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NU-LSHTM Joint Ph.D. Degree Programme

**Developing A Surface Cell Antigen A-based Serological Test
for Scrub Typhus Diagnosis**

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Nagasaki University “Doctoral Program for World-leading Innovative and Smart
Education” for Global Health

Declaration

I, Saho Takaya, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Diagnosis of scrub typhus is challenging. The main hurdle is the antigenic and geographical heterogeneity of *Orientia tsutsugamushi*. Serological tests have used antigen combinations based on locally circulating strains. Surface cell antigen A (ScaA) is an autotransporter protein, and specific antibody responses against its passenger domain in humans were previously reported. This study aimed to develop a new serological test to diagnose scrub typhus. The first objective was to develop a ScaA IgM enzyme-linked immunosorbent assay (ELISA) and estimate the sensitivity and specificity using Bayesian latent class models. The second objective was to narrow down the epitope region and develop a simplified ELISA based on peptide fragments. A ScaA IgM ELISA was developed with a recombinant 101-amino acid fragment of the ScaA autotransporter domain. This amino acid sequence had a high identity across *Orientia* strains. The new test was evaluated using two cohorts previously collected in Vietnam: a rickettsiosis cohort and a fever cohort. The estimated sensitivity and specificity of a ScaA IgM ELISA were 73.9% and 96.9% in the rickettsiosis cohort and 68.6% and 94.8% in the fever cohort. The antibody response against the recombinant ScaA protein was slower to develop than that against 56kDa type-specific antigen. The estimated sensitivity and specificity were interpreted in a clinical scenario of the scrub typhus prevalence of 3-30% in patients with acute febrile illnesses. To develop a ScaA peptide IgM ELISA, overlapping peptides of the 101-amino acid sequence were investigated. Narrowing down the epitope region reduced the sensitivity but increased the specificity. The sensitivities of the ScaA serological tests were not sufficient. This lack of sensitivity may have been due to the low antigenicity of ScaA and the quantity, timing, and location of its expression in the course of infection. The test sensitivity may improve by combining it with other conserved antigens.

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Table of content

Declaration.....	2
Abstract.....	3
Acknowledgement.....	4
Table of content.....	5
List of Tables.....	8
List of Figures.....	10
Abbreviations.....	13
PART I. Background to the thesis.....	16
1. Scrub typhus.....	16
1.1. Epidemiology.....	18
1.2. Vector and transmission.....	18
1.3. <i>Orientia tsutsugamushi</i>	19
1.3.1. Outer membrane proteins.....	21
1.3.1.1. 56kDa type-specific antigen.....	21
1.3.1.2. Surface cell antigen A.....	22
1.3.1.3. Other outer membrane proteins.....	25
1.3.2. Infection cycle.....	25
1.3.3. Human immune response to <i>Orientia tsutsugamushi</i> infection.....	27
1.4. Clinical disease.....	29
1.5. Laboratory diagnosis.....	30
1.5.1. Bacterial isolation.....	31
1.5.2. Nucleic acid amplification test.....	31
1.5.3. Serological test.....	32
1.5.4. Point-of-care test.....	34
1.6. Treatment and vaccine.....	35
2. Serological tests of infectious diseases.....	37
2.1. Antigen and antibody.....	37
2.2. Detection methods of serological tests.....	38
2.3. Advantages and disadvantages of serological tests.....	40
3. Evaluation of a new diagnostic test in the absence of a gold standard test.....	43
4. Aims and objectives.....	46
4.1. Aims.....	46

4.2.	Objectives.....	46
4.3.	Target product profile.....	46
4.4.	Expectations and intended value.....	47
5.	Progress before the Ph.D. study.....	49
PART II. Material and methods.....		51
6.	Samples.....	51
7.	Diagnostic tests used in the Ph.D. study.....	56
8.	Prediction of the ScaA protein structure and B-cell linear epitopes.....	59
9.	Statistical analysis.....	60
10.	Ethics approval.....	60
11.	Funding.....	61
PART III. Development of a ScaA-based ELISA.....		62
12.	Development of a recombinant ScaA-based ELISA.....	62
12.1.	Amino acid sequence conservation of the ScaA antigen region across strains.....	62
12.2.	Progress until the Qualifying Examination.....	66
12.3.	Purification of a recombinant ScaA antigen.....	69
12.4.	Recombinant ScaA-based ELISA protocol.....	73
13.	Evaluation of a recombinant ScaA-based ELISA.....	76
13.1.	Demography of the cohorts and summary of applied test results.....	76
13.2.	Cut-off values for an IgM IFA and a recombinant ScaA-based IgM ELISA.....	78
13.3.	Sensitivity and specificity of a recombinant ScaA-based ELISA against an IgM IFA and PCR assay as reference standard tests.....	84
13.4.	Cut-off index.....	87
13.5.	Characteristics of a recombinant ScaA-based ELISA.....	89
13.6.	Estimation of the sensitivity and specificity of a recombinant ScaA-based IgM ELISA by Bayesian latent class model.....	98
13.6.1.	Model description.....	98
13.6.2.	Definition and rationale of prior information.....	103
13.6.3.	Assessment of model fitness.....	105
13.6.4.	Results.....	106
13.7.	Summary and discussion.....	108
14.	Development of a ScaA peptide ELISA.....	116
14.1.	Overlapping peptide synthesis.....	116
14.2.	Prediction of the three-dimensional structure and B-cell linear epitopes.....	118

14.3.	ScaA peptide dot-blot	124
14.4.	ScaA peptide IgM ELISA protocol	125
14.5.	ScaA peptide IgM ELISA results	130
14.6.	Estimation of the sensitivity and specificity of a ScaA peptide IgM ELISA by Bayesian latent class model	137
14.7.	Summary and discussion	139
PART IV. Overall discussion and conclusions		144
15.	The main finding of the study.....	144
16.	Discussion and future directions	146
17.	Impact of the COVID-19 pandemic on the Ph.D. study	153
18.	Conclusions.....	154
REFERENCES.....		155
APPENDICES		168

List of Tables

Table 1. Different properties of intracellular and extracellular <i>Orientia tsutsugamushi</i>	27
Table 2. The sensitivity and specificity of diagnostic tests and a clinical sign reported in previous studies.....	35
Table 3. Target product profile for a recombinant ScaA-based IgM ELISA	47
Table 4. Summary information of the assay development, rickettsiosis, and fever cohorts.....	54
Table 5. The amino acid sequence identity of ScaA ATD, ScaA-ATD-A, ScaA-ATD-B, and ScaA-ATD-C of 14 <i>Orientia tsutsugamushi</i> strains compared to the amino acid sequence of the recombinant ScaA autotransporter domain antigen (BDP28846.1).	63
Table 6. Forward and reverse primers for the ScaA antigen gene cloning	69
Table 7. A recombinant ScaA protein purification protocol.....	71
Table 8. A recombinant ScaA-based IgM ELISA protocol.....	75
Table 9. Basic demography of the rickettsiosis cohort and fever cohort.....	77
Table 10. Basic characteristics of overall, scrub typhus, and non-scrub typhus patients in the rickettsiosis and fever cohorts diagnosed using an IgM IFA and/or 47kDa-based PCR assay as gold standard tests.	80
Table 11. Sensitivity, specificity, and Youden index of five cut-off values for the recombinant ScaA-based IgM ELISA in the rickettsiosis cohort	83
Table 12. Likelihood ratios for a positive test result for different OD value slices of the recombinant ScaA-based IgM ELISA.....	83
Table 13. Number of patients by combinations of test results in the rickettsiosis cohort.	84
Table 14. Number of patients by combinations of test results in the fever cohort.....	86
Table 15. Sensitivity, specificity, and Youden index of five cut-off values for the cut-off index of the recombinant ScaA-based IgM ELISA in the rickettsiosis cohort.....	87
Table 16. Sensitivities and specificities of a recombinant ScaA-based IgM ELISA using absolute OD values and cut-off indices in the rickettsiosis and fever cohorts.....	88
Table 17. Sensitivities of a TSA56- and ScaA-based IgM ELISAs by IgM IFA serotypes.	90
Table 18. Prevalence of scrub typhus, test sensitivities, and test specificities at admission and follow-up in the rickettsiosis cohort.	94
Table 19. Sensitivities of a recombinant ScaA-based and TSA56-based IgM ELISA in patients with severe and non-severe scrub typhus.....	95
Table 20. Positivity rates for a recombinant ScaA-based IgM ELISA, TSA56-based IgM ELISA, and IgM IFA in patients in whom other bacteria were isolated.	97

Table 21. Checklist of items for the Standards for the Reporting of Diagnostic Accuracy studies by the use of Bayesian Latent Class Models (STARD-BLCM) for a recombinant ScaA-based IgM ELISA.	101
Table 22. Assumptions on sensitivities and specificities of the four diagnostic tests and the presence of eschar.....	103
Table 23. Comparison of three latent class models in the rickettsiosis and fever cohorts.....	105
Table 24. A scrub typhus prevalence, test sensitivities, and test specificities estimated using an IgM IFA as a reference standard test or using Bayesian latent class models in the rickettsiosis cohort. .	106
Table 25. A scrub typhus prevalence, test sensitivities, and test specificities estimated using an IgM IFA and a PCR assay as reference standard tests or using Bayesian latent class models in the fever cohort.....	107
Table 26. Basic characteristics of nine ScaA peptides.	116
Table 27. List of B-cell linear epitopes in the ScaA antigen region predicted by Bepipred 2.0.....	122
Table 28. ScaA peptide IgM ELISA protocol.....	126
Table 29. Cut-off values for ScaA peptide IgM ELISA defined as the mean plus three standard deviations of healthy controls and by the receiver operating characteristic curve analysis.....	127
Table 30. Sensitivities of ScaA peptide ELISAs in scrub typhus patients with low and high background noise reactions.....	132
Table 31. IgM ELISA OD values to the recombinant ScaA antigen and ScaA peptides 1-9 of 69 scrub typhus patients with a positive IgM IFA result in the rickettsiosis cohort.....	132
Table 32. Number of patients by combinations of test results including a ScaA peptide 6 IgM ELISA in the rickettsiosis cohort.	134
Table 33. Sensitivities of a TSA56-based IgM ELISA, recombinant ScaA-based IgM ELISA, and ScaA peptide 6 IgM ELISA by IgM IFA serotypes.	135
Table 34. Sensitivity, specificity, and Youden index of three cut-off values for the cut-off index of the ScaA peptide 6 IgM ELISA in the rickettsiosis cohort.....	136
Table 35. Comparison of three latent class models in the rickettsiosis cohorts.	137
Table 36. A scrub typhus prevalence, test sensitivities, and test specificities estimated using an IFA as a reference standard test or using Bayesian latent class models in the rickettsiosis cohort.....	138

List of Figures

Figure 1. Description of tsutsugamushi disease in the book ‘Tousanjin’yawa - Ehonhyakumonogatari’.	17
Figure 2. Schematic representation of the phylogenetic relationships of major clades of <i>Orientia tsutsugamushi</i> based on DNA sequences of the <i>tsa56</i> gene and the geographical distribution of the genotypes based on <i>tsa56</i> sequences in the Asia-Pacific region.	20
Figure 3. The autotransporter protein biogenesis.....	22
Figure 4. Similarity plots of <i>tsa56</i> and <i>scaA</i> nucleotide and amino acid sequences of the Karp, Kato, and Gilliam strains compared to the sequences of the Boryong strain.....	24
Figure 5. The infection cycle of <i>Orientia tsutsugamushi</i>	26
Figure 6. Schematic representation of direct, indirect, and sandwich ELISAs.	39
Figure 7. Schematic explanation of the background noise reaction correction.....	42
Figure 8. IFA slides of FA Slide R. Tsutsugamushi Seiken (Denka Company Ltd, Japan).....	57
Figure 9. The amino acid sequence in FASTA format of ScaA autotransporter domain submitted to the SWISS-MODEL, I-TASSER, and IEDB programs.....	59
Figure 10. Alignment of amino acid sequences of the ScaA antigen region of 15 <i>Orientia tsutsugamushi</i> strains.	64
Figure 11. Alignment of amino acid sequences of a ScaA autotransporter domain of 12 <i>Orientia tsutsugamushi</i> strains.	65
Figure 12. IgM antibody responses to the recombinant ScaA protein synthesised using a pET43.1a(+) vector and a control protein.	66
Figure 13. A result report of the ScaA protein synthesis from CellFree Sciences.	68
Figure 14. ScaA purification products resolved by SDS-PAGE and stained with Coomassie Blue.	70
Figure 15. Analysis of the synthesised recombinant ScaA protein.....	72
Figure 16. Background noise reaction of the recombinant ScaA-based IgM ELISA with different blocking agents and microplates.	73
Figure 17. IgM antibody responses to the recombinant ScaA protein of different concentrations. ...	74
Figure 18. Study flow of participants to estimate test sensitivities and specificities by Bayesian latent class models.	76
Figure 19. Distribution of patients by IgM IFA titres in the rickettsiosis and fever cohorts.....	78
Figure 20. Distribution of patients by ScaA-based and TSA56-based ELISA OD values at admission and follow-up in the rickettsiosis cohort.	82

Figure 21. Receiver operating characteristics curve of the recombinant ScaA-based IgM ELISA in the rickettsiosis cohort.....	83
Figure 22. Receiver operating characteristics curves of the recombinant ScaA-based and TSA56-based IgM ELISAs in the rickettsiosis cohort.....	85
Figure 23. Receiver operating characteristics curve of the recombinant ScaA-based IgM ELISA in the rickettsiosis cohort when cut-off indices were used.	87
Figure 24. Distribution of IgM ELISA OD values and IgM IFA titres.	89
Figure 25. Scatter plots of ELISA OD values over day of illness.	92
Figure 26. Changes in antibody responses to ScaA and TSA56 from admission to follow-up.	93
Figure 27. Diagram representation of a latent class model for <i>Orientia tsutsugamushi</i> tests and sign.	98
Figure 28. Schematic representation of <i>Orientia tsutsugamushi</i> antigens used in an IFA, TSA56-based ELISA, and ScaA-based ELISA.....	100
Figure 29. Prior distributions for sensitivities and specificities of the four diagnostic tests and the presence of eschar.	104
Figure 30. Pre- and post-test probabilities of scrub typhus before and after applying a recombinant ScaA-based IgM ELISA and TSA56-based IgM ELISA.	109
Figure 31. Amino acid sequences of nine overlapping peptides 1-9.....	116
Figure 32. Results of amino acid sequence homology analysis of the ScaA peptides on the NCBI BLASTp website.....	117
Figure 33. A predicted three-dimensional structure of the ScaA autotransporter domain.....	119
Figure 34. Topology of overlapping peptides P1-P9 in the ScaA autotransporter domain.....	120
Figure 35. Amino acid sequences of the ScaA antigen region of 14 <i>Orientia tsutsugamushi</i> isolates and nine overlapping peptides.	121
Figure 36. Results of the B-cell linear epitope prediction of the ScaA autotransporter domain on the IEDB program.....	123
Figure 37. ScaA peptides IgM dot-blot results.....	124
Figure 38. IgM antibody responses to the ScaA peptides of different concentrations.....	125
Figure 39. Background noise reactions in the recombinant ScaA-based IgM ELISA and ScaA peptide IgM ELISA in healthy controls and the rickettsiosis cohort.....	129
Figure 40. IgM antibody responses to ScaA peptide 6 and background noise reactions in the ScaA-based peptide ELISA in healthy controls and patients in the rickettsiosis cohort.....	129
Figure 41. IgM antibody responses to ScaA peptides 1-9 in scrub typhus and non-scrub typhus patients in the rickettsiosis cohort.	130

Figure 42. Proportions of patients responding to peptides 1-9 and the recombinant ScaA protein in scrub typhus and non-scrub typhus patients in the rickettsiosis cohort.....	131
Figure 43. Receiver operating characteristics curve of the ScaA peptide 6 IgM ELISA in the rickettsiosis cohort when cut-off indices were used.	136
Figure 44. Pre- and post-test probabilities of scrub typhus before and after applying a ScaA peptide 6 IgM ELISA, recombinant ScaA-based IgM ELISA, and TSA56-based IgM ELISA.....	140
Figure 45. Schematic representation of the stepwise process to narrow down the ScaA antigen region.	141
Figure 46. Schematic representation of the factors that affect sensitivities of diagnostic tests for acute febrile illnesses.....	149

Abbreviations

AD	Antigen domain
AIC	Akaike information criterion
ANDEMIA	African Network for improved Diagnostics, Epidemiology and Management of Common Infectious Agents
ATD	Autotransporter domain
AUC	Area under the receiver operating characteristic curve
Bam	β -barrel assembly machinery
BCA	Bicinchoninic acid
BGN	Background noise
BSA	Bovine serum albumin
BSL	Biosafety level
CI	Confidence interval
COI	Cut-off index
CrI	Credible interval
CRP	C-reactive protein
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
FIEBRE	Febrile Illness Evaluation in a Broad Range of Endemicities
GBP	Great Britain pound
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
ICAM-1	Intercellular adhesion molecule 1
IFA	Indirect immunofluorescence assay
IFN- γ	Interferon gamma
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IIP	Indirect immunoperoxidase
IPC	Infection prevention and control
IQR	Interquartile range
JPY	Japanese yen
LB	Luria-Bertani
LCM	Latent class model
LFA	Lateral flow assay

LPS	Lipopolysaccharide
LR-	Likelihood ratio for a negative test result
LR+	Likelihood ratio for a positive test result
MAT	Microscopic agglutination test
MCMC	Markov chain Monte Carlo
MEXT	Ministry of Education, Culture, Sports, Science and Technology
MML5	Mixed-lineage leukemia 5
NAAT	Nucleic acid amplification test
NCGM	National Center for Global Health and Medicine
NIDIAG	Neglected Infectious Diseases DIAGnosis
NIHE	National Institute of Hygiene and Epidemiology
Ni-NTA	Nickel-nitrilotriacetic acid
NOD	Nucleotide oligomerisation domain
NU	Nagasaki University
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
POCT	Point-of-care test
PVDF	polyvinylidene fluoride
qSOFA	Quick sequential organ failure assessment
ROC	Receiver operating characteristic
Sca	Surface cell antigen
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate - polyacrylamide gel electrophoresis
STARD-BLCM	Standards for the Reporting of Diagnostic accuracy studies that use Bayesian Latent Class Models
STIC	Scrub typhus infection criteria
TLR	Toll-like receptor
TPP	Target product profile
TSA22	22kDa type-specific antigen
TSA47	47kDa type-specific antigen
TSA56	56kDa type-specific antigen
UT	The University of Tokyo
VCAM-1	vascular cell adhesion molecule 1
WHO	World Health Organization

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PART I. Background to the thesis

1. Scrub typhus

Fever is one of the main reasons why patients seek medical care in the tropics (1). The burden of fever is significant but challenging to estimate (2). According to the World Health Organization (WHO) publication, the incidence rate of fever in the rural tropic was estimated to be 9.8 per person-year in children under five years of age and 4.0 per person-year in people over five years (3, 4). The estimated incidence rates in urban settings were 2.4 and 0.6 per person-year for these two age groups. The medical community has been pathogen-oriented toward fever, and malaria has been the main target for a long time. Now that we have relatively accurate and affordable diagnostic tests for malaria and WHO recommends antimalarial therapy after confirmation of the diagnosis, it has come to be recognised that many acutely febrile patients do not have malaria (5, 6). We now face a new challenge of non-malarial fever.

We need a comprehensive approach toward a fever syndrome (7). As successes of a syndromic approach, we can learn from paediatric diarrhoea and pneumonia (8, 9). These successes were made possible by the robust understanding of the causative pathogens. Many studies on fever aetiology are ongoing such as FIEBRE (Febrile Illness Evaluation in a Broad Range of Endemicities), NIDIAG (Neglected Infectious Diseases DIAGnosis), and ANDEMIA (African Network for improved Diagnostics, Epidemiology and Management of Common Infectious Agents) (10-12). Studying fever aetiology is more complex than studying diarrhoea or pneumonia aetiology, primarily for two reasons. The first reason is the wide range of pathogens that cause fever. Fever aetiology depends on many factors: geographical locations, urban or rural settings, seasonality, vaccines included in the Expanded Program on Immunization and its coverage, human immunodeficiency virus (HIV) prevalence, exposure to insects and animals, and age. In Asia, dengue, typhoid fever, leptospirosis, scrub typhus, Japanese encephalitis, and melioidosis are common causes of acute non-malarial fever (4, 13, 14). Among patients with persistent fever lasting for seven days or longer, these infectious diseases, except for dengue, are still important causes (15). The second reason is the lack of accurate, simple, and affordable diagnostic methods for infections with many of these causative pathogens (15). Although statistical analysis that incorporates the concept that none of the diagnostic tests are perfect may correct this problem in research settings, the challenge remains for clinicians on the ground. Developing specific microbiological point-of-care tests (POCTs) is a priority (16). Rickettsiosis was listed as one of the target diseases for the diagnostic test development since patients with the disease need specific clinical management (7, 17).

Scrub typhus, or tsutsugamushi disease, is a febrile illness caused by the infection of a mite-borne bacterium, *Orientia tsutsugamushi*. 'Tsutsuga' means sickness or disaster, and 'mushi' means bugs in Japanese. In Japan, scrub typhus has been endemic in many parts of the country, and people have feared the disease since the Edo era (the 1600s). Tsutsugamushi was among the monstrous creatures described in the Japanese book, *Tousanjin'yawa - Ehonhyakumonogatari*, published in 1841 (Figure 1) (18).

According to a systematic review by Prasad et al., 8.0% of the patients with severe febrile illnesses who needed hospital admission had scrub typhus in Southeast Asia (19). Another narrative review by Shrestha et al. showed that the prevalence of scrub typhus among patients with acute febrile illnesses was 1.9-35.9% in South Asia and 3.6-7.8% in Southeast Asia (14). In Asia, scrub typhus is a major cause of acute fever, especially severe febrile illnesses (20).



Figure 1. Description of tsutsugamushi disease in the book 'Tousanjin'yawa - Ehonhyakumonogatari'. The Japanese writing on the up left: once upon a time, there was a monster called tsutsugamushi that killed many people. Therefore, we now say 'tsutsuga-nashi (no problem)' when things are all fine. Image from reference (18).

1.1. Epidemiology

It has been estimated that one billion people worldwide are at risk of *O. tsutsugamushi* infection, and one million scrub typhus cases occur per year worldwide (21). Despite this, scrub typhus and other rickettsial infections are not on the WHO's neglected tropical diseases list (22). Traditionally, scrub typhus has been described in the 'tsutsugamushi triangle' (23). The triangle includes Russia in the north, Pakistan in the west, Australia in the south, and Japan in the east. A systematic review by Xu et al. summarised the results of previous seroprevalence studies in many parts of the triangle. The seroprevalences among the general population were 2.6% in Australia, 23.7% in Bangladesh, 10.0% in China, 15.0-40.0% in India, 1.3% in Indonesia, 68.4% in Japan, 1.5-17.9% in Malaysia, 35.2% in South Korea, 27.3-38.7% in Sri Lanka, 4.2-21.0% in Thailand, and 1.1% in Vietnam (24). Some studies included in the review tested only a limited number of participants in a highly endemic region of a country, therefore these statistics need careful interpretation.

