9, 95% *CI*: 1.6–15.1), HH with poor sanitation/conditions (a*OR* = 7.9, 95% *CI*: 1.9–32.9), workplace environment with risk (a*OR* = 3.0, 95% *CI*: 1.2–7.6), always observing mice around home (a*OR* = 3.7, 95% *CI*: 1.4–9.9) and, as protective factor with low statistical evidence, use of PPE in the field (a*OR* = 0.4, 95% *CI*: 0.1–1.1).

Risk factors among cases-hospital controls (i.e. living in different communes) were not similar to those among cases-community controls (i.e. living in same commune). Among people living in different communes, always observing mice around home, workplace environment with risk were associated with increased scrub typhus risk (aOR = 5.4, 95% CI: 1.7–17.1, aOR = 4.9, 95% CI: 1.6–15.3, respectively). Changing clothes when at home was likely to protect from scrub typhus (aOR = 0.1, 95% CI: 0.02–0.6). Among persons in same commune, sitting/laying directly on HH floor and adult men were risk factors of scrub typhus (aOR = 35.3, 95% CI: 3.4–368.8, aOR = 6.3, 95% CI: 1.1–34.4, respectively). Use of PPE in the field was a potential factor to protect from bites of mites in same endemic communes (aOR = 0.21, 95% CI: 0.04–1.09) (Table S3.2 and Table S3.3).

Table 3.3. Risk factors of scrub typhus resulting from the conditional multivariable logistic regression*

Risk factors	**Cases (*n* = 44)** *n* **(%)**	**Controls (*n* = 152)** *n* **(%)**	**m*OR* (95% *CI*)[#]**	**a*OR* (95% *CI*)[##]**	***P*-value**
Sitting/laying directly on HH floor	23 (52.3%)	40 (26.7%)	3.9 (1.7–8.7)	4.9 (1.6–15.1)	0.006
Use of Personal Protective Equipment in the field	14 (31.8%)	86 (56.6%)	0.3 (0.2–0.7)	0.4 (0.1–1.1)	0.076
Household with poor sanitation/conditions	41 (93.2%)	96 (63.2%)	8.4 (2.4–29.1)	7.9 (1.9–32.9)	0.005
Workplace environment with risk	30 (68.18%)	58 (38.16%)	3.6 (1.7–7.7)	3.0 (1.2–7.6)	0.020
Always observing mice around home homeAlways	21 (47.73%)	37 (24.34%)	3.1 (1.5–6.6)	3.7 (1.4–9.9)	0.008
Sex (males)	27 (61.4%)	65 (42.8%)	2.0 (1.0–4.1)	1.4 (0.5–3.8)	0.518

** The model adjusted for: sex, field work group, use of personal protective equipment in the field, urinating in the forest/near bushes/field, using the same work clothes the next day, changing clothes when at home, always observing of mice around home, raising cattle, seeing chickens that you raise have mites, passing riverside, sitting/laying directly on household floor, household with poor sanitation/conditions, household surroundings with risk, workplace environment with risk.*

[#] mOR: matching odds ratio, using univariable conditional logistic regression, [##] aOR: adjusted odds ratio, using multivariable conditional logistic regression.

3.5 DISCUSSION

Scrub typhus is acquired through the bite of infected chiggers. Based on biological plausibility pathways, we present the discussion in order from proximal (direct chigger exposure) to distal risk factors (always observing mice around home). The five main factors for scrub typhus acquisition in this study were i) sitting/laying directly on HH floor, ii) use of PPE in the field, iii) HH with poor sanitation/conditions, iv) workplace environment with risk, and v) always observing mice around home.

3.5.1 Sitting/laying directly on the HH floor

Sitting/laying directly on HH floor was the most direct exposure to acquire scrub typhus in our endemic area (aOR = 4.9, 95% CI: 1.6–15.1). One likely explanation is that people were bitten by infected chiggers when sitting/laying directly on HH floor. Chiggers of mites of *Leptotrombidium deliense* and *Ascoschoengastia (Laurentella) indica* (species of *Trombiculidae* mite family) were key chigger species transmitting *Orientia* spp. in Southeast Asia and found on indoor and outdoor mice [1-3]. In Khanh Hoa, *L. deliense and A. indica* were found on house mice, accounting for 11.9% of all detected transmission mites [53]. Among house rodents, the proportion of mite infestation was for *Rattus norvegicus* 82,7%, followed by *R. flavipestus* (66,7%), and *R. exulans* (34,1%). Proportions of *R. norvegicus* and *R. flavipestus* with *Orientia* spp. positivity were 1.7% and 0.24%, respectively (Khanh Hoa, 2013-2014) [44]. One scrub typhus paediatric patient (10 months) in Khanh Hoa was confirmed by IPN in 2013 [36]. Evidence suggests that sitting/laying directly on HH floor, is strongly associated with an increased risk of acquiring scrub typhus.

3.5.2 Use of PPE in the field

Benefits of PPE usage in preventing scrub typhus were is reported. Most authors considered a benefit of wearing gumboots, aprons, long-sleeved shirt, long-sleeved clothes as separate items [73, 74, 211, 212]. In this study, we did not observe benefits of single measures -our findings rather suggest that an advantage lies in using the full PPE, including all 5 items: socks, boots, long/extra shirt, long/extra trousers, and gloves, potential to protect persons from bites of mites in the endemic area (a*OR* = 0.4, 95% *CI*: 0.1–1.1). This highlights that people at risk need a full protective equipment to prevent infected mites climbing up any body parts while working/sitting in the field.

3.5.3 Households with poor sanitation or conditions

Households with poor sanitation/conditions (HH with drainage on ground-not including grassy edges of water bodies, or HH with a muddy/sandy yard or HH with a muddy floor)

represented an important risk factor in this study. Water drainage or seepage in drier areas was defined as a crucial micro-ecology favoring the presence of chiggers and increased risk of acquiring scrub typhus [64, 79, 80]. Common chigger habitats are e.g. grassy edges, soil dampness, and a well nearby the household [79, 80, 213], but water drainage on the household ground/premises was not considered [77, 196, 214]. A sandy/muddy yard or a muddy house floor take up dampness through water drainage after rainfall, which fosters survival of chiggers. Chigger abundance could be maintained by sprinkling the ground with water after rains in Malaysia [215].

3.5.4 Workplace environment with risk

Workplace environment with risk (i.e. close to forest/hilly field/water bodies within 100 meters or bushes within 10 meters) was associated with scrub typhus acquisition (a*OR* = 3.0, 95% *CI*: 1.2–7.6). To note is that more importantly than occupation. Working in forests/hilly fields were common risk factors [39, 66, 73]. However, in previous studies, daily activities were often indicated as individual 'long-term' activities [66], rather than "workplace environment". In our study, we expressed "workplace environment with risk" based on the wide diversity of work activities in this surrounding and by duration (p. ex. 1 hour/day), rather than by one long-lasting single work. In Khanh Hoa, not being named by any specified job title, working close to forests/hilly fields included varied typical occupations (self-business/hired) such as herding cows, mowing grass for livestock, picking up firewood, planting vegetation into forest streams, wood truck driver, loading fruits/sugarcane. Therefore, "workplace environment with risk" was a more relevant and broader definition than a single type of occupation. This approach may be more useful for medical staffs to locate risk areas and initiate preventive measures, than focusing only on specific jobs/occupations.

Working near water bodies (lakes/ponds/streams/channels/wells/irrigation systems surrounded by vegetation or seepages within 100 meters) was a major risk factor for scrub typhus [79, 80, 213]. We identified typical local seepages such as sand fields, beaches, coastlines as a special working habitat of people planting garlic on sand, fishermen, throwing fishing nets, and catching seafood. This is geographically relevant for Khanh Hoa [202]. Sandy fields and beaches represent preferred environments for mites and chiggers [64]. *Orientia* spp. vectors and hosts have been described along sandy beaches in Malaysia [216]. *Leptotrombidium arenicola* spp. has been detected in vegetation alongside beaches in Southeast Asia [208, 217].

3.5.5 Farmer or agriculture work is a "vague" risk factor definition in Vietnam

Farmers represent a high risk population for scrub typhus due to their long time exposure in fields [66, 73, 218]. However, we could not find this, which could be explained by following reasons: i) different rates of pre-existing immunity through repeated exposure in highly endemic areas; ii) a patchy distribution of chiggers in the environment (chigger islands), which are dependent on rodent density in this region, iii) a different perception/definition of occupation as "farmer", and iv) multiple people perform multiple work-related activities, representing a "mixed" risk profile.

Association between farmer/occupation and scrub typhus varied across endemic areas, depending on both activity and environment in which cases were exposed to. A hospital-based study reported that agricultural labour was associated with an increased risk for scrub typhus in Jiangsu province, China (a*OR* = 2.9, 95% *CI*: 1.5–5.8) [219]. However, in Uttarakhand, India, housewives (52%) and students (28%) were the two major occupational subgroups among scrub typhus patients (while farmers were 11.11%), thus likely representing previously non-exposed people with no pre-existing immunity, that participated in local harvests in fields, rather than their usual urban-based occupations [220]. Among community-based studies, an association between farming and occurrence of scrub typhus was reported for India (a*OR* = 2.0, 95%*CI:* 1.1–3.5 [221], m*OR* = 10.0; 95% *CI*: 2.7–63.0 [73]) and for the Lao PDR (m*OR* = 2.1, 95% *CI*:1.0–4.2) [67]. However, in a scrub typhus outbreak in Guangzhou, China (2012), no patients engaged in agricultural activities. The outbreak was finally assigned due to outdoor exercises in a local park [72].

This study revealed that the term "farmer" or "agricultural work" is often perceived as a "vague" definition in Vietnam. This was more pronounced in rural study areas, and often people defined themselves as farmers when their family owned a field, even if they hired others for farming or did no agricultural work for a long time. In-depth clarification of our study revealed that a proportion of 25.5% agricultural labours did no farming within the preceding 3 weeks prior to onset. In contrast, 27.5% persons indicating that they were officers/factory workers/students/small businessmen, involved agricultural/forest activities during that period. Further, a person - hired for agricultural work (cultivating, harvesting for 2–10 days), described themselves as "work-for-hire", not as "farmer". Therefore, using occupation/farmer to examine risk of scrub typhus in Vietnam could introduce bias into associations.

People with multiple occupations/work activities were common in our study setting. A participant could have multiple jobs, i.e. a nurse after night work-shift had a day-off and mowed grass for cows; or a hotel waiter worked as gardener for the hotel and as tour guide. Over 50% participants had at least 3 varied activities in the preceding 3 weeks exposure

period, often including farming, cultivating crops (hired), cow herding (hired), fishing, planting acacia trees on hilly areas. A high mobility was noted among these patients. In the 3 weeks before disease onset, next to cultivating, a person could work as wood/sugarcane truck driver, loading fruits, herding cows, mowing grass, laying bricks (hired) or coastal fishing. Farming is a seasonal occupation, whereby most do not work full-time in the fields, even during the 3-4 months farming season. Therefore, in “non-farming time”, these people are occupied with other exposures/jobs/activities.

The collated data suggests that farmer/occupation is likely not a clear indicator of scrub typhus infection. Workplace environment is more practical than farmer/occupation to examine that association. Workplace environment, covering and representative for all activities in such risk area, is likely a better approach for a risk assessment of acquiring scrub typhus.

3.5.6 Always observing mice around home

“Always observing mice around home” was one of the risk factors for scrub typhus in this study, similar to other studies [72, 73] (a*OR* = 3.7, 95% *CI*: 1.4–9.9). This finding is supported by available evidence on the presence of *Orientia* spp. infected *Leptotrombidium deliense* spp. and *Ascoschoengastia indica* spp. among house rodents in Khanh Hoa [53].

3.5.7 Difference in risk factors between hospital and community controls

In independent analyses of case-community controls, we found that sitting/laying directly on HH floor was a risk factor of scrub typhus only among persons in the same commune, whereas “always observing mice around home” was not (a*OR* = 2.7, 95% *CI*: 0.7–10.3,

Table S3.2). These findings could be explained due to the fact that confirmed cases and their community controls lived in the same area (within 500 meters) and shared similar ecological characteristics such as mice/rodent density [64]. Therefore, we did not observe a difference in presence of mice around their houses. However, sitting/laying directly on HH floor was the most important risk factor among persons in the same communes. It was likely the strong evidence on association between direct infected mite exposure and scrub typhus, confirming transmission of *O. tsutsugamushi* spp. among mites and between mites-house mice in these hyper endemic communes. Independent analyses of case-hospital controls (i.e. living in different communes) showed that always observing mice around home was more clearly associated with *O. tsutsugamushi* infection, with a*OR* = 5.4, 95% *CI*: 1.7–17.1 (

Table S3.3). This finding was also supported for transmission of *O. tsutsugamushi* spp. among mites and house mice in cases’ communes.

3.5.8 Study highlights

Reported common risk factors for scrub typhus -are bushes around house/working place [53, 66, 69, 73, 75, 214, 222], always observing mice around home [72, 73], and working near forest/hilly fields [39, 66, 73]. In this study, we highlighted additional factors for Khanh Hoa, including (i) sitting/laying directly on HH floor; (ii) use of full PPE set (socks, boots, long/extra shirt, long/extra trousers, gloves) in the field, (iii) changing of clothes when at home; (iv) HH with sandy or muddy grounds/a muddy floor, or HH with drainage on ground; and (v) workplace near sandy fields. This study assessed ecological and behavioural risk factors, based on the full landscape framework [205] and an ecological epidemiology approach in a comprehensive in-depth questionnaire, which allowed the identification of proximal and more distal ecological factors.

3.5.9 Consequences for control and elimination

Facing challenges to detect scrub typhus cases in Vietnam despite limitations in diagnostics, the risk factors found in this study, combined with better known clinical symptoms and epidemiology, should be presented in training courses at hospitals and for doctors trained on the emerging scrub typhus. Epidemiological risk factors and illness case-detection have improved with this study.

The factors identified in this study are useful to support establishment of preventive measures, inform regional surveillance, and promote much-needed effective public health responses against scrub typhus after many decades of neglect in Vietnam. Currently, there are no disease prevention and control strategy for scrub typhus or rodents in Khanh Hoa and Vietnam -however, we found this an important risk factor. To note is that rodent control in settlements can also prevent other diseases such as plague or leptospirosis [223]. Facing current limitations in diagnostic capacity for scrub typhus in Central Vietnam, especially at primary health care levels, these findings will support local education and allow local people in hyper-endemic areas to increase their risk factor knowledge and protect themselves from scrub typhus.

3.5.10 Limitations of the study

This was an exploratory study given that no known set of risk factors for Vietnam could be confirmed. A retrospective study design is prone to recall bias. However, in this study, defining and enrolling participants to the study was no longer than 1 month since the date of the case confirmation and asking for a 3 week exposure period to minimise recall bias. The initial sample size was adjusted due to the COVID-19 pandemic, which reduces statistical power to detect associations between use of PPE in the field and scrub typhus.

However, the study was obviously sufficiently powered to detect several important risk factors. Further, our results might only apply for provinces in same ecological and endemic zone like Khanh Hoa, which are the 11 central provinces in Vietnam.

3.6 Conclusions

Ecological and household hygiene related factors such as HH with poor sanitation/conditions, always observing mice around home, and workplace environment with risk were associated with *Orientia* spp. infection, rather than individual-level exposure activities. Use of PPE in the field and changing clothes when at home were potential protective factors.

3.7 Abbreviations

PCR: Polymerase Chain Reaction; IFA: Indirect immunofluorescence assay EDTA: Ethylenediaminetetraacetic acid; ELISA: Enzyme-Linked Immunosorbent Assay; COVID-19: Coronavirus disease 2019; IgM: Immunoglobulin M; PPE: Personal protective equipment; ID: Identification number; HH: household; AIC: Akaike information criterion. IPN: Nha Trang Pasteur Institute

3.8 Appendix

3.8.1 Acknowledgments

We would like to express our deep gratitude to the directors, senior hospital managers, staffs working at the planing and general departments, medical doctors, nurses and patients at the 11 study hospital: Khanh Hoa Provincial Hospital; Ninh Hoa Hospital, Khanh Hoa Hospital for Tropical Diseases; the 87 Army Hospitals and 7 district hospitals for their generous support and interest in this project. Special thanks to Phung Tan Le, the Deputy Director, and Quan Hong Do of the Khanh Hoa Department of Health, for their approval of the study implemetation in Khanh Hoa province. We would like to give many thanks to Mlacha Yeromin for his help on the case and control distribution maps. Further, we are grateful to Mai Quang Vien and Hung Do Manh for their help and advice in setting up this study. Finally, we would like to give special thanks to all heads and staffs of the commune health centers, and the village health workers for their great support in verification of participants and assisting in the field study investigations.

3.8.2 Funding

Research reported in this publication is a part of Hanh Tran's PhD project "Scrub typhus in Khanh Hoa, Viet Nam: an eco-epidemiological approach". This ongoing PhD work is financially partly covered by the Swiss Government Excellence Scholarship Award from

9/2017 to 12/2020. The remaining salary is covered by Esther Schelling and Daniel H. Paris. Esther Schelling funded the data collection and field trips. Daniel H. Paris funded the laboratory assays and technical laboratory training course. This data collection in Vietnam was also supported by a Wolfermann-Nägeli-Stiftung Award for Project Tsutsugamushi-Fieber (scrub typhus) in Khanh Hoa, Viet Nam (Hanh Thi Duc Tran PhD Candidate). The content is solely the responsibility of the authors.

3.8.3 Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

3.8.4 Ethical approval and consent to participate

Ethical approval was granted by the Scientific and Ethical Committee in Biomedical Research, Hanoi University of Public Health (No.382/2018/YTCC-HD3 and No.329/2019/ YTCC-HD3) and by the Ethics Committee of Northwestern and Central Switzerland (Ethikkommission Nordwest- und Zentralschweiz, EKNZ) (BASEC-Nr-2018-00974). The study implementation was approved by the Provincial Health Department of Khanh Hoa at the document No 2192/ SYT-NVY signed 16 August 2018. All participants were asked for their consent before enrolling in the study and had the right to withdraw from the study at any time without any threat or disadvantage. All patients provided written informed consent prior to study enrollment and sample collection.

3.8.5 Competing interests

The authors declare that there are no competing interests.

3.8.6 Consent for publication

Not appliable.

3.8.7 Authors' contributions

Conceptualization and study design: ES, HTDT, JZ; Data collection: HTDT, HMD, HHH, TTH, HNL; Data curation: HTDT, JH; Data analysis: HTDT, JH, LTHV; Laboratory work: MHK; Writing – review & editing: HTDT, JH, LTHV, JZ, DHP, ES. All authors read and approved the final manuscript.

3.8.8 Author details

[1] Department of Epidemiology and Public Health, Swiss Tropical and Public Health Institute, Basel, Switzerland, [2] Department of Epidemiology, Hanoi University of Public Health, Hanoi, Vietnam, [3] University of Basel, Basel, Switzerland, [4] Department for Infectious Disease Control and Prevention, Nha Trang Pasteur Institute, Khanh Hoa, Vietnam, [5] Institute of Gastroenterology and Hepatology, Vietnam, [6] Department of Microbiology and Immunology, Nha Trang Pasteur Institute, Khanh Hoa, Vietnam, [7] Faculty of Medicine, University of Basel, Basel, Switzerland, [8] Department of Medicine, Swiss Tropical and Public Health Institute, Basel, Switzerland, [9] Vétérinaires Sans Frontières Suisse, Bern, Switzerland.

Table S3.1 Inclusion and exclusion criteria of scrub typhus suspected, confirmed cases and hospital controls, community controls

Suspected acute scrub typhus case	Confirmed acute scrub typhus cases	Hospital controls	Community controls
Demography			
Inclusion criteria: •Age ≥ 16 years old •Resident of Khanh Hoa province •Living in Khanh Hoa province for ≥ 6 months prior to study enrolment	**Inclusion criteria:** Same as suspects	**Inclusion criteria:** •Age ≥ 16 years old •Resident of Khanh Hoa province •Living in Khanh Hoa province for ≥ 6 months in the same district and, but different commune as the case, matching for urban – rural areas (according to classification of the statistical office) •+/- 10 years of the case •**Exclusion criteria:** •Army members, tourists	**Inclusion criteria:** •Age ≥ 16 years old •Resident of Khanh Hoa province •Living in Khanh Hoa province for ≥ 6 months in the same village as the case •Present in the commune 3 weeks prior to enrolment •+/- 10 years of the case **Exclusion criteria:** •Army members, tourists
Clinical			
Inclusion criteria •Patient with undifferentiated acute fever (temperature using axilla ≥37.5^{O}C) and having had at least one of the following twelve secondary symptoms: eschar, non-specific skin rash, headache, myalgia, retro-orbital pain, congestion of the conjunctival blood vessels, tinnitus, lymphadenopathy (regional/body), hepatomegaly, splenomegaly, dry cough, dyspnoea without upper respiratory tract discharge. **Exclusion criteria:** •Patients diagnosed with malaria, dengue fever, measles, influenza, bacterial pneumonia, urinary tract infections, based on strong clinical suspicion	**Inclusion criteria** Same as suspects	**Inclusion criteria:** •Hospitalized >24 hours •Diagnosed with any disease other than scrub typhus without fever or with ≥ 4 days of fever [1] •Hospitalized within 14 days since the case's enrolment date. **Exclusion criteria:** •Patients with dengue fever, malaria, HIV/AIDS and TB, •Patients with limited mobility caused by past surgeries or illness	**Inclusion criteria:** •People with without fever or with ≥ 4 days of fever, but without scrub typhus diagnosis **Exclusion criteria:** •People with dengue fever or malaria at the time of the study, HIV/AIDS and TB, •Patients with limited mobility caused by past surgeries or illness •People hospitalized within 1 month prior to study enrolment

Laboratory			
Exclusion criteria: • Positive (rapid) laboratory test results with malaria, dengue fever (confirmed by NS1), measles, influenza, bacterial pneumonia, urinary tract infections.	**Inclusion criteria:** • Positive of PCR on buffy coat and/or ELISA IgM with OD cut-off of 0.8 [155]	**Inclusion criteria:** • The ELISA IgM negative for scrub typhus with OD cut-off of 0.8 [155]	**Inclusion criteria:** • The ELISA IgM negative for scrub typhus with OD cut-off of 0.8 [155]
Exposure period			
	21 days (3 weeks) before symptoms/fever onset [1]	21 days before fever onset or 21 days before hospitalisation if controls have no fever [1]	21 days before fever onset or before study enrolment [1]

Table S3.2 Risk factors of scrub typhus resulting from case-hospital control analyses

Variables	Cases (N=44) n (%)	Controls (N=70) n (%)	aOR (95%CI)[#]	P-value
Gender (male)	27 (61.4%)	35 (50.0%)	0.9 (0.3-3.0)	0.890
Urinating in the forest/near bushes/field	25 (56.8%)	12 (17.1%)	2.3 (0.7-7.2)	0.151
Changing clothes when at home	34 (77.3%)	68 (97.1%)	0.1 (0.0-0.6)	0.014
Observation of mice around the home always	21 (47.7%)	15 (21.4%)	5.4 (1.7-17.1)	0.004
HH with poor sanitation/conditions	41 (93.2%)	37 (52.9%)	7.1 (1.6-32.1)	0.011
Workplace environment with risk	30 (68.2%)	16 (22.9%)	4.9 (1.6-15.3)	0.006
Age group				
≤30	12 (27.3%)	22 (31.4%)	-	
31-45	15 (34.1%)	23 (32.9%)	0.9 (0.3-3.4)	0.919
≥46	17(38.6%)	25 (35.7%)	1.0 (0.3-3.6)	0.977
District with mainly flat area				
Yes	25 (56.8%)	39 (55.7%)	1.7 (0.5-5.2)	0.373
No	19 (43.2%)	31 (44.3%)	-	

** The model adjusted for: sex, field work group, use of PPE in the field, urinating in the forest/near bushes/field, using the same work clothes the next day, changing clothes when at home, observation of mice around the home always, raising cattle, seeing chickens that you raise have mites, passing riverside, sitting/laying directly on HH floor, HH with poor sanitation/conditions, HH surroundings with risk, workplace environment with risk.*

[#] aOR: adjusted odds ratio, using multivariable normal logistic regression

Table S3.3 Risk factors of scrub typhus resulting from case-community control analyses

Variables	Cases n (%)	Controls n (%)	aOR (95%CI)[#]	P-value
Gender (male)	27 (61.4%)	30 (36.6%)	6.3 (1.1-34.4)	0.035
Use of PPE in the field	14 (31.8%)	43 (52.4%)	0.2 (0.0-1.1)	0.065
Urinating in the forest/near bushes/field	25 (56.8%)	22 (26.8%)	2.6 (0.7-10.0)	0.170
Observation of mice around the home always	21 (47.7%)	22 (26.8%)	2.7 (0.7-10.3)	0.146
Sitting/laying directly on the HH floor	23 (52.3%)	21 (25.6%)	35.3 (3.4-368.8)	0.003
HH with poor sanitation/conditions	41 (93.2%)	59 (72.0%)	9.7 (1.2-80.9)	0.035
Workplace environment with risk	30 (68.2%)	42 (51.2%)	3.3 (0.8-12.7)	0.086

** The model adjusted for: sex, field work group, use of PPE in the field, urinating in the forest/near bushes/field, using the same work clothes the next day, changing clothes when at home, observation of mice around the home always, raising cattle, seeing chickens that you raise have mites, passing riverside, sitting/laying directly on HH floor, HH with poor sanitation/conditions, HH surroundings with risk, workplace environment with risk.*

[#] aOR: adjusted odds ratio, using multivariable conditional logistic regression

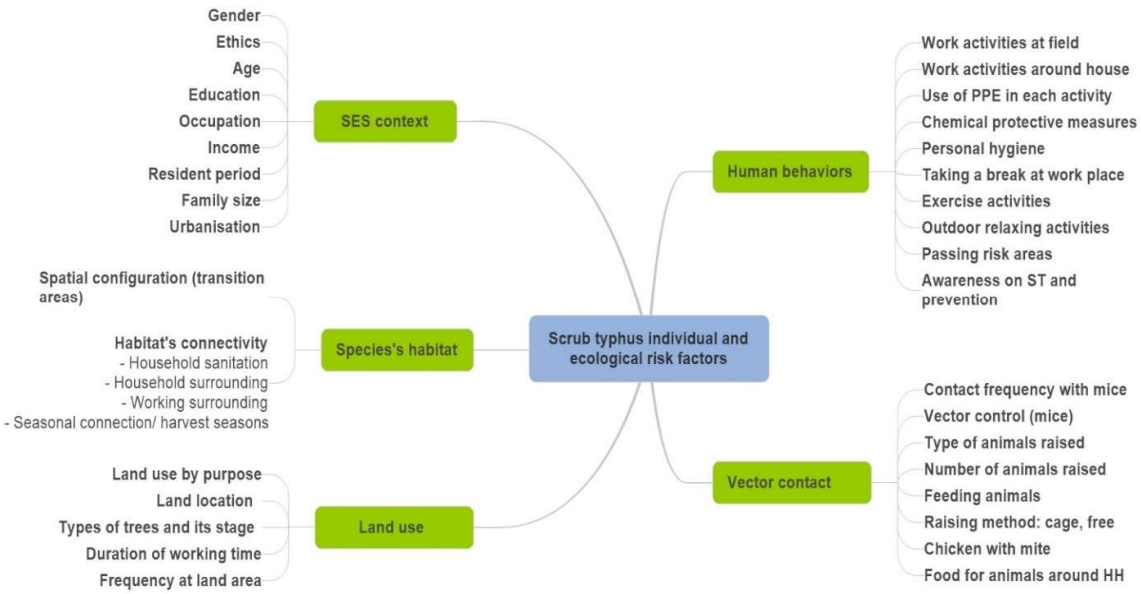


Figure S3.1: Visualisation of 5 main items regarding ST risk factors to be assessed in Khanh Hoa

CHAPTER 4. TEMPORAL DYNAMICS AND PERFORMANCE OF PCR AND SEROLOGICAL DIAGNOSTIC TESTS FOR SCRUB TYPHUS – IN KHANH HOA, CENTRAL VIETNAM

Temporal Dynamics and Performance of PCR and Serological Diagnostic Tests for Scrub Typhus – in Khanh Hoa, Central Vietnam

Hanh Tran[1,2,3], Ngo Thi Quyet[4], Nguyen Bao Trieu[4], Huynh Kim Mai[4], Jan Hattendorf [1], Vien Quang Mai[5], Do Thai Hung[5], Esther Schelling[6], Daniel H. Paris[2, 7]

[1] Department of Epidemiology and Public Health, Swiss Tropical and Public Health Institute, Basel, Switzerland.

[2] Department of Clinical Research, University of Basel, Basel, Switzerland.

[3] Department of Epidemiology, Hanoi University of Public Health, Hanoi, Vietnam.

[4] Department of Microbiology and Immunology, Nha Trang Pasteur Institute, Khanh Hoa, Vietnam.

[5] Nha Trang Pasteur Institute, Khanh Hoa, Vietnam,

[6] Vétérinaires Sans Frontières Suisse, Bern, Switzerland

[7] Department of Medicine, Swiss Tropical and Public Health Institute, Basel, Switzerland.

* corresponding author: daniel.paris@swisstph.ch

Manuscript in preparation for Clinical and Vaccine Immunology ASM Journals
(working paper)

4.1 ABSTRACT

Background: Scrub typhus is re-emerging after 40 years of neglect as an important treatable aetiology among of acute undifferentiated fever in Vietnam. Life-threatening complications are common among patients with scrub typhus experiencing diagnosis and treatment delays; severe complicated scrub typhus is reported in approximately 12%-14% of confirmed cases, with an estimated case-fatality rate of 6-8% if untreated, despite the easily treatable nature of the disease. In Vietnam, the estimated proportion of complicated disease is approximately 15%. Timely diagnosis is crucial, but often complicated by a limited availability of tests and incomplete understanding of the bi-phasic disease dynamics relating to diagnostic modalities. Early diagnosis and appropriate empirical treatment can deter potential morbidity and severe complications. In this study we investigated the temporal dynamics and performance over time for PCR-based and serological assays in early diagnosis of scrub typhus in Vietnam.