In recent years, increasing evidence has suggested the wider geographical distribution of the disease beyond the triangle. Seropositivity of *O. tsutsugamushi* infection in humans has been demonstrated in Kenya and Djibouti (25-27), and scrub typhus cases possibly acquired in the Republic of Congo and Cameroon were reported too (28, 29). The bacterial deoxyribonucleic acid (DNA) of *Orientia* spp. was detected in rodents in Senegal (30). In 2006, two cases of infection of *Orientia* species were detected outside of the triangle: one in the United Arab Emirates and another in southern Chile (31, 32). The first case from the Middle East, 'chuto' in Japanese, resulted in the identification of a new species, *Orientia chuto* (31). In 2016 three autochthonous cases of scrub typhus were reported in Chiloé Island, Chile, and it was proposed to designate the new species as *Candidatus Orientia chiloensis* (33, 34).

1.2. Vector and transmission

Humans contract *O. tsutsugamushi* via a bite of the larval trombiculid mite of the genus *Leptotrombidium*. Its life cycle has seven stages: egg, deutovum, larva, nymphochrysalis, nymph, imagochrysalis, and adult (35). Only the larva, also known as a chigger, is an ectoparasitic stage; the other six stages are free-living in the soil. Chiggers have a low host specificity and live on the body surface of many animals. Rodents are the most common host (35). The infection is passed on to the offspring through vertical transmissions: transovarial and transstadial. Chiggers feed only once on vertebrate animals therefore transovarial transmission has been considered the main route. The

transmission of *O. tsutsugamushi* from rodents to mites was only experimentally demonstrated (36). The transmission of the organism from infected mites to co-feeding non-infected mites was also experimentally proven (37). The larva inserts its mouthpart into the skin and sucks lymph and tissue for two to three days. The larva enlarges in size and falls off to the ground. The activity of chiggers depends on temperature and humidity. They are abundant in forest clearings, riverbanks, vegetative canopies, and rice fields (21, 38). Humans acquire *O. tsutsugamushi* infection when they are exposed to infected chiggers in these locations of their preference. The occupational risk of the infection is therefore high among farmers and those who work in vegetable and rice fields and participate in harvesting in autumn (38). The seasonality of the transmission depends on the activities of the chiggers and humans (21). There is still no evidence of human-to-human transmission.

1.3. *Orientia tsutsugamushi*

Orientia tsutsugamushi is a gram-negative obligate intracellular bacterium. It was previously called *Rickettsia tsutsugamushi*. It is classified as Class α -proteobacteria, Order Rickettsiales, Family Rickettsiaceae, and Genus *Orientia*. The order Rickettsiales includes two families: the Anaplasmataceae and the Rickettsiaceae. The former family includes *Anaplasma* spp., *Ehrlichia* spp., *Neorickettsia*, and *Wolbachia*; the latter includes *Rickettsia* spp. and *Orientia* spp. *Orientia tsutsugamushi* is 0.5-0.8 μm in diameter and 1.2-3.0 μm long (39). Although it had been thought that the cell wall of *O. tsutsugamushi* lacked peptidoglycan and lipopolysaccharide, Atwal et al. showed that the bacteria possessed a peptidoglycan-like structure at a low level (40). The shape of *O. tsutsugamushi* cells is less uniform than that of other rickettsia cells, likely due to the absence of the rigid cell wall (41). The reduced level of peptidoglycan is beneficial for the bacteria to avoid the recognition by cytosolic immune receptors such as nucleotide oligomerisation domain (NOD) like receptors.

Orientia tsutsugamushi has a 2.1-megabase single-chromosome genome, double the size of the genome of *Rickettsia* (41, 42). This difference in the genome size reflects the difference in the number of repeats, and 42% of the *Orientia* genome is composed of repeated sequences. These highly repetitive genomes make it difficult to generate complete genome assemblies with short-read sequencing (43). Two complete genomes of the Boryong and Ikeda strains were reported using short-read sequencing technology (44, 45). Complete genomes of six further strains, namely the Karp, Kato, Gilliam, TA686, UT76 and UT176 strains, were reported using long-read sequencing (43).

Antigenic variation among *Orientia* strains has been a critical challenge for scrub typhus diagnosis and vaccine development (23). Historically, strains were defined by serological methods with whole-cell antigens, such as a complement fixation test and an immunofluorescence assay, and *O. tsutsugamushi* was classified into various serotypes, including the Karp, Kato, and Gilliam prototypes (46-48). After 56kDa type-specific antigen (TSA56) was identified as a major surface antigen that reacted with strain-specific antibodies, genotypes have been increasingly used, and *Orientia* strains have been further classified (46, 49). DNA analysis of *tsa56* is currently the standard technique for the strain specification. There have been more than 20 strains reported, and the Karp strain occupies 40-50% of isolates, followed by the Gilliam strain in both serological and molecular analysis (Figure 2 left panel) (23). The geographical distribution of genotypes is shown in the right panel of Figure 2 (46). The information on the locally circulating strains is lacking in many parts of the *tsutsugamushi* triangle.

The difference in virulence between the strains was studied in a mouse model. Nagano et al. classified the four strains isolated in Gifu, Japan, into three groups: high, intermediate, and low virulence groups, by evaluating a 50% mouse lethal dose (50). The difference in disease presentation and severity by infected strains is poorly understood in humans.

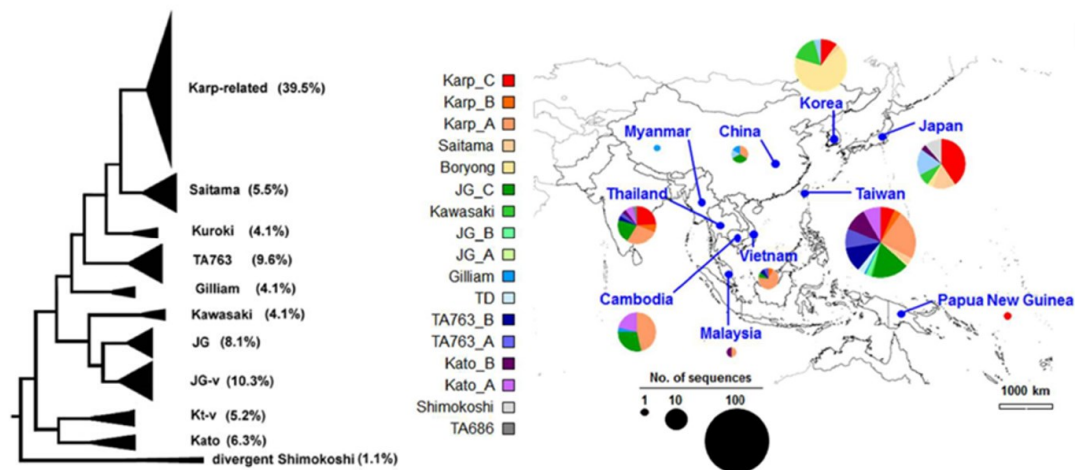


Figure 2. Schematic representation of the phylogenetic relationships of major clades of *Orientia tsutsugamushi* based on DNA sequences of the *tsa56* gene and the geographical distribution of the genotypes based on *tsa56* sequences in the Asia-Pacific region.

Left panel: percentages in the brackets represent the frequency of genotypes among the total sequences. Image from reference (23). Right panel: the pie charts show the relative proportion of each genotype in the country. Image from reference (46).

1.3.1. Outer membrane proteins

Outer membrane proteins play essential roles in the internalisation of Rickettsiales (42). These proteins have been targeted as antigens for developing diagnostic tests and vaccines. However, surface proteins are under selective pressure to avoid being recognised by immune systems. Some have considerable variation between strains and/or during the course of an infection (42).

Historically, the Western blot analysis of *O. tsutsugamushi* whole-cell lysates applied to patients' serum identified and differentiated antigenic outer membrane proteins with molecular weight (51, 52). Lin et al. showed that TSA56 had a higher antibody response against patients' serum compared to those of 22kDa type-specific antigen (TSA22) or 47kDa type-specific antigen (TSA47) in the Taiwanese patient cohort (53). Ha et al. analysed two *O. tsutsugamushi* genomes and identified another major outer membrane protein group, autotransporter proteins called surface cell antigens (Sca) (54).

1.3.1.1. 56kDa type-specific antigen

TSA56 is the most abundant protein that occupies 20% of the whole bacterial proteasome of *O. tsutsugamushi* (55). The protein is approximately 500-540 amino acids and consists of a signal sequence at the N-terminal end, three antigen domains (AD I-III), four variable domains, and a transmembrane domain at the C-terminal end. Seong et al. modelled its tertiary structure based on antibody binding patterns and predicted the secondary structure and transmembrane region in 1997 (56). The three-dimensional structure of TSA56 has yet to be experimentally confirmed. TSA56 is involved in bacterial adhesion and invasion to host cells. Fibronectin and syndecan 4 serve as host receptors. AD III and the adjacent C-terminal region of TSA56 bind to fibronectin (57). The binding triggers an integrin-mediated signalling cascade leading to actin remodelling and clathrin-mediated internalisation (58). TSA56 has high immunogenicity and contributes to the antigenic diversity of *O. tsutsugamushi*. The protein shows strain-specific antigenicity with weak cross-reactivity with heterologous antisera. Human immunoglobulin M (IgM) antibodies were shown to bind predominantly to the AD I and AD III, and immunoglobulin G (IgG) antibodies to the AD I (56).

1.3.1.2. Surface cell antigen A

Autotransporter proteins are part of the gram-negative bacteria's type Va secretion system (59). These proteins are expressed as a single polypeptide consisting of three domains: a signal sequence at the N-terminal end, a passenger domain, and an autotransporter domain (ATD) at the C-terminal end. The passenger domain exhibits considerable variation and functions as a virulence factor. While the ATD is diverse in amino acid sequence, its structure is highly conserved among gram-negative bacteria. The ATD comprises 250-300 amino acids and forms a β -barrel (59). The signal peptide initiates the translocation of the precursor polypeptide across the inner membrane via the Sec machinery (step 1 in Figure 3) (60). Chaperone proteins interact with the polypeptide in the periplasm and bind to its passenger domain (steps 2-3). The β -barrel assembly machinery (Bam) complex catalyses the binding, folding, and insertion of the ATD into the outer membrane (step 3). The passenger domain is translocated to the extracellular milieu through the ATD (step 4). The passenger domain is cleaved from or attached to the cell surface and mediates adhesion, autoaggregation, invasion, biofilm formation, and cytotoxicity (59). Autotransporter proteins have been tested and used as vaccine targets. The primary example is *Bordetella pertussis* pertactin, an essential component of acellular pertussis vaccines (59). Pertactin mediates bacterial adhesion to host cells.

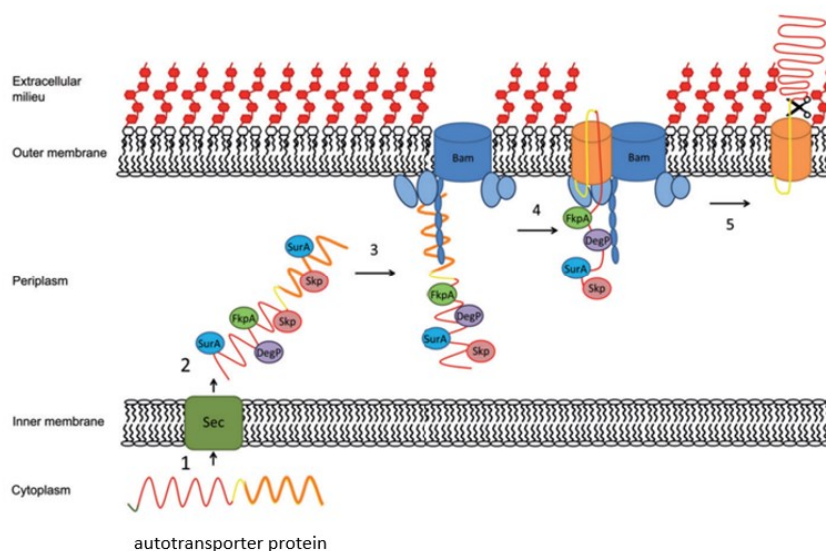


Figure 3. The autotransporter protein biogenesis.

Dark green sequence of an autotransporter protein: signal peptide, red: passenger domain, yellow: α -helix of the autotransporter domain, orange: β -barrel of the autotransporter domain. Bam: β -barrel assembly machinery. Image from reference (60).

Six Sca proteins (ScaA -ScaF) have been identified so far. Ha et al. revealed that *O. tsutsugamushi* encoded five Sca proteins (ScaA-ScaE) by genome sequence analysis of the Boryong and Ikeda strains in 2011 (54). Koralar et al. identified a sixth gene, *scaF*, in 2018 (61). While *scaA*, *scaC*, *scaD*, and *scaE* genes were detected among diverse *Orientia* isolates from broad geographical areas, *scaB* and *scaF* genes were detected in isolates in limited areas (61). This may suggest the heterogenous conservation of Sca proteins in the organism.

ScaA and ScaC are the most studied among the six *Orientia* Sca proteins. ScaA is the largest with approximately 1,500 amino acids. ScaC is the smallest with approximately 500 amino acids and is highly conserved across *Orientia* strains (54). Both proteins mediate attachment of the bacteria to host cells. The host receptor for ScaC is fibronectin. Nguyen et al. recently identified a splicing variant of mixed-lineage leukemia 5 (MLL5) protein as a host receptor for ScaA (62). The *MML5* gene was initially identified as a candidate for the myeloid leukaemia suppressor gene. Several alternative splicing variants of MML5 have been reported. These splicing variants have different functions depending on different locations, such as cell cycle progression, genomic maintenance, and spermatogenesis (63).

Ha et al. reported antibodies against ScaA and ScaC in human scrub typhus patients in 2012 (64). Their IgG enzyme-linked immunosorbent assays (ELISAs) used the passenger domains of ScaA and ScaC of the Boryong strain as antigens. Their reference test was an IgG indirect immunofluorescence assay (IFA) with the Boryong, Karp, and Gilliam strains. The sensitivity and specificity of their ScaA IgG ELISA were 100% and 73% in patients in the Republic of Korea where 80% of *O. tsutsugamushi* infection was due to the Boryong strain. The sensitivity and specificity of a ScaC IgG ELISA were 100% and 67%. Anti-ScaA and anti-ScaC antibodies were observed in patients with a high IFA titre of $\geq 1:640$, although anti-TSA56 antibodies were detected in patients with lower IFA titres.

Subsequent to the detection of specific anti-ScaA antibodies in human samples, Ha et al. showed that anti-ScaA serum neutralized the adhesion of *Escherichia coli* that expressed ScaA proteins to HeLa cells (65). Antibodies against ScaB, ScaC, and ScaE showed marginal or no neutralizing activity. They then moved on to the development of a ScaA vaccine using a mouse model. Mice were immunised with ScaA, ScaC, and TSA56 of the Boryong strain and challenged with the *O. tsutsugamushi* Boryong strain. The vaccination with either ScaA or TSA56 proteins provided protective immunity against an infection of a homologous strain, but the vaccination with ScaC did not. When mice were challenged with the Karp or Kato strains, the ScaA vaccination conferred lower but significant protection. Immunisation with both TSA56 and ScaA provided significantly higher protection against heterologous infections than that with either TSA56 or ScaA. Their experiment

used the purified ScaA passenger domain as a recombinant antigen to produce polyclonal antiserum and vaccine. Although they argued that the result of their ScaA vaccine was promising, the sequence variation of the ScaA passenger domain across the strains may affect its heterologous protective immunity (Figure 4).

The other four Sca proteins, ScaB, ScaD, ScaE, and ScaF, need further study. Recently ScaB was shown to be involved in adherence and invasion to host cells (66).

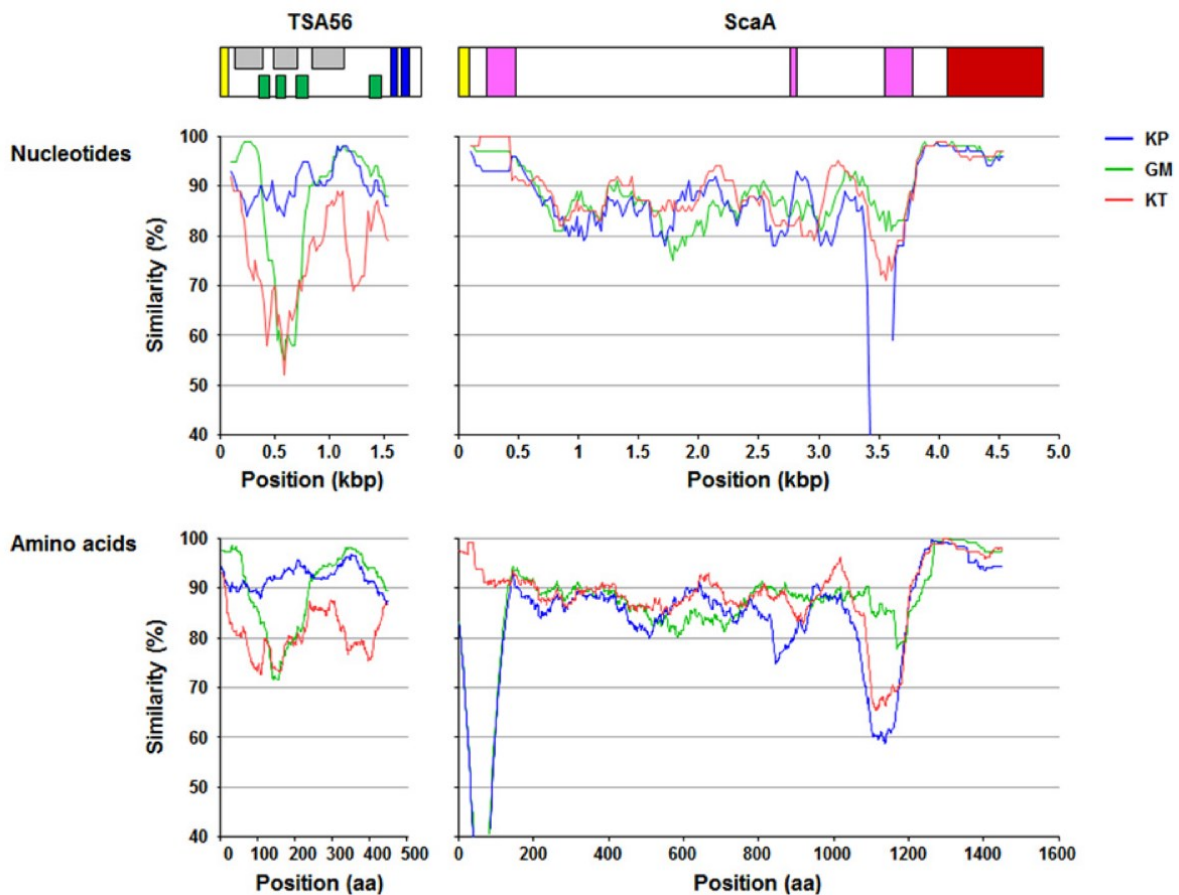


Figure 4. Similarity plots of *tsa56* and *scaA* nucleotide and amino acid sequences of the Karp, Kato, and Gilliam strains compared to the sequences of the Boryong strain.

Yellow box: signal peptide, grey box: antigenic domain, green box: variable domain, blue box: transmembrane domain, pink box: repeated sequences, and red box: autotransporter domain. KP: Karp, GM: Gilliam, KT: Kato. Image from reference (65).

1.3.1.3. Other outer membrane proteins

Other major TSA proteins are TSA47 and TSA22. TSA47 belongs to the high-temperature requirement protein (HtrA) family of serine proteases (67). TSA47 is highly conserved across *Orientia* strains. *HtrA* gene has been used for species identification. It has been shown that the protein induces antibody response, but its cross-reactivity with human serine protease could be an issue as a target for vaccines or diagnostic tests. The function of TSA22 is yet to be known, but it was reported that TSA22 had B-cell and T-cell epitopes (68).

Kim W et al. recently proposed a new outer membrane protein, 27kDa antigen, as a novel antigen for serological diagnosis of scrub typhus (69). The amino acid sequence identity of the antigen between the Boryong, Karp, Kato, Gilliam, and Ikeda was 85-94%. This antigen is a β -barrel protein expressed in the outer membrane. Although the sensitivity and specificity of the Western blotting assay were both high, this protein's diagnostic potential needs further investigation. The authors used 27kDa protein of the Boryong strain and applied the assay to a small number of patients of which 90% had the Boryong genotype.

1.3.2. Infection cycle

The target cells of *O. tsutsugamushi* infection are endothelial cells, dendritic cells, monocytes, and macrophages. The attachment of *O. tsutsugamushi* to the host cells is mediated by TSA56, ScaA, and ScaC as adhesin and fibronectin and syndecan 4 as host receptors (Figure 5) (41, 42). The binding triggers a clathrin-mediated zipper-like mechanism to enter the organism into host cells. *Orientia tsutsugamushi* then escapes from the endolysosomal pathway by disrupting the endosomal membrane and enters the cytosol within 30 minutes after attachment and internalisation (42, 70). After 24-48 hours post-infection in the host cytoplasm, *O. tsutsugamushi* moves to the perinuclear region where it replicates, by microtubule-mediated trafficking, unlike other Rickettsia that utilises actin-mediated processes (42, 71). It replicates in the perinuclear region to a level of >50 bacteria per cell, and the host cells can stay intact for seven days or longer (42). Approximately four days post-infection, a bacterial exit starts. The bacteria bud out from the cell as enveloped bacteria in the host plasma membrane (72). *Orientia tsutsugamushi* is unique among the Rickettsiaceae in this budding-out process, similar to envelope viruses (73).

Atwal et al. recently reported that intracellular *O. tsutsugamushi* and budded-out extracellular *O. tsutsugamushi* were two distinct populations regarding physical properties, metabolic activity, and

gene and protein expression profiles (Table 1) (74). Intracellular *O. tsutsugamushi* was shown to be translationally active, while extracellular *O. tsutsugamushi* was translationally inactive. ScaA proteins were expressed more in intracellular bacteria by 1.5 times than in extracellular bacteria, while ScaC proteins were expressed more in extracellular bacteria by 1.9 times. The expression levels of TSA56 and TSA47 in two forms were not significantly different. The authors also showed that ScaA protein started accumulating in intracellular bacteria shortly after the infection and speculated that *scaA* transcripts were prepared in extracellular bacteria for new infections. Depending on the bacterial location and stage in the course of infection, there may be a regulatory mechanism for the ScaA and ScaC protein expression on the outer membrane surface.

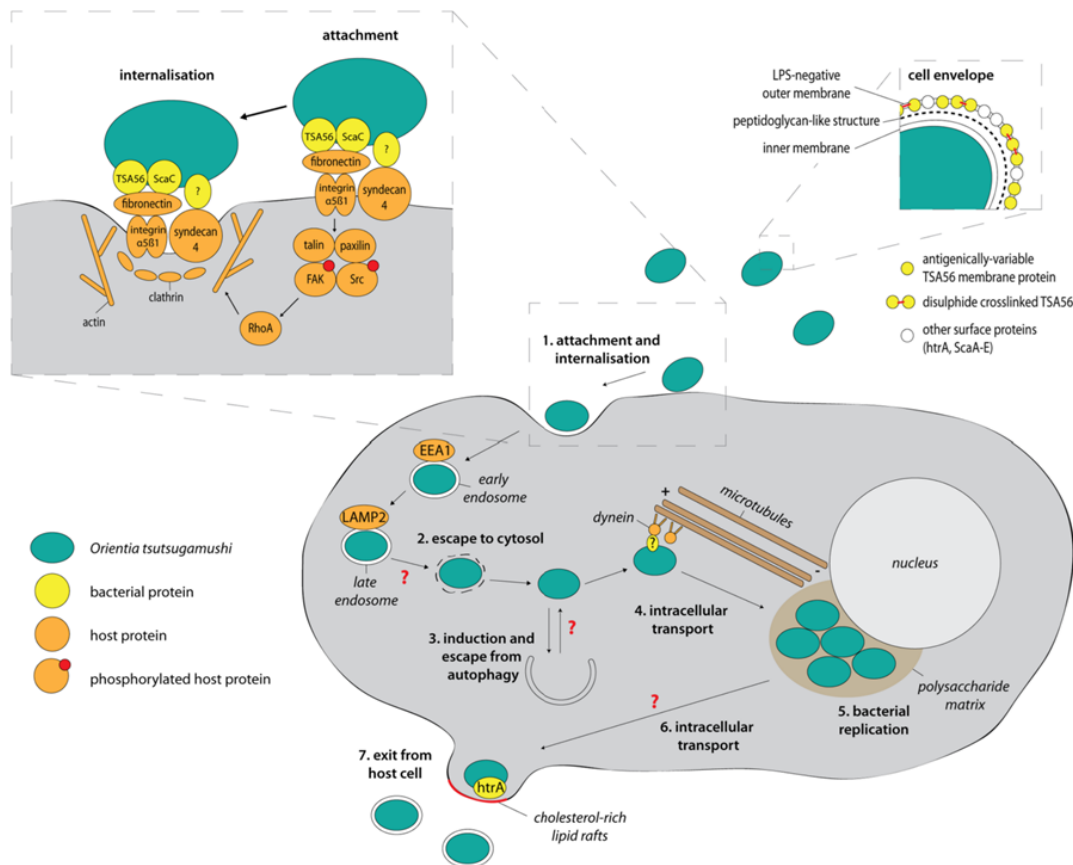


Figure 5. The infection cycle of *Orientia tsutsugamushi*.

LPS: lipopolysaccharide. Red question marks indicate unknown pathways. Image from reference (41).

Table 1. Different properties of intracellular and extracellular *Orientia tsutsugamushi*.

	Intracellular <i>O. tsutsugamushi</i>	Extracellular <i>O. tsutsugamushi</i>
Physical property	More peptidoglycan Elongated and round Less fragile	Less peptidoglycan Round More fragile
Metabolic activity	Translationally active	Translationally inactive
Disulphide bond formation of TSA56	Less	High
ScaA and ScaC protein expression	High ScaA Low ScaC	Low ScaA High ScaC
Mechanism to enter host cells	Clathrin-mediated endocytosis and macropinocytosis	Macropinocytosis

Retrieved and summarized from reference (74).

1.3.3. Human immune response to *Orientia tsutsugamushi* infection

Innate immune response

Dermal tissue is where the early immune responses against *O. tsutsugamushi* start. Following inoculation, *O. tsutsugamushi* is ingested by phagocytic cells. Paris et al. showed the tropism of *O. tsutsugamushi* in the eschar specimens for dendritic cells and monocytes (75). These phagocytic cells sense bacterial infection through pattern-recognition receptors such as Toll-like receptors (TLRs) and NOD-like receptors. Infection of dendritic cells results in impaired migration of these cells to lymph nodes, secretion of proinflammatory cytokines, and activation of type I interferon response. Infection of monocytes leads to perivascular infiltration of mononuclear cells and upregulation of proinflammatory genes. All these processes lead to the dissemination of *O. tsutsugamushi* from the initial inoculation site (75, 76). After the dissemination, endothelial cells are the important target of the pathogen, and endothelial activation is the hallmark of scrub typhus pathogenesis. The intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and other adhesion molecules become upregulated to promote endothelial activation (76). Otterdal et al. showed that increased monocyte/macrophage and endothelial-related markers were associated with the disease severity (77).

Adaptive immune response

As discussed in Chapter 1.3., *O. tsutsugamushi* has significant antigenic diversity that hampers the acquisition of heterologous immunity, although immunological memory is an important property of adaptive immune responses.

Humoral immunity plays a part primarily in protection against homologous infections (76). Our understanding of IgM and IgG kinetics after *O. tsutsugamushi* infection is still limited. This insufficient understanding could undermine our interpretation of serological test results. Shishido et al. studied patients with scrub typhus in Niigata, Japan, in the 1960s. They reported that antibodies detected by the complement fixation test persisted for five years after the infection and then disappeared (78). Saunders et al. reported that 61% of patients had negative seroconversion by an IFA one year after the onset in Malaysia in the 1980s (79). Kim DM et al. followed up 490 patients with scrub typhus and monitored their post-infection serological response for three years using an IFA in South Korea (80). IgM IFA titres gradually increased from the second to the third week of illness, peaked in the fourth week, and rapidly decreased during the fourth and fifth week. IgG IFA titres, on the other hand, rapidly increased in the first two weeks, peaked in the fourth weeks, and gradually decreased. After one year, none had an IgM IFA titre of $\geq 1:80$, but more than half had an IgG IFA titre of $\geq 1:128$. Varghese et al. followed optical density (OD) values of InBios Scrub Typhus Detect™ ELISA in 203 patients in South India (81). The mean IgM OD value gradually decreased and remained above the local cut-off value of 0.8 for 12 months after onset. The mean IgG OD value gradually increased, peaked 10 months after onset, and remained above their cut-off value of 1.8 for more than 36 months. Schmidt et al. also followed up with 197 scrub typhus patients in the same area as Varghese et al. using the same commercial ELISA kit (82). In this study, IgM OD values decreased rapidly after its initial peak, and half of the patients were below the cut-off value of 1.0 by 82 days after onset. Two years after the onset, half had an IgG OD value below the cut-off value of 1.5. As described, the available information is inconsistent. However, it is safe to say that the human immune protection acquired by *O. tsutsugamushi* infection is short-lived. Heterologous protection is especially short-lived, for less than one year, whereas homologous protection may last a few years (76, 83).

Due to the short-lived immunity against *O. tsutsugamushi*, its reinfection has been thought to be relatively common in endemic areas but rarely reported (84, 85). Bourgeois et al. categorised IgM and IgG antibody responses into two types using an IFA in Penghu, Taiwan (84). Type 1 responders had a rapid increase in IgM antibody responses by day 8 and a slower increase in IgG after 12 days. Type 2 responders had an increase in IgG first, and their IgM response was variable. Type 1

responders were younger than type 2 responders. The authors speculated that type 1 and 2 responses indicated primary infection and reinfection, respectively. An experimental study in 1952 by Smadel et al. demonstrated that patients with secondary infection may have less typical clinical presentations including a longer incubation period, a milder disease, and less eschar formation (86), although a contradictory result was published recently (87). This different clinical picture of secondary infection may have been one of the reasons why scrub typhus reinfection is seldom confirmed.

Most of the previous studies used whole-cell antigens or TSA56 as serological test antigens. The kinetics of antibody responses may differ by target antigens. Chen et al. examined antibody responses against conserved (TSA47) and variable (TSA56) antigens in 108 patients (88). They purified recombinant TSA47 proteins and truncated TSA56 proteins and used them as antigens in IgM and IgG ELISAs. The antibody response against TSA47 was slow and weak compared to that against TSA56. They discussed that this difference may have reflected the quantity of the proteins expressed on the cell surface and their immunogenicity.

Cell-mediated immunity plays a key role in both homologous and heterologous responses and is more significant in developing heterologous protection than humoral immunity (76). A type 1 cell-mediated immunity and interferon gamma (IFN- γ) production is essential for protection. Upregulation of IFN- γ and IFN- γ -inducing cytokines in the early phase of illness has been reported in human scrub typhus patients (89, 90). The cytotoxic activity of CD8+ T cells is important for eliminating cytosolic pathogens; however it has not been well studied in *O. tsutsugamushi* infection. A protective response against the bacteria requires efficient crosstalk between humoral and cell-mediated immunity; however the role of T-cells and cytotoxicity needs further research (76).

1.4. Clinical disease

Scrub typhus presents a wide range of severity from asymptomatic infection to a fatal disease (91). The incubation period is 10-12 days. Its clinical presentation is a non-specific acute febrile illness. Classic symptoms include fever, malaise, headache, rash, eschar, and lymphadenopathy. A maculopapular rash spreading from the trunk to the limb may be observed typically after the onset of fever. Eschar is a necrotic lesion of the skin at the site of arthropod inoculation. Although it is an important diagnostic clue, it could be associated with other rickettsial infections in areas where other tick-borne diseases occur. The reported prevalence of eschar is highly variable between studies. The difference in the prevalence of eschar is speculated to be due to skin colour and

infected *Orientia* strains (91, 92). Yoo et al. recently reported that the prevalence of eschar among scrub typhus patients was 78.7% in East Asia, 52.2% in Oceania, 41.4% in Southeast Asia, and 32.8% in South Asia (93). These differences also may have been attributable to different diagnostic criteria, the prevalence of other diseases that also form eschar, and how familiar clinicians are with the disease in different areas.

A proportion of patients progress to severe disease and present meningoencephalitis, myocarditis, pneumonitis, hepatitis, acute renal failure, and coagulopathy (94). According to the systematic review on mortality from untreated scrub typhus, the median mortality was 6.0%, with a wide range of 0-70% (94). Taylor et al. discussed that mortality was lower than previously thought, but its morbidity was expected to be significant due to the long duration of fever (median 14.4 days). The mortality from treated scrub typhus was estimated at 1.4% (95).

The severity of the disease depends on both pathogen- and host-related factors. Some studies showed different clinical presentations and laboratory findings among different strains. Kim DM et al. reported that malaise, conjunctival injection, eschar, and rash were more common in patients infected with the Boryong genotype than in those infected with Karp genotype in South Korea (96). Park et al. suggested that infected strains were not associated with the severity of the disease in the same country (97). Wei et al. reported that patients with the Gilliam genotype were more likely to present sustained fever and those with the Kato genotype were more likely to present with splenomegaly in Guangzhou, China (98). Geographical variation of disease severity may be associated with differences in locally circulating strains (99). The bacterial load was demonstrated to be associated with the severity in some studies. DNA load was significantly higher in fatal patients than in non-fatal patients in studies from Thailand and South Korea (99, 100). Devamani et al. reported that patients with a high initial IgG antibody level at diagnosis were at an increased risk of severe disease in South India and argued that this could be because severe infection caused a quicker IgG response or previous infection enhanced the severity (87).

1.5. Laboratory diagnosis

The common findings of blood count and basic biochemistry tests in scrub typhus patients include leukopenia, lymphopenia, thrombocytopenia, elevated liver enzymes, and elevated C-reactive protein (CRP) level, but all of these are observed in patients with other pathogens of non-malarial fever. For the laboratory diagnosis of scrub typhus, bacterial isolation, nucleic acid amplification tests (NAATs), and serological tests are available. Each test has strengths and weaknesses. A

combination of a polymerase chain reaction (PCR) assay and serological test has been the mainstay of scrub typhus diagnosis expecting to complement the shortcomings of each test and improve the diagnostic yield (101). The day of illness is the critical information for choosing diagnostic tests.

The sensitivity and specificity of the diagnostic tests and a clinical sign used in this Ph.D. study, namely an IgM IFA, InBios Scrub Typhus Detect™ IgM ELISA, a 47kDa-based real-time PCR assay, and the presence of eschar, are summarised in Table 2.

1.5.1. Bacterial isolation

Bacterial isolation of *O. tsutsugamushi* is performed in mammalian cell culture or inoculated mice and requires biosafety level (BSL) 3 facilities. In vitro isolation with established cell lines has been predominantly performed and is time-consuming and labour-intensive (102). The median time to in vitro isolation was reported to be 27 days (interquartile range [IQR]: 22.5-33.5) (103). Mouse inoculation is even more laborious. Although successful isolation provides solid evidence of the presence of the bacteria, it is not used for diagnostic purposes (104). Its specificity is expected to be 100%. Its sensitivity is dependent on assay protocols and sample processing.

1.5.2. Nucleic acid amplification test

PCR assays have been increasingly used but are still mainly offered in reference laboratories (105). The genes of TSA56, TSA47 (*htrA*), heat shock protein (*groEL*), and 16S ribosomal ribonucleic acid are common targets (101). The *tsa56* gene is unique to *O. tsutsugamushi* and is the most used target to differentiate infecting genotypes. However, its sequence diversity may affect primer annealing. PCR assays can be applied to whole blood, buffy coat, and eschars. A biopsy specimen or crust of eschars is a good sample for the assays if present. Testing of eschar samples may allow for the detection of the *Orientia* DNA even after the initiation of antibiotics (101). Loop-mediated isothermal amplification assays were shown to have comparable test sensitivity to real-time and nested PCR assays (106) but is not widely used. PCR assays complement serological assays, especially in the early phase of illness before the antibody level reaches a detectable level (105). A positive PCR test is strong evidence of the presence of the pathogen DNA (101). Generally, the specificity is expected to be very high around 95-100%. The sensitivity depends on the target gene, types of PCR assays, sample types, and the timing of sample collection.

1.5.3. Serological test

Indirect immunofluorescence assay

An IFA detects a fluorescent-labelled anti-human antibody that binds to specific antibodies bound to smears of *Orientia* antigens (107). Even though being considered a reference test, an IFA has critical problems.

First, an IFA lacks agreed methodologies and a definition of a cut-off titre (108). Due to the organism's antigenic diversity, cell lysates of multiple strains are usually combined as IFA antigens. In addition to the three prototypes, Karp, Kato, and Gilliam, locally circulating strains may be added. In Japan, three Japanese strains, namely the Irie/Kawasaki, Hirano/Kuroki, and Shimokoshi strains, are included in the assay at the National Institute of Infectious Diseases (109). The commercial IFA kit available in Japan, FA Slide R. Tsutsugamushi Seiken (Denka Company Ltd, Japan), includes only three prototypes. In South Korea, the Boryong strain is often added (110). Antibody isotype used in assays also varies (108). The choice may depend on the test's purpose and the availability of convalescent samples.

The choice of cut-off titres is another problem. A four-fold titre increase in paired samples is generally recommended. However obtaining convalescent samples from patients is often difficult. A single titre cut-off is commonly used in clinical practice (108). Brown et al. proposed an IgM IFA cut-off titre of $\geq 1:400$ at admission in the 1980s (111). It has been thought that an IgM IFA has a high false-positive rate due to cross-reactivity with antigens of other pathogens and the low cut-off value can exacerbate the confusion. Blacksell et al. reviewed IFA cut-off titres of 109 publications and revealed that the commonly used single-titre cut-off ranged from 1:10 to 1:400 (108). The cut-off values showed significant geographical variation, with the values in Japan and South Korea being the lowest (1:10 to 1:40) and the values in Thailand and Malaysia being the highest (1:50 to 1:400). In South Korea, the Korean Centre for Disease Control and Prevention IgM IFA yielded a sensitivity of 55.7% and specificity of 94.8% with a cut-off titre of 1:16 when compared to a combination of bacterial isolation and nested PCR assays (110). In northern Thailand, Lim et al. determined that an IgM IFA titre of $\geq 1:3,200$ at admission or a four-fold titre increase to $\geq 1:3,200$ in the convalescent phase yielded the highest sensitivity of 81.6% and specificity of 100% using Bayesian latent class models (LCMs) (112). The differences reflect the local scrub typhus endemicity but may also result from methodological differences and racial factors (110). The methodology and the choice of a cut-off value of an IFA can affect the prevalence of scrub typhus. As Blacksell et al. pointed out, the lack

of appropriate reporting of the methodologies and cut-off criteria in many publications limits the comparability of seroprevalences among studies (108).

Second, an IFA requires resources that are often unavailable where scrub typhus is a problem, such as fluorescence microscopy. BSL-3 laboratories are not required for performing the IFA but are necessary for preparing whole cell antigens for the IFA slides. The assay uses raw materials from in vitro culture of the pathogen as antigens. *Orientia tsutsugamushi* is classified as a Risk Group 3 organism, and its in vivo propagation and in vitro culture requires BSL-3 laboratories (104, 113). It has been argued that low-risk activities, for instances in vitro culture, could be performed safely in BSL-2 laboratories. The assay may still lack objectivity and reproducibility even with all the equipment available. Phetsouvanh et al. demonstrated inter- and intra-observer variation in IFA reading and emphasized that IFA slides needed to be read immediately after the processing by multiple observers (114).

Indirect immunoperoxidase assay

An indirect immunoperoxidase (IIP) assay is a modification of an IFA. The assay uses peroxidase instead of fluorescein and can be read with light microscopy. It has been shown that the test performance of an IIP assay is comparable to that of an IFA (115).

Enzyme-linked immunosorbent assay

An ELISA is a labelled immunoassay. Of multiple formats of ELISAs, an indirect ELISA is most widely used in clinical practice and scientific studies (116). For the serological diagnosis of scrub typhus, an indirect ELISA detects an enzyme-conjugated anti-human antibody that binds to antibodies in the patient's serum bound to *Orientia* antigens. Most assays use the recombinant TSA56 protein as antigens. Since TSA56 shows considerable antigenic diversity across strains, combining the recombinant TSA56 proteins of multiple strains is common. The variability of antigen combinations in different assays may hinder the comparability of the results across studies. InBios Scrub Typhus Detect™ immunoglobulin M (IgM) ELISA, the most evaluated and widely used commercial kit, contains recombinant TSA56 proteins of the Karp, Kato, Gilliam, and TA716 strains (117). In Taiwan, Yang et al. used recombinant TSA56 proteins of the three prototypes and four locally circulating strains as ELISA antigens. By combining antigens of seven strains, test sensitivity improved compared to only three prototypes (118). Chao et al. developed an IgM and IgG ELISA with recombinant TSA56

proteins of the Kato, Karp, Gilliam, and TA763 strains in Thailand (119). Phanichkrivalkosil et al. developed an IgM ELISA with whole-cell antigens of the Karp, Kato, and Gilliam strains (120, 121).

An ELISA has some advantages over an IFA. It is simple, objective, inexpensive, and high-throughput (101). However, the lack of standardisation on its methodologies and criteria of cut-off values is a problem, similarly to an IFA (122). Although the cut-off values need to be determined locally, there has yet to be a consensus on how to do so. A recent review by Saraswati et al. reported that the explanation of the cut-off OD value selection was insufficient or completely lacking in many studies (122). It was determined by the receiver operating characteristic (ROC) curve analysis or calculation of the mean plus 1-3 standard deviations (SDs) of non-scrub typhus patients or healthy controls. InBios Scrub Typhus Detect™ immunoglobulin M (IgM) ELISA uses a cut-off value of the mean plus 3 SDs of non-scrub typhus patients. The locally determined cut-off values of this assay were reported to be 0.5-0.6 in northern Thailand (123), 0.5 in central Nepal (124), 0.75-1.25 in Chittagong, Bangladesh (125), 0.8 in Vellore, India (81, 126), and 1.0 in Karnataka, India (127). The sensitivity and specificity of scrub typhus ELISAs depend on ELISA methodologies, cut-off values, availability of convalescent samples, reference tests, and statistical analysis. Therefore, it is difficult to summarise or compare results across studies. The sensitivity and specificity of InBios Scrub Typhus Detect™ immunoglobulin M (IgM) ELISA have been reported around 70-93% and 90-100% (38, 123-127).

Weil-Felix test

The Weil-Felix test is the oldest test for rickettsiosis diagnosis (102). The test is an agglutination assay that detects cross-reacting antibodies to *Proteus* antigens. *Proteus mirabilis* OXK antigen reacts with antibodies against *O. tsutsugamushi*, *P. vulgaris* OX19 antigen reacts with antibodies against the typhus group *Rickettsia*, and *P. vulgaris* OX2 antigens react with antibodies against the spotted fever group *Rickettsia* (128). The assay suffers low sensitivity and specificity (126, 127, 129). However, this inexpensive, less labour- and resource-intensive test remains a diagnostic option in resource-limited areas.

1.5.4. Point-of-care test

Scrub typhus typically affects people in rural areas. Accurate and affordable POCTs will be especially useful in such settings. However, a trade-off between desirable characteristics of POCTs, such as affordability, accuracy, rapidness, and user-friendliness, is inevitable (130). Available POCTs for scrub

typhus are lateral flow assays (LFAs) and immunoblot assays (101). Saraswati et al. reviewed 31 studies on scrub typhus POCTs and showed that the pooled sensitivity and specificity of commercial LFAs were 66.0% and 92.0%, while the heterogeneity between the studies was significant (131).

Table 2. The sensitivity and specificity of diagnostic tests and a clinical sign reported in previous studies.

	Recommended timing to test	Sensitivity	Specificity	References
IgM IFA	After the second week of illness	54-96%	74-100%	(110, 118, 126, 132)
InBios Scrub Typhus Detect™ IgM ELISA (TSA56-based)	After the second week of illness	70-93%	90-100%	(123-127)
47kDa-based real-time PCR assay	Up to day 10 of illness	63-97%	90-100%	(106, 132, 133)
Presence of eschar	-	Depending on geographical location 33-92%	Depending on the prevalence of other rickettsiosis >95%	(92, 93, 132, 134)

1.6. Treatment and vaccine

Doxycycline, azithromycin, and rifampicin are effective for scrub typhus and doxycycline is the most frequently used (135). β -lactam antibiotics are not effective. Rapid defervescence within 24 to 48 hours is clinically useful to differentiate scrub typhus from other febrile diseases, but the response can be delayed. The recently published result of the randomised controlled trial in India showed that the combination therapy of doxycycline and azithromycin might be better than monotherapy of either one for patients with severe scrub typhus (136).

Doxycycline resistance was initially reported in the 1990s. Strickman et al. investigated the in vitro susceptibility of doxycycline using an isolate that the authors defined as doxycycline resistant (137). Watt et al. reported doxycycline resistance in patients with mild scrub typhus from northern Thailand in 1996 (138). They performed antibiotic susceptibility testing of the isolates in mice and cell culture. In 2020, Wangrangsamakul et al. pointed out that the antibiotic susceptibility testing methods used in those studies did not reflect the pharmacokinetic-pharmacodynamic relationships

in obligate intracellular bacteria and that there had been no verification of the findings after these studies (139). They argued that following studies did not support the original findings and that doxycycline resistance was a misconception. The lack of consensus on how to define antibiotic resistance of *O. tsutsugamushi* is yet to be resolved (140, 141).