Methods: Active hospital surveillance was established for scrub typhus in Khanh Hoa, central Vietnam from January 2013 to December 2014 and from August 2018 to March 2020 in provincial referral hospitals. Whole-blood and eschar swab specimens were collected, suspected cases confirmed by PCR eschar, PCR buffy coat, and/or ELISA IgM (cutoff ≥ 0.8), and “days of fever upon admission” were collected. A cohort of 437 scrub typhus patients with a span of “days of fever” ranging from 1 to 23 days was collected and diagnostic data available for analysis.

Results: A total of 1,038 samples of 437 suspected patients were included in this study. Of these, 420 scrub typhus were confirmed by PCR buffy coat, ELISA IgM (cutoff ≥ 0.8) and an RDT, of which 151/420 (36%) also had PCR eschar available. PCR buffy coat performed best from day 1 to day 6, compared to ELISA and RDT, with an overall positivity rate of 73% during this early phase, but contributed to final diagnosis until day 14 of fever. ELISA IgM and RDTs performed better after day 7 of fever, with positivity rates of 90% and 81%, respectively, in the later phase – but contributed to diagnosis from day 3 of fever. The combination of PCR buffy coat with an RDT detected 93% to 100% of all positive cases during the first 14 days of fever. PCR using eschar specimens yielded higher positivity rates when compared directly to PCR from buffy coat in paired matched samples.

Conclusions: Although PCR performance is superior during the first 7 days of fever and serology superior to PCR beyond this time point, the use of both modalities during the first 14 days of fever improves overall diagnosis, when compared to the use of single modalities. The RDT shows great promise as a useful point-of-care test, particularly in the primary or remote settings and emergency departments. A combined training package for awareness, knowledge on risk factors and clinical predictors, as well as correct use of RDTs should be

implemented at the primary health care level where rapid diagnosis and early appropriate management can make a large impact on reducing severe scrub typhus and associated mortality.

4.2 INTRODUCTION

Scrub typhus is a major under-diagnosed cause of febrile illness in many parts of Asia [17, 31, 118, 120, 123, 124, 130, 224, 225]. This tropical rickettsial illness is increasingly recognized as an important cause of fever in Vietnam and adjacent countries in the region, including Thailand, Laos, and India [226-229]. In Vietnam, scrub typhus is re-emerging with cases increasing being reported after 40 years of clinical and scientific neglect [16, 17, 22, 206, 230]. In the 1960s, scrub typhus was a common disease among American veterans in Vietnam and recognised as an endemic disease in the midlands and mountainous forests [168]. Only few cases were registered after the discovery and broad usage of chloramphenicol in the 1970s, but since 2000 scrub typhus has been re-emerging and expanding its geographical and population distributions [16, 17]. In recent years, scrub typhus was detected in all regions of Vietnam and recorded as a leading treatable cause of undifferentiated febrile illness (31.1%-40.9%) in Vietnam [15-17, 130, 231, 232].

Timely diagnosis and appropriate empiric treatment in clinically suspected cases can deter potential morbidity and severe complications from scrub typhus [233]. In severe cases, the disease can progress to multi-organ failure, with pathologic lesions in lungs, kidneys, liver, and brain [188, 189]. These complications are mostly observed in patients with delayed and late diagnosis. All of the patients with acute hearing loss due to scrub typhus, a forgotten complication of this neglected disease, had duration of fever for more than 10 days [234]. Pradeep, et al. found that early diagnosis and treatment with doxycycline can prevent the occurrence of acute respiratory distress syndrome (ARDS) which is associated with a high morbidity and mortality among scrub typhus cases [235]. The approximate proportion of complicated scrub typhus was 10% with a median of 6 fever days upon admission. In a recent study in Vietnam, this proportion was estimated at 15%; 76.0% of confirmed patients were hospitalised after 7 days of symptoms, with altered mental status (45/251; 17.9%), jaundice or hyper bilirubinaemia in 42/251 (16.7%) and pulmonary pathology in 39/251 (15.5%) of cases, and three deaths in elderly individuals (3/251, 1.2%) presenting late to hospital with multi-organ failure - corresponding to a 1.2% mortality rate [17]. Complications were also common among scrub typhus patients who did not immediately seek treatment, leading delayed time from the onset of symptoms to treatment to a median of 9 days (IQR 7–11), including altered mental status (10, 9.7%), jaundice or hyperbilirubinemia (24, 40%), and pulmonary pathology (rales) (22, 21.4%) [130].

Accurate timely diagnosis is important to ensure early appropriate treatment. We performed this study to improve our understanding of the temporal aspects of the currently available different diagnostic modalities, in order to improve the early and complete coverage of diagnosis throughout the symptomatic period during which patients seek health care. The move away from the indirect immunofluorescence assay (IFA) to the ELISA format in serology, as well as increased use of molecular tools has already impacted early diagnostics in Thailand, Korea and China [191]. Unfortunately, the knowledge of adequate tools supporting early diagnosis is insufficient to positively impact incidence rates – these must also be employed at the community level where most people seek medical attention early to maximise the use of these tools. Unfortunately, the availability of point-of-care and reference diagnostics remains highly limited in Vietnam, and there is a great need for evaluating the temporal dynamics of DNA and serology based assays (ELISA and RDTs) and for promoting their use at the adequate respective health care setting levels in Vietnam. In this study we asked the following research questions:

1. Until how many days of fever is PCR performance superior over that of serology?
2. Are RDTs suitable for early presentation at rural health care centres?
3. How reliable is serology in the waning phase of bacteremia - and from which timepoint can it be reliably used?
4. Should PCR always be used in combination with a serological test?
5. How does the current RDT perform in direct comparison to ELISAs?

4.3 MATERIALS AND METHODS

4.3.1 Study design

Active hospital surveillance was established for scrub typhus in Khanh Hoa, central Vietnam from January 2013 to December 2014 and from August 2018 to March 2020 in 5 and 11 provincial referral hospitals, respectively (Figure 4.1) These were; Provincial Hospital, Ninh Hoa branch provincial hospital, Dien Khanh district hospital, 87 Army hospital, and Ninh Diem Hospital – and in the second period also the Khanh Hoa Hospital for Tropical Diseases (established in 2015) and Van Ninh, Khanh Son, Khanh Vinh, Cam Ranh, Cam Lam district hospitals. Patients with an undifferented febrile illness and who fulfilled the scrub typhus suspected case definitions (see Table 4.1) were invited for enrolment into the study. Informed consent was obtained from all patients or their guardians prior to sample collection.

4.3.2 Sample collection and preparation

For all patients who gave consent, eschar swabs and whole blood specimens were collected at the admission timepoint of hospitalization with a follow-up sample at discharge. All clinical and laboratory investigations during hospitalization admission were made by trained local physicians or laboratory staff.

Whole-blood specimens were collected from all enrolled patients at the time of admission, in 2 tubes (3 mL/tube), with and without Ethylenediaminetetra acetic acid (EDTA). Whole-blood specimens were collected from patients after leaving hospital in only EDTA tubes (3 mL/tube). These whole-blood specimens were centrifuged at 1800rpm in 15 minutes. After centrifugation, plasma and PBMCs were separated from red blood cells in EDTA tubes, respectively. Buffy coat was collected from EDTA blood and sera were separated from clots in sera tube. This separation was conducted within 24 hours after the sample collection and samples were preserved at -20^{o}C, in the respective laboratory units of the hospitals [152], Samples were subsequently transferred weekly in a cool box to the Pasteur Nha Trang Institute, Vietnam, for PCR, ELISA, and RDT testing [152].

Eschars swab specimens were performed by trained laboratory technicians. The patients participating in the study were thoroughly examined for the presence or absence of eschars. When an eschar was detected, the whole eschar area was scrubbed with a cotton swab wetted with saline (0.9% NaCl solution), and this swab as well as eschar crust if available were collected into tubes with 0,5 mL Phosphate buffered saline (PBS) buffer 1X.

4.3.3 Storing and preparation samples at Pasteur

Samples were checked with submission forms, then labelled and stored at 2-8^{o}C if they were tested within 3 days. Otherwise, the samples were stored at -80^{o}C until testing.

4.3.4 Eschar Specimens

The eschar sample was prepared by masticating it with 500 uL PBS buffer using a ceramic pestle and the ceramic bowl, before collecting it into a new screw-cap tube. Eschar swab samples were vortexed, pressed and the cotton swab removed, before transferring the supernatant into a new screw-cap tube. All samples were stored at -80^{o}C prior to DNA extraction. DNA was extracted from eschar specimens and buffy coat using the QIAamp DNA Mini kit (51304-Qiagen) and QIAamp DNA Blood Mini kit (51104-Qiagen) according to manufacturer's instructions, respectively.

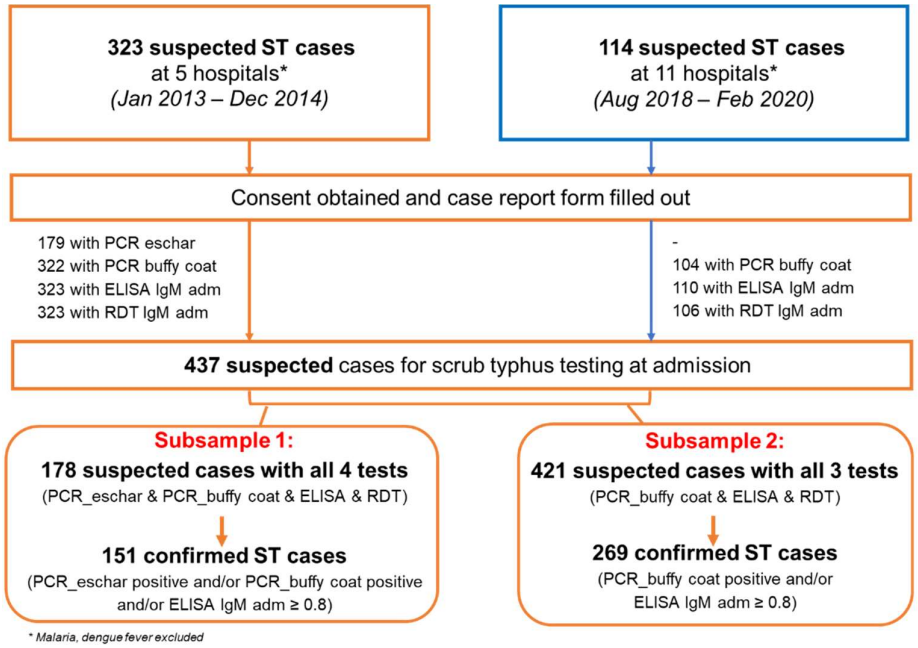


Figure 4.1 Study overview and flow chart for enrolment of suspected scrub typhus cases in Khanh Hoa.

4.3.5 Ethical approval

Ethical approval was given by the local research ethics committee, Scientific and Ethical Committee in Biomedical Research, Hanoi University of Public Health (No. 382/2018/YTCC-HD3 and No.329/2019/ YTCC-HD3) and by the Ethics Committee of Northwestern and Central Switzerland (Ethikkommission Nordwest- und Zentralschweiz, EKNZ) (BASEC-Nr-2018-00974). The study implementation was approved by the Provincial Health Department of Khanh Hoa at the document No 2192/ SYT-NVY signed on 16 August 2018. All participants had given their consent before being enrolled in the study, and they had the right to withdraw from the study at any time without any threats or disadvantages. They were provided written informed consent prior to sample collection.

Table 4.1 Inclusion and exclusion criteria of suspected scrub typhus cases

Surveillance study 1 January 2013 to December 2014	**Surveillance study 2** August 2018 to March 2020
Inclusion criteria: All patients are Khanh Hoa residents with at least one of five criteria: • Persisting fever despite the use of medicine • Presence of an eschar • A suspected case of dengue fever, but with a negative dengue RDT result (NS1-neg.) • A suspected case of malaria, but with a negative test result for malaria (Giemsa stained blood smear) • Long-lasting persisting fever (over 10 days or any form of undifferentiated fever).	**Inclusion criteria:** • Residence in Khanh Hoa • Age ≥ 16 years old • Patient with undifferentiated acute fever (temperature using axilla ≥37.5^{O}C) and having had at least one of the following twelve secondary symptoms: eschar, non-specific skin rash, headache, myalgia, retro-orbital pain, congestion of the conjunctival blood vessels, tinnitus, lymphadenopathy (regional/body), hepatomegaly, splenomegaly, dry cough, dyspnoea without upper respiratory tract discharge.
Exclusion criteria: Patients diagnosed with malaria (confirmed by Giemsa stained blood films method), dengue fever (confirmed by NS1)	**Exclusion criteria:** Patients diagnosed with malaria (confirmed by Giemsa staining method), dengue fever (confirmed by NS1), measles, influenza, bacterial pneumonia, urinary tract infections, based on strong clinical suspicion and/or (rapid) laboratory test results.

4.3.6 Diagnostic tests

Following diagnostic tests and sample specimens were used in this study: PCR using eschar swabs and buffy coat; ELISA (IgM) with serum; and RDTs (IgM) using full blood.

Real-time PCR: In 2013-2014, a qualitative SYBR Green real-time PCR with primers designed from the GroEL gene [153] was used to identify the presence of *O. tsutsugamushi* in 327 patients with 327 peripheral blood mononuclear cells (PBMCs) and 209 eschar samples. The samples were collected in 5 hospitals in Khanh Hoa province in 2013 and 2014. The real-time PCR was performed with 2 µL of DNA extracted from the PBMCs or eschar samples, 0.2 µM forward primer, 0.2 µM reverse primer, nuclease free water, and 10 µL master mix (iQ SYBR Green Supermix reagents, 11733038-Biorad) to obtain a final volume of 20 µL. The amplicon length was 160bp, and the limit of detection of the assay was < 3 copies/µL of *O. tsutsugamushi* [153].

Semi-nested PCR: During 2018-2019, an in-house semi nested PCR [152], validated by the qualitative SYBR green real-time PCR [153], for detection of partial 56-Kda outer membrane protein gene was used to identify the presence of *O. tsutsugamushi* in 28 patients with 28 buffy coat samples (3 patients did not provide buffy coat samples). Primers used for the semi-nested PCR includes 2 forwards primers with the sequence of (F1): CAATGTCTGCRTTGTCRTTG; (F2): CCKTTTTCIGCTRGTGCGATAG and 1 reverse primer with sequence of (R): ATAGYAGGYTGAGGHGGYGTAAG.

The PCR reaction mix contained 2 µL of 10X Taq buffer, 1.6 µL of MgCl2 (25mM), 0.4 µL dNTPs (20mM), 2 µL of each primer, 0.3 µL Taq DNA Polymerase, and 29.7 µL nuclease free water (QIAGEN). The first PCR step was performed with 2.5 µL of DNA extracted from the buffy coat or eschar. The second step was performed with 2.0 µL of the amplicon of the first PCR step.

The first PCR step was performed for 1 cycle of initial polymerase activation at 94°C for 3 min; followed by 35 cycles at 94°C for 20 s, 54°C for 20 s, and 72°C for 30 s; and a final elongation step was done at 72°C for 5 min. The second PCR step was performed at 94°C for 3 min; followed by 25 cycles at 94°C for 20 s, 55°C for 20 s, and 72°C for 30 s; and a final elongation step was done at 72°C for 5 min.

The PCR products were analyzed by electrophoresis on a 2% agarose gel; the products were stained with SYBR Safe and were observed under an UV transilluminator.

ELISA (IgM): The Scrub Typhus Detect ELISA system for IgM Test (part no. 500242, Lot no. XM5057; InBios International Inc., Seattle, WA, USA) was used for the detection of IgM antibodies to *O. tsutsugamushi* in 110 serum samples of 110 patients in 2018-2019 and 323 samples in 2014. The Scrub Typhus Detect ELISA system for IgM Test uses recombinant p56kD type specific antigens of *Orientia tsutsugamushi* Karp, Kato, Gilliam, and TA716 strains to detect anti-*O. tsutsugamushi* IgM antibodies. The manufacturer's methods were followed exactly. All sera were tested at a 1:100 dilution and the absorbance was determined at 450 nm (OD@450 nm) using a microplate reader to give a final optical density (OD) result. The test kit was validated and recommended for the diagnosis of scrub typhus in SE-Asian countries [154, 155]. An OD cut-off of 0.8 showed a sensitivity of 91.5%, specificity of 88.3% for admission samples, in the similar setting of high endemicity of Bangladesh – these criteria were applied to confirm our cases among suspected scrub typhus infection [155].

Rapid diagnostic test (RDT): Besides, a Scrub Typhus Detect IgM Rapid Test Casstte (Inbios) (Lot no. XE5255; InBios International Inc., Seattle, WA, USA) was used to detect rapidly IgM antibodies to *O. tsutsugamushi* in 323 serum samples in 2018-2019 and 106 serum samples in 2014 of suspected ST patients. The nitrocellulose strip is pre-coated with a mixture of novel recombinants (representing several geographical isolates) on the test

line region. During testing, the sample reacts with the dye conjugate (anti-human IgM colloidal gold conjugate) which has been pre-coated in the test device, then reacts with the *O. tsutsugamushi* derived recombinant antigens on the membrane and generates a red line. The process followed the manufacturer's instructions. The results were read after 15-17 minutes. A faint line was considered a positive.

4.3.7 Data analysis

Descriptive statistics included counts, proportions and percentages for qualitative variables, and medians and interquartile ranges (IQR) for quantitative variables used in the data analysis. All positive scrub typhus cases and their fever duration on admission (days) were used to present the numbers, proportions of the confirmed cases across the days of fever.

4.4 RESULTS

A total of 1,038 samples were included in this study from a total of 437 suspected scrub typhus cases; including 179 eschar samples, 426 buffy coat samples, and 433 serum samples. All clinical samples were received and tested by the Department of Microbiology and Immunology, Nha Trang Pasteur Institute, Khanh Hoa, Vietnam.

The study flow chart is shown in Figure 4.1. We screened 1,038 samples of 437 suspected patients who met the enrolment criteria. All patients gave consent prior to being enrolled into the study. A total of 179 patient admission samples were available for eschar PCR, 426 were available for buffy coat PCR and 433 for serologic testing. Of the enrolled patients, 66.6% (291/437) had a robust diagnosis of scrub typhus with one or more positive test results by PCR eschar, PCR buffy coat, and ELISA IgM ≥ 0.8 (Table 4.2). The median days of illness and IQR before presentation was 7 days (IQR 5-10 days).

A total of 178 suspected scrub typhus patients were tested by all of four test modalities including PCR eschar, PCR buffy coat, ELISA and RDT at admission (subsample 1). Among them, 72.5% had positive results for PCR eschar, 50.0% for PCR buffy coat, and 68.0% for ELISA (Table 4.2).

A total of 421 suspected scrub typhus patients were tested by all of three tests, PCR buffy coat, ELISA IgM and RDT, at admission (subsample 2). Among them, 40.9% had positive results of PCR buffy coat, and 69.8% had positive results of ELISA (Table 4.2)

Table 4.2 Overview of diagnostic results for all suspected scrub typhus cases included in this study (n=437)

	RDT adm (rapid test)		PCR buffy coat		ELISA Adm_08		PCR eschar	
2013-2014								
Positive/Yes	186	57.6%	149	46.3%	203	62.9%	130	72.6%
Negative/No	137	42.4%	173	53.7%	120	37.1%	49	27.4%
Total	**323**	**100.0%**	**322**	**100.0%**	**323**	**100.0%**	**179**	**100.0%**
2018-2020								
Positive/Yes	34	32.1%	23	22.1%	102	92.7%		
Negative/No	72	67.9%	81	77.9%	8	7.3%		
Total	**106**	**100.0%**	**104**	**100.0%**	**110**	**100.0%**		
All period								
Positive/Yes	220	51.3%	172	40.4%	305	70.4%	130	72.6%
Negative/No	209	48.7%	254	59.6%	128	29.6%	49	27.4%
Total	**429**	**100.0%**	**426**	**100.0%**	**433**	**100.0%**	**179**	**100.0%**

Subsample 1: Suspected cases with data for all 4 test modalities available (n=178): (pcr_es, pcr_bc, ELISA and RDT)

	RDT adm		PCR eschar		PCR buffy coat		ELISA Adm_08	
Positive/Yes	113	63.5%	129	72.5%	89	50.0%	121	68.0%
Negative/No	65	36.5%	49	27.5%	89	50.0%	57	32.0%
Total	**178**	**100.0%**	**178**	**100.0%**	**178**	**100.0%**	**178**	**100.0%**

Subsample 2: Suspected cases with data for 3 test modalities available (n=421): (pcr_bc, ELISA and RDT)

	RDT adm		PCR buffy coat		ELISA Adm_08	
Positive/Yes	216	51.3%	172	40.9%	294	69.8%
Negative/No	205	48.7%	249	59.1%	127	30.2%
Total	**421**	**100.0%**	**421**	**100.0%**	**421**	**100.0%**

Among confirmed cases with positive results for PCR buffy coat and/or ELISA (total n=269), CR buffy coat positivity was greater or equal to that of ELISA and/or RDT until day 6 (Figure 4.2, Figure 4.3, and Figure 4.4). During this early phase of 1-6 days of fever, PCR buffy coat positivity was found in 73%-100% of cases each day, while ELISA positivity ranged from 0%-78%, and RDT positivity ranged from 0%-75% (Figure 4.2 and Table S4.1).

From 7 days of fever onwards, the proportion of positives by ELISA and by RDT was consistently higher than that of PCR buffy coat Figure 4.2.

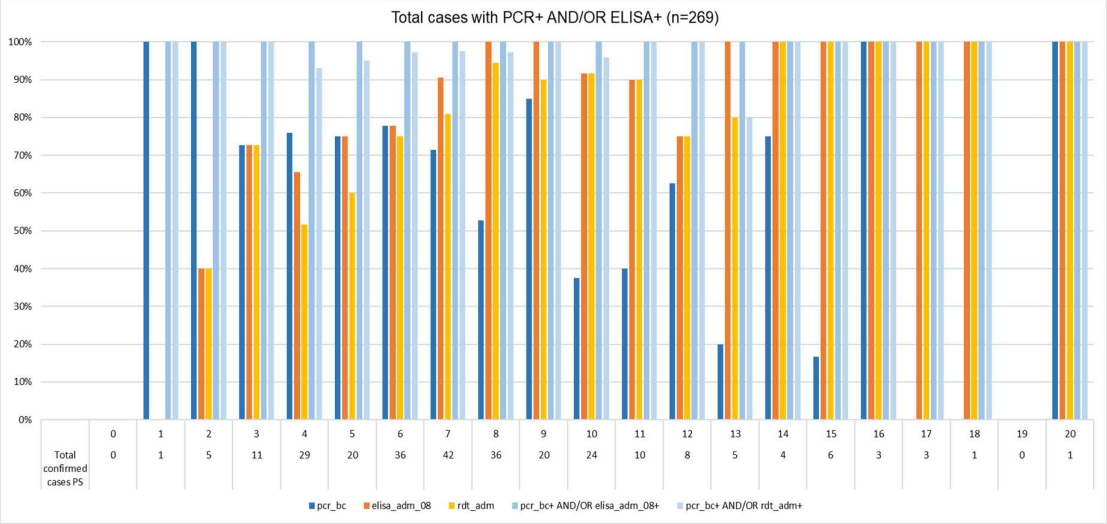


Figure 4.2 Proportions of positivity diagnosed by PCR buffy coat, IgM ELISA, IgM RDT over time.

The x-axis represents "Days of fever on admission" and "total confirmed case per day"; the first row states the days of fever reported on admission, and the second row states the total confirmed cases included per each respective day. The y-axis represents percentage.

All suspected scrub typhus patients (n=437) with diagnostic results available for PCR buffy coat, ELISA and RDT were collated in a temporal chart. The majority of patients presented with a history of 4-10 days of fever (median 7 days, IQR 6-10). From day 7 of "fever days on admission" serology tests clearly outperform PCR tests. The added benefit of PCR in addition to ELISA after day 7 however was not zero, as PCR diagnosed ELISA-negative cases on days 7, 10, 11, 12 - ranging from 0% to 25% of diagnoses per day in this period. Similarly, ELISA contributed to overall diagnosis in the first 6 "days of fever on admission", by detecting PCR-negative cases on days 3, 4, 5 and 6 – which ranged from 0% to 27% of diagnoses per day (Figure 4.5 and Table 4.3).

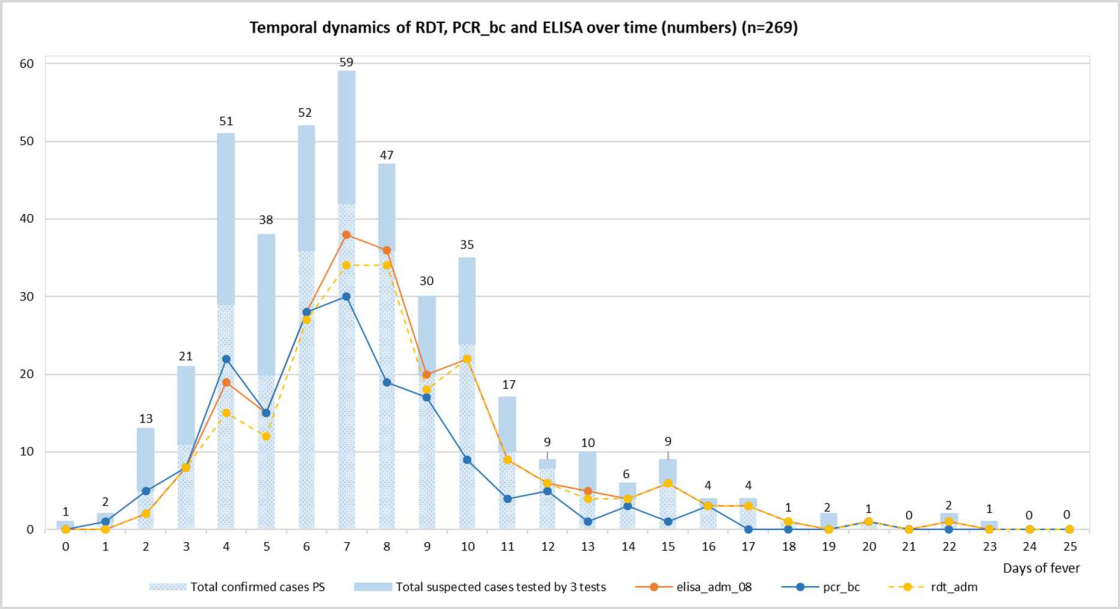


Figure 4.3 Temporal dynamics of RDT, PCR buffy coat and ELISA positive results in absolute numbers over time.

X-axis represents "days of fever on admission", y-axis represents absolute patient numbers. The number of suspected cases seen per each day is represented by the blue bar (exact number stated above), of which the pale shaded blue bar represents the proportion of cases confirmed by PCR and/or ELISA by (single positive RDT results were not included as confirmed cases).

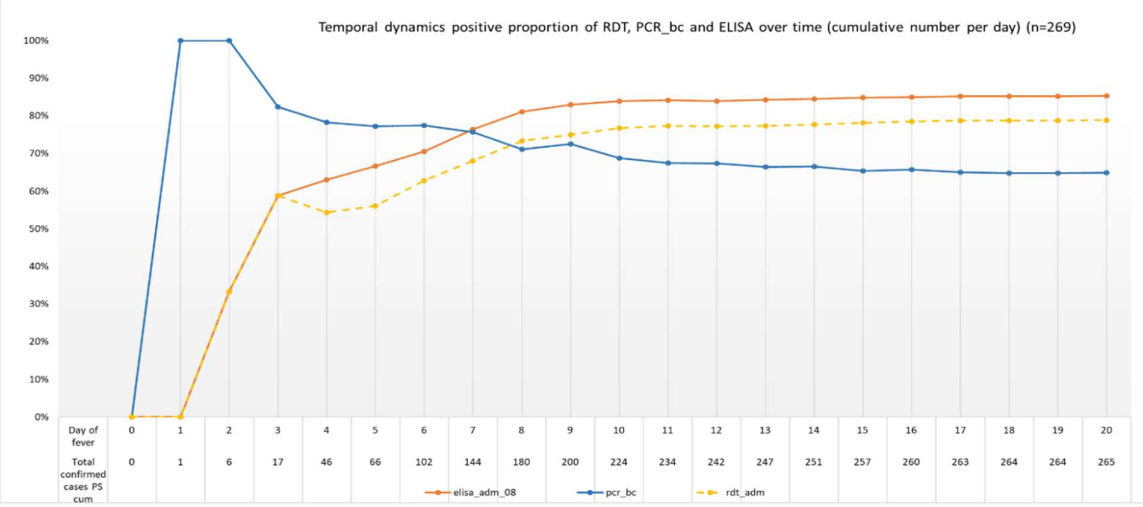


Figure 4.4 Temporal dynamics depicting the cumulative positivity proportions of RDT, PCR_bc and ELISA over time ("cumulative number of all positives by each diagnostic modality per day" divided by "cumulative number of all confirmed cases per day").

The x-axis represents "Days of fever on admission" and "total confirmed case per day"; the first row states the days of fever reported on admission, and the second row states the total confirmed cases included per each respective day. The y-axis represents percentage.

If all confirmed cases are added up day-by-day and the proportion diagnosed by PCR calculated as a cumulative positivity proportion, then the maximum proportion of diagnoses in this cohort by PCR-positivity plateaus out at 63%. This graph shows that the value of PCR is higher than serology during the early stages of disease (days of fever on admission), until day 7 (where ELISA is slightly superior) and day 8 (where RDT is superior for the first time) – after 7 DOF serology starts to outperform molecular diagnosis. Additionally this means that of all samples measured, a maximum of 65% are positive by PCR buffy coat, 85% for ELISA and 79% for RDT among the confirmed cases.