After 80 years since the first report of a vaccine against *O. tsutsugamushi* in the 1940s (142), effective vaccines have yet to be developed. Whole cells, TSA56, TSA47, and Sca proteins have been considered as vaccine antigens in human and animal models (65, 143). Although TSA56 has been most studied, its genetic diversity limits heterologous immunity. Valbuena et al. advocated three strategies for developing an effective vaccine against scrub typhus: a strong focus on T cell-mediated immunity, investigation of immunogenicity of proteins encoded by conserved genes, and vaccine effectiveness assessment using appropriate animal models that truly mimic human disease (143). Kim HI et al. recently demonstrated that immunisation with concatenated conserved blocks of TSA56 provided improved protective immunity against heterologous infections (144).

2. Serological tests of infectious diseases

Serum is a fluid component of blood without clotting factors. Serology studies serum, especially antigens and antibodies. Serological tests are widely used in medical practice and research. It is important to understand the characteristics of serological tests and interpret the results in the clinical and epidemiological context.

2.1. Antigen and antibody

Antigens are molecules recognised as foreign substances by a host immune system, such as proteins, polysaccharides, lipids, and nucleic acids (145). An epitope is a small portion of an antigen that binds to antibodies, B-cells, or T-cells. Two types of B-cell epitopes exist: continuous and conformational (146). Continuous epitopes are also known as linear epitopes formed by continuous residues. The majority of B-cell epitopes (90%-) are conformational epitopes consisting of amino acids that are discontinuous in the primary structure but brought together in space in the tertiary structure (145, 147). To be accessible to antibodies and B-cells, B-cell epitopes need to be on the surface of an antigen. T-cell epitopes are linear epitopes presented on the surface of an antigen-presenting cell by major histocompatibility complex molecules. An antigen is usually a large molecule and has many epitopes. Therefore, the response against an antigen results in a mixture of antibodies recognising different epitopes (148).

Antibodies or immunoglobulins are serum proteins involved in the clearance of pathogens and antigens. The roles of antibodies are neutralisation, opsonisation, and complement activation (149). Immunoglobulin molecules consist of two heavy chains and two light chains held together by the disulphide bonds. Each chain has variable and constant regions. The variable region is the loop of highly variable 110 amino acids at the amino end of the chains. The region is unique to each antibody and binds to a specific epitope of an antigen. The constant regions are the rest of the molecule (150). There are five isotypes of immunoglobulin: IgM, IgG, immunoglobulin A, immunoglobulin D, and immunoglobulin E. Most serological tests of infectious diseases target IgM and IgG antibodies. IgM is a pentamer with the largest molecular weight of 900kDa and therefore stays in the intravascular space. Its half-life in serum is 6-10 days. IgG is a 150kDa monomer and its half-life is 3-4 weeks. The serum IgM and IgG concentrations are 50-200 mg/dL and 800-1,600 mg/dL, respectively (148, 151). The response after initial exposure to a pathogen primarily results in an increase in IgM antibodies with a relatively low affinity to the antigen. Mature B-cells undergo

immunoglobulin class switching within several days to weeks and start producing IgG antibodies. On subsequent exposure to the same antigen, the increase in IgG antibodies is more rapid, robust, and prolonged (148). An increase in IgM antibodies is less significant in a secondary response compared to the primary one.

Affinity is the strength of attraction between a single antigen-binding site on an antibody molecule and a single epitope of the corresponding antigen. Avidity is the net strength of the antigen-antibody binding, that is, the sum of the affinities between antigen-binding sites on a multivalent antibody and epitopes on a multivalent antigen. Avidity is determined by affinity, valency, and structural arrangement of an antigen and antibody (150). Through affinity maturation, IgG antibodies acquire a higher affinity than IgM antibodies. However, IgM molecules being a pentamer have up to ten antigen-binding sites resulting in an improved avidity despite their low affinity (149).

2.2. Detection methods of serological tests

There are unlabelled and labelled techniques. Unlabelled techniques such as precipitation and agglutination are more straightforward but often lack sensitivity because a certain concentration of antigen or antibody is necessary to visualise a reaction. A complement fixation test uses immune haemolysis mediated by anti-erythrocytic antibodies with the presence of complement (148).

Labelled techniques use labels such as a colorimetric substrate, fluorescence, or luminescent molecule to generate a signal and detect substances at much lower concentrations using detectors. A chemiluminescent substrate yields optimal sensitivity followed by fluorescence (150). An enzyme immunoassay or ELISA uses enzymes as a label. One molecule of an enzyme can generate multiple molecules of the product which further improves the sensitivity of the assay (150). There are competitive and non-competitive ELISA formats, and the non-competitive formats include direct, indirect, and sandwich ELISAs (Figure 6). To detect antibodies in the specimen, indirect ELISAs are most commonly used. It employs a labelled secondary antibody to detect a primary antibody bound to the target antigen. The labelled secondary antibody is generic; therefore, the indirect format is economical. A direct ELISA requires specifically labelled antibodies to each antigen. A sandwich ELISA uses two types of antibodies: a capture antibody and a detection antibody. Two forms of antibody-capture ELISAs based on the detection principle exist: direct sandwich ELISA and indirect sandwich ELISA (152). A direct sandwich ELISA starts with the passive attachment of capture antibodies to the solid surface. These capture antibodies bind to antigens, to which labelled primary detection antibodies also bind. In an indirect sandwich ELISA configuration, antigens immobilised on the solid

surface by capture antibodies bind to unlabelled primary antibodies, to which labelled secondary antibodies subsequently bind. Streptavidin-biotin detection technology can be employed in a sandwich ELISA. The key advantages of a sandwich ELISA are its high sensitivity and specificity. The disadvantage is the optimisation of antibody pairs. As two types of antibodies are involved in binding to the target antigens, it is necessary to ensure that they bind to different epitopes of the target antigen and do not interfere with the binding capacity of one another. However, this optimisation process could be technically challenging. A double-antigen sandwich ELISA format is a variation of a sandwich ELISA that has been used to detect antibodies against many pathogens (153-155). Antigens coated on the solid surface function as the capturer in this format. The captured antibodies are detected using labelled secondary antigens. Similar to antibody-capture ELISAs, capture antigens and labelled antigens must bind to the two variable domains of the target antibody. The advantage of a double-antigen sandwich ELISA lies in its capacity to detect total antibodies as opposed to class-specific antibodies. In non-competitive ELISAs, the detected signal is directly proportional to the amount of antibodies in the sample (150).

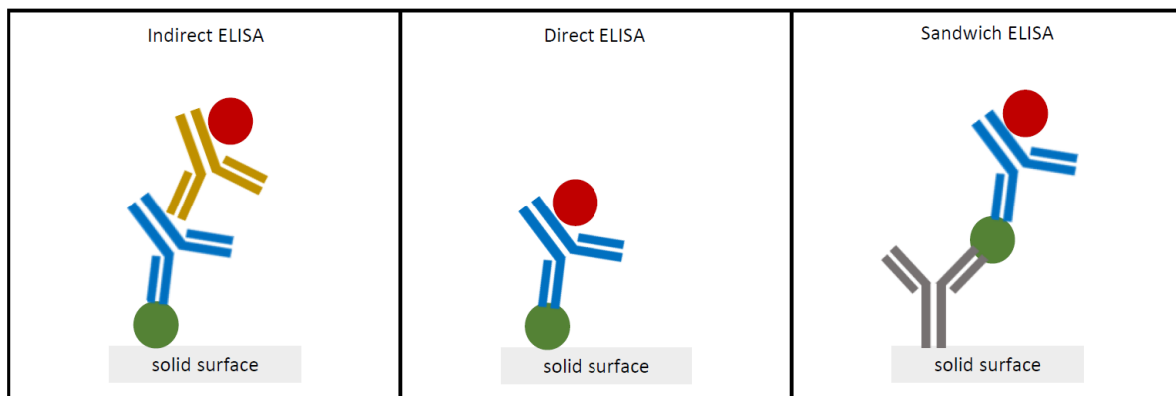


Figure 6. Schematic representation of direct, indirect, and sandwich ELISAs.

Green circle: a target antigen, red circle: an enzyme, blue immunoglobulin: a specific antibody to a target antigen, yellow immunoglobulin: an anti-human immunoglobulin antibody labelled with an enzyme, grey immunoglobulin: a capture antibody.

2.3. Advantages and disadvantages of serological tests

Serological tests have many advantages. The test can detect an infection of microorganisms that are either difficult or impossible to culture (148). Some microorganisms are difficult, time-consuming, and sometimes dangerous to culture. *Orientia tsutsugamushi*, being an intracellular bacterium, is one of them. The impact of previous antimicrobial exposure on serological tests is smaller than that on a microbiological culture and NAAT. Serological tests also offer insights into the infectious disease progression and response to therapy. High-throughput serological assays like an ELISA are useful for large-scale disease surveillance. An LFA is simple, rapid, user-friendly, and deliverable to end-users; therefore, this platform has been applied for many POCTs (156).

Serological tests, of course, have disadvantages. Firstly, the test is unable to detect an infection in the early phase of illness (148). In most infectious diseases, it takes 10-14 days to build up immune responses to a detectable level. It may take longer in some cases. Instead, serological tests can detect an infection even after the target microorganism is cleared from the body. In patients with immunodeficiency, the immune responses may be slower, weaker, or non-existent. Secondly, the presence of IgG antibody to a specific antigen only indicates an infection at some point in a patient's life (148). A high level of IgG antibodies implies that the patient is or was highly responsive to the antigen. The presence of a specific IgM antibody suggests a current or recent infection. The quantification of the IgG antibody concentration in acute and convalescent phases is therefore necessary. Acute and convalescent samples are tested as two-fold dilutions, and a four-fold titre increase is considered significant. The apparent inconvenience of convalescent sample testing is that patients must return for another blood test. The convalescent information does not contribute to the acute phase management either. Thirdly, cross-reactions can occur. This problem is more significant in serological tests for bacteria than viruses because bacteria share many genes in common (157). Some bacterial tests are even based on cross-reactions, for instance, the Wasserman test for syphilis and the Weil-Felix test for rickettsiosis. Lastly, non-specific reactions can cause a false-positive result. This was one of the major difficulties in this Ph.D. study to develop a new ELISA for scrub typhus. It is discussed here, focusing on an indirect ELISA system.

The use of serological tests depends on the assumption that antigen-antibody binding is highly specific. However, that is not always the case due to the non-specific reactions of antigen, antibody, and other components in the assay. Terato et al. argued that disregarding non-specific reactions was a common and critical problem in commercial and in-house ELISA systems (116). There are three non-specific reactions that cause false results in an indirect ELISA system: 1) background noise (BGN) reaction caused by the hydrophobic binding of immunoglobulin and immune-complex such as

immunoglobulin-rheumatoid factor complexes to plastic surfaces, 2) non-specific binding of immunoglobulins to target-antigens by protein-protein interactions, and 3) non-specific reactions caused by buffer components (158). The third reaction includes heterophile antibodies to animal proteins such as bovine serum albumin (BSA) (159, 160).

Among these three, the BGN reaction is the most intense false positive reaction, sometimes exceeding the true antibody-antigen reaction (116). The immunoglobulin concentration in serum is in the order of mg/ml, whereas the concentration of antigen-specific antibody immunoglobulins is in the order of ng or µg/ml. Non-antibody immunoglobulins can cause considerable non-specific reactions (158). It is essential to recognise that none of the currently available blocking buffers, such as skim milk, casein, and BSA have sufficient blocking ability, especially at low serum dilutions between 1:20 and 1:200 (161). Since BGN reactions depend on the immunoglobulin concentration in serum samples, they can be reduced in higher serum dilutions even with conventional blocking buffers. This issue has been actively discussed in the autoimmune diseases research community, where the diagnosis of such diseases may result in long-term, expensive, and potentially toxic treatment. Terato et al. proposed to measure the BGN reaction of individual samples in antigen-non-coated and blocked wells and subtract the signal of the BGN reaction from the signal of antigen-coated wells (Figure 7). The discussion on how to correct BGN reactions is still ongoing.

The important aspect of BGN reactions is that it is unique to individual samples. Guven et al. examined the non-specific binding in 70 patients with recent or ongoing bacterial infections. 43% (30/70) of the samples were non-specific binding sera defined as the IgG deposition on a non-coated well above the set cut-off (162). Among these 30 patients, the total IgG concentration was positively associated with the level of non-specific bindings. They speculated that the conformational change of immunoglobulins may have occurred under high temperatures induced either by febrile illnesses or sample treatment.

Scrub Typhus Detect IgM ELISA System (InBios International, Seattle, WA) is an indirect ELISA system (117). All wells of the polystyrene plate are pre-coated with the antigen and pre-blocked. It is instructed not to subtract blank background. It is unknown whether the assay uses capture antibodies to immobilise *Orientia* antigens. Although the blocking agent, wash buffer, and EnWash in the kit may be effective enough to prevent non-specific reactions, the problem is that we do not know because the BGN reactions are not measured.

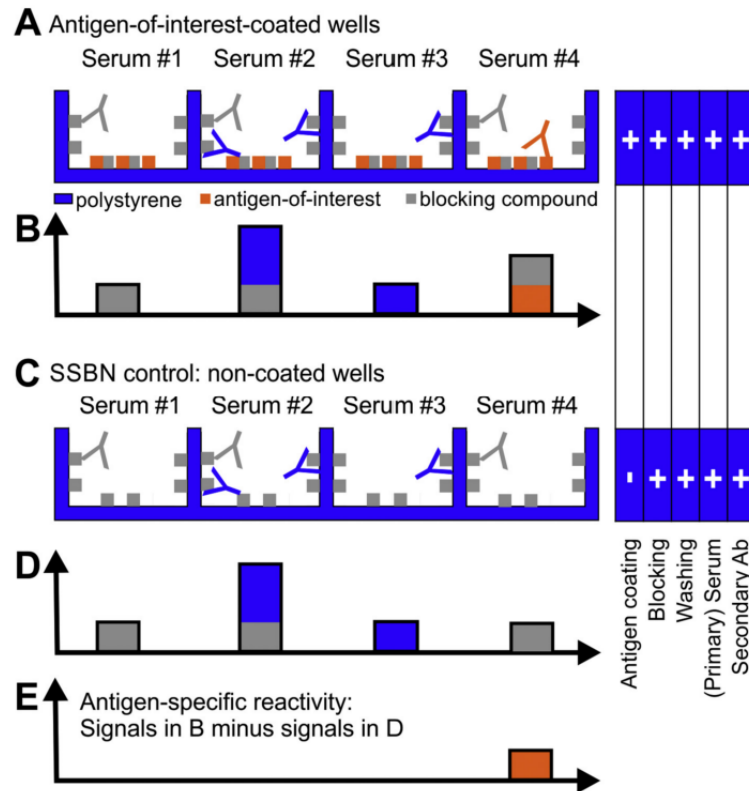


Figure 7. Schematic explanation of the background noise reaction correction.

Serum #1-4 are tested in antigen-coated wells (A) and overall signals are measured (B). Serum #1-4 are tested in antigen-non-coated wells (C) and background noise reactions are measured (D). Antigen-specific signals are calculated by subtracting a signal D from a signal B. SSBN: serum-specific background noise. Image from reference (163).

3. Evaluation of a new diagnostic test in the absence of a gold standard test

A perfect diagnostic test with both sensitivity and specificity of 100% does not exist for many diseases, likely not for any diseases. Even when there is such a test, it may not be feasible to use, too expensive, or too invasive. The best available test is often considered to be the 'gold standard' (164). Evaluation of a new test using a conventional test that is not truly a gold standard leads to biased estimates. An appropriate analysis method is necessary to overcome this lack of gold standard tests. In this Ph.D. study, Bayesian LCMs were used to estimate the sensitivity and specificity of ScaA-based tests. This chapter summarises the background of this analytical method.

Composite reference standard

When there is no perfect reference standard, the results of multiple imperfect tests are brought together (165). Composite reference standards are widely used to evaluate a new diagnostic test. The intuitive advantage is that it identifies more cases than any single test. Composite reference standards are based on either an OR or AND rule (166). Despite its widespread use, it is still unknown how many tests are necessary to define an adequate reference standard (167). However, adding more component tests to the standard could cause more misclassification. When component tests have nearly perfect specificities, an OR rule composite standard will have higher sensitivity than a single test. When component tests have imperfect specificities, false positive results are accumulated, and the sensitivity of a new test is underestimated (167).

Conditional dependence

In the previous paragraph, conditional independence between all tests was a premise. It was assumed that errors made by multiple tests are not correlated. This is not the case in practice. Diagnostic tests measure changes caused by a disease and are likely to be correlated. For instance, PCR assays may make the same false positive errors by picking up non-viable material or make the same false negative errors with low levels of bacteraemia or viremia. Serological tests may make the same false positive errors due to non-specific reactions or make the same false negative errors at an early stage of illness. Errors between multiple tests could be dependent on the true disease status. Ignoring conditional dependence can cause biased estimates of the disease prevalence, test

sensitivity, and test specificity. The bias can be significant under some circumstances, for example, population screening where the disease prevalence is low and the tests have low specificities (164). Several analytical methods, such as LCMs, fixed effects model, and random effects models, have been proposed to model conditional dependence between tests. The fixed effects model uses covariance between tests within the truly diseased and non-diseased populations (168). In a random effects model, sensitivities and specificities of tests are modelled as functions of subject specific variables.

Bayesian latent class models

Joseph et al. proposed the use of Bayesian LCMs as an analytical method for diagnostic test evaluation without gold standard tests in 1995 (169). Since then Bayesian LCMs have been increasingly used to estimate sensitivities and specificities of diagnostic tests for infectious diseases, for instance, scrub typhus, leptospirosis, melioidosis, malaria, and COVID-19 (132, 170-172). The true disease status (truly-diseased or non-diseased), which is not directly observable, is modelled as a latent variable. Instead of the standard two-latent class model, there is also a multiple latent variable model that assumes that tests measuring different biological mechanisms measure different latent variables, rather than the latent disease status (173). A Bayesian approach is necessary when non-identifiable LCMs are used (174). Bayesian statistics rely heavily on Markov chain Monte Carlo (MCMC) methods and it therefore requires considerable expertise and mathematical software, such as OpenBUGS (175). The selection of prior information is crucial in this approach because it will influence the final result. Selected prior information needs to be specified and justified.

The Standards for the Reporting of Diagnostic accuracy studies that use Bayesian Latent Class Models (STARD-BLCM) was published in 2017 (174). The key information to report includes the definition of latent disease status alongside its biological rationale, a detailed description of the model including its assumptions, and the justification of the prior distribution (176). This Ph.D. study reported the results following these standards.

Evaluation of diagnostic tests for scrub typhus

Scrub typhus provides a good example where the evaluation of a diagnostic test is very challenging due to the lack of gold standard tests. The so-called reference test, an IFA, is imperfect. The combination of PCR assays and serological tests (an IFA and/or ELISA) has been the mainstay of the

diagnosis in clinical practice. Paris et al. proposed a composite reference standard called scrub typhus infection criteria (STIC) in 2011 (106). In this criteria, confirmed scrub typhus cases need to fulfil: 1) positive cell culture isolation of *O. tsutsugamushi*; OR 2) an admission IgM titre of $\geq 1:12,800$; OR 3) a minimum four-fold increase in an IgM titre in paired serum samples; OR 4) a positive result in at least two of a nested TSA56-based PCR assay, 47kDa-based real-time PCR assay, or *groEL*-based real-time PCR assay.

Conditional dependence may need to be incorporated when we evaluate diagnostic tests for scrub typhus. In patients with a low level of antibody responses against *O. tsutsugamushi*, the infection is more likely to be missed by any serological assays including an IFA, ELISA, and LFA, compared to patients with a high-level response. This also applies to a bacterial culture and PCR assay with low levels of bacteraemia.

Lim et al. published their work on the evaluation of sensitivities and specificities of diagnostic tests for scrub typhus using Bayesian LCMs in 2015. The sensitivity and specificity of an IgM IFA estimated by Bayesian LCM (70.0% and 83.8%) were lower compared to when STIC was considered a gold standard (83.6% and 100%) (132). Instead, the sensitivity of the commercial LFA (Panbio ST ICT IgM) increased from 47.3% to 72.8%. The low specificity of an IgM IFA yielded false-positive results and led to an underestimation of the sensitivities of other tests.

4. Aims and objectives

4.1. Aims

The aim of the study was to develop a new serological test using a single conserved antigen for scrub typhus diagnosis.

4.2. Objectives

The objectives were:

- 1) to develop a recombinant ScaA-based IgM ELISA for scrub typhus diagnosis,
 - estimate sensitivity and specificity of a recombinant ScaA-based IgM ELISA in two Vietnamese cohorts (the rickettsiosis and fever cohorts) using Bayesian LCM, and
 - analyse the characteristics of a recombinant ScaA-based IgM ELISA.
- 2) to develop a simplified ScaA peptide IgM ELISA for scrub typhus diagnosis,
 - estimate sensitivity and specificity of a ScaA peptide IgM ELISA in the rickettsiosis cohort using Bayesian LCM, and
 - analyse the characteristics of a ScaA peptide IgM ELISA.

4.3. Target product profile

A target product profile (TPP) describes the minimum and optimal performance and operational characteristics of a new diagnostic test (177). TPPs serve as a guide for manufacturers to develop a medical test that would be actually used bedside. A TPP for a recombinant ScaA-based IgM ELISA was developed (Table 3). The intended use is the clinical diagnosis of patients with acute fever at medical facilities with laboratory capacity. Minimal sensitivity and specificity of 85% and 95% were targeted. These targets were equivalent to the sensitivities and specificities of the currently widely used serological tests (Table 2). The strengths of a recombinant ScaA-based IgM ELISA using a single conserved antigen discussed in the next chapter were thought to justify these targets.

Sensitivity and specificity are widely used indicators in TPPs for diagnostic tests (177), but these indicators need to be interpreted together. The sensitivity and specificity of 85% and 95% are converted to likelihood ratios for positive and negative test results (LR+ and LR-) of 17 and 0.16. The results would be interpreted with these LR+ and LR- and the expected prevalence of scrub typhus among patients with acute febrile illnesses in Asia. Evaluation of clinical utility and cost-effectiveness was considered important but beyond the scope of this study.

Table 3. Target product profile for a recombinant ScaA-based IgM ELISA

Goal of test	To detect specific IgM antibody responses against <i>Orientia tsutsugamushi</i> ScaA proteins	
Intended use	Diagnosis of patients	
Target patient	Patients with acute fever	
Target test setting	Medical facilities with laboratory capacity	
Results	Positive if an ELISA OD value is above a locally determined cut-off value	
Equipment	ELISA plate, refrigerator, phosphate buffered saline, Tween-20, BSA, anti-human IgM-HRP conjugate, chemiluminescent peroxidase substrate, hydrochloric acid, microplate reader	
Target use(s)	Testing patients with acute fever	
Reference	Bayesian latent class model analysis	
Performance	Minimal	Optimal
Clinical sensitivity	85%	100%
Clinical specificity	95%	100%
Quantification	OD value	

HRP: horseradish peroxidase.

4.4. Expectations and intended value

There are three expectations for the new test. First, the ELISA uses a single recombinant antigen that is conserved across *Orientia* strains; therefore, it is expected unnecessary to combine antigens of different circulating strains. Second, if a ScaA peptide ELISA is developed successfully, the test is expected to have practical strengths. Currently available commercial ELISA kits are expensive and in-house ELISA protocols require cell culture maintenance in a BSL3 laboratory or a purification of recombinant antigens. Antigenic peptides can be commercially synthesised, which is low-cost, stable,

and less labour-intensive. Third, if the new antigen is proven useful in an ELISA system, the antigen can be utilised for simpler serological techniques such as an LFA.

Scrub typhus is a treatable infectious disease. The hurdle is its diagnosis. An accurate, simple, and affordable diagnostic test for the disease will lead to appropriate treatment and improves patients' morbidity and mortality. It may also contribute to the understanding of aetiologies of febrile illnesses in the tropics.

5. Progress before the Ph.D. study

In 2012, Ha et al. reported the detection of human antibody responses against ScaA protein and proposed the protein as a potential antigen for scrub typhus diagnosis and vaccine development (64). They used a ScaA passenger domain. As demonstrated by Ha et al. (Figure 4) (65), a ScaA passenger domain exhibited more sequence variations than its autotransporter domain, which is generally expected for autotransporter proteins (59). Associate Professor Kozue Miura, the University of Tokyo (UT), started to explore the antigenicity of the ScaA ATD in 2016 because the ScaA ATD is more conserved across strains than the passenger domain. This chapter is a summary of Associate Professor Miura's work, and the data was provided by her (178). Initially, the ScaA ATD of 281 amino acids (GenBank accession number BDP28846.1) was divided into three regions of 101 amino acids (ScaA-ATD-A, B, and C) with 11 amino acids overlaps (Appendix 1.1). Nucleotide sequences of ScaA-ATD-A, B, and C of the *O. tsutsugamushi* Gilliam strain (LC720723.1) were amplified, cloned into a pET43.1a(+) vector (Novagen), and then expressed in *E. coli* BL21 (DE3). The recombinant ScaA-ATD-C protein could not be synthesised. In the Western blot analysis, both recombinant ScaA-ATD-A and -B proteins were recognised by hyperimmunized mouse serum (provided by Dr Mamoru Takahashi, Saitama Medical University, and Dr Seigo Yamamoto, Miyazaki Prefectural Institute for Public Health and Environment) (Appendix 1.2). The ScaA-ATD-A and B proteins were further divided into ScaA-ATD-A1, A2, B1, and B2 and purified in the same protocol (Appendix 1.3). ScaA-ATD-A1, B1, and B2 proteins were recognised by hyperimmunized mouse serum while ScaA-ATD-A2 protein was not in the Western blot analysis (Appendix 1.4).

A recombinant ScaA-based IgG ELISA with ScaA-ATD-A1, B1, and B2 proteins was developed and applied to a limited number of rat and human samples. The control protein was purified using an empty pET43.1a(+) vector. The ELISA protocol is in Appendix 1.5. In rat samples, a positive sample showed a dose-dependent antibody response to recombinant ScaA-ATD-A1, B1, and B2 proteins, while a negative sample had a dose-dependent antibody response only to a ScaA-ATD-A1 protein (Appendix 1.6). In human samples of two patients diagnosed with scrub typhus, one sample had a dose-dependent antibody response to ScaA-ATD-A1, B1, and B2. Another had high OD values to all antigens (Appendix 1.7).

From these preliminary results, it was suspected that the ScaA-ATD-A1 antigen might have caused a significant non-specific reactions. The assay development proceeded with ScaA-ATD-B (B1 and B2). However, the purification of recombinant ScaA-ATD-B protein was unstable and irreproducible. The development of the recombinant ScaA-based serological test had been halted until December 2018.

This Ph.D. study used ScaA-ATD-B; henceforth, ScaA-ATD-B was abbreviated as ScaA in the following chapters unless stated otherwise.

There were three limitations in the process before this Ph.D. study. First, the ScaA-ATD-C protein could not be synthesised and, therefore, was not tested as an antigen candidate. Second, only a limited number of samples were used to identify potentially antigenic regions. Third, animal samples were mainly used to guide the development process. Animals and humans may have different immune responses to *O. tsutsugamushi* infection.

PART II. Material and methods

6. Samples

Clinical information and blood samples from three cohorts previously collected in Hanoi, Vietnam, were used in this Ph.D. study. Basic information of the three cohorts is summarised in Table 4.

Assay development cohort

The original study was conducted by UT and the National Institute of Hygiene and Epidemiology (NIHE) at 105 Military Hospital in Hanoi (179). The study aimed to describe the *Orientia* genotypes circulating in northern Vietnam. Sixty-three patients who were suspected of having scrub typhus by the treating doctors and admitted to the hospital from July 2015 to September 2016 were enrolled. Verbal consent was obtained from all participants. Their blood samples were sent to NIHE for the confirmatory diagnosis as their routine clinical practice. Two PCR assays were performed: a nested TSA56-based PCR assay and a duplex PCR assay using the *groEL* gene to identify *O. tsutsugamushi* and other Rickettsiae. Of 63 patients, 42 (67%) tested positive for both assays. Nucleotide sequence analyses of the *tsa56* gene identified locally circulating genotypes: Karp (55%), TA763 (17%), Gilliam in Japan variant (17%), and Kato (12%).

Forty-six remaining serum samples stored at NIHE were transferred to UT with a material transfer agreement and 42 were used in this Ph.D. study. Serum samples were stored at -80°C until used. In the assay development process, scrub typhus cases were defined as those with an IgM IFA titre of $\geq 1:160$ and/or a positive result for a nested TSA56-based PCR assay. Sample size calculation was deemed unnecessary for this cohort because its use was for assay development but not for estimation of sensitivity and specificity of the new tests.

Rickettsiosis cohort

The original study was conducted by Bach Mai Hospital and Nagasaki University (NU) (180). The study aimed to describe the clinical epidemiology and characteristics of scrub typhus and murine typhus in northern Vietnam. Between March 2001 and February 2003, admission and follow-up blood samples were collected from hospitalised patients suspected of rickettsiosis at the

Department of Infectious Diseases, Bach Mai Hospital in central Hanoi. The hospital is a national referral hospital in northern Vietnam with approximately 2,000 beds. Patients were eligible for enrolment when they met all three criteria: 1) were 15 years or older; 2) had had an acute fever of $\geq 37.5^{\circ}\text{C}$ by axillary temperature measurement on and around the admission day without an apparent focus of infection identified by an initial evaluation of medical history, physical examination, and basic laboratory tests (complete blood counts and basic biochemistry); and 3) had had at least one of the following five signs: non-specific rash, multiple lymphadenopathy, eschar, hepatomegaly and/or splenomegaly, and no recovery after β -lactam antibiotic therapy. Patients who were diagnosed with malaria, dengue, and typhoid fever at the initial assessment were excluded from the study. Verbal consent was obtained from all participants. The samples collected in the second year between March 2002 and February 2003 were available for this Ph.D. study. Serum samples were stored at -80°C at the Institute of Tropical Medicine, NU.

In the original study, out of 207 patients, 72 (34.8%) were diagnosed with scrub typhus by an IgM ELISA (Panbio, Alere, Australia). This commercial ELISA kit detects IgM antibodies against the recombinant TSA56 antigens. The test sensitivity and specificity of the kit were reported to be 93% and 94% (181, 182). Eighty-four (40.6%) was diagnosed as murine typhus by the in-house IgG IFA using antigens of the *Rickettsia typhi* Wilmington strain. The test positivity was defined as either a single titre of $\geq 1:400$ or a four-fold increase in titre in paired samples. Seven patients had positive results for both diagnostic tests. In summary, 72.0% of the patients were diagnosed with rickettsiosis, and approximately 60% of the non-scrub typhus patients were diagnosed with murine typhus. There was no fatal case in this cohort.

From the preliminary results of the assay development cohort, the sensitivities of the ScaA-based IgM ELISA was expected to be around 70% compared to an IgM IFA as a gold standard. The sample size was calculated based on the confidence interval (CI) around the sensitivity of the new test (183). To produce a 95% CI of $\pm 10\%$, the number of scrub typhus patients needed to be 84. In the original study, the prevalence of the disease was approximately 35%; therefore, a total sample size of 240 was necessary. Unfortunately, the total number of patients whose serum samples were available was 207.

Fever cohort

The original study was conducted by Bach Mai Hospital and NU aiming to generate data on the proportion of scrub typhus cases among hospitalised patients with undifferentiated fever and to

analyse the characteristics of the cases in northern Vietnam (184). The study enrolled those admitted to the Department of Infectious Diseases, Bach Mai hospital between June 2012 and May 2013. The inclusion criteria were 1) aged 13 years or older and 2) axillary temperature of 37.5°C at any time from disease onset to admission. They excluded patients with clinically definitive diagnoses on admission. Patients with confirmed diagnoses on admission and patients with hepatitis were also excluded. Written informed consent was obtained from all participants.

A total of 1,127 patients were enrolled. Admission and follow-up blood samples were collected from 668 patients, and only admission samples were collected from 459. The follow-up samples were collected at least seven days after the admission samples. When patients were discharged within seven days, the samples were collected on the discharge day. Of 459 samples with only admission samples, samples of patients with positive results for any of the PCR assays had been used up in previous projects. Therefore, follow-up samples of 668 patients were used in this Ph.D. study. The samples were stored at -80°C at the Institute of Tropical Medicine, NU. 3.9% (26/668) of the patients were diagnosed as scrub typhus in the original study. An IIP assay (instead of an IFA) was applied to patients with positive results for a TSA56-based IgM ELISA and/or a 47kDa-based real-time PCR assay.

The clinical diagnosis made by the treating doctors at the time of discharge in the original study were: central nervous system infection (123, 18.4%), respiratory tract infection (89, 13.3%), bacteraemia (48, 7.2%), genitourinary system infection (20, 3.0%), skin and soft tissue infections (19, 2.8%), digestive system infection including liver abscess (17, 2.5%), cardiovascular system infections including infective endocarditis (8, 1.2%), HIV and opportunistic infection (56, 8.4%), tuberculosis without HIV infection (32, 4.8%), rickettsiosis (7, 1.0%), dengue (3, 0.4%), melioidosis (2, 0.3%), malaria (1, 0.1%), typhoid fever (1, 0.1%), other infections (11, 1.6%), connective tissue disorder such as systemic lupus erythematosus and adult-onset Still's disease (17, 2.5%), cancer (11, 1.6%), other non-infectious diseases (7, 1.0%), and undifferentiated (196, 29.3%). Two-thirds (437/668, 65.4%) were diagnosed as infectious diseases. The study highlighted that only one-fourth (7/26, 26.9%) of scrub typhus patients were clinically diagnosed during the admission (184).

Healthy controls

As healthy controls to set cut-off values for serological tests, blood samples of 68 visitors who received a general health examination between June 2012 and May 2014 at Bach Mai Hospital were used (184). These visitors had no fever or any other symptoms at the time of the blood test.

Table 4. Summary information of the assay development, rickettsiosis, and fever cohorts.

Cohort	Enrolment place	Enrolment year of the original study	Form of patient consent	Number of patients enrolled in the original study	Eligibility criteria of the original study	Number of patients enrolled in this Ph.D. study	Number and type of samples used for this PhD study	Day of illness at sample collection	Reference
Assay development	105 Military Hospital, Hanoi	2015-2016	Verbal	63 patients	<ul style="list-style-type: none"> - Hospitalised - Rickettsiosis suspected by treating doctors 	42 patients	<ul style="list-style-type: none"> - 42 admission samples - Serum 	<ul style="list-style-type: none"> - Data not available for most samples 	(179)
Rickettsiosis	Bach Mai Hospital, Hanoi	2001-2003	Verbal	579 patients	<ul style="list-style-type: none"> - Hospitalised - ≥ 15-year-old - Fever $\geq 37.5^{\circ}\text{C}$ on and around the admission day - No apparent focus of fever - Symptoms or signs suggestive of rickettsiosis - Patients diagnosed with apparent focus of infection, malaria, dengue, and typhoid fever at the initial assessment excluded 	207 patients <ul style="list-style-type: none"> - Enrolled in 2002-2003 - Samples were available 	<ul style="list-style-type: none"> - 189 admission samples and 152 follow-up samples - 55 patients with admission sample, 18 with follow-up samples, and 134 with both - Serum 	<ul style="list-style-type: none"> - Admission sample at median day 11 (IQR 8-12) - Follow-up sample at median day 15.5 (IQR 13-18) 	(180)
Fever	Bach Mai Hospital, Hanoi	2012-2013	Written	1,127 patients	<ul style="list-style-type: none"> - Hospitalised - ≥ 13-year-old - Fever $\geq 37.5^{\circ}\text{C}$ at any time from disease onset to 	668 patients <ul style="list-style-type: none"> - Provided both admission and follow-up 	<ul style="list-style-type: none"> - 668 admission samples and 668 follow-up samples 	<ul style="list-style-type: none"> - Admission sample at median day 8 (IQR 5-17) 	(184)

					admission - Patients with clinically definitive diagnoses on admission excluded	samples	- Plasma	- Follow-up sample at median day 14 (IQR 10-24)	
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7. Diagnostic tests used in the Ph.D. study

The following tests were performed as component tests to estimate the sensitivity and specificity of the new serological test. The reported sensitivity and specificity of each component test were summarised in Table 2. All tests were performed by Takaya S unless stated otherwise.

PCR assay

The authors of the original studies performed PCR assays. Their result data was used in this Ph.D. study. In the assay development cohort, a nested PCR assay for the *tsa56* gene and a conventional PCR assay for *groEL* gene of *Orientia* and *Rickettsia* were performed (179). The nested TSA56-based PCR assay was performed following the work by Furuya et al., and the duplex PCR assay was according to the protocol by Park et al. (185, 186). DNA samples were not available for the rickettsiosis cohort. In the fever cohort, a 47kDa-based real-time PCR assay using a QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems, Waltham, MA) was applied to all admission samples (184, 187).

IgM indirect immunofluorescence assay

Commercial FA Slide R. Tsutsugamushi Seiken (Denka Company Ltd, Japan) was used (Figure 8). The slides are pre-coated with whole cell lysates of one of the Gilliam, Karp, or Kato strains. Each sample was applied to three slides of three strains. The procedure was performed according to the manufacturer's protocol. Serum samples were serially diluted in phosphate buffered saline (PBS) and incubated in the antigen-coated wells in a humid condition at 37°C for 60 minutes. A positive and negative control samples were included in each slide. A positive control sample had a positive result for a nested TSA56-based PCR assay and an IgM IFA titre of $\geq 1:1280$. A negative control sample had a negative result for the PCR assay and an IgM IFA titre of $< 1:10$. The samples were then washed three times with PBS and air-dried. A fluorescein isothiocyanate-conjugated anti-human IgM antibody (Rockland Inc, Limerick, PA) was diluted in PBS and applied to the wells. The slides were incubated in a dark, moist chamber for 40 minutes at 37°C. The slides were washed three times and immediately examined under fluorescence microscopy (Zeiss) by two observers. One observer (Takaya S) had an experience with scrub typhus IFA slide reading for two years, and another (Miura K) had the experience for eight years. When the reading result was discordant between the two observers, it was re-examined and finalised by discussion between the two. Serum samples that

were positive at the titre of 1:80 were serially diluted to 1:160, 1:320, 1:640, 1:1,280, 1:2,560, 1:5,120, 1:10,240, 1:20,480 and applied to individual antigen strains to confirm the endpoint titre.

A single titre \geq 1:160 for any one or more of the three serotypes was considered positive. This cut-off titre was determined by applying the assay to the 68 healthy control samples and 20 samples diagnosed as other causes of acute febrile illnesses: three patients with tuberculosis and HIV infection, five patients with tuberculosis without HIV infection, five patients with bacteraemia, two patients with bacterial meningitis, one patient with infective endocarditis, three patients with dengue, and one patient with Japanese encephalitis (Appendices 2 and 3). These 20 samples were from 459 patients with only admission samples enrolled in the original fever study at Bach Mai Hospital in 2012-2013. Of the 68 healthy controls, four were positive at a titre of 1:10. The remaining 64 were negative at a titre of 1:10. Among the 20 febrile patients, one patient with pulmonary tuberculosis and HIV infection had a positive result at a titre of 1:80, and another with pulmonary tuberculosis had at a titre of 1:40. One with infective endocarditis had at a titre of 1:10. The remaining 17 patients had a negative result at a titre of 1:10.

The cost of materials to screen one patient sample was Japanese Yen (JPY) 880 (equivalent to Great Britain pound [GBP] 6). It cost JPY 2,360 (GBP 15) per strain to confirm the endpoint titre for one patient sample. For instance, when one sample had a positive result at the screening titre (1:80) for all three serotypes, three slides of three strains were used to confirm the endpoint titre, costing a total of JPY 7,960 (GBP 50).

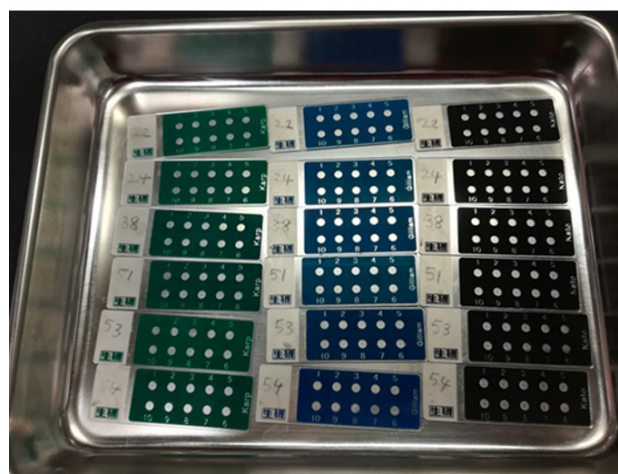


Figure 8. IFA slides of FA Slide R. Tsutsugamushi Seiken (Denka Company Ltd, Japan).

Ten wells on each slide are coated with whole cell lysates of one of the *Orientia tsutsugamushi* Karp (green), Gilliam (blue), or Kato (black) strains.

TSA56-based IgM and IgG ELISA

Commercial ELISA kits, Scrub Typhus Detect IgM ELISA System and Scrub Typhus Detect IgG ELISA System (InBios International, Seattle, WA) were used. The ELISA plate is pre-coated with recombinant TSA56 antigens of the Gilliam, Karp, Kato, and TA716 strains. The assay was performed according to the manufacturer's instructions. Of note, serum dilution was 1:100, and the signal of a blank background was not subtracted. IgM and IgG cut-off OD values were 0.513 and 0.4365, which were mean OD values plus 3 SDs of the 68 healthy controls following the instruction (Appendix 2) (184). The IgM ELISA results of the fever cohort and healthy controls were obtained from the original study. The material cost to screen one patient sample was JPY 600 (GBP 4) when a single well was used per sample.

8. Prediction of the ScaA protein structure and B-cell linear epitopes

Amino acid sequences of ScaA and TSA56 proteins were obtained from the NCBI database. JalView was used to align amino acid sequences. Protein BLAST (BLASTp) analysis was performed on the NCBI BLAST website. To predict the three-dimensional structures of the ScaA ATD and TSA56 protein, their amino acid sequences were submitted to the SWISS-MODEL (a homology modelling method) and I-TASSER (a threading method) programs. Homology modelling depends on the sequence similarity between the target and template proteins in the structure database. The use of a template protein sequence with a >30% identity is known to provide an accuracy equivalent to a low-resolution X-ray structure. The identity of less than 30% leads to substantial errors (188). A threading method that computes an energy function during the sequence alignment can be used even when there are no templates with sufficient sequence homology.

A ScaA ATD amino acid sequence (BDP28846.1, amino acid residues 1-281) (Figure 9) was submitted to the online SWISS-MODEL (<https://swissmodel.expasy.org/>) and I-TASSER (<https://zhanggroup.org/I-TASSER/>) programs. Protein structures were displayed using the PyMOL (Molecular Graphics System version 1.7.4.5 Edu Schrödinger, LLC, NY). For the prediction of B-cell linear epitopes, the ScaA ATD amino acid sequence was submitted to the IEDB program (<http://tools.iedb.org/main/bcell/>). All available methods on the website were used.

The amino acid sequence of the recombinant ScaA protein (residues 91-191) is highlighted in grey in Figure 9. This region of 101 amino acids is designated as the ScaA antigen region henceforth in this thesis.

```
>BDP28846.1 surface cell antigen A, partial [Orientia tsutsugamushi]
NINKQSWNIWADGFFSNVNQQDHENIQGYKTDISGIPIGADKHLKNNAIIGAAISYAKFDTKHTDSRIEK
INSNVYLLSLYGQYSFQSTTFIRGMVNVAKFSDDSKNSSLTLWQGHSYHGSLTAGHYFYPLKNNKLTLV
PTVGIRHSYFNTSGNNSVDSSSNKTIGDRSHKALEGIIIGISLEQLVANNANNLNLVSTVYGYVHHNLYD
CQDSAQLPNSNSTFNPDVITAHEQCLHKTFYQLGVKLAIKRNIMDIGIACDVYLAEKYISHTGIIYAKAS
F
```

Figure 9. The amino acid sequence in FASTA format of ScaA autotransporter domain submitted to the SWISS-MODEL, I-TASSER, and IEDB programs.

9. Statistical analysis

All tests were two-tailed and statistical significance was set at $p < 0.05$. Statistical analysis was performed using STATA 15.1 (Stata Corp., College Station, TX, USA). Bayesian LCM analysis was performed using OpenBUGS version 3.2.3., and its methodology is described in Chapter 13.6.

10. Ethics approval

This Ph.D. study was approved by the Observational/Interventions Research Ethics Committee, LSHTM (22791). Ethics approval was obtained from appropriate ethics committees for each cohort.

Assay development cohort

For the original enrolment, verbal consent was obtained from all participants, and an information sheet was provided to them. This form of consent was chosen because the collection of samples was a part of their routine clinical practice. The original project was approved by the life scientific committee, UT. NIHE requested UT to perform an ethics review. For this Ph.D. study, Associate Professor Miura discussed with 105 Military Hospital and NIHE again. 105 Military Hospital and NIHE agreed that an ethical review would be renewed at UT, and a material transfer agreement was signed between NIHE and UT. This process was done in consultation with the life science committee. The new ethics approval was obtained at the life science committee, UT (21-9). Following the recommendation from the committee, a new information sheet was sent to be posted at NIHE and 105 Military Hospital.

Rickettsiosis cohort

Verbal consent was obtained from all participants. The original study was approved in 2011 by the independent ethics committees of Bach Mai Hospital and the Institute of Tropical Medicine, NU. The committees did not require written informed consent with strict protection of patients' information as a condition. For this project, a new ethics review was applied and approved by the Institute of Tropical Medicine, NU (211216267).

Fever cohort and healthy controls

This project is part of the ‘Undifferentiated febrile illness study in Bach Mai Hospital.’ At the original enrolment, written informed consent was obtained from all participants. The study was approved by the institutional review board of NIHE (IRB-VN01057-31/2015) and the independent ethics committees of the Institute of Tropical Medicine, NU (12021085-6, 2020). The initial approval at NIHE was until December 2020 and extended until 2025 on September 16, 2020.