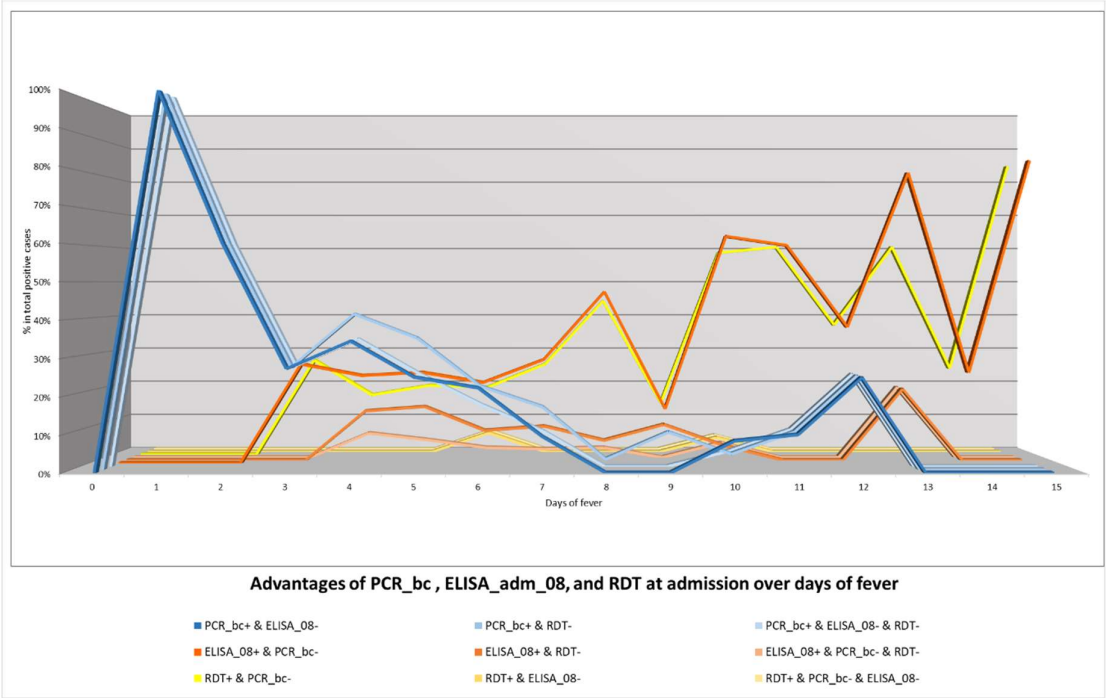


Figure 4.5 Combined diagnostic modalities of PCR_bc, ELISA and RDT depicted in a time course according to “days of fever on admission”.

The x-axis represents “Days of fever on admission” and the y-axis represents percentage positivity of all patients per day. Blue, orange and yellow lines represent PCR-positivity, ELISA-positivity and RDT-positivity, respectively.

These data reflect the single positivity value of a modality (e.g. PCR), when the other modality (e.g. serology) is negative. This graph highlights the added value of one diagnostic modality when the other is negative – i.e. more PCR-pos. AND ELISA-neg cases occur during the first 6 DOFs – highlighting the importance of using PCR as the preferred diagnostic modality during this period.

If PCR is not available, 60-100% of confirmed cases presenting with only two days of fever will be missed, about 30% would be missed in patients presenting with 3-5 DOF, and then 15% in patients presenting with 6-7 DOF. On the other hand, if ELISA is not available, approximately 30-50% of confirmed cases are missed in patients presenting with 7 or 8

days of fever, 15% for those with 9 DOF and over 60% for those with 10 DOF upwards. The dip in the ELISA curve at day 9 is the result of a high proportion of patients with a positive PCR result – leading to a reduced added value by performing ELISA at this time point (see Figure 4.2 for proportion positivity).

These time course single positivity data demonstrate the benefit and contribution of PCR to ELISA and/or RDT usage in diagnosing scrub typhus in patients with a history of 7 DOFs or higher, and vice versa an early benefit and contribution for ELISAs and/or RDTs in diagnosing scrub typhus can be seen in patients with a history of 7 DOFs or less.

Table 4.3 Overview of results from all possible combinations of diagnostic modalities

The different modalities were combined, and all results listed in this table. Data from day 7 onwards is shaded grey for ease of reading, data shown until day 15 "days of fever on admission".

Total confirmed cases per	**Total confirmed cases cumul**	**DOF**	***PCR_bc+ ELISA-***	***PCR_bc+ RDT-***	***PCR_bc+ ELISA-RDT-***	***ELISA_08+ PCR_bc-***	***ELISA_08+ RDT-***	***ELISA_08+ PCR_bc-RDT-***	***RDT+ PCR_bc-***	***RDT+ ELISA-***	***RDT+ PCR_bc-ELISA 08-***
0	0	**0**	0%	0%	0%	0%	0%	0%	0%	0%	0%
1	1	**1**	100%	100%	100%	0%	0%	0%	0%	0%	0%
5	6	**2**	60%	60%	60%	0%	0%	0%	0%	0%	0%
11	17	**3**	27%	27%	27%	27%	0%	0%	27%	0%	0%
29	46	**4**	34%	41%	34%	24%	14%	7%	17%	0%	0%
20	66	**5**	25%	35%	25%	25%	15%	5%	20%	0%	0%
36	102	**6**	22%	22%	17%	22%	8%	3%	19%	6%	6%
42	144	**7**	10%	17%	10%	29%	10%	2%	26%	0%	0%
36	180	**8**	0%	3%	0%	47%	6%	3%	44%	0%	0%
20	200	**9**	0%	10%	0%	15%	10%	0%	15%	0%	0%
24	224	**10**	8%	4%	4%	63%	4%	4%	58%	4%	4%
10	234	**11**	10%	10%	10%	60%	0%	0%	60%	0%	0%
8	242	**12**	25%	25%	25%	38%	0%	0%	38%	0%	0%
5	247	**13**	0%	0%	0%	80%	20%	20%	60%	0%	0%
4	251	**14**	0%	0%	0%	25%	0%	0%	25%	0%	0%
6	257	**15**	0%	0%	0%	83%	0%	0%	83%	0%	0%

4.4.1 Performance of PCR eschar versus PCR buffy coat

PCR assays using eschar sample specimens (either eschar swab and/or eschar scraping) were superior to PCR assays based on buffy coat specimens, with overall higher positivity rates in confirmed cases and longer duration of positivity (Figure 4.6, Figure 4.7, Table 4.4). In scrub typhus, a fever curve is a surrogate marker for bacteremia and PCR buffy coat results showed a remarkable apparent correlation with the median fever temperature curve when stratified by “days of fever on admission”.

Higher median fever values correlated with higher positivity rates when using PCR buffy coat specimens Figure 4.6. This similarity was observed predominantly in patients presenting during the early phase of scrub typhus – with up to days after fever (Figure 4.6), when the PCR buffy coat assay was on the most advantage (before ELISA took its turn to be the best assay since day 7, Figure 4.2). Fever does not affect to capacity of PCR eschar. Patient with both eschar and buffy coat specimens available were analysed in groups according to their days of fever. Figure 4.7 depicts a time course overview highlighting that – when stratified by fever days – PCR on eschar samples was significantly better (i.e. higher proportions of positivity) at diagnosing scrub typhus than when using buffy coat specimens. Of note, PCR positivity using eschar specimens demonstrated little to no fluctuation in positivity rates, and no correlation with median fever temperatures– representing a more reliable sample specimen, independent of bacteremia fluctuations – especially useful for patients presenting with higher days of fever on admission. Clearly, eschar specimens can only be used if available.

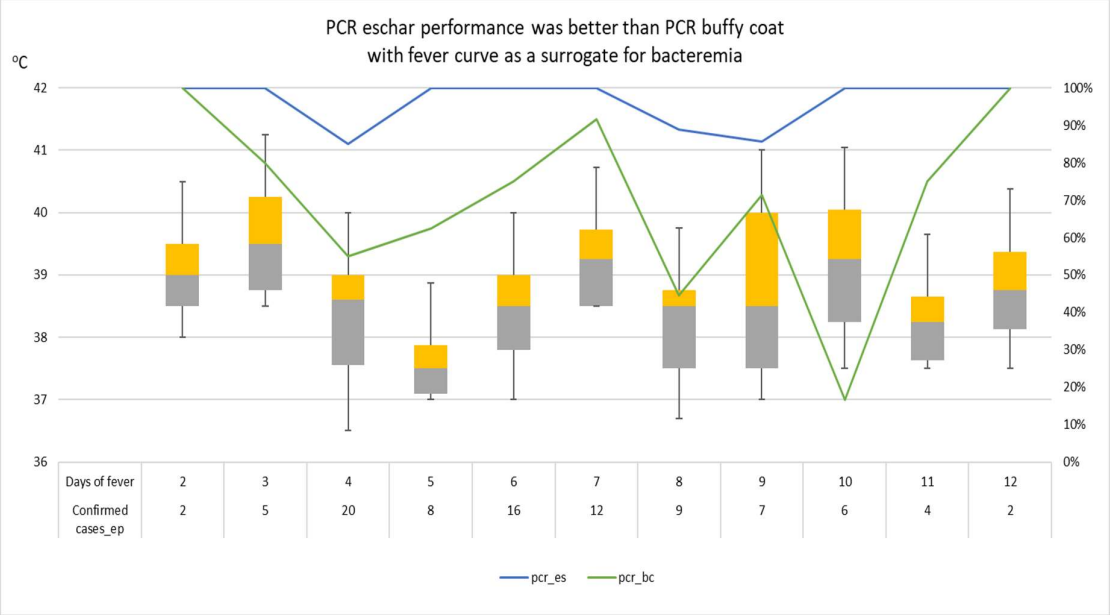


Figure 4.6 PCR eschar performance is superior to PCR buffy coat in patients with available PCR results for both eschar and buffy coat.

The median admission fever values of all patients when stratified by “days of fever on admission” and plotted against PCR-positivity, showed a remarkable apparent correlation [patients with a history of 2 to 12 “days of fever on admission” (n=96)]. PCR using eschar specimens demonstrated higher and more constant capacity of detecting O. tsutsugamushi DNA than assays using buffy coat. The data represents median fever temperature, with IQCs (box) and range (whiskers). The x-axis represents “Days of fever on admission” and “total confirmed case per day”; the first row states the days of fever reported on admission, and the second row states the total confirmed cases with both eschar and buffy coat PCR results available per each respective day. The y-axis represents the median body temperature measured in patients upon admission per day (left axis) and percentage (right axis).

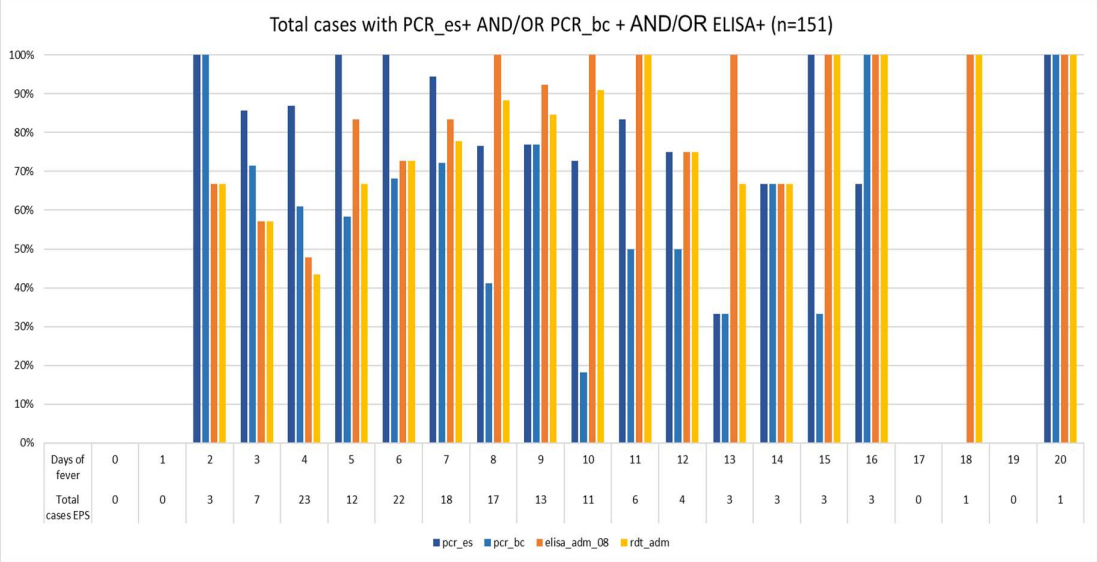


Figure 4.7 Time course plot of positivity proportions for PCR_es, PCR_bc, ELISA_adm and RDT_adm in confirmed patients with results for all four test modalities available (n=151). The x-axis represents “Days of fever on admission” and “total cases with a positive eschar-based PCR result per respective day”. The y-axis represents percentage.

Table 4.4 Comparisons between PCR on eschar versus buffy coat specimens and correlation with fever, stratified by “days of fever on admission”

Days of fever	Confirmed cases	pcr_es	(%)	pcr_bc	(%)	Median temperature (°C)
2	2	2	100	2	100	39.0
3	2	2	100	1	50	39.0
4	9	7	78	6	67	38.3
5	4	4	100	3	75	37.75
6	8	8	100	7	100	38.5
7	9	9	100	8	89	39.0
8	4	4	100	2	50	37.75
9	5	4	80	3	60	37.5
10	4	4	100	0	0	38.5

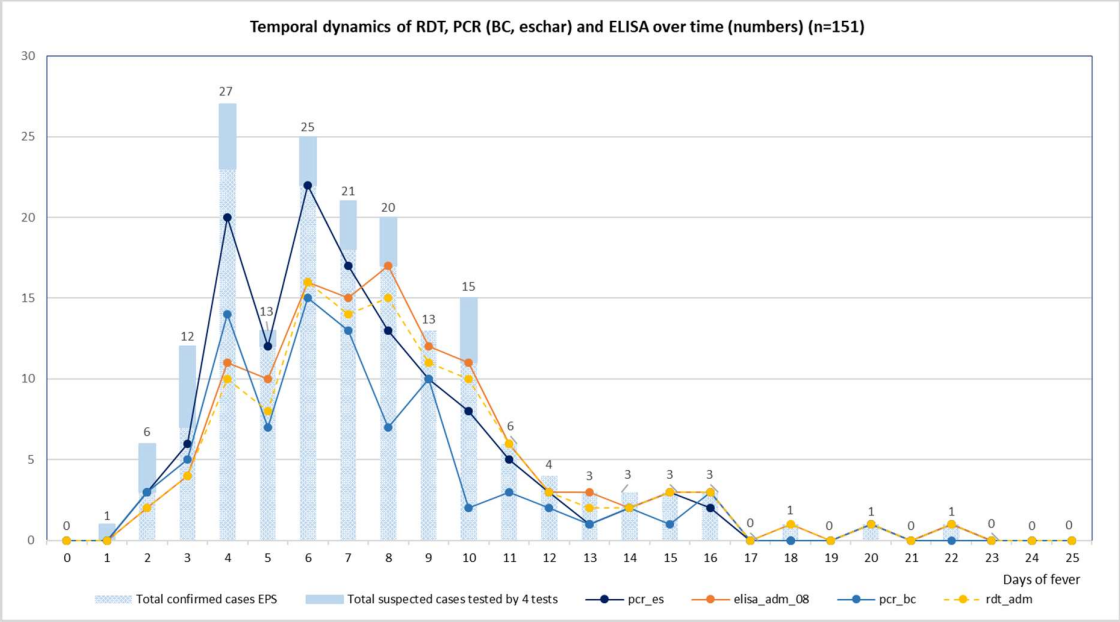


Figure 4.8 Temporal dynamics of ELISA, RDT, PCR buffy coat and PCR eschar positive results in absolute numbers over time.

Note: this graph includes eschar-positive cases. All cases with at least one or more modality testing positive using PCR eschar, PCR buffy coat and ELISA IgM among samples with all 3 tests available (n=151). RDT results are depicted as a dotted yellow line. X-axis represents “days of fever on admission”, y-axis represents absolute patient numbers. The number of suspected cases seen per each day is represented by the blue bar (exact number

stated above), of which the pale shaded blue bar represents the proportion of cases confirmed by PCR and/or ELISA by (single positive RDT results were not included as confirmed cases).

4.5 DISCUSSION

Infection with obligate-intravcellular *Orientia* spp. leads to a systemic impairment of the vasculature. The bi-phasic disease dynamics of scrub typhus include an early blood-borne dissemination and bacteremia, followed by a subsequent antibody phase. The diversity of strains and these disease dynamics have negatively affected the development of adequate diagnostic tools. The major diagnostic modalities for diagnosing scrub typhus include culture, nucleic acid amplification (PCR), and serological assays such as ELISA, rapid diagnostic tests (RDTs), and indirect immunofluorescence assays (IFAs).

For decades the IFA has been the gold standard serological reference assay – but it is cumbersome, requires labor-intensive preparation of microscope slides, high level of expertise and a fluorescence microscope. IFA is not easily implementable in the field and is gradually being replaced by the ELISA as reference serological assay of choice. It was not the aim of this study to perform diagnostic accuracy analyses, but rather trying to put the diagnostic modalities into the context of disease dynamics. For more in-depth diagnostic evaluation, additional diagnostic modalities would have been required – i.e. culture or IFA results in addition to the PCR and ELISA data would have allowed to evaluate the exact performance of the RDT used in this study. Unfortunately, this part of the thesis was not completed due to the COVID-19 pandemic.

In this study we focused on the diagnostic modalities PCR, ELISA and RDTs and their performance during the early bi-phasic disease dynamics. PCR in blood (buffy coat) is likely better to detect *O. tsutsugamushi* during the bacteremic phase, while ELISAs are likely better to detect antibodies in the subsequent humoral immune response. It has been established, that combining PCR with ELISA improves diagnostic accuracy and allows for earlier detection in scrub typhus and other rickettsial illnesses [191].

In order to bring these disease dynamics – namely the early bacteremic and subsequent antibody phases into a useful diagnostic context, the number of “Days of fever prior to admission” is a useful surrogate to inform on where the patient stands in terms of the disease phase, and could offer guidance on when to use which diagnostic modality.

4.5.1 Using PCR and ELISA as a combined reference diagnostic test modality

It became evident that generally, PCR buffy coat performed better than ELISA up to day 6 of fever on admission, and the ELISA became superior over PCR from 7 days of fever onwards. Bacteremia is closely correlated with febrile episodes and during the early

bacteremia prompting fever brings patients to seeking medical attention. The positivity rate of PCR with over 73% of cases detected during this early phase, suggests that the employment of field-usable PCR machines should be seriously considered, maybe even at the community level where possible. It is important to realise that the median fever duration prior to admission at hospital was 7 days (IQR 6-10 days of fever. As there is a switch of diagnostic modality exactly around this time point, it is crucial to dissect the dynamics for a better understanding.

Although ELISAs present a good steady increase of diagnostic sensitivity from day 3 of fever onwards, it only reached detection of 100% of cases on day 13 – after which ELISA was capable of detecting all cases reliably (Figure 4.2). In the early phase, the higher sensitivity of DNA based assays was described previously in Thailand, Bangladesh and India, among the scrub typhus cases with a median days of fever of 5 (range 3-7 days) or less than 7 days [115, 193, 236, 237] (compared to serology-based assays). Other studies also reported the InBios Scrub Typhus Detect ELISA IgM sensitivity at 93%, among patients with low median days of fever of 5.5 days (range 4-7 days) [238]. Similarly, an other study found that IgM antibodies starts appearing from day 7 of fever onwards [29], and ELISAs become the best assay approx. 10 days after fever onset [239].

In this study however, we were able to demonstrate the added benefit of the second modality to the contribution of the first. Although the Inbios ELISA IgM assay was superior in diagnosing scrub typhus from day 7 of fever onwards, it contributed to diagnosing approx. 20% of cases that were missed by PCR buffy coat during days 3 to 6 of fever (Figure 4.5). Thus the contribution of serology prior to day 7 of fever can be seen as substantial and important. Similarly, PCR buffy coat assays – although absolutely crucial in the first 3-4 days of fever, also contributed to increased diagnostic coverage of cases until day 12 of fever. Thus a case can be made that – in this cohort – a combination of PCR and ELISA represent the best diagnostic modality in the first 12 days of fever, after which the use of ELISA alone would be sufficient to diagnose all cases.

The cumulative positivity proportions for the modalities PCR, ELISA and RDT were estimated over 20 days of fever, and an overall maximum of 65% of cases are detected by PCR buffy coat, 85% by ELISA and 79% by RDT among the cohort (Figure 4.4). ELISA assays represent a strong contribution to diagnosing scrub typhus in this setting.

The exact diagnostic efficacy over 12 days of fever for single PCR buffy coat, ELISA IgM assay, and RDT IgM was 67% (163/242), 84% (203/242), and 77% (187/242) respectively - this data suggests that if we use only PCR buffy coat and (no ELISA IgM and/or RDT) for diagnosis, approximately 33% (79/242) of all cases will be missed. If only ELISA assay is used, then 16% (39/242) of all cases would be missed. Combining the PCR buffy coat and ELISA IgM assays increased the overall proportion of positivity to 100% across all 12 first

days of sample collection studied (Figure 4.2). A combined use of PCR buffy coat and RDT IgM test also improved the overall sensitivity and expanded the temporal spectrum of *O. tsutsugamushi* detection, ranging from 93% to 100% detection coverage per day of fever over the first 12 day acute phase (Figure 4.2). These findings suggest the true benefits of a combination DNA-based with serology-based methodology assays, at least with serology IgM RDT, in covering the spectrum of diagnostic positivity for all scrub typhus patients [115, 240].

4.5.2 The benefits and limitations of RDTs

The IgM-based RDT showed a slight lower sensitivity for detecting *Orientia* spp. over the whole incubation period, when compared to IgM-based ELISA. The cumulative proportion of positivity over 20 days of fever suggests a 7% reduced diagnostic detection in the present cohort - with 77% for RDTs versus 84% for ELISAs, respectively (Figure 4.4). In exact terms, during the 12 first days after onset, RDTs missed 19 ELISA-positive cases (8%).

In this study, patients presented with a median of fever days before admission of 7 days (IQR=6-10). The maximum of positivity found in this cohort, defined by PCR buffy coat and/or ELISA IgM ≥0.8 for RDT was 79% (Figure 4.4). This finding is consistent with similar previous studies about the performance of the same IgM RDT assay, IgM InBios rapid test for scrub typhus; the sensitivities reported were 93.9% among sera samples taken from 2 to 10 days after onset of illness in China [241], 90.2% among cases with median of fever of 6 days before admission (IQR:1–47 days) in Thailand [242], and 87.3% among confirmed ST cases with median of fever of 9 days before admission (IQR: 3-20 days) in India [237]. The positive rates of another IgM immunochromatographic test (CareStart assay; AccessBio IgM ICT) were 100% (among group with median days of fever before admission was7 days (IQR:5-10 days), 92% (23/25) (median: 9 days (IQR: 8-10.5 days), and 97% (median: 8 days (IQR: 6-11 days) [243]. The evidence above proved that RDT presented a good performance among suspected scrub typhus cases with median of days fever of about 7 days before admission. The high sensitivity of IgM InBios RDT for scrub typhus described in other study in Thailand during the early phase (< 4 days of fever) reached approximately 80%, but increased to 90% after 8 days of fever [242]. In contrast, among another case population with median duration of fever at the time of admission of 2 days (IQR: 2–3 days), another RDT CareStart Scrub Typhus IgM ICT just detected 23.3% of total confirmed cases [244].

During the first 12 days of fever, the cumulative number diagnosed by PCR buffy coat test was 163 cases (Figure 4.4). In the same period, the cumulative number of cases detected by PCR buffy coat but with a negative RDT result (PCR+ & RDT-) was 48 cases. Therefore, a clear benefit of combining PCR with RDTs was seen - if we do not employ PCR, we will miss 29% (48/163) of all positive cases during the period of the first 12 days of fever.

Nevertheless, the combination of PCR buffy coat and RDT detected at least 93% to 100% of all cases during the first 12 days of fever – which would represent an acceptable alternative if ELISA was not available, p.ex. at a community health centre. If the RDT was used alone the diagnostic positivity proportion beyond 7 days of fever was 81%. The employment of RDTs at community or district level would be beneficial, especially if "days of fever" is taken into consideration – even if no PCR or ELISA is available, the simple repeating of an RDT 24 or 48 hours later could lead to a clear diagnosis.

4.5.3 PCR eschar versus PCR buffy coat

PCR eschar performance was better than PCR buffy coat in patients with both PCR eschar and buffy coat assay for the whole study period. These results are consistent with the findings of another study where 17 (85%) of the eschar specimens and 5 (25%) of the whole blood specimens tested positive for *O. tsutsugamushi*, among 20 tested patients with both eschar and whole blood were reported [245]. Varghese, G. M., et al. (2015) and Nhiem, et al. (2017) also revealed similar findings that eschar samples are superior, when they are available and when compared to blood specimens [245, 246].

Our study also revealed the novel finding that higher fever correlates with higher capacity of PCR buffy coat assay or higher capacity of DNA detection in patient samples. These results align with a recent report in a non-human animal model where temporal dynamics were investigated and where bacteremia correlated with elevated temperatures, and temperature curves followed the same dynamics as bacteremia as measured by qPCR in blood [115]. In this study we quantitated the dramatic improvement if PCR is performed using eschar samples throughout the critical study period of the first 14 days of fever (Figure 4.7). Importantly the dead tissue of the eschar specimen represents a stable specimen not leading to the fluctuations of PCR performance when using blood-derived specimens (Figure 4.6). Eschars – both crust and swabs -also serve as ideal samples containing "preserved" *O. tsutsugamushi* DNA for PCR diagnosis, when empirical treatment has started and bacteremia in blood has declined.

4.5.4 Advantages of the study

Samples collected in this study and the assays employed reflect a real world situation and highlight practical issues regarding early diagnosis and treatment in the context of disease dynamics. Our findings present a basis to improve the current setting with available modalities, until new and improved diagnostic tests become available. All patient samples were collected at the admission time point, before medicine/antibiotic treatment was initiated, therefore these data are not affected by empirical treatment. Few false positive

results were obtained from the patients with dengue fever or falciparum malaria, but these were excluded in our study.

4.5.5 Limitations of the study

This study has some limitations. Firstly, the lack of local positivity cutoff titers in Vietnam may cause confusion about accuracy of the serology diagnostic tests with the indirect immunofluorescence method such as ELISA, RDT and even, IFA, the gold standard test for scrub typhus for decades, although an OD cut-off of 0.8 (a sensitivity of 91.5%, specificity of 88.3% for admission samples), in the similar setting of high endemicity of Bangladesh were applied to confirm our cases among suspected scrub typhus infection [155]. Secondly, the IgM antibody has been known to show some cross reactivity with many acute febrile illnesses, including leptospirosis, pulmonary tuberculosis, enteric fever, *Streptococcus viridans* septicaemia, and typhoid fever. Thirdly, discrepancies between archived samples and prospectively acquired samples may exist, therefore additional studies are required to determine the true diagnostic utility of the ICT. Fourthly, in hyperendemic areas, where a high level of background antibody positivity exists, IgG responses can be pronounced and early – with an accompanied blunted IgM response. Future studies should include both IgG and IgM serological assays, if the study is conducted in highly endemic areas. Fifthly, the study design was not able to systematically assess specificity.

4.6 What next

There is a need to implement better diagnostics for rickettsial illnesses (scrub and murine typhus – and possibly SFG rickettsiosis) – these are intrinsically difficult unfortunately the indirect immunofluorescent assays (IFA) remains in use in many referral hospital laboratories and is not available where diagnosis is needed most – at the district or community levels. The transition to using and employing RDTs and ELISAs is important, as is the development of field-usable PCR machines [30].

This is the third paper of Hanh Tran's publication series about scrub typhus in Vietnam. (The first paper is on the use of clinical and routine laboratory predictors in scrub typhus diagnosis and decision for empirical antibiotic treatment. The second paper is on ecological and behavioural risk factors of scrub typhus in central Vietnam: a case control study. The fourth paper evaluates disease ecology and epidemiology to improve diagnostics, prevention and integrated control of scrub typhus in Vietnam.

4.7 Conclusion

In summary, the RDTs hold great promise as a point-of-care test – even if its performance lies slightly under that of ELISA - particularly in the primary or remote settings and emergency departments, where rapid diagnosis and prompt initiation of appropriate

medication can prove life-saving. RDTs will be of practical benefit in primary and secondary care hospitals where tests such as ELISA or PCR for scrub typhus are not routinely performed.

This study has demonstrated the usefulness of the IgM RDT starting already at day 3 of fever. RDT presented a positivity rate range of 52%-75% from day 3 to day 6 of fever, and over 81% after day 7 of fever. Samples used in this study were collected at the admission time point during the acute phase of infection, before antibiotic treatment, which is the real world situation for early diagnosis and treatment. RDTs are easy to perform in point-of-care settings, and results can be obtained within 15 minutes for proper patient management. However, a combined package with clinical training, risk factors training, and RDTs should be recommended – especially at the primary health care level – to promote accurate diagnosis and treatment of undifferentiated fever in Vietnam [32]. In addition, serodiagnosis in endemic areas should be tailored to the target population/area based on the local cut off positivity titre and validated using a multi-modality standard reference assays approach [247].