11. Funding

This Ph.D. study was funded by Nagasaki University “Doctoral Program for World-leading Innovative and Smart Education” for Global Health, KENKYU SHIDO KEIHI. The funding source had not been involved in the study design, analysis, and interpretation of the data.

PART III. Development of a ScaA-based ELISA

12. Development of a recombinant ScaA-based ELISA

12.1. Amino acid sequence conservation of the ScaA antigen region across strains

The amino acid sequence identity of the ScaA antigen region in different *Orientia* strains was analysed. In addition to the sequence used in this study (BDP28846.1), 14 sequences with specified strains were retrieved from the NCBI website: Karp (AJC11312.1), Kato (AJC11313.1), Gilliam (AJC11311.1), Kuroki (BCZ49643.1), Kawasaki (BCZ49645.1), Ikeda (BAG40673.1), Shimokoshi (BCZ49644.1), Boryong (CAM79168.1), UT76 (KJV70937.1), UT144 (KJW06660.1), Sido (KJW03745.1), TA763 (KJV74865.1), TA716 (KJV77089.1), and TA686 (SPR02760.1).

The amino acid sequence identity of the ScaA antigen region was compared to the sequence used for the recombinant antigen (BDP28846.1) (Table 5). All sequences except for that of the Shimokoshi strain had a high identity of over 90%. As shown in Figure 10, the frequent amino acid differences were at six positions (48, 55, 66, 70, 92, and 97). As expected for an ATD of an autotransporter protein, the amino acid sequences of the whole ScaA-ATD had a high identity across strains (Table 5 and Figure 11). The amino acid sequence identities of ScaA-ATD-A and -C also exceeded 90% (Table 5).

The amino acid sequence of the ScaA antigen region used in this study (BDP28846.1) was not identical to the sequence of the other Gilliam strain analysed (AJC11311.1) (Table 5 and Figure 10). Observed amino acid differences between the two Gilliam strains were observed at five positions (48, 55, 66, 70, and 92). The frequent inter-strain amino acid differences were also seen intra-strain. This intra-strain diversity may hinder the detection of homologous antibodies.

Table 5. The amino acid sequence identity of ScaA ATD, ScaA-ATD-A, ScaA-ATD-B, and ScaA-ATD-C of 14 *Orientia tsutsugamushi* strains compared to the amino acid sequence of the recombinant ScaA autotransporter domain antigen (BDP28846.1).

Strain	ScaA-ATD	ScaA-ATD-A	ScaA-ATD-B	ScaA-ATD-C
Karp	95.4	98.0	92.1	93.1
Kato	98.0	99.0	98.0	96.0
Gilliam	97.7	98.0	95.0	99.0
Kuroki	-	-	95.0	-
Kawasaki	-	-	95.0	-
Ikeda	97.0	99.0	94.1	97.0
Shimokoshi	-	-	79.2	-
Boryong	96.0	96.0	95.0	95.0
UT76	96.7	96.0	98.0	95.0
UT144	97.0	96.0	98.0	96.0
Sido	96.7	97.0	95.0	96.0
TA763	97.7	98.0	95.0	99.0
TA716	97.3	98.0	95.0	98.0
TA686	94.4	96.0	91.1	91.1

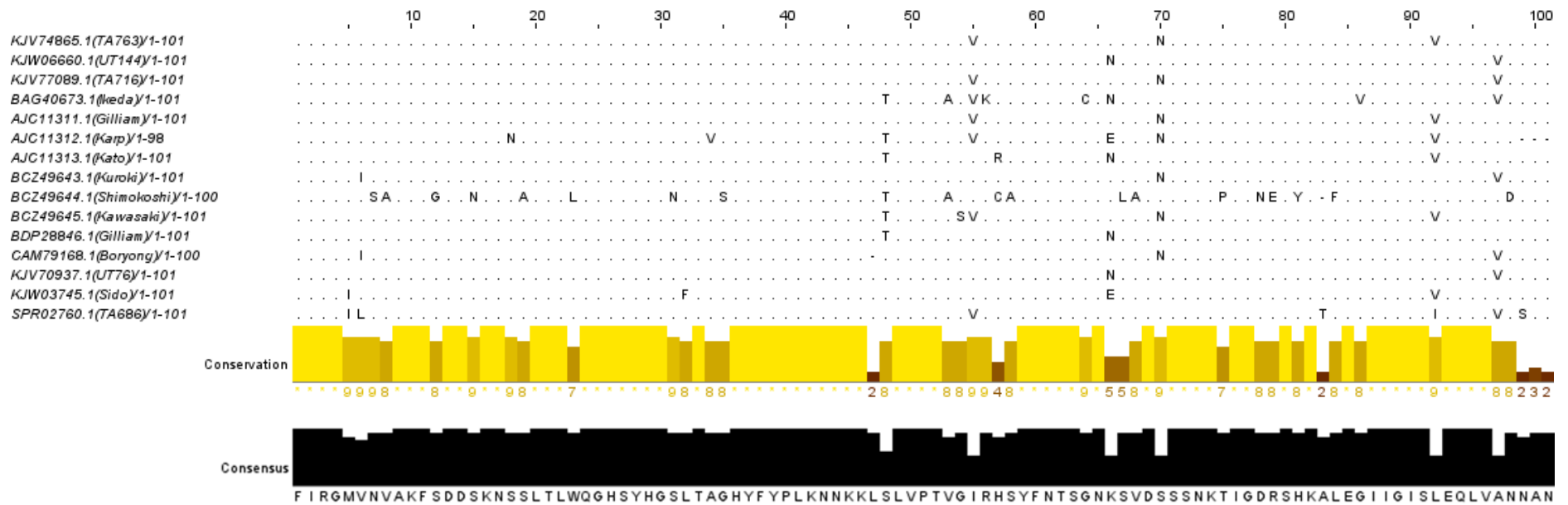


Figure 10. Alignment of amino acid sequences of the ScaA antigen region of 15 *Orientia tsutsugamushi* strains.

A ClustalW alignment of the translated amino acid sequences of 15 *Orientia* isolates is presented. Only amino acid differences relative to the consensus sequence are shown. BDP28846.1 was used as an antigen for a recombinant ScaA-based ELISA.

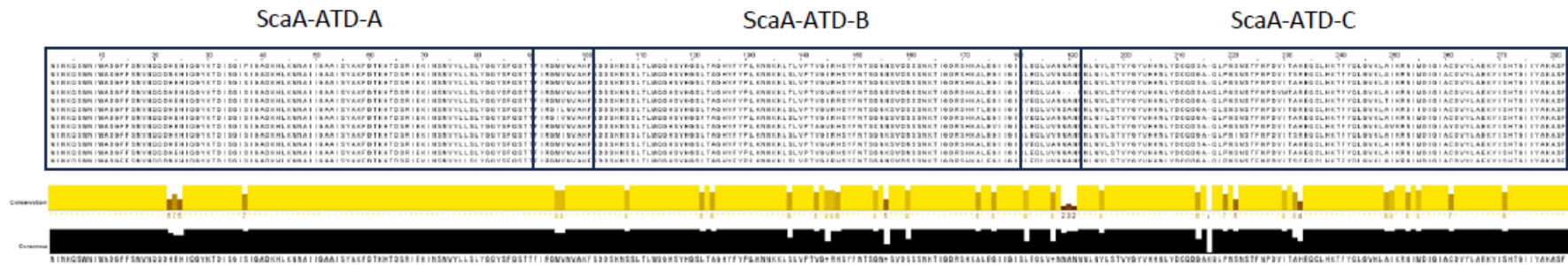


Figure 11. Alignment of amino acid sequences of a ScaA autotransporter domain of 12 *Orientia tsutsugamushi* strains.

A ClustalW alignment of the translated amino acid sequences of 12 *Orientia* isolates is presented. BDP28846.1 was used as an antigen for a recombinant ScaA-based ELISA.

12.2. Progress until the Qualifying Examination

As discussed in Chapter 5, the ScaA gene fragment was initially expressed into a pET43.1a(+) vector. This vector is designed to express proteins fused with a Nus•Tag™ protein (491 amino acids, 55kDa) that improves the solubility of the resultant protein. The expression and purification protocols are in Appendix 4. The recombinant ScaA protein was suspended in an imidazole-free Tris buffer. The control protein was purified using an empty pET43.1a(+) vector. The cleavage of the ScaA protein from the Nus•Tag™ protein with a recombinant enterokinase was not successful, possibly due to the insolubility of the cleaved ScaA protein.

The protocol of an IgM ELISA with a recombinant ScaA protein fused with a Nus•Tag™ is in Appendix 4. The IgM ELISA was applied to the serum samples of ten patients in the assay development cohort. Five of them had positive test results for both a TSA56-based nested PCR assay and an IgM IFA, and the remaining five patients had negative results for both tests (highlighted in grey in Appendix 5). The ELISA result is shown in Figure 12. Although some positive samples showed IgM antibody responses to the recombinant ScaA protein, the signal was not high and overlapped with that of negative samples.

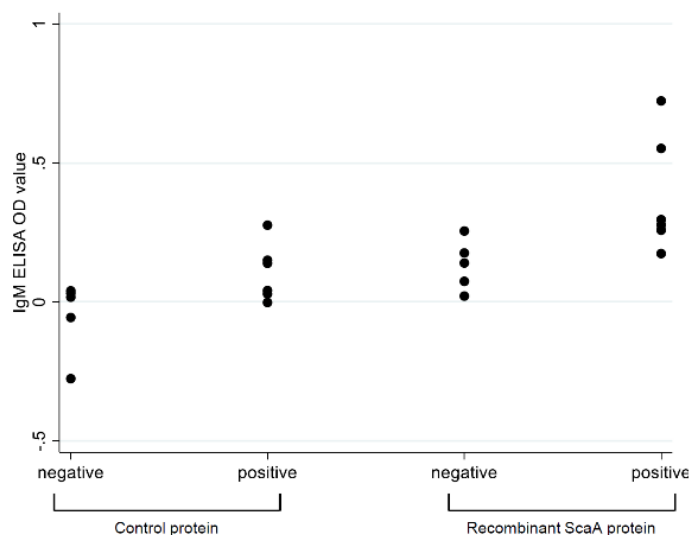


Figure 12. IgM antibody responses to the recombinant ScaA protein synthesised using a pET43.1a(+) vector and a control protein.

An IgM ELISA using the control protein and recombinant ScaA protein was applied to serum samples of five scrub typhus patients (positive) and five non-scrub typhus patients (negative).

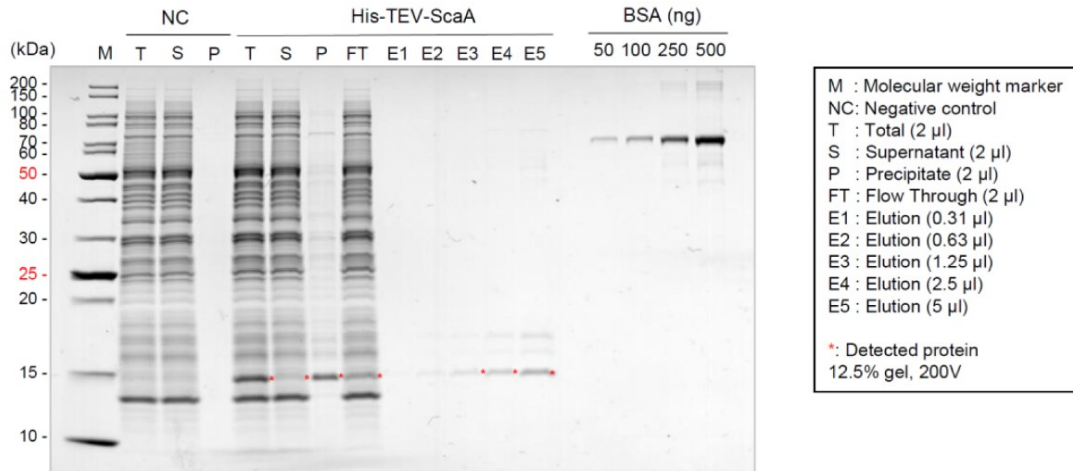
At the qualifying examination in December 2019, it was pointed out that the Nus•Tag™ protein was considerably larger than the fractional ScaA protein, and the recombinant protein may not be appropriate as an immunoassay antigen. The overlap of the antibody responses between positive and negative samples was another concern. Retrospectively, one of the potential reasons for the overlap could have been the poorly controlled BGN reactions on MaxiSorp plates with 3% skim milk as a blocking agent. It was also recommended to perform the prediction of the three-dimensional structure of ScaA proteins with online programs and identify the topology of the antigen region in the ScaA ATD.

A synthesis of the ScaA protein was also ordered to CellFree Sciences (Ehime, Japan) in February 2020 (189). Their protein synthesis system uses a wheat-germ cell-free expression method (190). The sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) result showed that the synthesised protein was primarily deposited in the precipitate and scarcely in the supernatant (Figure 13). To improve the protein yield, they proposed a collection of the precipitate or solubilization of the precipitate with detergents.

In summary, the recombinant ScaA protein was synthesised with a Nus•Tag™ protein but could not be collected once it was cleaved from the tag protein. The protein was deposited in the precipitate when it was synthesised by a wheat-germ cell-free expression method. These results suggested that the ScaA protein was insoluble. These results were plausible since ScaA is a membrane protein.

Following the advice at the qualifying examination, the expression and purification of the recombinant ScaA protein were resumed using a pET28.1a(+) vector in June 2020.

Fig.1 目的タンパク質の合成・可溶性および精製確認



サンプル名	分子量	総画分(6 ml)の目的タンパク質量	可溶化率	精製画分の目的タンパク質濃度	精製画分液量	精製画分の目的タンパク質量
His-TEV-ScaA	11 kDa	701 μ g	10%未満	17 μ g/ml	0.9 ml	15 μ g

* 総画分量・可溶化率は、粗画分のため参考値とする。

Solubilization rate <10%

精製タンパク質のBuffer組成:

20 mM Na-Phosphate pH7.5, 300 mM NaCl, 500 mM Imidazole

Figure 13. A result report of the ScaA protein synthesis from CellFree Sciences.

The synthesised ScaA protein was found primarily in the precipitate and scarcely in the supernatant. Of 701 μ g of the recombinant ScaA protein in 6 ml of the total sample, only 15 μ g was collected in 0.9 ml of the elute. The solubilization rate of the protein was less than 10%.

12.3. Purification of a recombinant ScaA antigen

ScaA antigen gene cloning

A nucleotide sequence of the ScaA gene fragment of the *O. tsutsugamushi* Gilliam strain (LC720723.1) was amplified by a PCR assay using the primers shown in Table 6. The amplified fragment was digested using *EcoR* I and *Hind* III restriction enzymes and then cloned into the *EcoR* I and *Hind* III site of a pET28.1a(+) vector (Novagen, Madison, WI). The partial ScaA gene fragment insert was confirmed by DNA sequencing.

Table 6. Forward and reverse primers for the ScaA antigen gene cloning

Forward	CCGAATTCTTTATTCGAGGAATGGTAAAT
Reverse	CGGAAGCTTATTTGCATTCATTTGCAACTAAC

Expression and purification of a recombinant ScaA protein

A nucleotide sequence encoding the ScaA antigen cloned into the expression vector was expressed in *E. coli* BL21 (DE3) (Novagen). The recombinant ScaA protein contained a 6×Histidine tag at its N-terminal, and the molecular weight was estimated at 17kDa. The SDS-PAGE analysis showed that the recombinant ScaA protein formed inclusion bodies (lane 3) (Figure 14). The inclusion body was solubilized in 8 M urea (lane 6). Refolding of the recombinant protein was not successful. As discussed in Chapter 12.2, this could have been due to the recombinant protein's insolubility. Therefore, the recombinant ScaA protein was collected in a denatured condition. The purification protocol is in Table 7. The recombinant protein yield was approximately 2,000-4,000 µg of the ScaA protein from 500 ml of the *E. coli* culture medium.

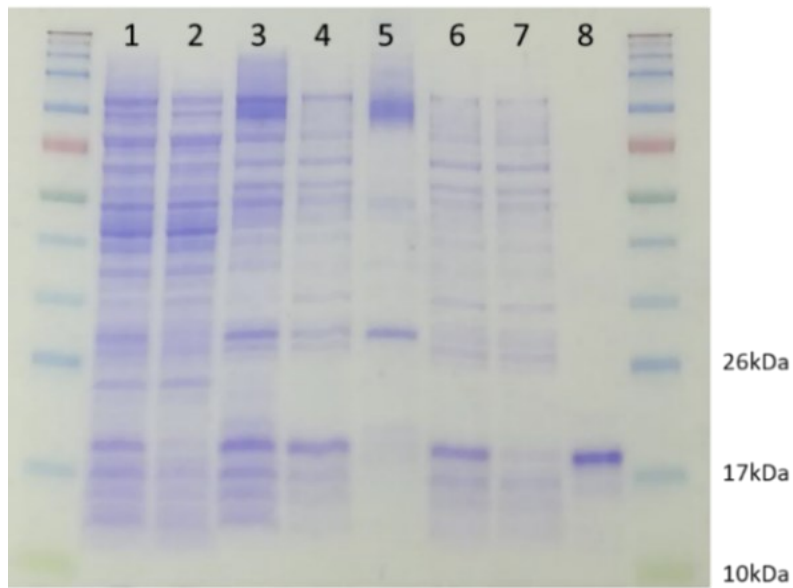


Figure 14. ScaA purification products resolved by SDS-PAGE and stained with Coomassie Blue. Lane 1: sonicated *E. coli* culture, lane 2: supernatant of lane 1, lane 3: precipitate of lane 1 (=inclusion bodies), lane 4: solubilised inclusion bodies, lane 5: precipitate of lane 4, lane 6: supernatant of lane 4 (to be applied to Ni-NTA agarose), lane 7: flow through from the Ni-NTA agarose, lane 8: elute (=final product). Ni-NTA: nickel-nitrilotriacetic acid.

Table 7. A recombinant ScaA protein purification protocol

1. Overnight culture	<ul style="list-style-type: none"> - Autoclave a Luria-Bertani (LB) medium. - Add kanamycin to a concentration of 100 µg/ml into the medium. - Pick 1-2 colonies of <i>E.coli</i> BL21 (DE3) of an expressed strain and place them into the medium. - Incubate the medium with shaking at 37 °C overnight.
2. Expansion of starter culture	<ul style="list-style-type: none"> - Centrifuge the overnight culture medium (2,500 g, 10 °C, 10 minutes). - Discard the supernatant. - Add the deposit into another autoclaved LB medium until the O.D.₆₀₀ value of the medium reaches 0.2. - Incubate with shaking at 37 °C for 1-2 hours until the O.D.₆₀₀ value of the culture reaches 0.7-0.8.
3. Induction	<ul style="list-style-type: none"> - Add isopropyl β-D-thiogalactopyranoside to a final concentration of 0.4 mM to the culture. - Shake the culture overnight at 25 °C. - Centrifuge the culture (2,500 g, 10 °C, 10 minutes). - Collect the deposit and resuspend in 1/20 volume of the culture of a sonification buffer (20 mM Tris, 50 mM NaCl, pH 8.0).
4. Sonification	<ul style="list-style-type: none"> - Sonicate the sample on ice for 5 minutes. - Centrifuge the sample (10,000 g, 4 °C, 10 minutes). - Collect the pellet.
5. Wash and isolation of inclusion body	<ul style="list-style-type: none"> - Resuspend the pellet in a washing buffer (20 mM Tris, 500 mM NaCl, 2% Triton X-100, pH 8.0). - Centrifuge the sample (10,000 g, 4 °C, 10 minutes) and collect the pellet. - Repeat the wash step twice and collect the pellet (=inclusion body).
6. Solubilization	<ul style="list-style-type: none"> - Resuspend the inclusion body in a solubilization buffer (20 mM Tris, 500 mM NaCl, 20 mM imidazole, 8 M urea, 1 mM 2-mercaptoethanol, pH 8.0). - Stir at 4 °C overnight. - Centrifuge the sample (10,000 g, 4 °C, 10 minutes) and collect the supernatant.
7. Elution	<ul style="list-style-type: none"> - Apply 1 ml of His Ni-NTA agarose resin (Fujifilm, Japan) to the supernatant and stir at 4 °C for 30 minutes. - Centrifuge the sample (1,000 g, 4 °C, 5 minutes) and discard the supernatant. - Wash the resin with the solubilization buffer. Repeat washing twice. - Apply 2 ml of an elution buffer (20 mM Tris, 500 mM NaCl, 500 mM imidazole, 8 M urea, 1 mM 2-mercaptoethanol, pH 8.0) and stir at 4 °C for 30 minutes. - Centrifuge the sample (1,000 g, 4 °C, 5 minutes) and collect the elute. - Measure the concentration of the ScaA antigen by a BCA protein assay kit with BSA protein standards.

BCA: bicinchoninic acid

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis and Western blot analysis

The recombinant ScaA protein was analysed by SDS-PAGE (Figure 15a). The polyacrylamide gel was stained with Coomassie Blue. The gel was transferred onto a polyvinylidene fluoride (PVDF) membrane by a gel transfer device for Western blot analysis. Mouse anti-6xHistidine tag IgG antibody (1:10,000 dilution) and anti-mouse IgG antibody (1:10,000 dilution) were used as primary and secondary antibodies. An anti-6xHistidine tag antibody recognised the protein (Figure 15b).

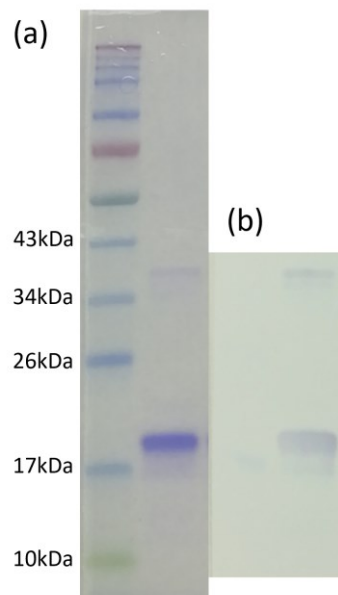


Figure 15. Analysis of the synthesised recombinant ScaA protein.

(a) the recombinant ScaA protein resolved by SDS-PAGE and stained with Coomassie Blue, (b) Western blot analysis using an anti-6xHistidine tag antibody.

12.4. Recombinant ScaA-based ELISA protocol

A blocking agent, microplate, and antigen concentration were confirmed step by step. The dilution of human serum samples (1:500) was the same as the preliminary results discussed in Chapter 12.2. Serum samples from the same ten patients in the assay development cohort (highlighted in grey in Appendix 5) were used to set up the protocol.

First, skim milk, BlockAce (191), Hammarsten grade casein, and BSA were tested as a blocking agent using Nunc MaxiSorp microplates. BSA showed the highest blocking ability (lowest OD values in antigen-non-coated wells) (Figure 16). Second, BGN reactions on Nunc MaxiSorp and PolySorp microplates were compared using BSA buffers. Nunc PolySorp microplates had lower BGN reactions (Figure 16). MaxiSorp plates are optimised for absorbing high amounts of hydrophilic proteins such as immunoglobulin, while PolySorp plates are optimised for absorbing hydrophobic molecules (192). Third, the concentrations of the antigen (1, 2, and 4 µg/ml) was set by a serial dilution (Figure 17). Among the three concentrations, the antigen concentration of 4 µg/ml yielded the highest signal to noise ratio and was chosen for the final protocol.

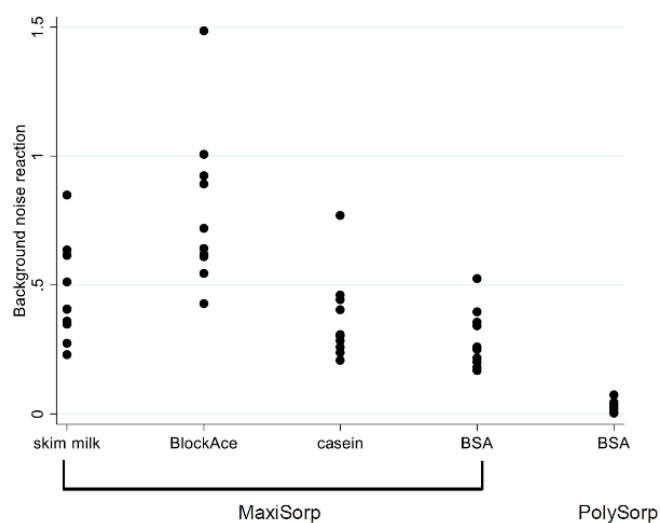


Figure 16. Background noise reaction of the recombinant ScaA-based IgM ELISA with different blocking agents and microplates.

Background noise reactions were measured in antigen-non-coated wells on a MaxiSorp microplate using skim milk (5% in PBS), BlockAce (1% in distilled water), Hammarsten casein (1% in PBS), and BSA (3% in PBS) as blocking agents. Ten human serum samples were diluted to 1:500, and the secondary antibody was a goat anti-human IgM-HRP conjugate diluted to 1:20,000. The same protocol using BSA as a blocking agent was performed with a PolySorp microplate.

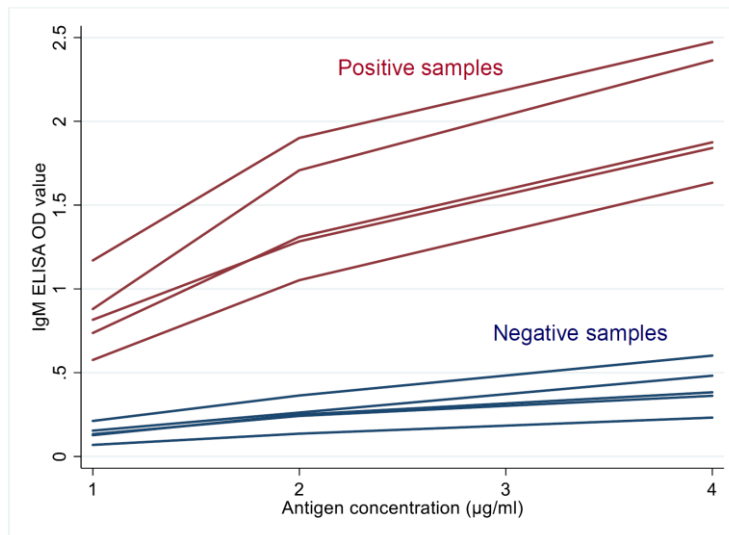


Figure 17. IgM antibody responses to the recombinant ScaA protein of different concentrations. A recombinant ScaA-based IgM ELISA was applied with three concentrations of the ScaA antigen (1, 2, and 4 µg/ml). 3% BSA in PBS-T was used as a blocking buffer. Ten human serum samples were diluted to 1:500, and the secondary antibody was a goat anti-human IgM-HRP conjugate diluted to 1:20,000.

The final protocol of the recombinant ScaA-based IgM ELISA is shown in Table 8. In this protocol, the influence of BGN reactions was minimised by three approaches: 1) use of PolySorp microplates, 2) dilution of the serum samples to 1:500, and 3) subtraction of the BGN reactions from the signal in antigen-coated wells. BGN reactions were measured for each patient using two antigen-non-coated wells. A positive and negative controls were included in every plate. A positive control had an IgM IFA titre of $\geq 1:20,480$ and a negative control had an IgM IFA titre of $< 1:80$. An IgG ELISA followed the same protocol except for using a goat anti-human IgG-horseradish peroxidase (HRP) conjugate (Bethyl Laboratories, Inc) diluted to 1:10,000 as a secondary antibody.

Table 8. A recombinant ScaA-based IgM ELISA protocol.

1. Coat an immunoassay plate (PolySorp 96-well flat-bottom microplates; Nunc) in duplicate with 100 μ l/well of
 - the recombinant ScaA protein at a concentration of 4 μ g/ml in 8 M urea.
 - 8 M urea (= non-coated wells for the measurement of the BGN reactions).
2. Incubate the plate at 4 $^{\circ}$ C overnight.
3. Wash the plate with a washing buffer (PBS-T: 0.05% Tween-20 in PBS) five times.
4. Block the wells with a blocking buffer (3% BSA in PBS-T) at room temperature (20-25 $^{\circ}$ C) for 1 hour.
5. Wash the plate with the washing buffer three times.
6. Apply 100 μ l/well of sample serum diluted to 1:500 in a dilution buffer (3% BSA in PBS-T) to non-coated and antigen-coated wells.
7. Incubate at room temperature for 1 hour.
8. Wash the plate with the washing buffer five times.
9. Apply 100 μ l/well of goat anti-human IgM-HRP conjugate (QED Bioscience, Inc.) diluted to 1:20,000 in the dilution buffer.
10. Incubate at room temperature for 1 hour.
11. Wash the plate with the washing buffer five times.
12. Apply 200 μ l/well of SIGMAFAST OPD (Sigma-Aldrich) and allow the colour to develop at room temperature for 30 minutes.
13. Apply 50 μ l/well of 3 M hydrochloric acid and stop the reaction.
14. Measure the OD values at 492 nm with a plate reader (Thermo Scientific Multiskan FC).
15. Subtract the mean OD value of two blank wells from the mean OD values of non-coated and antigen-coated wells.
16. Subtract the mean OD value in the non-coated wells from the mean OD value in the antigen-coated wells of each test sample.

13. Evaluation of a recombinant ScaA-based ELISA

13.1. Demography of the cohorts and summary of applied test results

The study flow of participants is shown in Figure 18. Time intervals between sample collection and application of diagnostic tests were 18 years for the rickettsiosis cohort and 8 years for the fever cohort. During those intervals, the blood samples were stored at -80°C , and there were no interventions or treatments for the samples. Until all the diagnostic tests had been applied, the clinical information was unavailable to the performers/readers (Takaya S and Miura K). There was no missing data for any diagnostic test results or the presence of eschar in either cohort.

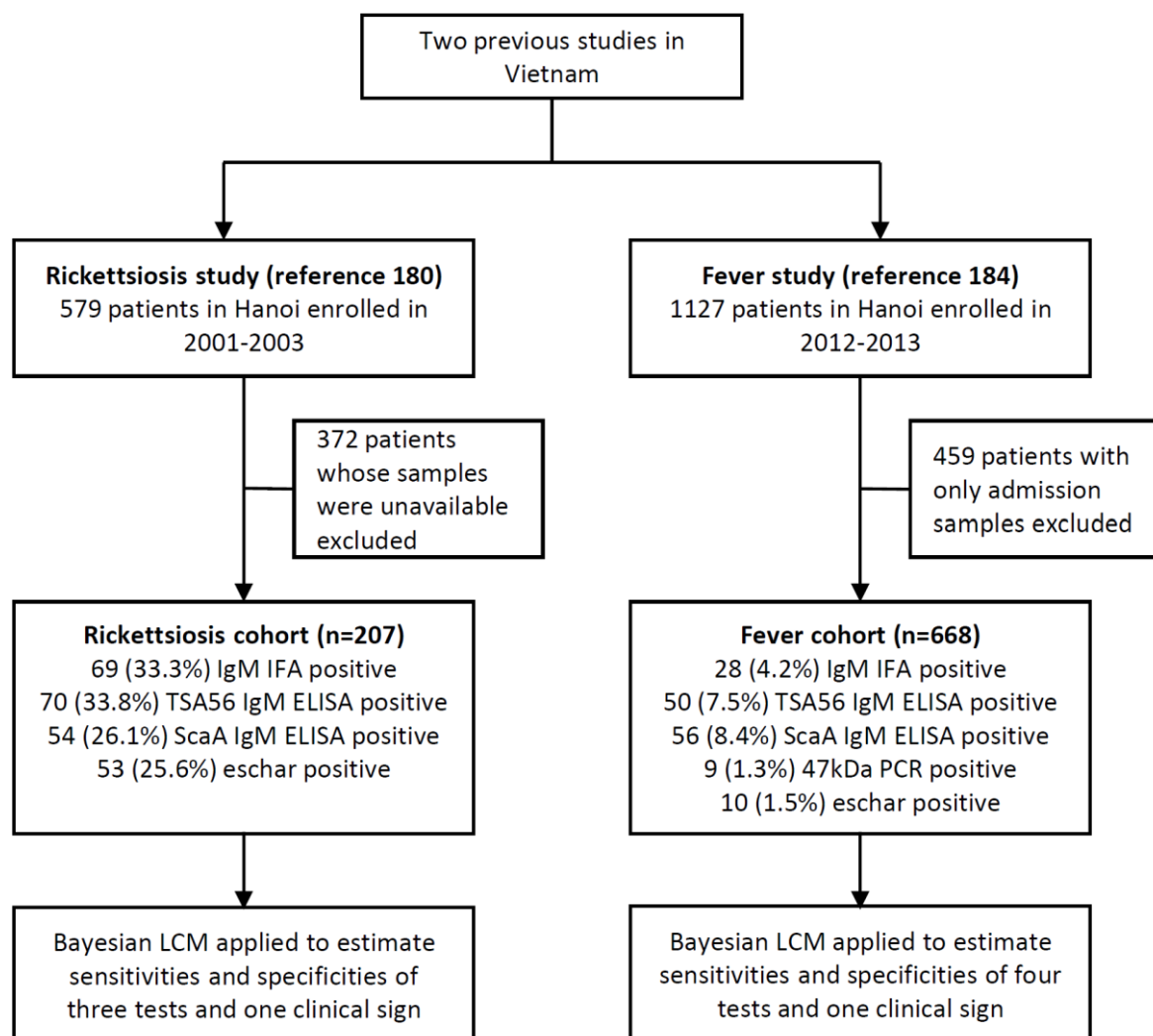


Figure 18. Study flow of participants to estimate test sensitivities and specificities by Bayesian latent class models.

The basic demography of the rickettsiosis and fever cohorts is in Table 9. The quick sequential organ failure assessment (qSOFA) was used as a proxy for severe infection. The qSOFA serves as a predictor of poor outcomes in patients with confirmed or suspected infectious diseases. The criteria use three variables, a Glasgow coma scale of <15, a respiratory rate of ≥ 22 per minute, and a systolic blood pressure of ≤ 100 mmHg. A patient with any two of these three is considered qSOFA positive (193).

In the rickettsiosis cohort, the median age was 46 years, and 65.2% were male. Approximately two-thirds of the patients (134, 64.7%) had both admission and follow-up samples; 55 (26.6%) had only admission samples, and 18 (8.7%) had only follow-up samples. Admission and follow-up samples were collected on median day 11 and day 15.5. 25.6% and 44.0% of the patients had eschar and rash. In the fever cohort, the median age was 47.5 years, and 56.4% were male. Admission and follow-up samples were collected on median day 8 and day 14. Only 1.5% of the patients had eschar. The rickettsiosis cohort included patients with a high clinical suspicion of scrub typhus whereas the fever cohort was a more heterogeneous with a diverse range of acute febrile illnesses.

Table 9. Basic demography of the rickettsiosis cohort and fever cohort.

	Rickettsiosis cohort (n=207)	Fever cohort (n=668)
Age	46 (37-58)	47.5 (31-58)
Male	135 (65.2)	377 (56.4)
Residence in Hanoi	53 (25.6)	200 (30.0)
Farming	88 (42.5)	217 (32.5)
Enrolled in rainy season (May-October)	121 (58.5)	329 (53.7)
Duration of fever (days)	10 (7-11)	7 (4-15)
Fever duration >7 days	148 (71.5%)	293 (43.9)
Temperature ≥ 38.0 °C	175 (84.5)	370 (55.4)
qSOFA score ≥ 2	16 (7.7)	41 (6.1)
Eschar	53 (25.6)	10 (1.5)
Rash	91 (44.0)	79 (11.8)
Admission and follow-up samples	134 (64.7)	668 (100.0)
White blood cell count $\geq 10,000$ / μ L	53 (25.6)	336 (50.3)

Median (IQR) for continuous variables, number (%) for categorical variables.

13.2. Cut-off values for an IgM IFA and a recombinant ScaA-based IgM ELISA

A cut-off value for a scrub typhus IgM IFA

In the rickettsiosis and fever cohorts, 33.3% (69/207) and 4.2% (28/668) had an IgM IFA titre of $\geq 1:160$ (Figure 19). In the former cohort, the distribution of the IgM IFA titre has a bimodal distribution with peaks at a titre of $<1:80$ and a titre of $\geq 1:20,480$. A significant proportion of patients, 4.4% (9/207) in the rickettsiosis cohort and 6.0% (40/668) in the fever cohort, had an IgM IFA titre of 1:80.

The cut-off value of 1:160 was set from the IgM IFA results of the healthy controls and febrile patients enrolled in 2013-2014. However, according to colleagues at Bach Mai Hospital, they have seen fewer scrub typhus cases over time. The baseline immunity against *O. tsutsugamushi* in this area may have been higher when the rickettsiosis cohort was enrolled in the early 2000s. Therefore it was examined whether more stringent cut-off values resulted in significantly fewer cases. This was not the case: IgM IFA cut-off titres of 1:320 or 1:640 led, respectively to 67 or 63 patients being diagnosed as scrub typhus, instead of 69 patients.

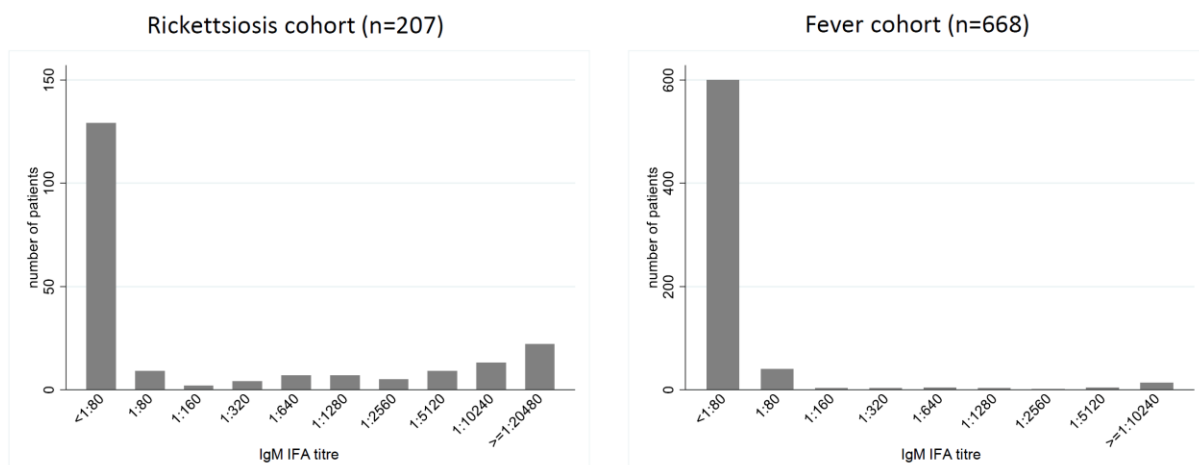


Figure 19. Distribution of patients by IgM IFA titres in the rickettsiosis and fever cohorts.

To clinically assess the validity of the IgM IFA cut-off value of $\geq 1:160$, the demographic and clinical information of scrub typhus patients and non-scrub typhus patients was examined in two cohorts (Table 10). There was no significant difference in age in the rickettsiosis cohort, but non-scrub typhus patients were more likely to be male. The proportion of patients residing in Hanoi was lower and the proportion of patients who contracted the infection during the rainy season was higher in

the scrub typhus group. The majority had a high fever of $\geq 38.0^{\circ}\text{C}$ in both groups. Clinical presentations differed in two groups: the proportion of patients with a qSOFA score of ≥ 2 was higher in the scrub typhus group; eschar was more common in the scrub typhus group; and rashes were more common in the non-scrub typhus group. These differences between the groups were consistent with the original study that used a different commercial IgM ELISA kit (Panbio, Alere, Australia) to diagnose scrub typhus. 60.1% (83/138) of non-scrub typhus patients in the rickettsiosis cohort were diagnosed with murine typhus. Therefore, the characteristics of the non-scrub typhus group reflected that of murine typhus, which is transmitted by fleas of urban rodents, mostly self-limiting, and not associated with eschars (194-196). In the fever cohort, statistically significant differences were observed only in the proportions of patients residing in Hanoi and patients with eschar. This could have been due to the heterogeneous aetiology of fever in the non-scrub typhus group and a low scrub typhus prevalence in this cohort.

These results seemed clinically plausible, and the IgM IFA cut-off of $\geq 1:160$ was assessed as reasonable.

Table 10. Basic characteristics of overall, scrub typhus, and non-scrub typhus patients in the rickettsiosis and fever cohorts diagnosed using an IgM IFA and/or 47kDa-based PCR assay as gold standard tests.

	Rickettsiosis cohort				Fever cohort			
	Total (n=207)	Scrub typhus patients (n=69)	Non-scrub typhus patients (n=138)	<i>P</i> -value*	Total (n=668)	Scrub typhus patients (n=28)	Non-scrub typhus patients (n=640)	<i>P</i> -value*
Age, years	46 (37-58)	45 (30-62)	47 (39-57)	0.448	47.5 (31-58)	43.5 (27.5-53.5)	48 (32-58)	0.173
Male	135 (65.2)	37 (53.6)	98 (71.0)	0.020	377 (56.4)	12 (42.9)	365 (57.0)	0.139
Residence in Hanoi	53 (25.6)	7 (10.1)	46 (33.3)	<0.001	200 (29.9)	3 (10.7)	197 (30.8)	0.021
Farming	88 (42.5)	34 (49.3)	54 (39.1)	0.181	217 (32.5)	14 (50.0)	203 (31.7)	0.061
Rainy season (May-October)	121 (58.5)	54 (78.3)	67 (48.6)	<0.001	329 (53.7)	15 (53.6)	344 (53.8)	1.000
Duration of fever (days)	10 (7-11)	10 (8-12)	9 (7-10)	0.029	7 (4-15)	7 (5-10)	7 (4-16)	0.883
Fever duration >7 days	148 (71.5%)	56 (81.2%)	92 (66.7%)	0.020	293 (43.9)	9 (32.1)	284 (44.4)	0.139
Temperature ≥ 38.0 °C	175 (84.5)	60 (87.0)	115 (83.3)	0.547	370 (55.4)	15 (53.6)	355 (55.5)	0.849
qSOFA score ≥ 2	16 (7.7)	13 (18.8)	3 (2.2)	<0.001	41 (6.1)	0 (0.0)	41 (6.4)	0.407
Eschar	53 (25.6)	52 (75.4)	1 (0.7)	<0.001	10 (1.5)	4 (14.3)	6 (0.9)	<0.001
Rash	91 (44.0)	15 (21.7)	76 (55.1)	<0.001	79 (11.8)	2 (7.1)	77 (12.0)	0.763
Admission and follow-up sample	134 (64.7)	50 (72.4)	84 (60.9)	0.123	-	-	-	-
White blood cell count $\geq 10,000$ / μ L	53 (25.6)	32 (46.4)	21 (15.2)	<0.001	336 (50.3)	12 (42.9)	324 (50.6)	0.446

Median (IQR) for continuous variables, number (%) for categorical variables. *Mann–Whitney U test for continuous variables, Fisher's exact test for categorical variables.

A cut-off value for a recombinant ScaA-based IgM ELISA

The distributions of OD values of a recombinant ScaA-based and TSA56-based ELISAs of 207 patients in the rickettsiosis cohort are shown in Figure 20. The median OD values of the BGN reactions were zero in both cohorts (rickettsiosis, 0.01 [IQR 0.01-0.02]; fever, 0.01 [IQR 0.01-0.04]).

The distribution of IgM antibody responses against TSA56 had a bimodal distribution with peaks at very low (≤ 0.2) and high (> 3.0) levels at both admission and follow-up. It was assumed that the peak at a high level represented the patients with scrub typhus. The distribution of IgM antibody responses against ScaA did not have a clear peak at a high level at admission. At follow-up, an increased number of patients had elevated IgM levels. The distribution of patients by IgM ELISA OD values in the fever cohort is in Appendix 6.1.

The cut-off value of 0.3721 was defined by calculating a mean plus 3 SDs of the OD values of 68 healthy controls. To validate this cut-off value, the ROC curve and Youden indices were analysed against a scrub typhus IgM IFA as a gold standard test (Figure 21 and Table 11). The cut-off values between 0.3 and 0.4 yielded the highest proportion of patients correctly classified as scrub typhus and the highest Youden indices in the rickettsiosis cohort. The results were similar in the fever cohort. The ROC curve and Youden indices in the fever cohort are in Appendices 6.2. and 6.3.

Although both sensitivity and specificity are important, specificity was prioritised over sensitivity to avoid considerable false-positive results, when the cut-off value was confirmed. In the management of fever in Asian settings, there is a wide range of differential diagnoses. False-positive results could take away an opportunity to diagnose other potentially fatal diseases. Therefore, the cut-off value was not lowered from 0.3721.

With a cut-off value of 0.3721, a recombinant ScaA-based IgM ELISA had an LR+ of 19.7 and an LR- of 0.3. Instead of having one cut-off value, slices of the test results can be directly translated into an LR+. This way, clinicians can use post-test probabilities depending on the ELISA OD values. An LR+ and post-test probabilities were calculated for four slices of the IgM ELISA OD values (Table 12).