4.8 Acknowledgments

We would like to express our deep gratitude to the directors, senior hospital managers, staffs working at the planing and general departments, medical doctors, nurses and patients at the 11 study hospital: Khanh Hoa Provincial Hospital; Ninh Hoa Hospital, Khanh Hoa Hospital for Tropical Diseases; the 87 Army Hospitals and 7 district hospitals for their generous support and interest in this project. Special thanks to Phung Tan Le, the Deputy Director, and Quan Hong Do of the Khanh Hoa Department of Health, for their approval of the study implemetation in Khanh Hoa province. Further, we are grateful to Mai Quang Vien, Hung Do Manh, Hung Do Thai for their help and advice in setting up this study.

4.9 Author contributions

Conceptualization and study design: DHP, HTTD; Local cordinator: MVQ, HDT; Data analysis: DHP, HTDT, JH; Laboratory work: QNT, BNT, MHK; Writing – review & editing: HTTD, QNT, BNT, MHK, DHP. Funding: ES, DHP. All authors read and approved the final manuscript.

Table S4.1 Number of cases and Proportions of positivity diagnosed by PCR buffy coat, IgM ELISA, IgM RDT over time.

DOF	PCR_bc	%	ELISA_adm_08	%	RDT_adm	%	PCR_bc+ AND/OR ELISA_adm_08+	%	PCR_bc+ AND/OR RDT_adm+	%	Total cases PS*	%
0	0	0%	0	0%	0	0%	0	0%	0	0%	0	100%
1	1	100%	0	0%	0	0%	1	100%	1	100%	1	100%
2	5	100%	2	40%	2	40%	5	100%	5	100%	5	100%
3	8	73%	8	73%	8	73%	11	100%	11	100%	11	100%
4	22	76%	19	66%	15	52%	29	100%	27	93%	29	100%
5	15	75%	15	75%	12	60%	20	100%	19	95%	20	100%
6	28	78%	28	78%	27	75%	36	100%	35	97%	36	100%
7	30	71%	38	90%	34	81%	42	100%	41	98%	42	100%
8	19	53%	36	100%	34	94%	36	100%	35	97%	36	100%
9	17	85%	20	100%	18	90%	20	100%	20	100%	20	100%
10	9	38%	22	92%	22	92%	24	100%	23	96%	24	100%
11	4	40%	9	90%	9	90%	10	100%	10	100%	10	100%
12	5	63%	6	75%	6	75%	8	100%	8	100%	8	100%
13	1	20%	5	100%	4	80%	5	100%	4	80%	5	100%
14	3	75%	4	100%	4	100%	4	100%	4	100%	4	100%
15	1	17%	6	100%	6	100%	6	100%	6	100%	6	100%
16	3	100%	3	100%	3	100%	3	100%	3	100%	3	100%
17	0	0%	3	100%	3	100%	3	100%	3	100%	3	100%
18	0	0%	1	100%	1	100%	1	100%	1	100%	1	100%
19	0	0%	0	0%	0	0%	0	0%	0	0%	0	100%
20	1	100%	1	100%	1	100%	1	100%	1	100%	1	100%

* Total cases were confirmed by PCR buffy coat and/or ELISA

CHAPTER 5. SUMMARY DISCUSSION

From disease ecology and epidemiology to improved diagnostics, prevention and integrated control of scrub typhus in Vietnam

Hanh Thi Duc Tran[1, 2, 3*], Jan Hattendorf [1, 3], Esther Schelling [4], Jakob Zinsstag [1, 3], Daniel H. Paris [5, 6]

[1] Department of Epidemiology and Public Health, Swiss Tropical and Public Health Institute, Basel, Switzerland, [2] Department of Epidemiology, Hanoi University of Public Health, Hanoi, Vietnam, [3] University of Basel, Basel, Switzerland, [4] Vétérinaires Sans Frontières Suisse, Bern, Switzerland, [5] Faculty of Medicine, University of Basel, Basel, Switzerland, [6] Department of Medicine, Swiss Tropical and Public Health Institute, Basel, Switzerland.

* corresponding author: thiduchanh.tran@unibas.ch, hanhtranch@gmail.com

Manuscript in preparation for PLoS Neglected Tropical Diseases (working paper)

5.1 Introduction: Acute febrile illnesses in Vietnam and scrub typhus

An acute undifferentiated febrile illness (UFI) is defined as a rapid onset of fever and non-specific symptoms such as headache, chills or muscle and joint pains. In Asia, diseases contributing to UFI include influenza, dengue, scrub typhus, leptospirosis, murine typhus, typhoid, and malaria. UFIs are responsible for major health problems in Vietnam over the last decade. Influenza and dengue are the top most common acute febrile illnesses recorded in Vietnam healthcare facilities, with incidence in 2018 of 559 and 150 per 100,000 population respectively. From 2010 to 2018, malaria incidence experienced a decline from 62 to 07 per 100,000, and typhoid fever decreased from 1 to 0.01 per 100,000 population. Leptospirosis was reported at a very low prevalence morbidity in 2018 (lower than 0.001/100,000 population), although this disease is not actively sought for in rural areas, where it is most commonly found [29, 248].

In Vietnam, scrub typhus is not included in the national infectious disease surveillance, even though scrub typhus is increasingly reported as a leading etiology among patients with UFI, hence current morbidity data is not available. Scrub typhus is likely the most misdiagnosed and/or underestimated disease among non-malarial and non-dengue fevers, in the recent decade. In 2000s, scrub typhus re-emerged after 40 years of neglect and it's endemicity confirmed in 32/63 provinces in Vietnam [11-14]. Given the establishment of dengue awareness and access to diagnostics, recent studies report scrub typhus as the major cause of "fevers of unknown origin" in Vietnam, followed by murine typhus and leptospirosis [17, 22, 130]. A study at two national tropical disease hospitals in Vietnam (2002) reported that in 579 UFI patients the most frequent cause was mite-borne scrub typhus (caused by *Orientia tsutsugamushi* spp.) in 237 patients (40.9%), followed by flea-borne murine typhus (caused by *Rickettsia typhi)* in 193 patients (33.3%), after excluding malaria, dengue, and typhoid fever [5, 7]. A follow-up study at the same hospitals conducted 13 years later in 2015, revealed that in 302 AUF patients, scrub typhus was the leading cause with 103 (34.1%) of patients and murine typhus in 12 patients (3.3%). In 2016, a similar study on AFIs at the province level showed that in 378 AUF patients with negative screening tests for dengue and malaria in Binh Thuan provincial hospital, southern Vietnam, rickettsial illnesses contributed to 10.8% of cases; scrub typhus in 21/378 (5.5%), murine typhus in 18/378 (4.8%), *Rickettsia felis* infection in 2/378 (0.5%) and leptospirosis in 18/378 (4.8%) [22].

Scrub typhus diagnosis remains poorly available and accessible and remains a serious challenge, at both the provincial or national healthcare settings, contributing to under diagnosis, under appreciation and under awareness of the impact scrub typhus in Vietnam levels [18, 245]. Due to the absence of specific clinical symptoms, scrub typhus in Vietnam

continues to be diagnosed mainly based on the presence of an eschar in the clinical examination, which is often incompletely done due to eschars being in intimate body areas and the finding missed by physicians. Most health care facilities are not well-equipped with diagnostic tools, especially at the provincial level; resulting in scrub typhus patients being referred from 24 provinces to the national Bach Mai hospital as UFI suspected cases [16]. The delay in diagnosis and initiating appropriate treatment was associated with complications in 15% referred patients, including altered mental status (45/251; 17.9%), jaundice or hyper bilirubinaemia in (42/251; 16.7%) and pulmonary pathology in 39/251 (15.5%) of cases. Scrub typhus accounted for 3/251 deaths (~ 1.2% mortality rate) in elder patients who were admitted late to hospitals with multiorgan failure [10]. The under appreciated impact of scrub typhus becomes more serious in the context of Vietnam - a dengue hyper endemic country, with limited dengue test availabilities – even at primary health care levels, despite one third of the UFI presenting at public primary health services is dengue [31, 32]. Dengue and scrub typhus are sympatric illnesses with similar clinical symptoms, but requiring different management strategies, hence the lack of adequate diagnostics in hotspot regions likely results in misdiagnosis and inappropriate treatment [33-36].

All the reasons above place Vietnam at risk of underestimating the impact and burden of scrub typhus and highlight the need for timely implementation of awareness-prevention programs and diagnosis improvement interventions, in all provinces in Vietnam. The purpose of this paper is to summarize the findings of a recent research project and to propose a a framework towards improved diagnostics and One Health prevention and integrated control of scrub typhus in Vietnam

5.2 Recent research findings on scrub typhus in Vietnam

During a recent research project as part of the PhD requirement of the first author three major topics relating to scrub typhus in Vietnam were investigated; i) assessing the risk factors for acquiring the disease (epidemiology-ecology), ii) identifying clinical predictors to differentiate scrub typhus from dengue, and iii) characterising the temporal disease dynamics in relation to diagnostic modalities.

5.2.1 Theme 1: risk factors for acquiring scrub typhus

Using a case control approach, we identified risk factors associated with *O. tsutsugamushi* infection in humans: Several factors were significantly associated with acquisition of scrub typhus, including sitting/laying directly on the household floor (adjusted OR=4.9, 95%CI:1.6–15.1, p=0.006), household with poor sanitation/conditions (aOR=7.9, 95%CI:1.9–32.9, p=0.005), workplace environment with risk (aOR=3.0, 95%CI:1.2–7.6, p=0.020), observation of mice around the home always (aOR=3.7, 95%CI:1.4–9.9,

p=0.008), and use of personal protective equipment in the field (aOR=0.4, 95%CI:0.1–1.1, p=0.076).

5.2.2 Theme 2: clinical predictors to differentiate scrub typhus from dengue

Using CART and M-LR models, the most relevant predictors of scrub typhus capable of reliably differentiating scrub typhus from dengue based on clinical-laboratory findings were: Eschar, regional lymphadenopathy, an occupation in nature, high days of fever on admission, high neutrophil count, low ratio of neutrophils/lymphocytes, and age over 40. Sensitivity and specificity of predictions based on these seven factors reached 93.7% and 99.5%, respectively. When excluding the “eschar” variable, the values dropped to 76.3% and 92.3%, respectively.

5.2.3 Theme 3: Temporal disease dynamics in relation to diagnostic modalities for Early Detection of Scrub Typhus

The currently available reference tests to diagnose scrub typhus are PCR (whole blood, buffy coat or eschar swab/biopsy) and ELISA or IFA (serum, plasma). The point-of-care RDTs are under evaluation and apart from InBios RDT not readily available. I analysed a total of 437 suspected cases of scrub typhus and confirmed a total of 269 cases in clinical samples (eschar swab, buffy coat and serum/plasma). In this study among the confirmed cases (defined as PCR buffy coat AND/OR ELISA positive); 65% were positive by PCR buffy coat, 85% by ELISA and 79% by RDT. PCR buffy coat performed best from day 1 to day 6, compared to ELISA and RDT, with an overall positivity rate of 73% during this early phase, but contributed to final diagnosis until day 14 of fever. ELISA IgM and RDTs performed better after day 7 of fever, with positivity rates of 90% and 81%, respectively, in the later phase – but contributed to diagnosis from day 3 of fever. The combination of PCR buffy coat with an RDT detected 93% to 100% of all positive cases during the first 14 days of fever. PCR using eschar specimens yielded higher positivity rates when compared directly to PCR from buffy coat in paired matched samples.

5.3 Towards improved diagnostics and One Health prevention and integrated control of scrub typhus in Vietnam

Increasing recognition and growing evidence suggest that the two infectious diseases - scrub typhus and dengue - amount to more than half of acute febrile illnesses in Vietnam. It is high time that official scrub typhus reports are registered in the national health information system or national scrub typhus surveillance system for non-malarial and non-dengue fevers diagnosed reliably in hospitals and by laboratories [249]. These reports will be useful to monitor interventions, specify exact morbidity and mortality numbers, increase

general awareness, identify hotspots for scrub typhus and help build diagnostic capacity in areas of high incidence. This evidence coupled with available information from dengue surveillance, will allow better understanding of the spacio-temporal distribution of sympatric scrub typhus and dengue, identifying regional hot spots. This represents a strong foundation for conceiving control and management programmes.

Based on the above summarised knowledge on the acquisition and transmission of scrub typhus, and what is needed to improve early reliable diagnosis we propose a One Health integrated framework for improved diagnosis, prevention and control at the different layers of the social-ecological system [250]. Elements of our approach are also termed "implementation research" [251], which we expand by quantifying intervention effectiveness [252] and by a formal inter-sectoral One Health approach. One Health can be defined as the added value in terms of better health of humans and animals, financial savings and sustainable environmental services from a closer cooperation of human and animal health sectors and related disciplines [253]. The particular ecology and epidemiology of scrub typhus is particularly suited for a One Health conceptual thinking. We structure the approach to integrated One Health prevention and control on: a) multi-layered social resilience framework Figure 5.1 by Obrist et al. [254], b) the conceptual framework of equity-effectiveness in access to health care [255, 256] and c) a transdisciplinary approach of co-production of transformational knowledge between academic and non-academic actors for policy formulation and implementation of interventions [257, 258].

The multi-layered resilience framework emphasis the interactions between enabling factors and capacities operating at different levels of society, like the household level, the intermediate levels like districts, or public service providers and the national and international levels (Figure 5.1). Enabling factors help to master threats and capacities lead social actors to cope with adverse conditions and to proactive responses, creating competences and pathways for mitigation [254]. The access to health care framework addresses five dimensions that influence the course of the health-seeking process: Availability, Accessibility, Affordability, Adequacy and Acceptability Table 5.1. The respective degree of access depends on the above levels of societal organisations in terms of service provision, policies and institutions. To optimize the transformation from knowledge to effective implementation a transdisciplinary (TD) dialogue between scientists, authorities and communities is needed as a participatory stakeholder process, as also promoted by the Organization for Economic Cooperation and Development (OECD) [259]. In such a participatory process, the respective competences of the different levels of societal organization and their interactions with the other levels are negotiated and specified. Similarly, the above-mentioned dimensions of access to health care are adjusted to the

perceptions and needs of health care seekers and providers to maximize the overall intervention effectiveness in an iterative way analogous to [256].

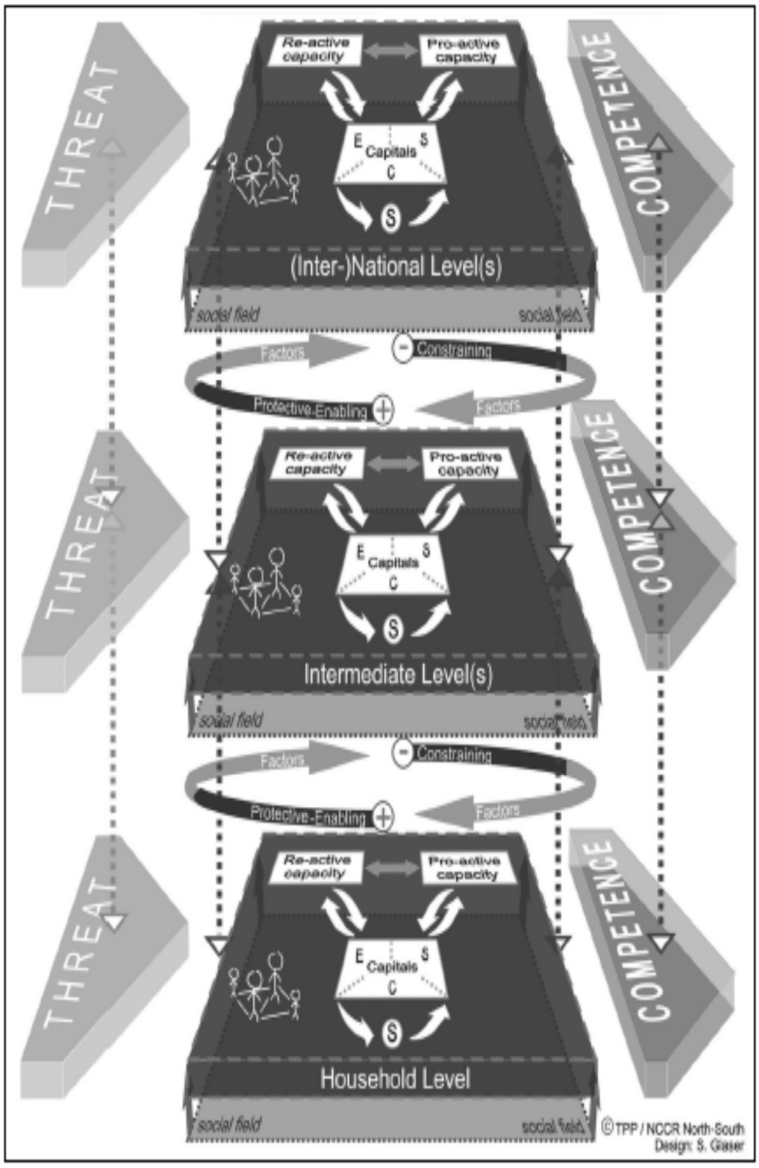


Figure 5.1 Multi-layered social resilience framework as proposed by Obrist et al.[254]

Below we apply the resilience and access frameworks, including the TD approach to the diagnosis, prevention and control of scrub typhus Table 5.2. The overall framework is incomplete and requires further rounds of iterative participatory approaches between the involved actors.

5.4 Diagnosis of scrub typhus

Following the results of our studies, summarized in part 2, five 5 interventions are proposed for improving scrub typhus diagnosis quality in the hotspot areas and are indicated in Table 5.2.

5.4.1 Interventions for scrub typhus diagnosis in the hotspot areas

1. Clinical training for medical staff should include basic knowledge on UFI – with special emphasis on scrub typhus and dengue fever
2. Training about risk factors for scrub typhus, incl. training in clinical epidemiology (why?)
3. Knowledge of clinical and laboratory predictors for early identification of suspected cases of scrub typhus
4. Training in the use of and broad implementation of RDTs at CHCs
5. Implementation of PCR and ELISA based diagnostics at regional laboratories and hospitals throughout the region.

Table 5.1 Five Dimensions of Access to health care services [255] and intervention effectiveness [256].

Drug, vaccine efficacy	Pharmacological properties
Targeting accuracy	How well health care providers and stakeholders identify the true health problem.
Availability (Adoption [251])	The existing health services and goods meet clients' needs.
Accessibility	The location of supply is in line with the location of clients.
Affordability	The prices of services fit the clients' income and ability to pay.
Adequacy (Appropriateness [251])	The organization of health care meets the clients' expectations.
Acceptability	The characteristics of providers match with those of the clients.
Provider compliance	How well the provider initiates the correct procedures.
Consumer adherence	Howe well the recipient follows the medical advice given.

Provider compliance - How can doctors be informed on the importance of scrub typhus? how can their awareness be improved? And how can access to diagnostics be facilitated?
Given the challenge in detecting scrub typhus cases early in the disease course, the appropriate clinical symptoms, epidemiology, combined with the predictors and risk factors found in this study, should be presented and taught in training courses at hospitals (district and provincial) and for the medical students via the infectious disease training program in the health science universities. The understanding of epidemiological risk factors and disease case-detection have improved dramatically in staff associated with this study. However, better physicians' awareness and improved access to scrub typhus diagnostics holds potential to reduce complications and death associated with delayed diagnosis and referral.
Emphasis on the timely response to signs and symptoms should be prioritised in the educational program in high-risk areas during the peak transmission season – for the public, CHCs and hospitals/laboratories.

- In the public this would be to raise awareness about basic clinical symptoms (eschar) that seeking diagnosis sooner is associated with better chances of successful treatment or more rapid referral upon manifestation of flu-like symptoms.
- In CHCs this would be the use of RDTs to identify more cases earlier.
- In hospitals and laboratories this would be the timely supply of PCR and ELISA test kits as well as therapeutic drugs (e.g. doxycycline, azithromycin), especially in high-risk areas.

Table 5.2 One Health socially-layered diagnosis, prevention and control framework of scrub typhus in Vietnam

Bold and Italic: interventions are proposed based on study results. *Italic: context for interventions*.

Social layer[254]		**Diagnostic interventions (5.1 and 5.2)**	**Diagnostic interventions with dual hotspots**	**Access dimensions of diagnostic tests [255]**	**Interventions against scrub typhus**	**Prevention of scrub typhus**	**Access dimensions of Interventions and prevention [255]**
International						CDC Disease prevention and control guidelines	
National level	Government ministry of health				Recognition as an important disease National surveillance	Disease prevention and control guidelines	Acceptability
	Government wildlife authority				Restriction of wildlife trade and consumption	Disease prevention and control guidelines	Acceptability
	National reference hospitals	Clinical, diagnostic and epidemiological training Supply of diagnostic tests	Provider compliance / Affordability *PCR not covered by insurance*	Provider compliance / Affordability			
National – Intermediate level					active cooperation of medical doctors and preventive health staff		
Intermediate levels	Provincial	Clinical, **ST-DF diagnostic,** epidemiological training **Supply RDT (PCR)**	Provider compliance / Affordability *RDT $5.3*	Provider compliance / Affordability	**ST service provision & recording in health information systems** *Epi studies - Province*		Availability RDT in 2/63 provinces

Social layer[254]		**Diagnostic interventions (5.1 and 5.2)**	**Diagnostic interventions with dual hotspots**	**Access dimensions of diagnostic tests [255]**	**Interventions against scrub typhus**	**Prevention of scrub typhus**	**Access dimensions of Interventions and prevention [255]**
	District	Clinical, **ST-DF diagnostic,** epidemiological training **Supply RDT (PCR)**	Provider compliance / Affordability *RDT $5.3*	Provider compliance / Affordability	**ST service provision & recording in health information systems** *Epi studies - District*		Availability Routine lab: 93% district
	Community Health Centres	Clinical, epidemiological training **Supply RDT (check 24h-72h later)**	Provider compliance *RDT $5.3*	Provider compliance	**ST service provision**	Clinical, diagnostic training **Risk factors - Health education campaigns**	Availability
Household		**Raise awareness on clinical symptoms and early diagnosis**					Adequacy Acceptability
					Rodent control	**Household (concrete floors) + workplace preventions + PPE**	Accessibility, Affordability Adequacy, Acceptability, Consumer adherence

5.4.2 Adequacy (Appropriateness) of diagnostic tests

5.4.2.1 Technical characteristics

5.4.2.1.1 Predictors for scrub typhus and dengue:

ST-DF predictors included neutrophil, lymphocytes count indicators, therefore this intervention is suitable to implement in hospitals with hematological blood laboratory.

5.4.2.1.2 Technical Requirements of RDT – ELISA – PCR (Samples – technician qualify - machines)

RDT assay (Scrub Typhus Detect IgM Rapid Test, InBios International Inc., Seattle, WA, USA) uses serum, plasma or whole blood sample specimens and presents results in 15 minutes. It should be stored at room temperature (20-28℃), and its shelf life lasts 24 months [260]. It requires no machine and can performed easily by healthcare staff at the primary health care centres.

ELISA assay (Scrub Typhus Detect™ IgM ELISA Kit, InBios International Inc., Seattle, WA, USA) uses serum or plasma, with a total incubation time of approx. 75 minutes. The 96-Well ELISA Microplates need to be stored at 2-8℃ and their shelf life is 12 months [261]. ELISAs need to be performed by trained laboratory technicians with the ELISA semi-automated/automated machine system: centrifugal machine, ELISA washer, ELISA microplate reader. (Up to date, the ELISA and RDT assays are for research use only)

PCR assay (the semi-nested PCR gel-based developed by the Nha Trang Pasteur Institute) uses buffy coat as its preferred sample specimen. PCR assays require trained laboratory technicians in a spohisticated laboratory with standard equipment: biological safety cabinets level 2, thermal cycler, centrifugal machine, vortex mixer, water bath, gel electrophoresis chamber, DNA visualization system [152]. However, newer PCR assays and machines are becoming available for more simplified use without loosing diagnostic accuracy.

5.4.2.1.3 Sensitivity of RDT – ELISA - PCR

In our study, 65% are positive by PCR buffy coat, 85% for ELISA and 79% for RDT among the confirmed cases can be diagnosed by PCR buffy coat AND/OR ELISA. PCR buffy coat was the best from day 1 to day 6, compared to ELISA and RDT, with the positive rate of above 73% over this early phase. ELISA IgM is the best since the day 7 of fever, compared to PCR buffy coat and RDT, with the positive rate of 90%. RDT presented the positive rate of over 81%, since the day 7 of fever.

Comparision of the accuracy and performance characteristics of assays for acute diagnostic of scrub typhus infection presented in the Table 5.3 (and see details at Supplement Table S5.1. Sensitivity and Specificity of different diagnostic tests: Polymerase chain reaction (PCR)-Enzyme immunosorbent assay (ELISA) and Rapid diagnostic tests (RDT)).

5.4.2.2 Provider compliance of diagnostic tests and cost considerations (Affordability)

This section addresses the implementation probability of the proposed interventions across 4 levels of Vietnam health care levels: i) community health care facilities, ii) district, ii) provincial and iv) national health care centres. Two scenarios are discussed: a) with/without RDT test availability, and b) for dual hotspots with high scrub typhus-dengue co-endemicity *versus* hotspots predominantly for scrub typhus.

5.4.2.2.1 At commune level

If RDT tests are available, it is preferable to implement for screening scrub typhus at community health care centres (CHC), as it is based on whole blood and requires no equipment. The RDT test can be conducted by CHC staff, no licensed laboratory technician is required. Our data showed that RDTs display adequate performance (sensitivity is over 50%) starting at 3-4 days of fever [262] (RDT appropriateness).

However, the two interventions – i) training for infectious diseases with clinical epidemiology and ii) the introduction of RDTs - showed little impact on the improvement of diagnostic resolution and accuracy of the presumptive diagnosis for undifferentiated fevers at the CHC level. Only the combined approach, training plus RDTs, improved diagnostic accuracy for dengue diagnosis among AUFs [32].

The introduction of RDTs for infectious diseases such as dengue, through free market principles, does improve the quality of the diagnosis and decreases antibiotics prescriptions at the primary heath care level (PHC). However, without training, RDTs lead to an excess of costs [32]. Therefore, a combined package with clinical training, risk factors training, and RDTs should be implemented at CHC level to promote accurate diagnostic for scrub typhus.

Table 5.3 Comparisons of the accuracy and performance characteristics of assays for acute diagnostic of scrub typhus infection*

Assay	Days of fever on admission median (IQR)	Sensitivity	Specificity	Cost per test	Ease	Time	Setting	Comments	Reference
RDT IgM									
RDT IgM InBios International Inc., Seattle WA, USA	7 (5-10)	79		+ (US$ 5.3)					this study in Vietnam
RDT IgM InBios International Inc., Seattle WA, USA (various cut-off and timing)	various	82-92	89-98						[237, 263]
RDT SD Bioline IgM (/IgG/IgA)	various	21-92	74-98	+	+++	<30 mins	Primary health care facilities	Sensitivity dependent on timing Does not require specialised equipment, Rapid and simple	[237, 244, 264, 265]
RDT ImmuneMed IgM	NA	92-99	92-98						[237, 264]
RDT Access Bio CareStart Scrub Typhus test (Somerset, NJ) IgM	2 (2-3)	23.3	81.4						[244]
RDT immunochromatographic test IgM	various	61-94	86-100						[30, 111, 115]
* See more at Table S5.1. Sensitivity and Specificity of different diagnostic tests: Polymerase chain reaction (PCR)-Enzyme immunosorbent assay (ELISA) and Rapid diagnostic tests (RDT) PCR-ELISA-RDT Sensitivity and Specificity									
ELISA IgM									
ELISA IgM InBios International Inc., Seattle WA, USA	7 (5-10)	85		++ (US$ 7.5 if conducted all 96 wells, US$ 59.8 if conducted individually)	++	2 h	Reference lab/Hospital	Need gold standard, expensive equipment, Requires infrastructure, Sensitivity dependent on sample type and timing, Possible contamination issues	this study in Vietnam
ELISA IgM (various antigen, cut-off/reference test, and timing)	various	84-100	73-99	++	++	2 h	Reference lab/Hospital		[30, 111, 154, 155, 237, 238]

PCR using blood sample									
Semi-nested PCR (56-Kda)	7 (5-10)	65		++ (US$ 30.9)	+		Reference lab/Hospital	Expensive equipment, Requires infrastructure, Sensitivity dependent on sample type and timing, Possible contamination issues	this study in Vietnam
Conventional PCR 56kDa protein gene	9 (3–20)	75.32	100	++	+				[237]
Nested PCR (gen targets: 56-kDa and 47-kDa)	5 (3–7)	83	88	++	+				[115]
Realtime PCR (gene targets 47 Kda protein gene, GroEL)	various	77-97	94-100	++	+	3 h			[115, 237]
Loop mediated isothermal amplification (LAMP assay) 1 (GroEL)	9 (3–20)	91.77	77.22	++	++	2h	Primary hospital	Simple, inexpensive, Possible contamination issues	[237]

Regarding the cost comparison in Vietnam, as Scrub Typhus Detect IgM Rapid Test, InBios International Inc., Seattle, WA, USA is not available in Vietnam, we collected cost data of SD Bioline Tsutsugamushi, Standard Diagnostics, InC, Korean with the same function for example and assumed that they have the same costs. The cost per test for SD Bioline Tsutsugamushi, Standard Diagnostics, InC, Korean is US$ 5.3 (listed by a provincial hospital in Vietnam). This cost is considerably lower than specific ELISA testing or PCR for *Orientia Tsutsugamushi* spp. The cost per test for Scrub Typhus Detect™ IgM ELISA Kit (InBios International Inc., Seattle, WA, USA), for research purpose, is US$ 7.5 (US$717/96 wells). If being performed individually, it costs US$ 59.8 per test per person (US$ 7.5 x 8 wells). These prices do not include expenses of ELISA semi-automated/automated machine system and payments for technicians (quoted from a scrub typhus researcher in Thailand). The price for PCR for *Orientia Tsutsugamushi* spp US$ 30.9 per test (service by Pasteur Nha Trang Institute). Moreover, the price of US$ 5.3 per rapid test for scrub typhus also equal to the price of US$ 5.5 for NS1Ag rapid test for dengue in Vietnam (listed by a district hospital, Vietnam) [266]. This price is accepted and affordable for patient. Considered all factors above, it is suggested that RDT cost of US$ 5 is the most reasonable price and can be affordable by patient at primary health care level, compared to ELISA and PCR.

With appropriateness regarding to technical issues and work force, affordable price for patient, RDT is feasible to conducted in 11.083 community health centers in Vietnam (coverage)

Other methods, SF-DF predictors and ELISA/PCR, cannot be applied at CHC level, due to the limited laboratory equipment and number of technicians. SF-DF predictors such as neutrophil; lymphocytes count required haematological blood laboratory which is not available at CHCs. Furthermore, there is no ELISA/PCR machines available at CHCs. Regarding to health workforce report, only 2% (250/11100) among CHC facilities in Vietnam have licensed laboratory technician [267]. The cost for ELISA/PCR also cannot be accepted at the primary health care facilities as above.

An RDT-only approach (without ELISA/PCR) is likely to lead to a significant proportion of false negatives in early phase of disease, due to its nature of detecting antibodies. To limit this disadvantage, RDTs are recommeded to be repeated after 24h-72h again, for more accurate results (Table 5.2). An empirical doxycycline treatment strategy started upon clinical suspicion, before the RDT becomes positive (usually after 5-6 days of fever/symptoms), was proposed as a comprehensive approach to tackle scrub typhus in areas of high endemic scrub typhus incidence. However, this approach shoud be considered carefully in Vietnam until a better defined epidemiological understanding of scrub typhus in hyper endemic areas is available. As humans are dead-end hosts, the induction of doxycycline resistance against scrub typhus is less likely, but overuse of

doxycycline could result in development of resistance against other pathogens. Vietnam is a potential hot spot for the emergence of antimicrobial resistance (AMR) due to the high burden of infectious diseases and the relatively unregulated access to antimicrobials for humans and importantly also for animals and in agriculture [268, 269].

In addition, dengue fever, which mimics the symptoms of scrub typhus, is responsible for approximately one third of the acute undifferentiated fevers (AUF) presented to the public primary health services in Vietnam [31]. An empirical doxycycline strategy is likely to bring disadvantages such as increase in prescription of antibiotics, and costs for patients in cases where scrub typhus is misdiagnosed in the primary health care setting, especially in commune health facilities. Alternatively, the accuracy of the combination of two methods, the simple clinical and laboratory predictors (with a sensitivity of 93.7% and a specificity 99.5% if eschar presents; a sensitivity of 77.4% and a specificity of 90.7% without eschar) [270] and RDTs, need to be considered to combat scrub typhus and improve diagnosis at primary health care levels (district hospitals) in Vietnam. A simplification of DNA-based diagnostic methods for scrub typhus need to be developed for the future – similar to dengue NS1 antigen assays or combination of dengue NS1 antigen and IgM antibody assays for the early diagnosis of acute dengue cases [142-144], which has dramatically increased the sensitivity of admission diagnosis.

The availability, technical requirements (samples – technician qualify - machines) and conclusion on appropriateness of ST-DF predictors (neutrophil, lymphocytes count), RDT – ELISA – PCR are summarised in Table 5.4.

5.4.2.2.2 At the district hospitals

If RDT test is introduced, in the area with a dual endemic of scrub typhus and dengue fever, implementation of a package including clinical training, SF-DF predictors training, risk factors training and RDTs, is recommended to improve diagnostic accuracy for scrub typhus. SF-DF predictors model with sensitivity of 77.4% and specificity of 90.7% (paper 1) propose high accuracy to differentiate scrub typhus and dengue fever. In term of equipment, haematological blood laboratory machine is ready (working) in over 90% of these health care facilities, ensuring the high feasibility - coverage - sustainability for this intervention [271] (SF-DF predictors aprroriateness). The technician work force is sufficient at this level. According to the national health statistics in 2018, Vietnam had an average of 6.3 technicians working per hospital (5,956 technicians work in the 943 district hospitals). At the district hospitals, haematological blood test is routine and cost covered 100% by Vietnam national health insurance (SF-DF predictors cost). All elements suggested that SF-DF predictors model is highly feasible at the district level in Vietnam. In our study [262] the median duration of scrub typhus illness prior hospitalization was 7 days. The sensitivity of

RDT in our study at day 7 of fever is over 80%, proposing for a few false negative results, and thus fewer cases of disease are missed, a high screening performance of RDT for scrub typhus at district level.

In summary, the package intervention including clinical training, SF-DF predictors training, risk factors training and RDTs with reasonable cost, proposes a high diagnosis accuracy and high feasibility, coverage and sustainability implementation in at the district hospitals in Vietnam. In areas with only scrub typhus hotspots, SF-DF predictors training is excluded, the package including clinical training, risk factors training plus RDTs is recommended. If RDT test is not available, a package of clinical training, SF-DF predictors training, risk factors training for a dual endemic area, or a package of clinical training plus risk factors training for only scrub typhus hotspots is suggested.

5.4.2.2.3 At provincial hospitals

The same package is recommended to provincial hospitals with/without the available test across the types of hotspots. In addition, real-time PCR for scrub typhus could be applied at this level. Real-time PCR for scrub typhus was applied in Quang Nam provincial Hospital in 2016 [22]. This method is likely to be applied in 34 provincial hospitals equipped with real-time PCR system and reagents (updated to 20 January 2020) under the Vietnam improving laboratory capability program for COVID-19 [272]. However, with a high cost of US$ 30.9 per test for PCR for *Orientia Tsutsugamushi* spp, it should be thoroughly considered before implementation because the cost is not covered by health insurance, the patient must pay out of pocket (PCR cost).

Either ELISA or PCR seem not suitable to apply both at district and provincial hospital, although technician workforce is available and sufficient, in the context of lacking local scrub typhus epidemiology information

Due to limitations in clinical knowledge and the current laboratory diagnostic capacity, few cases of scrub typhus are diagnosed in hospitals. As scrub typhus is an endemic disease predominantly in rural areas, only is a limited number of patients seek medical advice in hospitals. Moreover, due to the lack in understanding and evidence for the true scrub typhus epidemiology in Vietnam, the awareness of doctors and local people alike is minimal. This context brings difficulties to implement ELISA or PCR for scrub typhus in Vietnam, as the general (false) perception is that only few cases were diagnosed and only a limited number of patients seek hospital-based health services per week. Because of all these reasons, in Vietnam at present, PCR or ELISA testing should be conducted for an individual, causing higher test costs and are perceived as not cost-effective investments. To conduct ELISA test for an individual, 8 wells will be used at 1 time, and it costs US$ 59.8 per test (US$ 7.5

x 8 wells). The Standard Operating Procedure for ELISA is complicate with many steps and take at least 75 minutes for total incubation time. A technician may have to spend a full 2 hours in per ELISA test. For a hospital, the cost of time and work force are considered substantial. ELISA is a complicated process also resulting in higher expenses for technicians. The present immunology laboratory machine available at district and provincial hospitals does not include scrub typhus in the system. Therefore, if conducting scrub typhus ELISA test, a new expensive ELISA semi-automated/automated machine system must be bought. Similarly, the semi-nested PCR gel-based developed by the Nha Trang Pasteur Institute for PCR assay is not suitable in (district and) provincial hospital due to its complicated procedure and machine requirements. In summary, due to the waste of time and workforce, high payment for staff and machine system, ELISA is not likely to be chosen to apply at either district or provincial hospitals in Vietnam. In addition, fee for ELISA and PCR not covered by health insurance, the patient must to pay it by their own pocket. Therefore, it should be thoroughly considered before implementation of the two tests.

In summary, to improve the quality of diagnosis in the dual hotspot areas of scrub typhus and dengue fever, the introduction of RDTs for scrub typhus is a crucial element. The package comprising clinical training, risk factors training, and the use of RDTs is recommended for commune heath centres. A package of clinical training, SF-DF predictors training, risk factors training, RDTs is recommended for district/provincial hospitals. However, although a big need for RDTs is easy to use, cheap and needed in the field, it needs to be validated in Vietnam patients and manufactured based on the local validated diagnostic cut-off value. The implementation package recommended for health care facilities at each level in the scrub typhus hotspot areas are presented in Table 5.5.

Table 5.4 Availability, technical requirements and conclusion on appropriateness of RDT – ELISA – PCR in Vietnam health care facilities

	Commune Health Centre (CHC)				District Hospital				Provincial Hospital				National Hospital			
	Sample	Machine	Lab technician	Conclusion	Samples	Machine	Lab technician	Conclusion	Sample	Machine	Lab technician	Conclusion	Sample	Machine	Lab technician	Conclusion
Clinical training				Yes				Yes				Yes				Yes
Risk factors and Epidemiology training				Yes				Yes				Yes				Yes
SF-DF predictors training		NA* (haematological blood machine)	NA	No	Yes (whole blood)	Yes (hematological blood machine)	Yes*	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
RDT	Yes (whole blood)			Yes	Yes (whole blood)			Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
ELISA				NR**				NR	Yes (serum)	NA (ELISA machines)	Yes	No	Yes	Yes	Yes	Yes
PCR (gel based)	[130]			NR				NR	Yes	NA	Yes	No (Yes if real time PCR [22])	Yes	NA	Yes	No (Yes, if Real time PCR [130]

*NA: Not available ** NR: Not recommended (setting in Table 5.2)*

Table 5.5 Implementation package recommended for health care facilities in the scrub typhus hotspot areas

	Commune Health Centre (CHC)	**District Hospital**	**Provincial Hospital**	**National – no area risk applied**
If test available				
In the dual hotspots of ST and DF	Clinical training + SF-DF predictors training (clinical indicators) + risk factors training + RDTs	Clinical training + SF-DF predictors training + risk factors training + RDTs	Clinical training + SF-DF predictors training + risk factors training + RDTs (real time PCR for confirmation)	Clinical training + SF-DF predictors training + risk factors training + RDTs for screening (real time PCR for confirmation)
Only hotspots of ST	Clinical training + risk factors training + RDTs	Clinical training + risk factors training + RDTs	Clinical training + risk factors training + RDTs (real time PCR for	
If test not available				
In the dual hotspots of ST and DF	Clinical training + risk factors training	Clinical training + SF-DF predictors training + risk factors training	Clinical training + SF-DF predictors training + risk factors training	
Only hotspots of ST	Clinical training + risk factors training	Clinical training + risk factors training	Clinical training + + risk factors training	

5.5 Implementation of measures for scrub typhus endemic hotspots in Vietnam

5.5.1 Acceptability

The perception that an intervention is agreeable among stakeholders (policy makers, hospitals, preventive health facilities, and local persons) is the initial indicator for successful prevention and diagnosis implementations.

Agreement from the Vietnam Ministry of Health (MoH) is the first crucial indicator for the roll-out of scrub typhus interventions. The Vietnam MoH acknowledged scrub typhus as an important under appreciated disease at the Vietnam National Health Conference 2020, and confirmed to take its "re-emergence" after a long period of neglect seriously, due to the number of cases hospitalised in severe conditions or/with complications in recent years [273]. As a first response to what was initially thought to be sporadic outbreaks in Vietnam, the scrub typhus rapid diagnostic test (RDT, SD Bioline Tsutsugamushi) was issued with registration number for sale by Vietnam Ministry of Health via official Decision No 2671/QD-BYT, signed and dated 17th June 2016.

Vietnam Government Commitment on regulation on restricting wildlife trade and consumption regulation to prevent animal to human transmission disease based on One-health approach, and international support.

Scrub typhus is a zoonotic infectious disease caused by obligate intracellular bacterium *Orientia* spp. Humans can be exposed to this bacterium through bites of larval-stage *trombiculid* mites (chiggers), which are found mainly in rodents of forests and rice fields [184-187]. Chiggers have a large variety of hosts, including maintenance hosts i.e. small mammals (rodents, gerbils, hamsters and shrews), ground-dwelling birds, and incidental hosts i.e. other birds and larger mammals including humans [64]. Therefore, based on One-health approach, restricting commercial wildlife trade and consumptions help to prevent animal to human transmission disease such as scrub typhus. On 22 December 2020, a letter from the Pandemic Prevention Taskforce Group representatives by Ambassadors from Australia, European Union, United Kingdom of Great Britain and Northern Ireland, and United State of America, together with the Food and Agriculture Organisation of the United Nations (FAO), and the World Health Organisation (WHO) was sent to the Vietnam Prime Minister. It expressed their strong support for Vietnam to commit to regulate wildlife trade and consumption as a part of future pandemic prevention of zoonotic infectious disease [274]. This strong support is the fundamental issue to management the animal to human transmission disease in Vietnam.

The active cooperation of medical doctors (in hospitals) and preventive health staff in the health information system and their full participation in scrub typhus research efforts, along with support from the provincial health departments - as stated in the document - reflect the acceptability of scrub typhus interventions among local health care staff.

The Vietnamese communicable disease surveillance system is a nationwide effort under the responsibility of the General Department of Preventive Medicine - Ministry of Health, with 28 monthly reported diseases, including: cholera, typhoid, bacillary dysentery, amoebic dysentery, diarrhea, viral encephalitis, dengue fever, malaria, viral hepatitis, rabies, meningococcal meningitis, chickenpox, diphtheria, whooping cough, neonatal tetanus, tetanus (not neonatal tetanus), acute flaccid paralysis suspected of polio, measles, mumps, rubella, flu, influenza A (H5N1), disease caused by adenovirus, plague, anthrax, leptospira, hand-foot and mouth disease, streptococcus suis disease in human [275].

Although scrub typhus is not included in the national infectious disease health information/surveillance system, the ongoing concerns about outbreaks and apparent high mortality in defined areas of Khanh Hoa, led to recording of clinical scrub typhus cases in the hospital health information system as case reports to support early warning in the case of outbreaks or rising incidence.

The documented incidence of cases reported via the health information system also informs the health departments on the developments of local endemic diseases and their hotspots in the provinces. Following the remarkable rise in incidence reported (from 127 cases in 2008 to 179 cases in 2009) [276], scrub typhus became a priority research topic in Khanh Hoa in 2011. The Khanh Hoa Provincial Health Department encouraged and approved three large studies to generate scientific evidence towards supporting scrub typhus prevention and control activities in the province.

These three studies were:

1) The Epidemiological Characteristics of Scrub typhus in Khanh Hoa 2013-2014 (clinical characteristics, epidemiology, and vector), investigated by the Nha Trang Pasteur Institute, ordered by Khanh Hoa Provicial Department of Health [206, 277].

2) Scrub typhus in Khanh Hoa: an eco-epidemiological approach, 2017-2020 (this PhD project), including scrub typhus and dengue clinical characteristics, risk factors, diagnostic test accuracy evaluation, conducted by SwissTPH and the Hanoi University of Public Health

3) A hospital and community based investigation of rickettsial diseases, scrub typhus and Q fever in Viet Nam, conducted by the National Hospital of Tropical Disease, with supports from US Naval Medical Research Centre (HACIRD project), including estimation of incidence, identifying vectors, and laboratory reference diagnostics [278].

The infectious diseases specialists and preventive health staff gave their full support and active participation to these projects, expressing their interest for and agreement with scrub

typhus interventions. The general increase of experiencing difficulties in diagnostics and multiple referrals to different hospitals (with severe febrile illnesses not responding to antimicrobials and/or complications), scrub typhus became a feared disease in the patient's perception and a growing concern for people in these areas. These experiences circulated and worried people in the affected regions about the severe consequences of miss-managed scrub typhus, prompting them for knowledge about risk factors and preventive measures.

5.5.2 Availability

Availability, the intention, decision or action to try to employ a new intervention, is the second indicator of successful implementation. There are five components that reflect the potential for adoption of scrub typhus preventive and diagnostic interventions in Vietnam, these include: i) a great interest and readiness regarding scrub typhus interventions by medical doctors and preventive health care staff in health care facilities; ii) regular and reliable feedback to all health facilities about laboratory results; iii) great interest of community health care staff; iv) scrub typhus has become a priority research topic in Khanh Hoa; v) the national surveillance platform demonstrating increasing interest. There is a great interest in and readiness of doctors and preventive health care staff for scrub typhus interventions, as both the clinical and laboratory diagnoses of scrub typhus remain challenging and the patient may develop severe illness with organ failure, which can be fatal if adequate treatment is delayed or missed.

Scrub typhus is treatable if diagnosed timely and correctly, but the diagnostic procedures of this illness are not straightforward. Additionally, the clinical and laboratory features are non-specific with considerable overlap between scrub typhus, dengue, leptospirosis and other rickettsial infections [10]. After 40 years of neglect, scrub typhus is not included in official medical training programs and infectious disease short courses, and demand for increased emphasis on training for healthcare providers for earlier recognition, prevention, and treatment of rickettsial diseases in Vietnam was proposed [130]. A lack of awareness in clinicians leads to insufficient consideration, which is reflected by the low number of diagnostic tests performed, resulting in misdiagnosis and consequently miss-management (40.9% UFIs accounted for scrub typhus). Further, as *O. tsutsugamushi* is not responsive to cephalosporin antibiotics, which are often prescribed pre-emptively – an increasing rate of complicated scrub typhus is seen across Vietnam, even at the national tertiary hospital level [17, 18].

Overcoming the enormous challenges to create improved awareness is not a small task, and to date the successful diagnosis of a scrub typhus case reflects a great effort of infectious disease specialists often seeking medical consultations abroad. The diagnosis of

scrub typhus, even based on clinical symptoms alone (an eschar being the most vital diagnostic clue) attracts great interest of doctors and health facilities. Scrub typhus case reports and their occurrence are published on the official website of the provincial health department/hospital/preventive health facilities to inform local people and provide additional information for prevention of scrub typhus [279-283]. The hospitals are active in proposing to buy scrub typhus RDTs (SD Bioline Tsutsugamushi) for the provincial health departments. In Quang Ngai and Hai Duong provinces, the health department approved and included this RDT in the provincial annual purchasing lists 2020 [284, 285].

The full participation of health care staff in the above mentioned scrub typhus projects over almost ten years reflects the interest and willingness to improve detection, management and design interventions. In Khanh Hoa, in this almost ten year period, all regional hospitals send samples to the Nha Trang Pasteur Institute (reference laboratory performing validated PCR and ELISAs) upon clinical suspicion of scrub typhus, and receive rapid laboratory results as a standard diagnostic procedure in some years. The turn around of confirmed results from Nha Trang Pasteur Institute helps the clinician to improve their clinical differential diagnosis and encourages them to update their knowledge. Meanwhile, the district preventive health and commune health centres (CHC) are also participating in the projects – especially in risk factor investigations. CHC is the first level health care facility where patients go to when they get fever. Scrub typhus is receiving more attention by CHC staffs, which play an important role in conducting case investigation and health education for local people.

Scrub typhus has become a priority research topic in Khanh Hoa. The three scrub typhus studies conducted in the Khanh Hoa province in recent years have encouraged health care staff to be more vigilant for detecting scrub typhus in Khanh Hoa. Similarly, the national scrub typhus surveillance among UFI patients in the 28 provinces [278] will further encourage health care staff across Vietnam to participate in scrub typhus research and implementing interventions.

5.5.3 Appropriateness – Feasibility – Fidelity – Cost – Coverage – Sustainability

Scrub typhus is not transmitted between humans as humans represent dead-end hosts - but the larval stage of *Leptotrombidium* mites can transmit *Orientia* between rodents and to humans. Among the most effective preventive measures are avoidance of exposure to the vector and vector control [245]. The context of the limited diagnostic capacity and lack of an effective vaccine for scrub typhus, the most reasonable measures to reduce the incidence of scrub typhus, is prevention through education at primary health care levels, rodent control and facilitating early diagnosis (leading to subsequent adequate treatment). By systematically informing the populations at highest risk of acquiring scrub typhus in Central

Vietnam, already a substantial impact could be achieved. Currently, no prevention and control programs are available for Khanh Hoa province in particular or for Vietnam in general. This may also have contributed to the re-emergence in newly identified foci and outbreaks.

The previously described risk factors for acquiring scrub typhus and the newly identified risk factors from this research in Central Vietnam represent an important fundament to support education of local people in hyper-endemic areas to improve their knowledge and understanding of protecting themselves from scrub typhus. This is all the more important in the context of the limited diagnostic capacity for scrub typhus at primary health care levels. Our findings provide useful information to compile an updated and detailed guideline for the Centre for Disease Control and Prevention (CDC) on scrub typhus in endemic areas, such as Khanh Hoa and other scrub typhus hotspots in Vietnam [175].

There is a high demand for effective disease prevention and control guidelines based on reduction or elimination of these risk factors, supported by policy-makers and health preventive providers in hotspot provinces. Improving working and household environments (i.e. preventing mud/sand yards, a mud house floor or drainage on ground) should be emphasized in scrub typhus prevention guidelines in the region. Based on One Health perspectives, enforcing rodent control (especially house mice) represents a relevant approach to preventing infected mites on mice to transmit *Orientia tsutsugamushi* spp. to humans [223, 286]. Traps and cats around homes and rural villages are basic mouse control measures that are easily implementable for families with small children in scrub typhus hyper endemic areas.

Our study shows that community prevention programs should aim to educate people about the risk factors of scrub typhus (e.g. direct communication campaigns and distribution of leaflets), and improve the household surrounding conditions - these need to be conducted in hotspot communes.

Based the study results previously detailed in parts 1 and 2 of the thesis - the three following interventions are proposed for scrub typhus prevention in scrub typhus hotspot areas.

5.6 Prevention

5.6.1 Interventions for scrub typhus prevention in hotspot areas

The major interventions proposed are:

1. Preparing updated prevention guidelines on scrub typhus by the Centre for Disease Control and Prevention (CDC)
2. Training CHC staff in identifying the early clinical symptoms and predictors of scrub typhus including risk factor assessments

3. Implementing a community-based set of preventive interventions to educate people about risk factors of scrub typhus (i.e. a direct health education campaign and leaflet distribution) and instructions on how to improve the household surroundings
4. Rodent control

The identified scrub typhus risk factors:

1. taking a break / sitting directly on the HH floor (adjusted OR=4.9, 95%CI:1.6–15.1)
2. use of personal protective equipment in the field (aOR=0.4, 95%CI:0.1–1.1)
3. household with poor sanitation and hygienic conditions (aOR=7.9, 95%CI:1.9–32.9)
4. work place surrounded by water/vegetation (aOR=3.0, 95%CI:1.2–7.6)
5. observation of rodents around the home (aOR=3.7, 95%CI:1.4–9.9).

The clinical and laboratory predictors for scrub typhus:

1. Eschar
2. Regional lymphadenopathy
3. An occupation in nature
4. Increased days of fever on admission
5. Increased neutrophil count
6. Decreased ratio of neutrophils/lymphocytes
7. age over 40

5.6.2 Adequacy

Community-based preventive interventions aiming at educating people about risk factors and improving household surroundings conditions should be initiated at the community level. The interventions focus on avoiding sitting/laying directly on the household floor, wearing 5 labour clothes with 5 items (socks, boots, long/extra shirt, long/extra trousers, and gloves), improving household surrounding conditions, rodent control measures around household. These interventions are suitable, simple and easy to follow as they are built around the daily life of local people, and no new techniques need be introduced.

Training CHC staff about the initial clinical symptoms of scrub typhus and risk factors will improve early case-detection and community-targeted health care measures at the community front line health care facilities - the first health care facility patients seek care for when they get fever. CHCs play an important role in conducting case investigation and health education for local people. These simple interventions are in line with CHC's duties on early disease detection and health care education stipulated in the "Decision on CHC functions and duties" issued by Vietnam Ministry of Health [287].

The health education campaign of scrub typhus prevention for communities was conducted at 30 communes in Khanh Hoa, by the Provincial Health Department and the Nha Trang Pasteur Institute in 2014. These activities involved over 600 CHC staff and achieved broad outreach to local people at risk, with reasonable costs and without additional burden to the health system [288]. Therefore, a direct communication campaign on risk factors prior to and during the main scrub typhus season (September-January, raining season) for people living in high endemicity areas could bring high benefits and impact.

Such a program could not only educate people on the prevention of risk factors, but CDC staff could distribute leaflets to the population and recruit local staff to inform and assist people to implement preventive actions such as cleaning up their HH floors, changing their sandy/muddy yard or muddy house floor, filling drainage on ground, using rodent traps and raising cats to decrease the rodent density in villages and homes. People could equip themselves with work clothes with full PPE set: socks, boots, long/extra shirt, long/extra trousers, and gloves.

5.6.3 Feasibility

Integrating health education on scrub typhus risk factors into the routine health education program on communicable diseases for CHC at hotspot area would improve the feasibility of the health education campaign. Improving the availability of point-of-care diagnostics in rural areas and at the community level would improve awareness and support the acceptance of systematic identification of risk factors.

Scrub typhus if not diagnosed and treated early can lead to complications and is associated with increased mortality. Children are at risk of scrub typhus [71]. There were one scrub typhus paediatric patient (10 months) and other 10 cases among children under 6 years old in Khanh Hoa confirmed by Nha Trang Pasteur Institute in 2013 [206]. Improving children' health is a priority for parents, and a high motivational factor to consider learning about risk factors, and implementing better household sanitation and conditions. People voluntarily changed their sandy ground to a cemented one, after consultations on risk assessment for scrub typhus in this study, to protect their children (Figure 5.2).

Figure 5.2 Improvement of houses with cemented floors

5.6.4 Coverage

These simple interventions could easily be applied in hotspots in provinces with similar ecological and endemic zones like those in Khanh Hoa province, i.e., the 11 central provinces of Vietnam.

5.6.5 Implementation costs

In this section, the estimated costs of a disease prevention program at the community level and the minimum costs for treatment at hospital were presented in Table 5.6 and Table 5.7. The estimated total cost for a health education campaign with 600 participants at 30 hotspot communes (12 sessions) amounted to $3'167 which covered the whole province of Khanh Hoa. This contrasts against the costs of $6'454 to treat 57 suspected scrub typhus cases in a district hospital (hospitalised for 7 days), without any diagnosis test cost. A part of these costs are covered by health insurance ($4'036) and the rest by patients ($2'418). The latter consists of a non-medical direct costs for transport ($251) and indirect costs due to lost wages ($2'167) (estimated derived from 57 suspected cases of scrub typhus hospitalised in 2019 with a median hospitalisation of 7 days). This treatment cost was estimated for basic services related to the study findings: bed cost, diagnosis fee, routine hematological blood laboratory, and doxycycline. (see Table 5.6, Table 5.7).

The true treatment cost is likely to be higher when services such other costs such as laboratory services (serum biochemistry test, urine test), X-ray, dengue NS1 test, etc. were

included. Scrub typhus diagnosis test cost was not included into this estimation because it was not available in the province.

The hospital-associated costs are at least 2.0-fold higher than those for the health education campaign. Similar results were indicated by a Cost-Benefit Analysis of the *Tsutsugamushi* Disease Prevention Program in South Korea. The ratio of benefit to cost was found to be 5.5, making the economic value of the program significantly higher. The net benefit should increase if the *tsutsugamushi* disease prevention program is continued and the implementation period is expanded to 10 years [289].

Table 5.6 The estimated total cost for a health education campaign with 600 audience at 30 hotspot communes in Khanh Hoa (estimated based on payment guidance for 2020)*

Items	Contents	Unit cost	Amount	Total
Perdiem for 600 participants at 30 hotspots	$2.1/participant x 600 participants	2.1 [290]	600	1288
Water	$0.4/bottle x 600 bottles	0.4	600	258
Lecturer	$17.2/section x 12 sessions	17.2 [291]	12	206
Renting hall	$42.9/section x 12 sessions	42.9	12	515
Projectors	$21.5/section x 12 sessions	21.5	12	258
Design poster				215
Market poster				43
Printing cost of poster	$1.7/posters x 100 posters	1.7	100	172
Leaflets	$0.2/leaflet x 1000 leaflets	0.2	1000	215
Total				**3167**

**30 hotspot communes organised into 12 groups for communication (close communes were combined into 1 group). 1 communication section/group x 12 groups = 12 sections*

** 2 participants/village x 5 village/commune x 30 communes + 10 key persons of unions/communes x 30 communes = 600 participants

Table 5.7 Estimated total cost for 57 suspected scrub typhus cases treatment per year in a district hospital in 7 days in Khanh Hoa, 2020 (cost of basic services*)

Hospitalization	Contents	Cost for 1 patient ($)	Cost for 57 patients ($)	Source
Medical cost		**70.8**	**4'036**	
Bed cost per visit	$8.0 per day x 7 days	56.2	3'204	[292]
Diagnostics	$1.4 + $0.4/time x 1 time/day x 6 days ($1.4 for the first diagnostic and 0.4/time for next 6 days)	3.7	213	[292]
Routine hematological blood laboratory	$1.7/time x 6 times	10.4	593	[293]
Doxycycline	$0.02/pill x 2 pills/dose x 2 doses/day x 7 days	0.5	26	[292]
Nonmedical costs		**42.4**	**2'418**	
Nonmedical direct costs (transport)	Local transpot within district	4.4	251	[294]
Indirect costs (lost wages)*		38.0	2167	[295]
Total		**113.2**	**6'454**	

Cost of basic services which are related to the study findings: bed cost, diagnosis fee, routine hematological blood laboratory, and doxycycline.

*** Indirect costs (lost wages) was estimated by monthly average income per capita in 2019 at current prices for Khanh Hoa (US$ 163 per month, 30 days ~ US$ 5.4 per day ~ US$ 38.0 for 7 days of hospitalization*

5.7 ANNEXES AND APPENDICES

Table S5.1. Sensitivity and Specificity of different diagnostic tests: Polymerase chain reaction (PCR)-Enzyme immunosorbent assay (ELISA) and Rapid diagnostic tests (RDT)

	Code of test	Assays	Days of fever before admission Median (IQR)	Sensitivity	Specitivity	
		RDT IgM				
RDT		**RDT IgM InBios International Inc., Seattle WA, USA (various cut-off and timing)**		**82-92**	**89-98**	**[237, 263]**
RDT	16	Scrub Typhus Detect IgM rapid test; InBios International Inc., Seattle WA, USA) cut-off >1:1,600	NA	82	98	[263]
RDT	19	InBios rapid test for scrub typhus IgM	9 (3–20)	87.34	89.24	[237]
RDT	17	Scrub Typhus Detect IgM rapid test; InBios International Inc., Seattle WA, USA) cut-off >1:6,400	NA	92	95	[263]
RDT		**RDT SD Bioline IgM (/IgG/IgA)**		**21-92**	**74-98**	**[237, 244, 264, 265]**
	23	SD Bioline RDT IgM	NA	84.4	96.3	[264]
RDT	25	commercially available test (SD Bioline Tsutsugamushi assay) IgG/IgM/IgA	6 (1-47)	66.7	98.4	[265]
RDT	18	Standard Diagnostics (SD) BioLine Tsutsugamushi-Assay (SD BioLine, Korea) IgG/IgM/IgA antibodies	9 (3–20)	91.77	86.08	[237]

RDT	32	SD BIOLINE Tsutsugamushi test (Kyonggi-do, Republic of Korea) (IgG, IgM, or IgA)	2 (2-3)	20.9	74.4	[244]
RDT		**RDT ImmuneMed IgM**	**NA**	**92-99**	**92-98**	**[237, 264]**
RDT	28	ImmuneMed scrub typhus RDT IgM in Korea	NA	98.6	98.2	[264]
RDT	30	ImmuneMed scrub typhus RDT IgM/IgG in Sri Lanka	NA	92.1	96.1	[264]
RDT	20	ImmuneMed Scrub Typhus Rapid IgM/IgG	9 (3–20)	91.77	92.41	[237]
RDT	**31**	**RDT Access Bio CareStart Scrub Typhus test (Somerset, NJ) IgM**	**2 (2-3)**	**23.3**	**81.4**	**[244]**
RDT		**RDT immunochromatographic test IgM**		**61-94**	**86-100**	**[30, 111, 115]**
	33	The scrub typhus immunochromatographic test IgM (PanBio, Australia) (ST ICT)	5 (3–7)	61	88	[115]
RDT	27	Immunochromatographic IgM RDT	NA	82–94	86–100	[30]
RDT	21	PanBio (Brisbane, Australia) Scrub Typhus IgM RapidIgM Rapid Immunochromatographic	NA	83	93	[111]
		ELISA IgM (various antigen, cut-off/reference test, and timing)		**84-100**	**73-99**	**[30, 111, 154, 155, 237, 238]**
ELISA	6	InBios Scrub Typhus Detect IgM enzyme-linked immunosorbent assay (ELISA)	5.5 (4-7)	84	98	[238]
ELISA	7	ST Detect IgM ELISA kit (InBios International, Seattle, USA	9 (3–20)	92.41	93.67	[237]

ELISA	8	Scrub Typhus Detect™ IgM ELISA (Cat# STMS-1, InBios International Inc., Seattle WA, USA) (ELISA vs. IFA	10 (7-15)	91.5	92.4	[155]
ELISA	11	Scrub Typhus Detect IgM ELISA kits from InBios International (Seattle, WA, USA)	NA	85.3	95.5	[154]
ELISA	12	pooled antigen ELISA IgM (1:400 cutoff)	NA	94	91	[111]
ELISA	13	r56 Recombinant-antigen ELISA IgM (1:400 cutoffcut-off)	NA	93	94	[111]
ELISA	15	ELISA IgM	NA	84–100	73–99	[30]
PCR	**1**	**Conventional PCR 56kDa protein gene**	**9 (3–20)**	**75.32**	**100**	**[237]**
PCR	**5**	**Nested 56-kDa and 47-kDa PCR**	**5 (3–7)**	**83**	**88**	**[115]**
		Real time PCR (47 Kda protein gene, GroEL)		**77-97**	**94-100**	**[115, 237]**
PCR	2	Real time PCR 47 Kda protein gene	9 (3–20)	97.47	100	[237]
PCR	3	47 kDa based real-time PCR	5 (3–7)	79	90	[115]
PCR	4	GroEL-based real-time PCR	5 (3–7)	77	94	[115]
		Loop mediated isothermal amplification (LAMP assay)	9 (3–20)	91.77	77.22	[237]

CHAPTER 6. CONCLUSIONS AND OUTLOOK

6.1 Conclusions

This thesis work presented updated results to improve empirical diagnostic strategies and practical prevention of the seriously neglected and potentially life-threatening disease - scrub typhus in Vietnam. The current availability of adequate diagnostics remains very limited. The findings show that basic clinical symptoms and routine hematological blood laboratory tests support medical staff in the often-challenging clinical decision process for differentiating bacterial scrub typhus from viral dengue infections. Ecological and household hygiene-related factors were associated with an increased acquisition risk of scrub typhus. The rapid test, combined with clinial symptoms and risk factor indicators, shows great promise as a point-of-care approach, particularly in the primary health care system, in remote settings or emergency departments of hospitals, where rapid diagnosis and prompt initiation of appropriate medication can prove life saving.

Scrub typhus prevention and control programmes should be established in Vietnam aiming to estimate the population-based incidences, the true impact of scrub typhus, as well as improved identification of aetiologies in acute undifferentiated fevers [145]. In the first place, simple pragmatic measures are needed to reduce its serious life-threatening complications – which is primarily associated with enabling early diagnosis. This conclusion is essentially not applicable only for Viet Nam, but also for other low and middle income countries with limited laboratory diagnostic facilities, where scrub typhus is an overlooked cause of morbidity, mortality, and economic losses in marginalized populations [296].

6.2 Outlook

Despite advancing knowledge of the diagnosis and prevention of scrub typhus in Vietnam, this work has some limitations that call for further research.

6.2.1 IgM ELISA/ RDT validation in Vietnam

The lack of local positivity cutoff titers causes a lot of confusion about accuracy of the serology diagnostic tests with the indirect immunofluorescence method such as ELISA, RDT and even, IFA, the gold standard test for scrub typhus for decades. The choice of defined and varied positivity cutoff titers across countries raises concern about the appropriateness of the cutoffs for positive IFA results that are chosen for diagnosis of acute scrub typhus infection [297]. However, establishing a validated diagnostic cutoff is often overlooked, especially in endemic areas [30]. In Vietnam, there is no evidence about the

validity of the IgM ELISA/RDT and IFA assay for scrub typhus and their cut-off values [197]. Therefore, serodiagnosis should be assessed and tailored to the Vietnamese target population based on the local cutoff titres [247].

6.2.2 Determination of the true epidemiological picture and impact of scrub typhus in Vietnam

Studies presenting the true epidemiological picture and impact of endemic scrub typhus, and other rickettsial diseases, should be conducted in Vietnam, across the varied ecological areas. Propositions to solve this include i) defining scrub typhus incidence of confirmed cases among suspected cases, using prospective febrile illness studies and/or based on stored samples at the hospitals - employing locally validated PCRs, IgM ELISAs and RDTs; ii) defining the scrub typhus incidence of confirmed cases among acute undifferentiated fevers, in prospective studies and using stored samples at the hospitals with validated PCRs, IgM ELISAs and RDTs, iii) establishing a national surveillance system using validated diagnostic assays; iv) conducting population-based surveys to estimate the under-estimation of scrub typhus.

6.2.3 Evaluating efficacy of a combined package with measures

Evaluating a combined package with training in clinical predictors, risk factors, and adequate use of RDTs is recommended for health care facilities, to promote accurate diagnosis and (empirical) treatment of acute undifferentiated fevers and scrub typhus in Vietnam. Fever is a common medical problem for attending health care facilities in Vietnam. Response of health care providers to patients with fever commonly consists of making a presumptive diagnosis and proposing corresponding treatment. In Vietnam, where malaria was brought under control, notably scrub typhus and dengue represent the main causes of acute undifferentiated fever, but they are often misdiagnosed and inappropriately treated with antibiotics. Therefore, a future study should investigate if educating health care providers and introducing RDTs, especially at the primary health care level, could i) improve diagnostic resolution and accuracy in the management of acute undifferentiated fevers (thus reducing complicated and severe scrub typhus), ii) reduce prescription of antibiotics (preventing mis-use) and iii) lower the associated medical costs for health care systems as well as non-medical costs for patients.

References

1. Paris, D.H., A.L. Richards, and N.P.J. Day, *Chapter 112 - Orientia*, in *Molecular Medical Microbiology (Second Edition)*, M. Sussman, et al., Editors. 2015, Academic Press: Boston. p. 2057-2096.
2. Service, M.W., *Scrub typhus mites (Order Prostigmata: Family Trombiculidae)*, in *A Guide to Medical Entomology*, M.W. Service, Editor. 1980, Macmillan Education UK: London. p. 171-174.
3. Gillespie, J.J., et al., *Plasmids and Rickettsial Evolution: Insight from Rickettsia felis.* PLOS ONE, 2007. **2**(3): p. e266.
4. Izzard, L., et al., *Isolation of a novel Orientia species (O. chuto sp. nov.) from a patient infected in Dubai.* J Clin Microbiol, 2010. **48**(12): p. 4404-9.
5. Richards, A.L. and J. Jiang, *Scrub Typhus: Historic Perspective and Current Status of the Worldwide Presence of Orientia Species.* Trop Med Infect Dis, 2020. **5**(2).
6. Xu, G., et al., *A review of the global epidemiology of scrub typhus.* PLOS Neglected Tropical Diseases, 2017. **11**(11): p. e0006062.
7. Kelly, D.J., et al., *The past and present threat of rickettsial diseases to military medicine and international public health.* Clin Infect Dis, 2002. **34**(Suppl 4): p. S145-69.
8. Barrett, O.J. and F. Stark, *Rickettsial diseases and leptospirosis. In: Ognibene AJ, Barrett O'N Jr, eds.* General Medicine and Infectious Diseases, 1982. **2**: p. 133–158.
9. Neel, S., *Medical Support of the US Army in Vietnam 1965–70.* In: Vietnam Studies, Washington DC: Department of the Army, 1973: p. 34–137.
10. Wisseman, C., *Report of the Commission on Rickettsial Diseases*, in *Armed Forces Epidemiology Board Meeting, Walter Reed Army Institute of Research*. 1972, US Department of the Army: Washington, DC. p. 79–81.
11. Vietnam Ministry of Health, *An atlas of communicable diseases in Vietnam from 2000 to 2011*. 2012. p. 125.
12. Chau Nguyen Van, *Investigation on rats, chiggers and orientia tsutsugamushi in some foci in the center and western highland of Vietnam.* The Medicine Journal of Ho Chi Minh City, 2010. **Vol. 14**(Supplement of No 2): p. 207 - 213.
13. Chien., B.T., et al., *Some clinical and epidemiological characteristics of Scrub typhus caused by Orientia Tsutsugamushi in central Vietnam, 2009 - 2010.* Vietnam Journal of Preventive Medicine, 2014. **XXIV** (2 (150)).
14. Binh., L.T., et al., *Description of clinical and laboratory characteristes of scrub typhus in children.* Medical Journal of Ho Chi Minh City 2006. **10**: p. 1 – 7

15. Trung, N.V., et al., *Seroprevalence of Scrub Typhus, Typhus, and Spotted Fever Among Rural and Urban Populations of Northern Vietnam.* Am J Trop Med Hyg, 2017. **96**(5): p. 1084-1087.
16. Nadjm, B., et al., *Scrub typhus in the northern provinces of Vietnam: an observational study of admissions to a national referral hospital.* Trans R Soc Trop Med Hyg, 2014. **108**(11): p. 739-40.
17. Hamaguchi, S., et al., *Clinical and Epidemiological Characteristics of Scrub Typhus and Murine Typhus among Hospitalized Patients with Acute Undifferentiated Fever in Northern Vietnam.* Am J Trop Med Hyg, 2015. **92**(5): p. 972-8.
18. Katoh, S., et al., *Challenges in diagnosing scrub typhus among hospitalized patients with undifferentiated fever at a national tertiary hospital in northern Vietnam.* PLOS Neglected Tropical Diseases, 2019. **13**(12): p. e0007928.
19. Hang, N.L.K. and N.V. Tinh, *Prevalence Orientia tsutsugamushi among hospitalized patients with possible Scrub typhus in Hanoi, 2015-2016.* Vietnam Journal of Preventive Medicine, 2016. **8 (181)** (26): p. 55-60.
20. Chien, B.T., P. Buchy, and T.T.X. Mai, *Some clinical and epidemiological characteristics of Scrub typhus caused by Orienta tsutsugamushi in central vietnam, 2009 - 2010.* Vietnam Journal of Preventive Medicine, 2014. **2 (150)**(24): p. 9-15.
21. Quyet, N.T., N.B. Trieu, and T.H. Long, *Survey on Scrub typhus due to Rickettsia tsutsugamushi in Khanh Hoa province, Vietnam.* Vietnam Journal of Preventive Medicine, 2017. **8**(27): p. 579-585.
22. Le-Viet, N., et al., *Prospective case-control analysis of the aetiologies of acute undifferentiated fever in Vietnam.* Emerging Microbes & Infections, 2019. **8**(1): p. 339-352.
23. Le Van, N., et al., *Clinical Features, Laboratory Characteristics and Prognostic Factors of Severity in Patients with Rickettsiaceae at Two Military Hospitals, Northern Vietnam.* Infect Drug Resist, 2020. **13**: p. 2129-2138.
24. Nguyen, L.T., et al., *Impact of Climate Variability and Abundance of Mosquitoes on Dengue Transmission in Central Vietnam.* International journal of environmental research and public health, 2020. **17**(7): p. 2453.
25. Bett, B., et al., *Spatiotemporal analysis of historical records (2001-2012) on dengue fever in Vietnam and development of a statistical model for forecasting risk.* PLoS One, 2019. **14**(11): p. e0224353.
26. Hung, T.M., et al., *The Estimates of the Health and Economic Burden of Dengue in Vietnam.* Trends Parasitol, 2018. **34**(10): p. 904-918.
27. European Centre for Disease Prevention and Control. *Geographical distribution of dengue cases reported worldwide, 2020*. 2021 [cited 2021 07 May]; Available from:

https://www.ecdc.europa.eu/en/publications-data/geographical-distribution-dengue-cases-reported-worldwide-2020.

28. World Health Organisation in Western Pacific Region, *Update on the Dengue situation in the Western Pacific Region* in *Dengue Situation Update Number 533*. 2018.
29. Health, V.M.o., *Health Statistics Yearbook* 2018.
30. Paris, D.H. and J.S. Dumler, *State of the art of diagnosis of rickettsial diseases: the use of blood specimens for diagnosis of scrub typhus, spotted fever group rickettsiosis, and murine typhus.* Current Opinion in Infectious Diseases, 2016. **29**(5): p. 433-439.
31. Phuong, H.L., et al., *Dengue as a cause of acute undifferentiated fever in Vietnam.* BMC infectious diseases, 2006. **6**: p. 123-123.
32. Phuong, H.L., et al., *Randomised primary health center based interventions to improve the diagnosis and treatment of undifferentiated fever and dengue in Vietnam.* BMC Health Services Research, 2010. **10**(1): p. 275.
33. Gulati, S. and A. Maheshwari, *Dengue fever-like illnesses: how different are they from each other?* Scand J Infect Dis, 2012. **44**(7): p. 522-30.
34. Leelarasamee, A., et al., *Etiologies of acute undifferentiated febrile illness in Thailand.* J Med Assoc Thai, 2004. **87**(5): p. 464-72.
35. Ellis, R.D., et al., *Causes of fever in adults on the Thai-Myanmar border.* Am J Trop Med Hyg, 2006. **74**(1): p. 108-13.
36. Watt, G., et al., *Differentiating dengue virus infection from scrub typhus in Thai adults with fever.* Am J Trop Med Hyg, 2003. **68**(5): p. 536-8.
37. Ma, C.J. and G.J. Oh, *Differences in agricultural activities related to incidence of scrub typhus between Korea and Japan.* 2017. **39**: p. e2017051.
38. Singh, S.I., et al., *An outbreak of scrub typhus in Bishnupur district of Manipur, India, 2007.* Trop Doct, 2010. **40**(3): p. 169-70.
39. Varghese, G.M., et al., *Epidemiology & risk factors of scrub typhus in south India.* Indian J Med Res, 2016. **144**(1): p. 76-81.
40. Tilak, R. and R. Kunte, *Scrub typhus strikes back: Are we ready?* Med J Armed Forces India, 2019. **75**(1): p. 8-17.
41. Bonell, A., et al., *Estimating the burden of scrub typhus: A systematic review.* PLoS Negl Trop Dis, 2017. **11**(9): p. e0005838.
42. Li, Z., et al., *Epidemiologic changes of scrub typhus in China, 1952-2016.* Emerging Infectious Diseases, 2020. **26**.

43. Xin, H.L., et al., *Evaluation of scrub typhus diagnosis in China: analysis of nationwide surveillance data from 2006 to 2016.* Infect Dis Poverty, 2019. **8**(1): p. 59.
44. Kinoshita, H., et al., *Descriptive epidemiology of rickettsial infections in Japan: Scrub typhus and Japanese spotted fever, 2007–2016.* International Journal of Infectious Diseases, 2021. **105**: p. 560-566.
45. Kweon, S.S., et al., *Rapid increase of scrub typhus, South Korea, 2001-2006.* Emerging infectious diseases, 2009. **15**(7): p. 1127-1129.
46. Kim, S., J.S. Kim, and H. Lee, *Epidemiological Characteristics of Scrub Typhus in Korea, 2009.* Osong Public Health and Research Perspectives, 2010. **1**(1): p. 55-60.
47. Rawat, V., et al., *Epidemiological, clinical and laboratory profile of scrub typhus cases detected by serology and RT-PCR in Kumaon, Uttarakhand: a hospital-based study.* Trop Doct, 2018. **48**(2): p. 103-106.
48. Bandyopadhyay, B., et al., *Epidemiology, clinical profile and laboratory parameters of scrub typhus cases in West Bengal.* IP International Journal of Medical Microbiology and Tropical Diseases, 2019. **5**: p. 150-154.
49. Stephen, S., et al., *Outbreak of scrub typhus in Puducherry & Tamil Nadu during cooler months.* The Indian journal of medical research, 2015. **142**(5): p. 591-597.
50. Takhar, R.P., et al., *Scrub typhus: A prospective, observational study during an outbreak in Rajasthan, India.* Natl Med J India, 2017. **30**(2): p. 69-72.
51. Behera, B., et al., *Clinico-epidemiological analysis of scrub typhus in hospitalised patients presenting with acute undifferentiated febrile illness: A hospital-based study from Eastern India.* Indian J Med Microbiol, 2019. **37**(2): p. 278-280.
52. Bureau of Epidemiology of Thai Lan, *Scrub typhus*. 2021: Thailand.
53. Gautam, R., K. Parajuli, and J.B. Sherchand, *Epidemiology, Risk Factors and Seasonal Variation of Scrub Typhus Fever in Central Nepal.* Trop Med Infect Dis, 2019. **4**(1).
54. Lee, Y.S., et al., *Epidemiology of scrub typhus in eastern Taiwan, 2000-2004.* Jpn J Infect Dis, 2006. **59**(4): p. 235-8.
55. Lai, C.H., et al., *Epidemiology of acute q Fever, scrub typhus, and murine typhus, and identification of their clinical characteristics compared to patients with acute febrile illness in southern taiwan.* J Formos Med Assoc, 2009. **108**(5): p. 367-76.
56. Weerakoon, K., et al., *Revisiting clinico-epidemiological pattern of human rickettsial infections in the central region of Sri Lanka: a hospital based descriptive study.* BMC Research Notes, 2017. **10**.

57. Cosson, J.F., et al., *Detection of Orientia sp. DNA in rodents from Asia, West Africa and Europe.* Parasites & vectors, 2015. **8**: p. 172-172.
58. Acharya, K.P., N. Adhikari, and M. Tariq, *Scrub typhus: A serious public health issue in Nepal.* Clinical Epidemiology and Global Health, 2020. **8**(3): p. 815-817.
59. Suputtamongkol, Y., et al., *Epidemiology and Clinical Aspects of Rickettsioses in Thailand.* 2009. **1166**(1): p. 172-179.
60. Government of Nepal and Nepal Health Research Council, *Descriptive Epidemiology of Scrub Typhus in Nepal, 2017*. 2017.
61. Taylor, A.J., D.H. Paris, and P.N. Newton, *A Systematic Review of Mortality from Untreated Scrub Typhus (Orientia tsutsugamushi).* PLOS Neglected Tropical Diseases, 2015. **9**(8): p. e0003971.
62. JR, A., *The ecology of scrub typhus. In: May JM, editor. Studies in disease ecology*. 1961, New York: Hafner Publishing Company, Inc.
63. Fletcher, W., J.E. Lesslar, and R. Lewthwaite, *The ætiology of the tsutsugamushi disease and tropical typhus in the Federated Malay States.* Transactions of The Royal Society of Tropical Medicine and Hygiene, 1928. **22**(2): p. 161-174.
64. Elliott, I., et al., *Scrub typhus ecology: a systematic review of Orientia in vectors and hosts.* Parasites & Vectors, 2019. **12**(1): p. 513.
65. Kweon, S.-S., et al., *Rapid Increase of Scrub Typhus, South Korea, 2001–2006.* Emerging Infectious Disease journal, 2009. **15**(7): p. 1127.
66. Lyu, Y., et al., *A case-control study of risk factors associated with scrub typhus infection in Beijing, China.* PLoS One, 2013. **8**(5): p. e63668.
67. Vallee, J., et al., *Contrasting spatial distribution and risk factors for past infection with scrub typhus and murine typhus in Vientiane City, Lao PDR.* PLoS Negl Trop Dis, 2010. **4**(12): p. e909.
68. Tay, S.T., et al., *Antibody prevalence and factors associated with exposure to Orientia tsutsugamushi in different aboriginal subgroups in West Malaysia.* PLoS Negl Trop Dis, 2013. **7**(8): p. e2341.
69. Abhaykumar Ambilkar, A.J., Subhash Thakre, S D Nimgade, V M Chauragade, BR Patle, *Determinants of Scrub Typhus: A Case Control Study in Tribal Region of Eastern Maharashtra.* The Journal of Medical Research, 2019. **5**(2): p. 94-97.
70. Musa, T.H., *Risk factors associated with scrub typhus infection: A case-control study from Luhe, China*. 2019, In Review.
71. Rose, W., et al., *Risk factors for acquisition of scrub typhus in children admitted to a tertiary centre and its surrounding districts in South India: a case control study.* BMC Infectious Diseases, 2019. **19**(1): p. 665.

72. Wei, Y.H., et al., *A city park as a potential epidemic site of scrub typhus: a case-control study of an outbreak in Guangzhou, China.* Parasites & Vectors, 2014. **7**.
73. Puran Kumar Sharma, *Risk factors for scrub typhus and typhoid in the Kurseong sub-division of Darjeeling district, West Bengal, India, 2005-2006*, in *National Institute of Epidemiology (Indian Council ofMedical Research),*. 2006, National Institute of Epidemiology (Indian Council ofMedical Research),: Chennai, India. p. 99.
74. Kweon, S.S., et al., *A community-based case-control study of behavioral factors associated with scrub typhus during the autumn epidemic season in South Korea.* Am J Trop Med Hyg, 2009. **80**(3): p. 442-6.
75. Thangaraj, J.W.V., et al., *Risk Factors for Acquiring Scrub Typhus among Children in Deoria and Gorakhpur Districts, Uttar Pradesh, India, 2017.* Emerg Infect Dis, 2018. **24**(12): p. 2364-2367.
76. Khan, F., et al., *Prevalence of Scrub Typhus - A Cause of concern in Uttarakhand Region, India.* international journal of current microbiology and applied sciences, 2015: p. 101-109.
77. Kamble, S., et al., *Seroprevalence & seroincidence of Orientia tsutsugamushi infection in Gorakhpur, Uttar Pradesh, India: A community-based serosurvey during lean (April-May) & epidemic (October-November) periods for acute encephalitis syndrome.* Indian J Med Res, 2020. **151**(4): p. 350-360.
78. Hengbin, G., et al., *The foci of scrub typhus and strategies of prevention in the Spring in Pingtan Island, Fujian Province.* Ann N Y Acad Sci, 2006. **1078**: p. 188-96.
79. Audy, J.R. and J.L. Harrison, *A review of investigations on mite typhus in Burma and Malaya, 1945-1950.* Trans R Soc Trop Med Hyg, 1951. **44**(4): p. 371-404.
80. Audy, J.R., *Practical Notes on Scrub Typhus in the Field.* Journal of the Royal Army Medical Corps, 1949. **93**(6): p. 273.
81. Traub, R. and C.L. Wisseman, Jr., *Current concepts of the ecology of chigger-borne rickettsiosis (scrub typhus).* Jpn J Med Sci Biol, 1974. **27**(1): p. 1-5.
82. Traub, R. and C.L. Wisseman, Jr., *The ecology of chigger-borne rickettsiosis (scrub typhus).* Journal of Medical Entomology, 1974. **11**(3): p. 237-303.
83. Silpapojakul, K., *Scrub typhus in the Western Pacific region.* Ann Acad Med Singap, 1997. **26**(6): p. 794-800.
84. Yi, K.S., et al., *Scrub typhus in Korea: importance of early clinical diagnosis in this newly recognized endemic area.* Mil Med, 1993. **158**(4): p. 269-73.
85. Ben, R.J., N.H. Feng, and C.S. Ku, *Meningoencephalitis, myocarditis and disseminated intravascular coagulation in a patient with scrub typhus.* J Microbiol Immunol Infect, 1999. **32**(1): p. 57-62.

86. Hsu, Y.H. and H.I. Chen, *Pulmonary pathology in patients associated with scrub typhus.* Pathology, 2008. **40**(3): p. 268-71.
87. Park, J.S., et al., *Acute respiratory distress syndrome associated with scrub typhus: diffuse alveolar damage without pulmonary vasculitis.* J Korean Med Sci, 2000. **15**(3): p. 343-5.
88. Ohashi, N., A. Tamura, and T. Suto, *Immunoblotting analysis of anti-rickettsial antibodies produced in patients of Tsutsugamushi disease.* Microbiol Immunol, 1988. **32**(11): p. 1085-92.
89. Seong, S.Y., et al., *Induction of neutralizing antibody in mice by immunization with recombinant 56 kDa protein of Orientia tsutsugamushi.* Vaccine, 1997. **15**(16): p. 1741-7.
90. Shirai, A., et al., *Host defenses in experimental scrub typhus: role of cellular immunity in heterologous protection.* Infect Immun, 1976. **14**(1): p. 39-46.
91. Chu, H., et al., *Orientia tsutsugamushi infection induces CD4+ T cell activation via human dendritic cell activity.* J Microbiol Biotechnol, 2013. **23**(8): p. 1159-66.
92. Hickman, C.J., et al., *Murine T-cell response to native and recombinant protein antigens of Rickettsia tsutsugamushi.* Infect Immun, 1993. **61**(5): p. 1674-81.
93. Hu, M.-L., et al., *Abnormal liver function in scrub typhus* The American Journal of Tropical Medicine and Hygiene Am J Trop Med Hyg, 2005. **73**(4): p. 667-668.
94. Rikihisa, Y. and S. Ito, *Effect of antibody on entry of Rickettsia tsutsugamushi into polymorphonuclear leukocyte cytoplasm.* Infect Immun, 1983. **39**(2): p. 928-38.
95. Ni, Y.-S., et al., *Protection against scrub typhus by a plasmid vaccine encoding the 56-kd outer membrane protein antigen gene* The American Journal of Tropical Medicine and Hygiene Am J Trop Med Hyg, 2005. **73**(5): p. 936-941.
96. Nacy, C.A. and J.V. Osterman, *Host defenses in experimental scrub typhus: role of normal and activated macrophages.* Infection and immunity, 1979. **26**(2): p. 744-750.
97. Smadel, J.E., et al., *Persistence of Rickettsia tsutsugamushi in tissues of patients recovered from scrub typhus.* Am J Hyg, 1952. **56**(3): p. 294-302.
98. Shirai, A., et al., *Host defenses in experimental scrub typhus: effect of chloramphenicol.* Infect Immun, 1977. **18**(2): p. 324-9.
99. Kobayashi, Y., S. Kawamura, and T. Oyama, *Immunological studies of experimental tsutsugamushi disease in congenitally athymic (nude) mice.* Am J Trop Med Hyg, 1985. **34**(3): p. 568-77.
100. Bourgeois, A.L., et al., *Humoral and cellular responses in scrub typhus patients reflecting primary infection and reinfection with Rickettsia tsutsugamushi.* Am J Trop Med Hyg, 1982. **31**(3 Pt 1): p. 532-40.

101. Paris, D., A. Richards, and N. Day, *Orientia*. 2015. p. 2057-2096.
102. Richards, A.L., *Worldwide detection and identification of new and old rickettsiae and rickettsial diseases.* FEMS Immunol Med Microbiol, 2012. **64**(1): p. 107-10.
103. Saunders, J.P., et al., *The longevity of antibody to Rickettsia tsutsugamushi in patients with confirmed scrub typhus.* Trans R Soc Trop Med Hyg, 1980. **74**(2): p. 253-7.
104. Kelly, D.J., et al., *Detection of Rickettsia tsutsugamushi by gene amplification using polymerase chain reaction techniques.* Ann N Y Acad Sci, 1990. **590**: p. 564-71.
105. Kim, C.M., et al., *Accuracy of Conventional PCR Targeting the 16S rRNA Gene with the Ot-16sRF1 and Ot-16sRR1 Primers for Diagnosis of Scrub Typhus: a Case-Control Study.* J Clin Microbiol, 2016. **54**(1): p. 178-9.
106. Lee, J.H., et al., *Differentiation of rickettsiae by groEL gene analysis.* J Clin Microbiol, 2003. **41**(7): p. 2952-60.
107. Kim, D.M., et al., *Comparison of conventional, nested, and real-time quantitative PCR for diagnosis of scrub typhus.* J Clin Microbiol, 2011. **49**(2): p. 607-12.
108. Horinouchi, H., et al., *Genotypic identification of Rickettsia tsutsugamushi by restriction fragment length polymorphism analysis of DNA amplified by the polymerase chain reaction.* Am J Trop Med Hyg, 1996. **54**(6): p. 647-51.
109. Kim, D.M., et al., *Clinical usefulness of eschar polymerase chain reaction for the diagnosis of scrub typhus: a prospective study.* Clin Infect Dis, 2006. **43**(10): p. 1296-300.
110. Ching, W.M., et al., *Early diagnosis of scrub typhus with a rapid flow assay using recombinant major outer membrane protein antigen (r56) of Orientia tsutsugamushi.* Clin Diagn Lab Immunol, 2001. **8**(2): p. 409-14.
111. Coleman, R.E., et al., *Comparative evaluation of selected diagnostic assays for the detection of IgG and IgM antibody to Orientia tsutsugamushi in Thailand.* Am J Trop Med Hyg, 2002. **67**(5): p. 497-503.
112. Kim, Y.J., et al., *Improvement of the diagnostic sensitivity of scrub typhus using a mixture of recombinant antigens derived from Orientia tsutsugamushi serotypes.* J Korean Med Sci, 2013. **28**(5): p. 672-9.
113. Kala, D., et al., *Diagnosis of scrub typhus: recent advancements and challenges.* 3 Biotech, 2020. **10**(9): p. 396.
114. Koh, G.C.K.W., et al., *Diagnosis of scrub typhus.* The American journal of tropical medicine and hygiene, 2010. **82**(3): p. 368-370.
115. Paris, D.H., et al., *Diagnostic accuracy of a loop-mediated isothermal PCR assay for detection of Orientia tsutsugamushi during acute Scrub Typhus infection.* PLoS Negl Trop Dis, 2011. **5**(9): p. e1307.

116. Dussart, P., et al., *Evaluation of two new commercial tests for the diagnosis of acute dengue virus infection using NS1 antigen detection in human serum.* PLoS neglected tropical diseases, 2008. **2**(8): p. e280-e280.
117. Blacksell, S.D., et al., *Evaluation of the Panbio dengue virus nonstructural 1 antigen detection and immunoglobulin M antibody enzyme-linked immunosorbent assays for the diagnosis of acute dengue infections in Laos.* Diagn Microbiol Infect Dis, 2008. **60**(1): p. 43-9.
118. Mueller, T.C., et al., *Acute Undifferentiated Febrile Illness in Rural Cambodia: A 3-Year Prospective Observational Study.* Plos One, 2014. **9**(4).
119. Phuong, H.L., et al., *Acute undifferentiated fever in Binh Thuan province, Vietnam: imprecise clinical diagnosis and irrational pharmaco-therapy.* Trop Med Int Health, 2006. **11**(6): p. 869-79.
120. Shepard, D.S., E.A. Undurraga, and Y.A. Halasa, *Economic and disease burden of dengue in Southeast Asia.* PLoS Negl Trop Dis, 2013. **7**(2): p. e2055.
121. Kingston, H.W., et al., *Rickettsial Illnesses as Important Causes of Febrile Illness in Chittagong, Bangladesh.* Emerg Infect Dis, 2018. **24**(4).
122. Mayxay, M., et al., *Causes of non-malarial fever in Laos: a prospective study.* Lancet Glob Health, 2013. **1**(1): p. e46-54.
123. Luvira, V., et al., *Etiologies of Acute Undifferentiated Febrile Illness in Bangkok, Thailand.* Am J Trop Med Hyg, 2019. **100**(3): p. 622-629.
124. Suttinont, C., et al., *Causes of acute, undifferentiated, febrile illness in rural Thailand: results of a prospective observational study.* Ann Trop Med Parasitol, 2006. **100**(4): p. 363-70.
125. Hamaguchi, S., et al., *Clinical and Epidemiological Characteristics of Scrub Typhus and Murine Typhus among Hospitalized Patients with Acute Undifferentiated Fever in Northern Vietnam.* Am J Trop Med Hyg, 2015. **92**(5): p. 972-978.
126. Wangrangsimakul, T., et al., *Causes of acute undifferentiated fever and the utility of biomarkers in Chiangrai, northern Thailand.* PLOS Neglected Tropical Diseases, 2018. **12**(5): p. e0006477.
127. Maude, R.R., et al., *Serosurveillance of Orientia tsutsugamushi and Rickettsia typhi in Bangladesh.* The American Journal of Tropical Medicine and Hygiene, 2014. **91**(3): p. 580-583.
128. Watthanaworawit, W., et al., *Scrub typhus infection in pregnancy: the dilemma of diagnosis and treatment in a resource-limited setting.* Clinical case reports, 2016. **4**(6): p. 584-588.

129. McGready, R., et al., *Pregnancy Outcome in Relation to Treatment of Murine Typhus and Scrub Typhus Infection: A Fever Cohort and a Case Series Analysis.* Plos Neglected Tropical Diseases, 2014. **8**(11).
130. Trung, N.V., et al., *Clinical Manifestations and Molecular Diagnosis of Scrub Typhus and Murine Typhus, Vietnam, 2015-2017.* Emerg Infect Dis, 2019. **25**(4): p. 633-41.
131. Smadel, J.E. and R. Traub, *Chloramphenicol in the chemoprophylaxis of scrub typhus; results with volunteers exposed in hyperendemic areas of scrub typhus.* Am J Hyg, 1949. **50**(1): p. 75-91.
132. Hazlett, D.R., *Scrub typhus in Vietnam: experience at the 8th Field Hospital.* Mil Med, 1970. **135**(1): p. 31-4.
133. Wangrangsimakul, T., et al., *Causes of acute undifferentiated fever and the utility of biomarkers in Chiangrai, northern Thailand.* PLoS Negl Trop Dis, 2018. **12**(5): p. e0006477.
134. Taylor, A.J., D.H. Paris, and P.N. Newton, *A Systematic Review of Mortality from Untreated Scrub Typhus (Orientia tsutsugamushi).* PLoS Negl Trop Dis, 2015. **9**(8): p. e0003971.
135. Walker, D.H., *Scrub Typhus - Scientific Neglect, Ever-Widening Impact.* N Engl J Med, 2016. **375**(10): p. 913-5.
136. Vo, T.Q. and T.T. Phuong Pham, *Revisiting dengue-related knowledge, attitudes and practices: A cross-sectional study in Ho Chi Minh City, Vietnam, 2018.* J Pak Med Assoc, 2019. **69(Suppl 2)**(6): p. S108-S117.
137. Nguyen, P.V., et al., *Dengue fever in Southern of Vietnam: A survey of reported knowledge, attitudes, and practices.* J Pak Med Assoc, 2019. **69(Suppl 2)**(6): p. S118-S130.
138. Vietnam Ministry of Health, *Health Statistic Yearbook*. 2001. p. 20.
139. Vietnam Ministry of Health, *Health Statistics Yearbook* 2011.
140. Yuhana, M., et al., *Melioidosis and scrub typhus co-infection in a patient presenting with acute undifferentiated febrile illness.* Jurnal Kedokteran dan Kesehatan Indonesia, 2019. **10**: p. 86-90.
141. Lubell, Y., et al., *Modelling the Impact and Cost-Effectiveness of Biomarker Tests as Compared with Pathogen-Specific Diagnostics in the Management of Undifferentiated Fever in Remote Tropical Settings.* PLoS One, 2016. **11**(3): p. e0152420.
142. Tontulawat, P., et al., *Evaluation of rapid immunochromatographic NS1 test, anti-dengue IgM test, semi-nested PCR and IgM ELISA for detection of dengue virus.* Southeast Asian J Trop Med Public Health, 2011. **42**(3): p. 570-8.

143. Jayathilaka, D., et al., *Role of NS1 antibodies in the pathogenesis of acute secondary dengue infection.* Nat Commun, 2018. **9**(1): p. 5242.
144. Shukla, M.K., et al., *Utility of dengue NS1 antigen rapid diagnostic test for use in difficult to reach areas and its comparison with dengue NS1 ELISA and qRT-PCR.* J Med Virol, 2017. **89**(7): p. 1146-1150.
145. Paris, D.H., et al., *Unresolved Problems Related to Scrub Typhus: A Seriously Neglected Life-Threatening Disease.* The American Journal of Tropical Medicine and Hygiene, 2013. **89**(2): p. 301-307.
146. Government, K.H. *Khanh Hoa Overview* 2019; Available from: https://en.khanhhoa.gov.vn/vi/introduction/khanh-hoa-overview.
147. Mai, V.Q., et al., *Prevalence and Risk Factors of Dengue Infection in Khanh Hoa Province, Viet Nam: A Stratified Cluster Sampling Survey.* J Epidemiol, 2018. **28**(12): p. 488-497.
148. Vietnam General Department of Preventive Medicine, *Report of Dengue Prevention and Control National Program 2020*. 2020.
149. Deaton, J.G., *Febrile Illnesses in the Tropics (Vietnam).* Military Medicine, 1969. **134**(12): p. 1403-1408.
150. Institue of Malariology Parastology and Entomology Quy Nhon, V.M.o.H. *Tsutsugamushi disease in Khanh Hoa province*. 2011 [cited 2020 15.09]; Available from: http://www.impe-qn.org.vn/impe-qn/en/portal/InfoDetail.jsp?area58&cat=1041&ID=597.
151. Ngo Thi Quyet, N.B.T., Trinh Hoang Long, Nguyen Duc Duy, Ngo Dang Nghia, Vien Quang Mai,, *Scrub typhus caused by Orientia tsutsugamushi survey in Khanh Hoa* Vietnam Journal of Preventive Medicine, 2017. **8**(27): p. 579.
152. Pasteur Institute of Nha Trang, *SOP - The in-house semi-nested polymerase chain reaction (semi-nested PCR) for Orientia tsutsughamushi spp. antigen* 2014.
153. Paris, D.H., et al., *A highly sensitive quantitative real-time PCR assay based on the groEL gene of contemporary Thai strains of Orientia tsutsugamushi.* Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases, 2009. **15**(5): p. 488-495.
154. Koraluru, M., et al., *Diagnostic validation of selected serological tests for detecting scrub typhus.* Microbiol Immunol, 2015. **59**(7): p. 371-4.
155. Blacksell, S.D., et al., *Diagnostic Accuracy of the InBios Scrub Typhus Detect™ ELISA for the Detection of IgM Antibodies in Chittagong, Bangladesh.* Tropical medicine and infectious disease, 2018. **3**(3): p. 95.
156. World Health, O., *Dengue haemorrhagic fever : diagnosis, treatment, prevention and control*. 1997, World Health Organization: Geneva.

157. Wikipedia. *Monte Carlo method*. 2020 6 October 2020, at 22:46 (UTC). [cited 2020 10 October]; Available from: https://en.wikipedia.org/wiki/Monte_Carlo_method.
158. Lasko, T.A., et al., *The use of receiver operating characteristic curves in biomedical informatics.* Journal of Biomedical Informatics, 2005. **38**(5): p. 404-415.
159. The Open Data Kit community. *Collect data anywhere*. 2020 [cited 2020 23.09]; Available from: https://getodk.org/.
160. Maduka, O., G. Akpan, and S. Maleghemi, *Using Android and Open Data Kit Technology in Data Management for Research in Resource-Limited Settings in the Niger Delta Region of Nigeria: Cross-Sectional Household Survey.* JMIR Mhealth Uhealth, 2017. **5**(11): p. e171.
161. Tom-Aba, D., et al., *Innovative Technological Approach to Ebola Virus Disease Outbreak Response in Nigeria Using the Open Data Kit and Form Hub Technology.* PLoS One, 2015. **10**(6): p. e0131000.
162. World Health Organisation. *Guide to using Open Data Kit (ODK) eQuestionnaire Materials*. [cited 2020 23.09]; Available from: https://www.who.int/hiv/pub/guidelines/Guide-to-usingOpenDataKit.pdf?ua=1.
163. World Health Organization Department of Communicable Disease Surveillance and Response, *WHO Recommended Surveillance Standards. Vol 2*. 1999: Geneva.
164. Zimmerman, R.K., et al., *Classification and Regression Tree (CART) analysis to predict influenza in primary care patients.* BMC Infectious Diseases, 2016. **16**(1): p. 503.
165. Marshall, R.J., *The use of classification and regression trees in clinical epidemiology.* J Clin Epidemiol, 2001. **54**(6): p. 603-9.
166. Aguiar, F.S., et al., *Classification and regression tree (CART) model to predict pulmonary tuberculosis in hospitalized patients.* BMC Pulmonary Medicine, 2012. **12**: p. 40-40.
167. Andreas, M., A. Nancy, and T.T. Ming, *Negative Versus Positive Schizophrenia*. 1991: Springer-Verlag.
168. Alexander, A.D., et al., *Zoonotic infections in military scout and tracker dogs in Vietnam.* Infect Immun, 1972. **5**(5): p. 745-9.
169. Paris, D.H., et al., *Orientia tsutsugamushi in human scrub typhus eschars shows tropism for dendritic cells and monocytes rather than endothelium.* PLoS Negl Trop Dis, 2012. **6**(1): p. e1466.
170. Paris, D.H., et al., *A nonhuman primate scrub typhus model: protective immune responses induced by pKarp47 DNA vaccination in cynomolgus macaques.* J Immunol, 2015. **194**(4): p. 1702-16.

171. Silpapojakul, K., B. Varachit, and K. Silpapojakul, *Paediatric scrub typhus in Thailand: a study of 73 confirmed cases.* Trans R Soc Trop Med Hyg, 2004. **98**(6): p. 354-9.
172. Fan, M.Y., et al., *Rickettsial and serologic evidence for prevalent spotted fever rickettsiosis in inner Mongolia.* Am J Trop Med Hyg, 1987. **36**(3): p. 615-20.
173. Kim, D.M., et al., *Distribution of eschars on the body of scrub typhus patients: a prospective study.* Am J Trop Med Hyg, 2007. **76**(5): p. 806-9.
174. Bhat, N.K., R. Jindal, and M. Dhar, *Eschar of Scrub Typhus Hidden in Umbilicus.* The Indian Journal of Pediatrics, 2018. **85**(3): p. 247-248.
175. Centers for Disease Control and Prevention. *Scrub Typhus*. 2019 [cited 2020 06 September]; Available from: https://www.cdc.gov/typhus/scrub/index.html.
176. Cross, R., et al., *Revisiting doxycycline in pregnancy and early childhood--time to rebuild its reputation?* Expert Opin Drug Saf, 2016. **15**(3): p. 367-82.
177. Quan, T.M., et al., *Evidence of previous but not current transmission of chikungunya virus in southern and central Vietnam: Results from a systematic review and a seroprevalence study in four locations.* PLoS neglected tropical diseases, 2018. **12**(2): p. e0006246-e0006246.
178. Quyen, N.T.H., et al., *Chikungunya and Zika Virus Cases Detected against a Backdrop of Endemic Dengue Transmission in Vietnam.* The American journal of tropical medicine and hygiene, 2017. **97**(1): p. 146-150.
179. World Health Organisation. *Zika virus infection – Viet Nam*. 2016 [cited 2020 06 September]; Available from: https://www.who.int/csr/don/12-april-2016-zika-viet-nam/en/.
180. Centers for Disease Control and Prevention. *Lyme Disease*. 2019 [cited 2020 20 December]; Available from: https://www.cdc.gov/lyme/index.html.
181. Newton, P.N., et al., *A Prospective, Open-label, Randomized Trial of Doxycycline Versus Azithromycin for the Treatment of Uncomplicated Murine Typhus.* Clin Infect Dis, 2019. **68**(5): p. 738-747.
182. McClain, J.B., et al., *Doxycycline therapy for leptospirosis.* Ann Intern Med, 1984. **100**(5): p. 696-8.
183. Alikhani, A., et al., *Comparison of azithromycin vs doxycycline prophylaxis in leptospirosis, A randomized double blind placebo-controlled trial.* J Infect Dev Ctries, 2018. **12**(11): p. 991-995.
184. Lv, Y., X.-G. Guo, and D.-C. Jin, *Research Progress on Leptotrombidium deliense.* The Korean journal of parasitology, 2018. **56**(4): p. 313-324.

185. Peng, P.Y., et al., *An updated distribution and hosts: trombiculid mites (Acari: Trombidiformes) associated with small mammals in Yunnan Province, southwest China.* Parasitol Res, 2016. **115**(5): p. 1923-38.
186. Traub, R. and C.L. Wisseman, Jr., *Ecological considerations in scrub typhus. 2. Vector species.* Bull World Health Organ, 1968. **39**(2): p. 219-30.
187. Paris, D.H., et al., *A Brief History of the Major Rickettsioses in the Asia-Australia-Pacific Region: A Capstone Review for the Special Issue of TMID.* Trop Med Infect Dis, 2020. **5**(4).
188. Koh, G.C., et al., *Diagnosis of scrub typhus.* Am J Trop Med Hyg, 2010. **82**(3): p. 368-70.
189. Cracco, C., et al., *Multiple organ failure complicating probable scrub typhus.* Clin Infect Dis, 2000. **31**(1): p. 191-2.
190. Wangrangsimakul, T., et al., *Clinical Characteristics and Outcome of Children Hospitalized With Scrub Typhus in an Area of Endemicity.* J Pediatric Infect Dis Soc, 2020. **9**(2): p. 202-209.
191. Paris, D.H. and J.S. Dumler, *State of the art of diagnosis of rickettsial diseases: the use of blood specimens for diagnosis of scrub typhus, spotted fever group rickettsiosis, and murine typhus.* Curr Opin Infect Dis, 2016. **29**(5): p. 433-9.
192. Luce-Fedrow, A., et al., *Strategies for detecting rickettsiae and diagnosing rickettsial diseases.* Future Microbiol, 2015. **10**(4): p. 537-64.
193. Kingston, H.W., et al., *Comparative Accuracy of the InBios Scrub Typhus Detect IgM Rapid Test for the Detection of IgM Antibodies by Using Conventional Serology.* Clin Vaccine Immunol, 2015. **22**(10): p. 1130-2.
194. Xu, G., et al., *A review of the global epidemiology of scrub typhus.* Plos Neglected Tropical Diseases, 2017. **11**(11).
195. Chau Nguyen Van, *Trombiculidae mite and association with scrub typhus in Quang Ninh, 2003*. 2003, National Institute of Malariology, Parasitology and Entomology: Hanoi.
196. Hung Do Manh, N.N.M., Anh The Dao, Dieu Vo, Trang Le, Mai Vien Quang,, *Epidemiological Characteristics of Scrub typhus in Khanh Hoa 2013-2014* 2014, Nha Trang Pasteur Institute
197. Katoh, S., et al., *Challenges in diagnosing scrub typhus among hospitalized patients with undifferentiated fever at a national tertiary hospital in northern Vietnam.* PLoS Negl Trop Dis, 2019. **13**(12): p. e0007928.
198. Ma, C.J., et al., *Differences in agricultural activities related to incidence of scrub typhus between Korea and Japan.* Epidemiol Health, 2017. **39**: p. e2017051.

199. Varghese, G., et al., *Epidemiology & risk factors of scrub typhus in south India.* Indian Journal of Medical Research, 2016. **144**(1): p. 76-81.
200. Kelly, D.J., et al., *The Past and Present Threat of Rickettsial Diseases to Military Medicine and International Public Health.* Clinical Infectious Diseases, 2002. **34**(Supplement_4): p. S145-S169.
201. Pasteur Institute of Nha Trang, *Scrub typhus Clinical Characteristics in Khanh Hoa, Viet Nam*. 2017.
202. The People's Committee of Khanh Hoa Province. *Khanh Hoa Introduction* 2015 [cited 2021 21 Jan]; Available from: https://www.khanhhoa.gov.vn/english-portal.
203. National Hydro-Meteorological Service, M.o.N.R.a.E., *Monthly Climate data, 1979-2014*. 2014.
204. Vietnam Ministry of Health, *National guideline on diagnosis and treatment some infectious diseases*. 2016, Medical Publisher: Hanoi.
205. Lambin, E.F., et al., *Pathogenic landscapes: interactions between land, people, disease vectors, and their animal hosts.* Int J Health Geogr, 2010. **9**: p. 54.
206. Ngo Thi Quyet, T.H.L., Nguyen Duc Duy, Nguyen Bao Trieu, Ngo Dang Nghia, Vien Quang Mai,, *Survey on scrub typhus due to Rickettsia tsutsugamushi in Khanh Hoa province, Vietnam.* Vietnam Journal of Preventive Medicine, 2017. **27**(8): p. 110.
207. Audy, J.R., *Scrub typhus as a study in ecology.* Nature, 1947. **159**(4035): p. 295.
208. Audy, J. *A Summary Topographical Account of Scrub Typhus 1908-1946. Studies in the Distribution and Topography of Scrub Typhus, Number 1*. 1949.
209. Suzuki, T., *Studies on the bionomics and chemical control of tsutsugamushi (scrubtyphus mites). II. Trombicula scutellaris Nagayo et al. in southern Kanto of Japan.* Jpn J Exp Med, 1954. **24**(4): p. 181-97.
210. Kuo, C.L., Y. Duan, and J. Grady, *Unconditional or Conditional Logistic Regression Model for Age-Matched Case-Control Data?* Front Public Health, 2018. **6**: p. 57.
211. Wei, Y., et al., *A city park as a potential epidemic site of scrub typhus: a case-control study of an outbreak in Guangzhou, China.* Parasit Vectors, 2014. **7**: p. 513.
212. Sharma, P.K., et al., *Scrub typhus in Darjeeling, India: opportunities for simple, practical prevention measures.* Trans R Soc Trop Med Hyg, 2009. **103**(11): p. 1153-8.
213. Audy JR, B.J., Browning HC, Bullock AA, Cockings KL, Ford W, et al., *Scrub typhus investigations in South East Asia. A report on investigations on scrub typhus by G.H.Q. (India) Field Typhus Research Team, and the Medical Research Council Field Typhus Team, based on the Scrub Typhus Research Laboratory, South East Asia Command, Imphal. Part 1. General account. Part 2. Illustrations. Part 3. Appendices. London: War Office; .* 1947.

214. Rose, W., et al., *Risk factors for acquisition of scrub typhus in children admitted to a tertiary centre and its surrounding districts in South India: a case control study.* BMC Infect Dis, 2019. **19**(1): p. 665.
215. Gentry, J.W., O.W. Phang, and C. Manikumaran, *The effects of rainfall on trombiculid (Acarina:trombiculidae) larval populations in peninsular Malaysia.* Southeast Asian J Trop Med Public Health, 1977. **8**(2): p. 217-20.
216. Traub, R., *Two new species of chiggers of the genus Leptotrombidium (Acarina, Trombiculidae).* Malaysian parasites, 1960. **XLV Stud Inst Med Res Malaya**(29): p. 198–204.
217. Dohany, A.L., B.L. Lim, and D.L. Huxsoll, *Vectors of scrub typhus and their hosts on a mature oil palm estate.* Southeast Asian J Trop Med Public Health, 1979. **10**(4): p. 510-3.
218. Zhang, W.Y., et al., *Scrub typhus in mainland China, 2006-2012: the need for targeted public health interventions.* PLoS Negl Trop Dis, 2013. **7**(12): p. e2493.
219. Hu, J.L., et al., *Clinical Characteristics and Risk Factors of an Outbreak with Scrub Typhus in Previously Unrecognized Areas, Jiangsu Province, China 2013.* Plos One, 2015. **10**(5).
220. Pathania, M., et al., *Scrub typhus: Overview of demographic variables, clinical profile, and diagnostic issues in the sub-Himalayan region of India and its comparison to other Indian and Asian studies.* J Family Med Prim Care, 2019. **8**(3): p. 1189-1195.
221. Varghese, G., et al., *Epidemiology & risk factors of scrub typhus in south India*. Vol. 144. 2016. 76.
222. Trowbridge, P., et al., *Prevalence and risk factors for scrub typhus in South India.* PLoS Negl Trop Dis, 2017. **22**(5): p. 576-582.
223. Morand, S., et al., *Changing landscapes of Southeast Asia and rodent-borne diseases: decreased diversity but increased transmission risks.* 2019: p. e01886.
224. Mayxay, M., et al., *Causes of non-malarial fever in Laos: a prospective study.* The Lancet. Global health, 2013. **1**(1): p. e46-e54.
225. Spicer, P.E., T. Taufa, and A.L. Benjamin, *Scrub typhus (Orientia tsutsugamushi), spotted fever (Rickettsia australis) and dengue fever as possible causes of mysterious deaths in the Strickland Gorge area of Southern Highlands and West Sepik Provinces of Papua New Guinea.* P N G Med J, 2007. **50**(3-4): p. 172-83.
226. Varghese, G.M., et al., *Molecular epidemiology and genetic diversity of Orientia tsutsugamushi from patients with scrub typhus in 3 regions of India.* Emerg Infect Dis, 2015. **21**(1): p. 64-9.

227. Parola, P., et al., *Emerging rickettsioses of the Thai-Myanmar border.* Emerg Infect Dis, 2003. **9**(5): p. 592-5.
228. Phongmany, S., et al., *Rickettsial infections and fever, Vientiane, Laos.* Emerg Infect Dis, 2006. **12**(2): p. 256-62.
229. Chheng, K., et al., *A prospective study of the causes of febrile illness requiring hospitalization in children in Cambodia.* PLoS One, 2013. **8**(4): p. e60634.
230. Deller, J.J. and P.K. Russell, *An Analysis of Fevers of Unknown Origin in American Soldiers in Vietnam.* Annals of Internal Medicine, 1967. **66**(6): p. 1129-1143.
231. Kramme, S., et al., *Orientia tsutsugamushi bacteremia and cytokine levels in Vietnamese scrub typhus patients.* J Clin Microbiol, 2009. **47**(3): p. 586-9.
232. Le Van, N., et al., *Clinical Features, Laboratory Characteristics and Prognostic Factors of Severity in Patients with Rickettsiaceae at Two Military Hospitals, Northern Vietnam.* Infection and drug resistance, 2020. **13**: p. 2129-2138.
233. PicKard, A.L., et al., *A study of'febrile illnesses on the Thai-Myanmar border: predictive factors of rickettsioses.* Southeast Asian J Trop Med Public Health, 2004. **35**(3): p. 657-63.
234. Premaratna, R., et al., *Acute hearing loss due to scrub typhus: a forgotten complication of a reemerging disease.* Clin Infect Dis, 2006. **42**(1): p. e6-8.
235. Venkategowda, P.M., et al., *Scrub typhus: Clinical spectrum and outcome.* Indian journal of critical care medicine : peer-reviewed, official publication of Indian Society of Critical Care Medicine, 2015. **19**(4): p. 208-213.
236. Paul, D., et al., *Clinical Performance Analysis and Evaluation of Quantitative Real Time PCR for Diagnosis of Scrub Typhus in North East India*. 2020.
237. Kannanid, K., et al., *Performance of molecular and serologic tests for the diagnosis of scrub typhus.* PLoS neglected tropical diseases, 2020. **14**.
238. Blacksell, S.D., et al., *Diagnostic Accuracy of the InBios Scrub Typhus Detect Enzyme-Linked Immunoassay for the Detection of IgM Antibodies in Northern Thailand.* Clin Vaccine Immunol, 2016. **23**(2): p. 148-54.
239. Karthikeyan, P.A., S.L. Hoti, and R. Kanungo, *Evaluation of loop-mediated isothermal amplification assay for detection of scrub typhus in patients with acute febrile illness presenting to a Tertiary Care Center in Puducherry, India.* Journal of laboratory physicians, 2019. **11**(1): p. 82-86.
240. Kingston, H.W., et al., *Rickettsial Illnesses as Important Causes of Febrile Illness in Chittagong, Bangladesh.* Emerging Infectious Diseases, 2018. **24**(4): p. 638-645.
241. Zhang, L., et al., *Comparison of a rapid diagnostic test and microimmunofluorescence assay for detecting antibody to Orientia tsutsugamushi in scrub typhus patients in China.* Asian Pac J Trop Med, 2011. **4**(8): p. 666-8.

242. Silpasakorn, S., et al., *Development of new, broadly reactive, rapid IgG and IgM lateral flow assays for diagnosis of scrub typhus.* Am J Trop Med Hyg, 2012. **87**(1): p. 148-52.
243. Blacksell, S.D., et al., *Accuracy of AccessBio Immunoglobulin M and Total Antibody Rapid Immunochromatographic Assays for the Diagnosis of Acute Scrub Typhus Infection.* Clin Vaccine Immunol, 2010. **17**(2): p. 263-6.
244. Watthanaworawit, W., et al., *Diagnostic Accuracy Assessment of Immunochromatographic Tests for the Rapid Detection of Antibodies Against Orientia tsutsugamushi Using Paired Acute and Convalescent Specimens.* The American journal of tropical medicine and hygiene, 2015. **93**(6): p. 1168-1171.
245. Le Viet, N., et al., *Use of eschar swabbing for the molecular diagnosis and genotyping of Orientia tsutsugamushi causing scrub typhus in Quang Nam province, Vietnam.* PLOS Neglected Tropical Diseases, 2017. **11**(2): p. e0005397.
246. Varghese, G.M., et al., *Molecular epidemiology and genetic diversity of Orientia tsutsugamushi from patients with scrub typhus in 3 regions of India.* Emerging infectious diseases, 2015. **21**(1): p. 64-69.
247. Prakash, J.A.J., *Scrub typhus: risks, diagnostic issues, and management challenges.* Res Rep Trop Med, 2017. **8**: p. 73-83.
248. Vietnam Ministry of Health, *Health Statistics Yearbook 2010*. 2010.
249. Jiang, J., et al., *International Rickettsia Disease Surveillance: An Example of Cooperative Research to Increase Laboratory Capability and Capacity for Risk Assessment of Rickettsial Outbreaks Worldwide.* 2021. **8**(94).
250. Zinsstag, J., et al., *From "one medicine" to "one health" and systemic approaches to health and well-being.* Prev.vet.med., 2011. **101**: p. 148-156.
251. Peters, D.H., et al., *Implementation research: what it is and how to do it.* BMJ : British Medical Journal, 2013. **347**: p. f6753.
252. Muthiani, Y., et al., *Low coverage of central point vaccination against dog rabies in Bamako, Mali.* Prev Vet Med, 2015. **120**(2): p. 203-9.
253. Zinsstag, J., Schelling E., Crump, L., Whittaker, M., Tanner, M., and Stephen, C., *One Health: The Theory and Practice of Integrated Health Approaches*. 2nd ed. 2021, Wallingford, UK: CAB International.
254. Obrist, B., Pfeiffer, C., Henley, R., *Multi-layered social resilience: a new approach in mitigation research.* Progress in Development Studies, 2010. **10**(283).
255. Obrist, B., et al., *Access to health care in contexts of livelihood insecurity: a framework for analysis and action*, in *PLoS Medicine*. 2007. p. 1584-1588.

256. Mosimann, L., et al., *A mixed methods approach to assess animal vaccination programmes: The case of rabies control in Bamako, Mali.* Acta Trop, 2017. **165**: p. 203-215.
257. Hirsch Hadorn, G., et al., *Handbook of Transdisciplinary Research*, ed. G. Hirsch Hadorn, et al. 2008: Springer. 1-448.
258. Hitziger, M., et al., *Knowledge integration in One Health policy formulation, implementation and evaluation.* Bull World Health Organ, 2018. **96**(3): p. 211-218.
259. OECD, *Addressing societal challenges using trandisciplinary research*, in *OECD Science, Technology and Industry Policy Papers*, O. publishing, Editor. 2020, OECD: Paris. p. 80.
260. InBios International, I. *Scrub Typhus Detect™ IgM Rapid Test, For Research Use Only*. 2021 [cited 2021 09 April]; Available from: https://inbios.com/scrub-typhus-detecttm-igmrapid-test-usa/.
261. InBios International, I. *Scrub Typhus Detect™ IgM ELISA Kit, For Research Use Only*. 2021 [cited 2021 09 April]; Available from: https://inbios.com/scrub-typhus-detect-igm-elisa-system/.
262. Hanh Tran, et al., *Temporal Dynamics and Performance of PCR and Serological Diagnostic Tests for Scrub Typhus – in Khanh Hoa, Central Vietnam.* Plos neglected tropical diseases (in press), 2021.
263. Kingston, H.W., et al., *Comparative Accuracy of the InBios Scrub Typhus Detect IgM Rapid Test for the Detection of IgM Antibodies by Using Conventional Serology.* 2015. **22**(10): p. 1130-2.
264. Kim, Y.-J., et al., *Clinical Evaluation of Rapid Diagnostic Test Kit for Scrub Typhus with Improved Performance.* Journal of Korean Medical Science, 2016. **31**(8): p. 1190-1196.
265. Silpasakorn, S., et al., *Performance of SD Bioline Tsutsugamushi assays for the diagnosis of scrub typhus in Thailand.* J Med Assoc Thai, 2012. **95 Suppl 2**: p. S18-22.
266. Center, D.K.D.H. *Costs of Medical and Labratory services at Dien Khanh District Health Center (applied from 12/2018)*. 2019; Available from: https://trungtamytedienkhanh.com/bang-gia-dich-vu-trong-kham-chua-benh-bhyt-nam-2018-hieu-luc-tu-15122018-gia-theo-thong-tu-39.html.
267. Vietnam Ministry of Health, *Vietnam National Health Statistic 2018*. 2019. p. 20.
268. Mao, W., et al., *Systematic review on irrational use of medicines in China and Vietnam.* PLoS One, 2015. **10**(3): p. e0117710.
269. Ganguly, N., et al., *Situation analysis antibiotic use and resistance in India.* 2011.

270. Hanh Thi Duc Tran, et al., *Simple clinical and laboratory predictors to improve empirical treatment strategies in areas of high scrub typhus and dengue endemicity, central Vietnam.* Plos neglected tropical diseases (printing), 2021.
271. Bank, W., *Quality and Equity in Basic Health Care Services in Vietnam: Findings from the 2015 Vietnam District and Commune Health FacilitySurvey*. 2015. p. 142.
272. Vietnam Ministry of Health. *Report on the Vietnam improving laboratory capability program for COVID-19*. 2020; Available from: https://kcb.vn/wp-content/uploads/2020/10/10_Tinh-hinh-trien-khai-XN-SARS-CoV-2-VAMS-trong-BV-01.10.2020.pptx.
273. Vietnam Ministry of Health. *Vietnam National Health Conference 2020*. 2020 [cited 2021 14 April]; Available from: https://moh.gov.vn/documents/174521/605005/1.+Tai+lieu+HN+Y+te+toan+quoc+06.01.2020.pdf/280a8947-91a8-4b3f-a378-2e673e05b85e.
274. Pandemic Prevention Taskforce Group, *Letter to Vietnam Prime Minister: Pademic prevention by managing the human-animal-ecosystem interface, with particular focus on restricting commercial wildlife trade and consumptions*. 2020.
275. Vietnam Ministry of Health, *Guidance on infectious disease report in Vietnam (Circular No 48/2010/TT-BYT, issused on 31 Dec 2010)*. 2010.
276. Quy Nhon Institute of Malaria and Parasites. *Scrub typhus in Khanh Hoa*. 2011 [cited 2021 23 May]; Available from: http://www.impe-qn.org.vn/impe-qn/vn/portal/InfoPreview.jsp?ID=4535.
277. Vien Quang Mai, N.T.Q., Nguyen Dinh Huy, Trinh Hoang Long, Nguyen Duc Duy, Nguyen Bao Trieu,, *Evaluation for test results of scub typhus caused by Rickettsia tsutsugamushi in Khanh Hoa province, during 2013 - 2014.* Vietnam Journal of Preventive Medicine, 2015. **XXV**(5 (165), Special Issue): p. 110.
278. Vietnam Ministry of Health. *A hospital and community based investigation of ricketsia diseases, crub typhus and Q fever in Viet Nam*. 2017 [cited 2021 14 April]; Available from: https://moh.gov.vn/web/ministry-of-health/top-news/-/asset_publisher/EPLuO8YEhk19/content/a-hospital-and-community-based-investigation-of-ricketsia-diseases-crub-typhus-and-q-fever-in-viet-nam?inheritRedirect=false.
279. Center, V.N.D.P.H. *Pediatric Department - Successful treatment for scrub typhus pediatric patients*. [cited 2021 14 April]; Available from: http://www.trungtamytevanninh.com.vn/TinChiTiet/tabid/105/id/947/Default.aspx.
280. Khanh Vinh District Preventive Health Center. *Treatment for scrub typhus*. 2020 [cited 2021 14 April]; Available from: http://trungtamytekhanhvinh.khanhhoa.gov.vn/pddt/348-sm.html.

281. Thua Thien Hue Health Department. *Do not confuse scrub typhus with malaria*. 2011 [cited 2021 14 April]; Available from: https://syt.thuathienhue.gov.vn/?gd=35&cn=90&tc=884.
282. Tuyen Quang Health Department. *Warning of danger fever - Scrub typhus*. 2016 [cited 2021 14 April]; Available from: http://soytetuyenquang.gov.vn/tin-tuc-su-kien/kham-chua-benh/canh-bao-nguy-hiem-tu-benh-sot-mo.html.
283. Hanoi Health Department. *Successful treatment for scrub typhus patients with multiorgan failure complications*. 2020 [cited 2021 14 April]; Available from: https://soyte.hanoi.gov.vn/tin-tuc-chung/-/asset_publisher/4IVkx5Jltnbg/content/-ieu-tri-thanh-cong-cho-benh-nhan-sot-mo-bien-chung-suy-a-tang.
284. Heath, Q.N.D.o., *Report on results of selection of agencies of chemicals and tests for public health facilities in Quang Ngai 2020-2021* 2021.
285. Heath, H.D.D.o., *Report on results of selection of agencies of chemicals and tests for public health facilities in Hai Duong 2020-2021 (Decision 1830/QD-UBND dated 03/7/2020)*. 2021.
286. Hanh Thi Duc Tran, et al., *Ecological and behavioural related risk factors of scrub typhus in central vietnam: a case control study.* Infectious Diseases of Poverty BMC (Under review), 2021.
287. Health, V.M.o., *Providing guidelines for functions and duties of commune-level healthcare stations (Circular 33/2015/TT-BYT, dated 27 Oct 2015)*. 2015.
288. Nha Trang Pasteur Institute, *Scrub typhus health education campaign*. 2014.
289. Kim, J., E. Lee, and H.C. Rhee, *Cost-benefit analysis of the tsutsugamushi disease prevention program in South Korea.* Jpn J Infect Dis, 2012. **65**(3): p. 222-7.
290. Vietnam Ministry of Finance, *Regulation of payment for workshop (No. 40/2017/TT-BTC, signed 28 April 2017)*. 2017.
291. Vietnam Ministry of Finance, *Regulation of payment for training (No. 139/2010/TT-BTC, signed 21 September 2010)*. 2010.
292. Ninh Hoa Hospital. *List cost of Health service at Ninh Hoa Hospital 2020 (applied from 30/11/2018-present)*. 2020 [cited 2021 11 April]; Available from: https://benhvienninhhoa.khanhhoa.gov.vn/Chitiettin/tabid/65/id/765/BANG-GIA-DICH-VU-Y-TE-(BENH-VIEN-HANG-II)/Default.aspx.
293. Ninh Hoa Hospital. *List cost of Laboratory service at Ninh Hoa Hospital 2020 (applied from 30/12/2019-present)*. 2020 [cited 2021 11 April]; Available from: https://benhvienninhhoa.khanhhoa.gov.vn/Portals/0/Gia_BHYT/2021/2%20Khoa%20XN_2020.pdf.

294. Fischer, T.K., et al., *Health care costs of diarrheal disease and estimates of the cost-effectiveness of rotavirus vaccination in Vietnam.* J Infect Dis, 2005. **192**(10): p. 1720-6.
295. General Statistics Office of Vietnam, *Statistical Yearbook of Vietnam 2019*, General Statistics Office of Vietnam, Editor. 2020, Statistical Publishing House: Hanoi. p. 850.
296. Salje, J., et al., *Rickettsial infections: A blind spot in our view of neglected tropical diseases.* PLOS Neglected Tropical Diseases, 2021. **15**(5): p. e0009353.
297. Blacksell, S.D., et al., *Scrub typhus serologic testing with the indirect immunofluorescence method as a diagnostic gold standard: a lack of consensus leads to a lot of confusion.* Clin Infect Dis, 2007. **44**(3): p. 391-401.

Curriculum Vitae – December 20, 2021

Tran Thi Duc Hanh

Mobile in Switzerland: (+41)779887487

Mobile (Vietnam): (+84) (0) 91986 4183

hanhtranch@gmail.com

Vietnamese Citizen

Research Fields

Epidemiology, global health, emerging diseases, migrant heath, evaluation study designs, innovation, implementation research.

Education

09.2017 - 12.2021 PhD Doctor of Philosophy (PhD) in Epidemiology, Swiss Tropical and Public Health Institute, University of Basel, Switzerland

06.2011 - 12.2012 Master of Epidemiology (Mepi), University of Queensland, Australia

09.2003 - 08.2007 Bachelor of Public Health, Hanoi School of Public Health, Vietnam

Fellowships and Awards

2020 PhD Scholarship: Swiss Government Excellence Scholarship for Foreign Scholar (ESKAS) for extending PhD work 01.2021- 03.2021

2017 PhD Scholarship: Swiss Government Excellence Scholarship for Foreign Scholar (ESKAS) for PhD in Switzerland

2012 Dean's Commendation for High Achievement, University of Queensland, Australia

2011 Dean's Commendation for High Achievement, University of Queensland, Australia

2011 Master Scholarship: Australia Government Development Scholarship (ADS)

2008 Successful Candidate, the Sixth Vietnam Innovation Day Competition, sponsored by the World Bank (Wedding borax out of our life project)

2007 The first in Hanoi School of Public health (2003-2007 academic years)

Positions held

2008 – Present Researcher and Lecturer: Hanoi University of Public Health (formerly called as Hanoi School of Public Health - HSPH)

Department of Epidemiology

Responsibilities: teaching, student supervision, research, consultant, course coordinator (epidemiology, communicable disease epidemiology, One-health, non-communicable disease epidemiology, evaluation study design)

2007-2008 Scientific Research Management Department: HSPH

Responsibilities: managing projects/research, university scientific abroad secretary

2006 – 2007 DKT International, Vietnam

Responsibilities: research assistant, admin

Research Experiences

2017-present **Principle Investigator,** Scrub typhus in Khanh Hoa, Viet Nam: an eco-epidemiological approach (PhD thesis)

2017 **National coordinator/Researcher,** Analysing the policy and governance environment for NCD control, and identifying potential policy options (Iran, Pakistan, Tunisia, Bangladesh, Nepal, Vietnam, Afghanistan, United Kingdom), 2017-2018, funded by Medical Research Council

2016 **National coordinator/Researcher,** Engaging community health workers (CHWs) in Combating Chronic Diseases in Asia (Bangladesh, China, Nepal, Vietnam), 2016-2017, funded by WHO Asia Pacific Observatory Research Hub

National coordinator/Researcher, Feasibility Assessment of Invigorating grassrooTs primary Healthcare for cardiovascular prevention and management in low-resource settings in China, India, Vietnam and Kenya (FAITH study), 2016-2017, funded by WHO Asia Pacific Observatory Research Hub

2015 **Principle Investigator**, Epidemiological distribution of non-communicable risk factors among male aged 24-65 in Long Bien, Hanoi, funded by Atlantic Philanthropies – Hanoi School of Public Health

Researcher, Impact of tobacco smoking to reducing morbidity of non-communicable diseases in Vietnam: a modeling study, funded by Atlantic Philanthropies – Hanoi School of Public Health

Researcher, Reproductive health status , need for reproductive health care and model interventions to enhance access to reproductive health services for women migrant workers in industrial parks", Vietnam Ministry of Health – Hanoi School of Public Health

Researcher, Review report: Policies, health program for sustainable development and poverty reduction in Vietnam, General and Health Policy Division, Ministry of Health – Hanoi School of Public Health

2013 – 2014 **Co-Principle Investigator**, Cervical cancer and Risk factors in Bac Ninh and Can Tho, Viet Nam, 2013", funded by Vietnam Health national target program

Project Coordinator, MHealth information for migrants: a pilot project to increase health information accessibility for migrants in Vietnam ("M2 project")", funded by Grand Challenges Canada.

Principle Investigator, Public Awareness of Land, Health Insurance and Minings", prepared for Oxfam in Vietnam

Researcher, Literature review on Service Delivery Models for Non-communicable Diseases Prevention and Control in Vietnam", prepared for World Health Organization in Vietnam

2012 **Principle Investigator,** Correlates of mortality among middle-aged women: results from a nationally representative prospective Australian cohort study (Master of Epidemiology Thesis)

2010- 2011 **Researcher,** Dengue hemorrhagic fever, a epidemiological study in Bach Mai Hospital, Hanoi, Vietnam", Hanoi School of Public Health

Researcher, Distribution and risk factors of Cervical Human Papilloma Virus (HPV) Infection among women in Vietnam", Hanoi School of Public Health

Research assistant, “Integrated Health, Social and Economic Impacts of Extreme Events: Evidence, Methods and Tools” Project, EU Commission – Hanoi School of Public Health

2007 – 2009 **Principle Investigator**, “Wedding borax out of our life” Project 2008-2009, funded by World Bank - Vietnam Innovation Day 2008, June 2008

Researcher, “Implementing knowledge into practice for improved neonatal survival; a community-based trial in Quang Ninh province, Vietnam”, Uong bi Hospital – Vietnam Ministry of Health

Researcher, “Reproductive health in ethnic minorities in Vietnam, A review for the period 2000 – 2007”, prepared for UNFPA

Reaseacher, “Enforcement and implementation of smoke-free policy at work places and public venues in Hanoi” Project

Reaseacher, “Evaluating the satisfactoriness of patients with the services of St Paul hospital”, Department of nursing, St Paul hospital

2006 - 2007 **Reaseacher**, “Participatory Rapid Assessment on avian influenza risk behaviors for developing intervention in Bac Ninh, Thanh Hoa and Dong Thap”, Hanoi school of public heath

Reaseacher, “Meta-evaluation of information, education, communication (IEC) studies and knowledge, attitudes and practices (KAP) surveys and other Avian Influenza assessments research”, Hanoi school of public heath, April 2007, donated by UNICEF

Reaseacher, “Developing and testing communication materials for prevention and control trachoma in 4- 5 graded students of My Tien primary school, Myloc, Namdinh in May 2007” (Thesis)

Reaseacher, “Increase of parents’ support in health care for their pre-school aged children with disabilities in Ha Tay”, Hanoi school of public health

Reaseacher, “Increase knowledge, attitudes and practices (KAP) in integrating care of mothers to their 0-5 child with acute respiratory infections (ARI) in a commune, Haiduong, Vietnam in June 2006”, Hanoi school of public health

Research Funding

Granted:

2019 -2021 **Wolfermann-Nägeli-Stiftung Award for Project Tsutsugamushi-Fieber (scrub typhus) in Khanh Hoa, Viet Nam**

Pricipal Investigator: Hanh Tran Thi Duc [CHF 20,000]

2016-2017 **Alliance for Health Policy and System Research, WHO Special Programme for Research and Training in Tropical Diseases (TDR)**

Strengthening Capacity for Implementation Research (SCAPIR)

Co-investigators (CIs): Ha Bui, Anh Le Thi Kim, **Hanh Tran** [USD 115,000]

2015-2016 **UNICEF/UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR)**

Capacity building for Implementation Research in TB in Viet Nam (CaR-TB)

CIs: Vinkeles Melchers Natalie, **Hanh Tran**, Ha Bui [USD 49,977]

2014-2015 **Hanoi School of Public Health cross collaboration funding**

Epidemiological distribution of non-communicable Risk factors among male aged 24-65 in Long Bien, Hanoi

CIs: **Hanh Tran**, Ngoc Le, Anh Le Thi Kim, Van Nguyen [USD 5,000]

2008 – 2009 **World Bank in Vietnam - Vietnam Innovation Day 2008**

Wedding borax out of our life Project 2008-2009

CIs: **Hanh Tran**, Anh Nguyen, Huong Le, Trang Dao [USD 10,000]

Training/Workshops

1. *Migration Health training course*, organised by SSPH+ PhD Program in Public Health, 06-08 Dec 2021, Basel, Switzerland (hybrid format)

2. *Health Policy Analysis Training Workshop*, organised by University College London, and School of Public Health, Tehran University of Medical Science, 8-12 July, 2017, Dubai, UAE

3. *Implementation Research for Practitioners and Implementers,* organised by the Alliance for Health Policy & Systems Research/ BRAC James P Grant School of Public Health, January 15-17, 2017, Dhaka, Bangladesh

4. *The 2nd Annual Conference of Chinese Consortium of Universities for Global Health (CCUGH)*, organized by Duke Kunshan University, 14-15 Oct 2016, Kunshan, China (brief presentation)

5. *Implementation Research Training Course,* given by Alliance for Health Policy and Systems Research (AHPSR), Hanoi, Vietnam, 23-27 May, 2016

6. *One Health and Eco-Health workshop*, hold by The Center for Public Health and Ecosystem Research, Hanoi School of Public Health (CENPHER) and Vietnam One Health University Network (VOHUN), Hanoi, Vietnam, 17-18 August, 2015

7. *International Journal Publication training workshop*, given by Hanoi School of Public Health, Hanoi, Vietnam, 25-27, November, 2014

8. *Regression Methods in Biostatistics training workshop,* given by Center for Disease Control and Prevention (CDC), Hanoi, Vietnam, 28 March – 1 April, 2011.

9. *HIV Surveillance for Most-at-Risk Populations training workshop,* given by Center for Disease Control and Prevention (CDC), Hanoi, Vietnam, 13-17 December, 2010.

10. *Public Health Evaluation: choosing, using and justifying mixed method training workshop,* given by Center for Disease Control and Prevention (CDC), Hanoi, Vietnam, 13-16 September, 2010.

11. *Asian Symposium on disaster impact and its asseessment in Aisa,* Hue College of Economics, Hue University, 25-27 August, 2010

12. *The 10th Annual Summer Evaluation Training,* organized by the National Center for Chronic Disease Prevention and Health Promotion (NCCDPHP) in collaboration with

the CDC and the American Evaluation Association (AEA), Atlanta, USA, June 13-16, 2010.

13. *Geospatial Technologies and Public Health training course,* given by Preventive medicine and Environment Department, Ministry of health, 3-6 May 2010.

14. *Integrated Health, Social and Economic Impacts of Extreme Events: Evidence, Methods and Tools Workshop,* Jakarta, Indonesia, 10–14 October, 2009.

15. *Training of trainer course on HIV/AIDS prevention and Control*, given by Hanoi School of Public Health, 15-19 December , 2008.

16. *International Health Winter School, Emerging and re-emerging infectious deceases in South East Asia*, given by Hanoi School of Public Health, 10-21 November, 2008.

17. *XVII International AIDS Conference, Mexico City,* Vietnam Delegate, 3-8 August 2008.

18. *Building Skills on designing and implementing community project with efficiency*, given by World Bank, Hanoi, June 2008,

19. Epidemiology in Management training course, given by Hanoi School of Public Health, 24-28 September, 2007.

Publications

Tran, H.T.D., et al., Ecological and behavioural risk factors of scrub typhus in central Vietnam: a case-control study. Infectious Diseases of Poverty, 2021. 10(1): p. 110.

2. Luong, H.M., **Hanh Tran** et al., Oro-Dental Health and Primary Nephrotic Syndrome among Vietnamese Children. Children, 2021. 8(6).

3. **Hanh Thi Duc Tran**, C.S., et al., Simple clinical and laboratory predictors to improve empirical treatment strategies in areas of high scrub typhus and dengue endemicity, central Vietnam. Plos neglected tropical diseases (printing), 2021.

4. Long, H., **Hanh Tran** et al., Engaging village health workers in non-communicable disease (NCD) prevention and control in Vietnam: A qualitative study. Glob Public Health, 2020. 15(4): p. 611-625.

5. Gong, E., **Hanh Tran** et al., Feasibility assessment of invigorating grassrooTs primary healthcare for prevention and management of cardiometabolic diseases in resource-limited settings in China, Kenya, Nepal, Vietnam (the FAITH study): rationale and design. Glob Health Res Policy, 2019. 4: p. 33.

6. Vu, L.T.H., **Hanh Tran** et al., Community-Based Screening for Cervical Cancer Using Visual Inspection With Acetic Acid: Results and Lessons Learned From a Pilot Study in Vietnam. Journal of public health management and practice : JPHMP, 2018. 24 Suppl 2 Supplement, Public Health in Vietnam: p. S3-S8.

7. **Tran, T.D.H**., et al., Premarital sex, contraceptive use among unmarried women migrant workers in industrial parks in Vietnam, 2015. Health Care Women Int, 2018. 39(4): p. 377-388.

8. Pham, C.V., **H.T.D. Tran,** and N.T. Tran, Alcohol Consumption and Binge Drinking Among Adult Population: Evidence From the CHILILAB Health and Demographic Surveillance System in Vietnam. J Public Health Manag Pract, 2018. 24 Suppl 2 Supplement, Public Health in Vietnam: p. S67-s73.

9. Le, A.T.K., **Hanh Tran** et al., Reproductive tract infection and related factors among female migrants working in industrial zones in Vietnam 2013-2014. Health Care Women Int, 2018. 39(4): p. 389-403.

10. Vu, L.T., **Hanh Tran** et al., mHealth information for migrants: an e-health intervention for internal migrants in Vietnam. Reprod Health, 2016. 13(1): p. 55.

11. **Tran Thi Duc Hanh**, et al., Alcohol consumption status, pattern and related risk factors among males aged 25 - 64 years, in Long Bien district, Hanoi, 2015. Vietnam Journal of Public Health, 2016. 13(40).

12. **Tran Thi Duc Hanh**, Le Thi Kim Anh, and Bui Thi Thu Ha, Premarital sex, contraceptive use and related factors among single female workers working in industrial parks in Vietnam, 2015. Vietnam Journal of Practical Medicine, 2016. 1005.

13. Le Thi Kim Anh, **Tran Thi Duc Hanh**, and Le Bich Ngoc, Difficulties in the implementation of health policies ralated to the sustainable poverty reduction for people in Dien Bien, Kontum and Quang Tri in 2014. Vietnam Journal of Public Health, 2016. 39.

14. Le Thi Kim Anh, **Tran Thi Duc Hanh**, and Bui Thi Thu Ha, Reproductive tract infection and its related factors among female migrants working in industrial zones in Vietnam 2013-2014. Asia-Pacific Journal of Public Health, 2016. reviewed.

15. **Tran Thi Duc Hanh**, V.T.H.L., Le Tu Hoang, Nguyen Thuy Linh, Bui Thi Thu Ha.,, Risk factors for cervical cancer among women aged 30-65 years: results of case-control study in Bac Ninh anc Can Tho, 2013. Vietnam Journal of Preventive Medicine, 2015. XXV(No 3 (163)).

16. **Tran Thi Duc Hanh**, V.T.H.L., Le Tu Hoang, Nguyen Thuy Linh, Bui Thi Thu Ha.,, Diagnostic value of Cervical Cancer Screening using VIA and Pap Smear methods for wome aged from 30 to 65 in Bac Ninh and Can Tho, 2013, Vietnam Journal of Public Health. Vietnam Journal of Public Health, 2015. 36.

17. Nguyen Thuy Linh, **Tran Thi Duc Hanh**, Vu Thi Hoang Lan, Nguyen Thanh Binh, Bui Thi Thu Ha.,, Cervical cancer screening by Pap smear and Visual inspection of the cervix with acid acetic (VIA) test among women aged 30-65 in Luong Tai, Bac Ninh, Vietnam, 2013. Vietnam Journal of Preventive Medicine, 2014. XXIV (No 6 (155)).

18. Nguyen Thanh Binh, **Tran Thi Duc Hanh**, Vu Thi Hoang Lan, Bui Thi Thu Ha,, Cervical canver screening using Visual inspection of the cervix with acid acetic (VIA) method in Bac Ninh and Can Tho, Vietnam, 2013, . Vietnam Journal of Preventive Medicine, 2014. Vol XXIV, No 3 (152)

19. Nguyen Thanh Binh, **Tran Thi Duc Hanh**, Vu Thi Hoang Lan, Bui Thi Thu Ha.,, Cervical cancer screening by Visual inspection of the cervix with acid acetic (VIA) in Bac Ninh, Vietnam, 2013: Advantages and Difficulties. Vietnam Journal of Practical Medicine, 2014. No 5 (919)

20. Le Tu Hoang, **Tran Thi Duc Hanh**, Vu Thi Hoang Lan, Bui Thi Thu Ha.,, Effectiveness of screening for cervical cancer among woment aged 30-55 using Visual inspection of the cervix with acid acetic (VIA) and Pap smear methods in Can Tho province, Vietnam, 2013. Vietnam Journal of Preventive Medicine 2014. XXIV (No 5 (154)).

21. Bui TTH, V.T., Le TTH, La NQ, **Tran TDH**, Nguyen N, Le XH, Nguyen VN, Pham TT, Nguyen TY.,, Epidemiology of Infectious Diseases. Vietnam Journal of Preventive Medicine. 2014, Hanoi: Medical Publication.

22. Vu, L., **Hanh Tran** et al., Prevalence of cervical human papillomavirus infection among married women in Hanoi, Vietnam, 2010. Asia Pac J Public Health, 2012. 24(2): p. 385-90.

23. **Tran, H.D.T**., Correlates of mortality among middle-aged women: results from a nationally representative prospective Australian cohort study, in Epidemiology 2012, University of Queensland: Queensland, Australia.

24. Bich, T.H., **Hanh Tran** et al., Impacts of flood on health: epidemiologic evidence from Hanoi, Vietnam. Glob Health Action, 2011. 4: p. 6356.

25. Bich.T.H, Q.L.N., Ha.L.T.T, **Hanh.T.T.D** Flooding and its impact on health: epidemiologic evidence from Hanoi, Vietnam, Asian Symposium on disaster impact and its assessment in Asia, in Asian Symposium on disaster impact and its assessment in Aisa. 2010.

26. Bich.T.H, Q.L.N., Ha.L.T.T, **Hanh.T.T.D** The difference between flood affected and flood non-affected households in mortality and morbidity patterns; social and economic aspects, in Health for the million. 2010.

27. **Tran, H.D.T**., Developing and testing communication materials for prevention and control trachoma in 4- 5 graded students of My Tien primary school, Myloc, Namdinh in May 2007, in Epidemiology. 2007, Hanoi University of Public Health: Hanoi.

Professional skills

Good judgment, high sense of responsibility, organizational and analytical skills.

Research Skills:

- Strong research, analytical and report writing skills.
- Advanced data analysis
- Strong skills at project management
- Strong skills at program implementation at local.
- Field coordination and supervision
- Project Evaluation

Language: - English: IELTS: 6.5

- Vietnamese: Native language, excellent in oral and writing language

Computer software:

- Excellent in Stata, SPSS,
- Excellent in QGIS/ArcGIS
- Excellent in MindJet Pro, Microsoft Excel, Word, PowerPoint
- Epi Info (good)