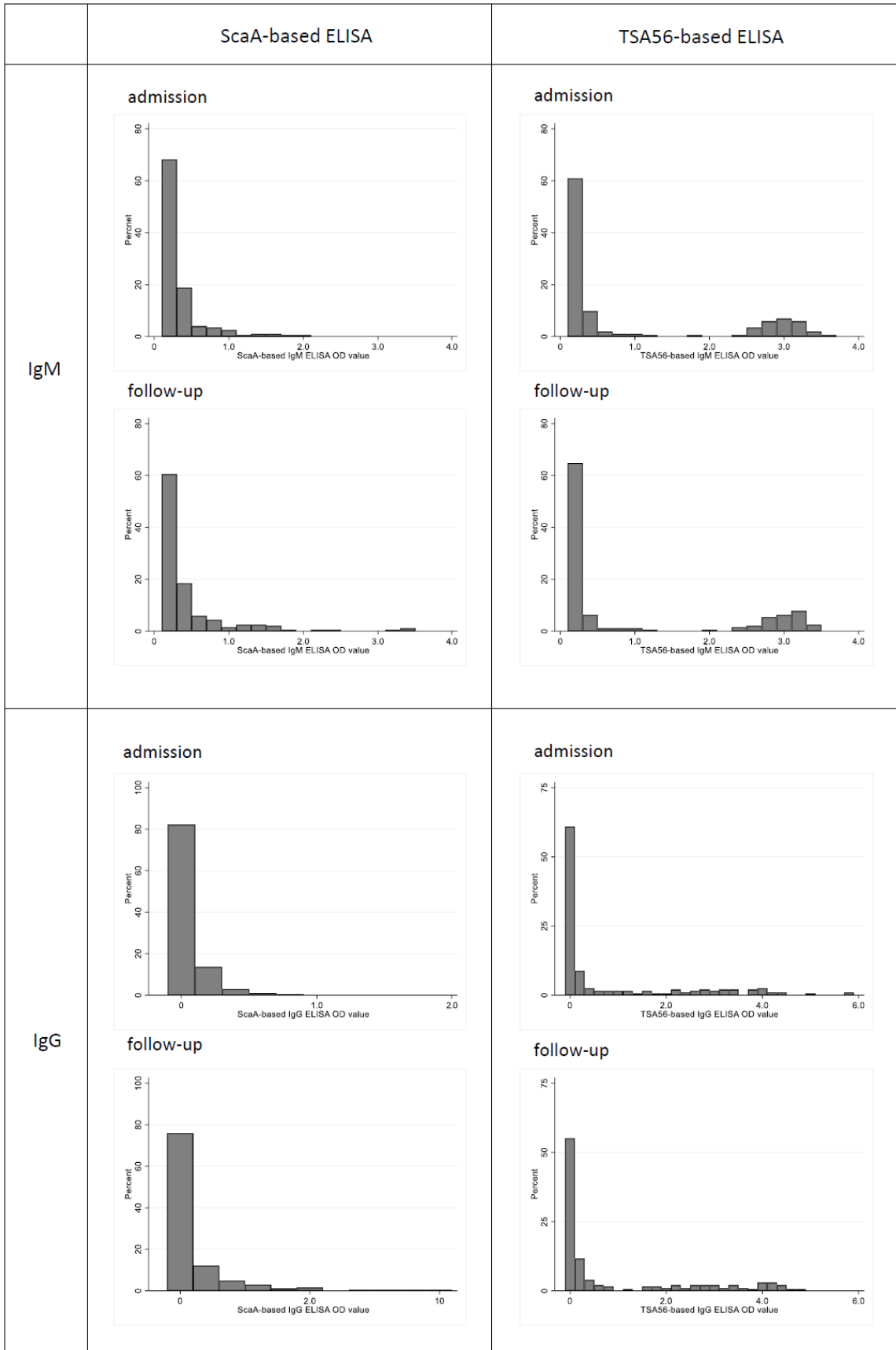


Figure 20. Distribution of patients by ScaA-based and TSA56-based ELISA OD values at admission and follow-up in the rickettsiosis cohort.

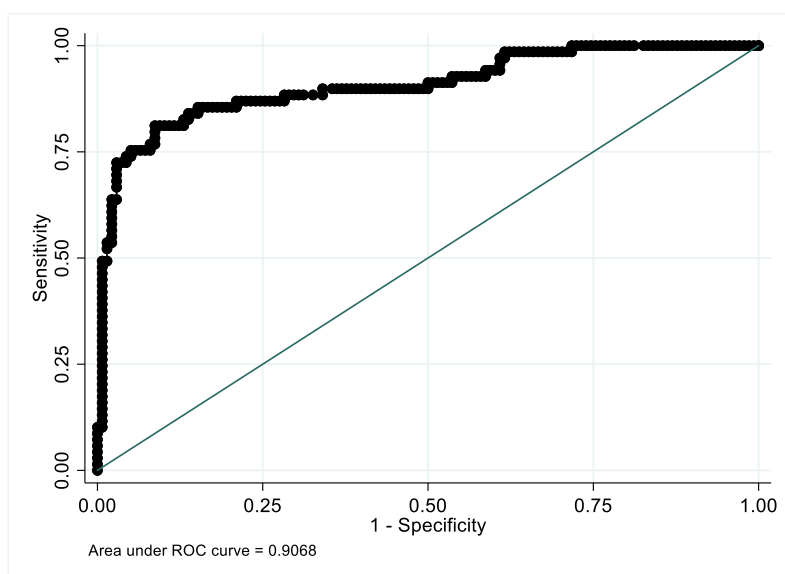


Figure 21. Receiver operating characteristics curve of the recombinant ScaA-based IgM ELISA in the rickettsiosis cohort.

Table 11. Sensitivity, specificity, and Youden index of five cut-off values for the recombinant ScaA-based IgM ELISA in the rickettsiosis cohort

Cut-off value	Sensitivity (95% CI)	Specificity (95% CI)	Youden index
0.2	87.0% (76.7-93.9)	72.5% (64.2-79.7)	0.595
0.3	75.3% (63.5-84.9)	92.0% (86.2-96.0)	0.673
0.3721	71.0% (58.8-81.3)	96.4% (91.7-98.8)	0.674
0.4	68.1% (55.8-78.8)	97.1% (92.7-99.2)	0.652
0.5	56.5% (44.0-68.4)	97.8% (93.8-99.5)	0.543

Table 12. Likelihood ratios for a positive test result for different OD value slices of the recombinant ScaA-based IgM ELISA.

ScaA IgM ELISA OD value	Number of scrub typhus patients	Number of non-scrub typhus patients	Likelihood ratio for a positive test result	Post-test probability when a pre-test probability is 10%
≤0.3	17	127	0.26	2.9%
>0.3 & ≤0.45	8	8	2	18.2%
>0.45 & ≤0.6	10	2	10	52.6%
>0.6	34	1	68	88.3%
Total	69	138		

13.3. Sensitivity and specificity of a recombinant ScaA-based ELISA against an IgM IFA and PCR assay as reference standard tests

Rickettsiosis cohort

In the rickettsiosis cohort, of 16 ($=2^4$) possible combinations of test results with three dichotomous diagnostic tests and one clinical sign, 12 patterns were observed (Table 13). ‘All tests negative’ (61.8%) and ‘all tests positive’ (18.8%) were the two most common patterns. When a scrub typhus IgM IFA was considered as a gold standard, the prevalence of scrub typhus was 33.3% (69/207) (grey rows in Table 13). The sensitivity and specificity of the recombinant ScaA-based IgM ELISA was 71.0% (58.8-81.3) and 96.4% (91.7-98.8). The 95% CI of the sensitivity could not achieve the target range of $\pm 10\%$ because the number of the samples was short of the calculated sample size. The sensitivity and specificity of the TSA56-based IgM ELISA were 94.2% (85.8-98.4) and 96.4% (91.7-98.8). The ROC curves of the recombinant ScaA-based and TSA56-based IgM ELISAs are shown in Figure 22. The area under the ROC curve (AUC) of the new test was significantly smaller than the AUC of the TSA56-based test (ScaA, 0.91 [0.86-0.95]; TSA56, 0.99 [0.97-1.00]; $p=0.001$).

Table 13. Number of patients by combinations of test results in the rickettsiosis cohort.

IgM IFA	TSA56-based IgM ELISA	Recombinant ScaA-based IgM ELISA	Presence of eschar	Observed number of patients (%)
-	-	-	-	128 (61.8)
-	-	-	+	1 (0.5)
-	-	+	-	4 (1.9)
-	+	-	-	4 (1.9)
-	+	+	-	1 (0.5)
+	-	-	-	2 (1.0)
+	-	-	+	1 (0.5)
+	-	+	-	1 (0.5)
+	+	-	-	5 (2.4)
+	+	-	+	12 (5.8)
+	+	+	-	9 (4.4)
+	+	+	+	39 (18.8)

Grey rows show patients confirmed as scrub typhus when an IgM IFA was considered as a gold standard.

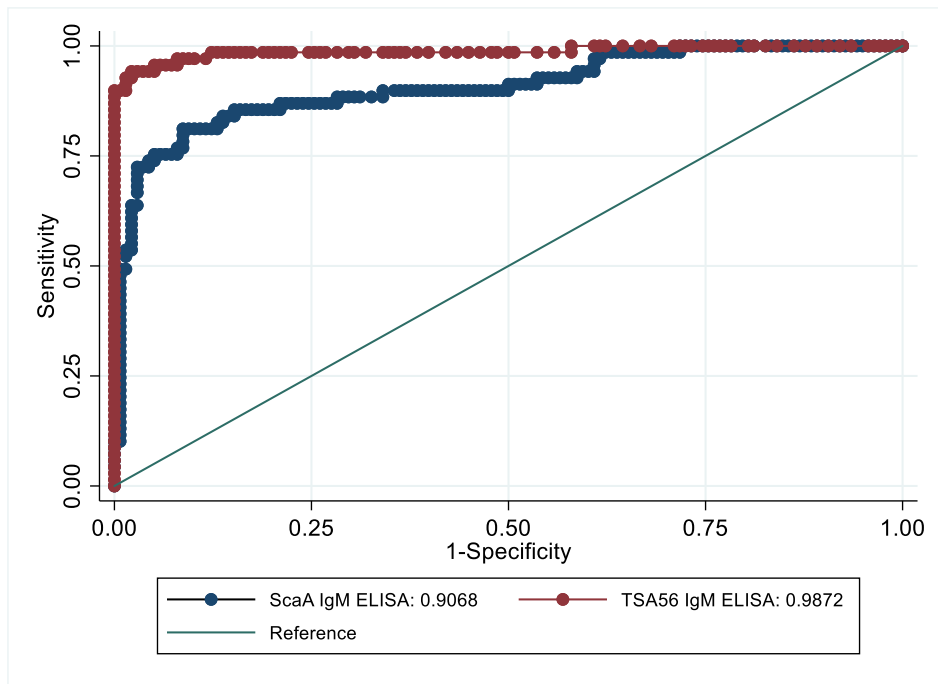


Figure 22. Receiver operating characteristics curves of the recombinant ScaA-based and TSA56-based IgM ELISAs in the rickettsiosis cohort.

Fever cohort

In the fever cohort, of 32 ($=2^5$) possible combinations of test results with four diagnostic tests and one clinical sign, 13 patterns were observed (Table 14). 'All tests negative' (86.5%) was the most common pattern, followed by 'only a recombinant ScaA-based ELISA positive' (4.8%). When a scrub typhus IgM IFA and/or 47kDa-based real-time PCR assay were considered gold standard tests, the sensitivity and specificity of the recombinant ScaA-based IgM ELISA was 57.1% (37.2-75.5) and 93.8% (91.6-95.5). The sensitivity and specificity of the TSA56-based IgM ELISA were 92.9% (76.5-99.1) and 96.3% (94.5-97.6). The ROC curves of the two ELISAs are in Appendix 6.4. The AUC of the ScaA test was significantly smaller than the AUC of the TSA56 test in this cohort, too (ScaA, 0.85 [0.78-0.93]; TSA56, 0.99 [0.97-1.00]; $p < 0.001$).

Table 14. Number of patients by combinations of test results in the fever cohort.

IgM IFA	TSA56-based IgM ELISA	Recombinant ScaA-based IgM ELISA	47kDa real-time PCR	Presence of eschar	Observed number of patients (%)
-	-	-	-	-	578 (86.5)
-	-	-	-	+	6 (0.9)
-	-	+	-	-	32 (4.8)
-	+	-	-	-	16 (2.4)
-	+	+	-	-	8 (1.2)
+	-	-	-	-	2 (0.3)
+	+	-	-	-	6 (0.9)
+	+	-	-	+	1 (0.2)
+	+	-	+	-	3 (0.5)
+	+	+	-	-	9 (1.4)
+	+	+	-	+	1 (0.2)
+	+	+	+	-	4 (0.6)
+	+	+	+	+	2 (0.3)

Grey rows show patients confirmed as scrub typhus when an IgM IFA and/or a 47kDa-based real-time PCR assay were considered as gold standard tests.

13.4. Cut-off index

To correct interplate variations, cut-off indices (COIs) were calculated as follows: (an OD value of the test sample – an OD value of the negative control) / (an OD value of the positive control – an OD value of the negative control) (152). To determine the COI cut-off value, the ROC curve and Youden index were analysed against an IgM IFA as the gold standard test (Figure 23 and Table 15). The cut-off values between 0.2 and 0.25 yielded the highest proportion of patients correctly classified as having scrub typhus and the highest Youden indices in the rickettsiosis cohort.

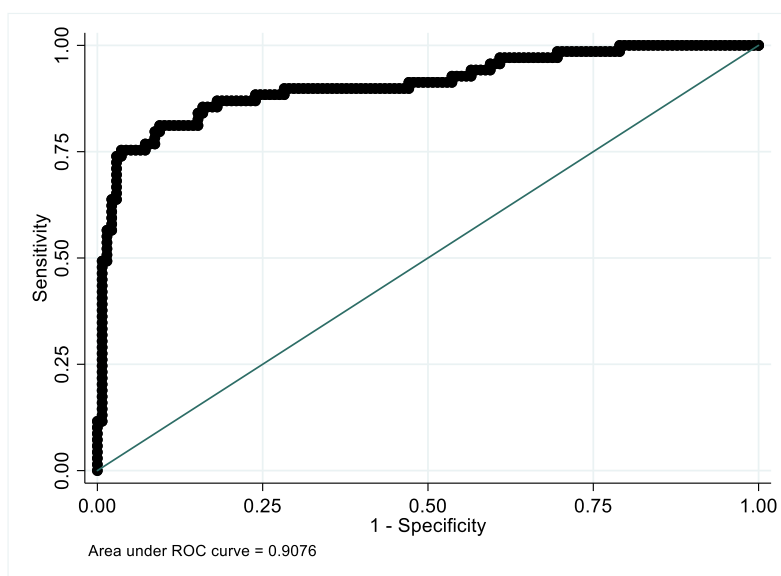


Figure 23. Receiver operating characteristics curve of the recombinant ScaA-based IgM ELISA in the rickettsiosis cohort when cut-off indices were used.

Table 15. Sensitivity, specificity, and Youden index of five cut-off values for the cut-off index of the recombinant ScaA-based IgM ELISA in the rickettsiosis cohort.

Cut-off COI	Sensitivity (95% CI)	Specificity (95% CI)	Youden index
0.1	87.0% (76.7-93.9)	76.1% (68.1-82.9)	0.631
0.2	76.8% (65.1-86.1)	92.8% (87.1-96.5)	0.696
0.25	72.5% (60.4-82.5)	97.1% (92.7-99.2)	0.696
0.3	65.2% (52.8-76.3)	97.1% (92.7-99.2)	0.623
0.4	53.6% (41.2-65.8)	98.6% (94.9-99.8)	0.522

With a COI cut-off value of 0.25, the sensitivity and specificity of the recombinant ScaA-based IgM ELISA were 72.5% (60.4-82.5) and 97.1% (92.7-99.2) in the rickettsiosis cohort (Table 16). The test had an LR+ of 25 and an LR- of 0.28. The ROC curve and Youden indices in the fever cohort are in Appendices 6.5. and 6.6. With the COI cut-of value of 0.25, the sensitivity and specificity of the test were 57.1% (37.2-75.5) and 95.9% (94.1-97.3) in the fever cohort.

Table 16. Sensitivities and specificities of a recombinant ScaA-based IgM ELISA using absolute OD values and cut-off indices in the rickettsiosis and fever cohorts.

	Absolute OD values		Cut-off indices	
	Sensitivity	Specificity	Sensitivity	Specificity
Rickettsiosis	71.0% (58.8-81.3)	96.4% (91.7-98.8)	72.5% (60.4-82.5)	97.1% (92.7-99.2)
Fever	57.1% (37.2-75.5)	93.8% (91.6-95.5)	57.1% (37.2-75.5)	95.9% (94.1-97.3)

13.5. Characteristics of a recombinant ScaA-based ELISA

Throughout this chapter, 69 scrub typhus patients of the rickettsiosis cohort were analysed unless stated otherwise. Scrub typhus patients were defined as those who had a positive result for an IgM IFA as Bayesian LCM estimates the true prevalence of scrub typhus but does not provide information on which patients were truly infected.

Scrub typhus IgM IFA titre

Figure 24 shows the correlations between IgM ELISA OD values and IgM IFA titres in the rickettsiosis cohort. The distribution of the OD value of a TSA56-based test was S-shaped, while that of a ScaA-based test was not. Positive associations were observed between ScaA IgM OD values and IgM IFA titres. The Pearson correlation coefficient was 0.60 ($p < 0.001$), and the Spearman correlation coefficient was 0.69 ($p < 0.001$). A stronger positive correlation was observed between TSA56 IgM OD values and IgM IFA titres (the Pearson correlation coefficient 0.96 [$p < 0.001$]; the Spearman correlation coefficient 0.77 [$p < 0.001$]). The sensitivity of the TSA56-based IgM ELISA was over 95% (98.4%, 61/62) when an IgM IFA titre was $\geq 1:640$ and was 100% (56/56) when it was $\geq 1:1280$. Antibody response to the recombinant ScaA antigen was also more significant when an IgM IFA titre was high. The sensitivity was over 80% (84.1%, 37/44) when an IgM IFA titre was $\geq 1:5120$. This result is similar to the previous study that observed a specific antibody response against the ScaA passenger domain predominantly in patients with high IFA titres (64).

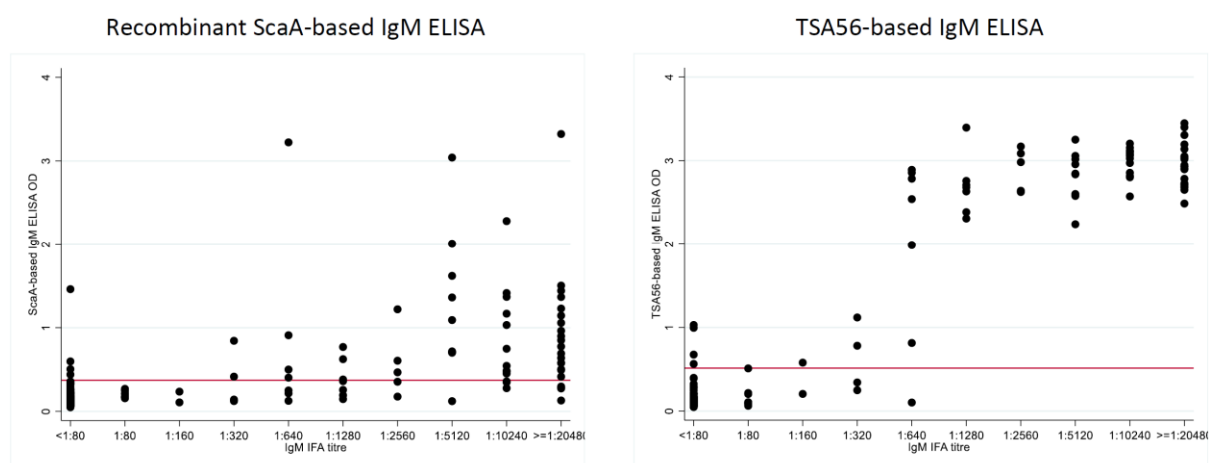


Figure 24. Distribution of IgM ELISA OD values and IgM IFA titres.

The red line is the cut-off value for each ELISA.

***Orientia tsutsugamushi* serotype and genotype**

One of the expected strengths of a recombinant ScaA-based IgM ELISA was that the assay could detect the infection regardless of infecting *Orientia* serotypes/genotypes. Sensitivities of two IgM ELISAs were examined by scrub typhus IFA serotypes (Table 17).

An IgM IFA was performed at serum dilutions from 1:80 to 1:20,480 in the rickettsiosis cohort and at dilutions from 1:80 to 10,240 in the fever cohort. For this analysis, 97 patients with an IgM IFA titre of $\geq 1:160$, 69 from the rickettsiosis cohort and 28 from the fever cohort, were included to increase the number of patients infected with each serotype. Serotypes were determined between the titres of 1:160 and 1:10,240. Cross-reactivity among *Orientia* serotypes is a common phenomenon in serological tests. 32.0% (31/97) of the samples showed cross-reactivity, and serotypes could not be confirmed for these samples. Sixty-six samples with confirmed serotypes (highlighted in grey in Table 17) were analysed to examine whether the sensitivities of IgM ELISAs differed by the serotypes. There was no statistically significant difference in the sensitivity of either IgM ELISAs. Since the commercial TSA56-based ELISA kit uses recombinant TSA56 proteins of the Karp, Kato, Gilliam, and TA716 strains, the assay is expected to be able to detect the infection of all these strains.

The fever cohort had nine cases with confirmed genotypes by the *tsa56* gene sequence. Six out of 8 patients infected with the Karp genotype had a positive result for the ScaA-based IgM ELISA. One patient with the TA763 genotype had a negative result for the ScaA test. All nine patients had a positive result for an IgM IFA and TSA56-based IgM ELISA.

Table 17. Sensitivities of a TSA56- and ScaA-based IgM ELISAs by IgM IFA serotypes.

Serotype	Number of patients (%)	TSA56 ELISA positive (%)	<i>P</i> -value*	ScaA ELISA positive (%)	<i>P</i> -value*
Karp	26	23 (88.5)	1.000	15 (57.7)	0.856
Kato	35	32 (91.4)		23 (65.7)	
Gilliam	5	5 (100.0)		3 (60.0)	
Karp and Kato	4	4 (100.0)		2 (50.0)	
Karp and Gilliam	4	4 (100.0)		1 (25.0)	
Kato and Gilliam	10	10 (100.0)		9 (90.0)	
Karp, Kato and Gilliam	13	13 (100.0)		12 (92.3)	
Total	97	91 (93.8)		65 (67.0)	

* Fisher's exact test.

Day of illness

The OD values of a recombinant ScaA-based and TSA56-based ELISAs over the day of illness are shown in Figure 25. Most patients had IgM and IgG antibody responses against TSA56 above the cut-off values after the first week of illness (Figures 25 c and d). The cut-off value for a TSA56-based IgG ELISA (0.4365) was calculated according to the manufacturer's instruction. The OD values of a TSA56-based IgM ELISA were concentrated around 3.0, while the level of the IgG responses varied. The sensitivity and specificity of a recombinant TSA56-based IgG ELISA were 92.8% (83.9-97.6) and 89.1% (82.7-93.8).

IgM responses against ScaA appeared after the first week and increased gradually along the day of illness (Figure 25a). IgG antibody responses against ScaA also increased over time. However, the proportion of patients with IgG responses above the cut-off was lower compared to the proportion of patients with IgM responses (Figure 25b). The cut-off value for a ScaA-based IgG ELISA was set to be 0.3447 by calculating the mean plus 3 SDs of the healthy controls. Against an IgM IFA as a gold standard test, a recombinant ScaA-based IgG ELISA had a sensitivity and specificity of 43.5% (31.6-56.0) and 97.1% (92.7-99.2).

Of 69 scrub typhus patients, 48 had both admission and follow-up samples and complete data on the date of both sample collections. The changes in antibody responses to TSA56 and ScaA from admission to follow-up are shown in Figure 26. These results suggested that IgM antibody responses to ScaA were slower to build up compared to that against TSA56.

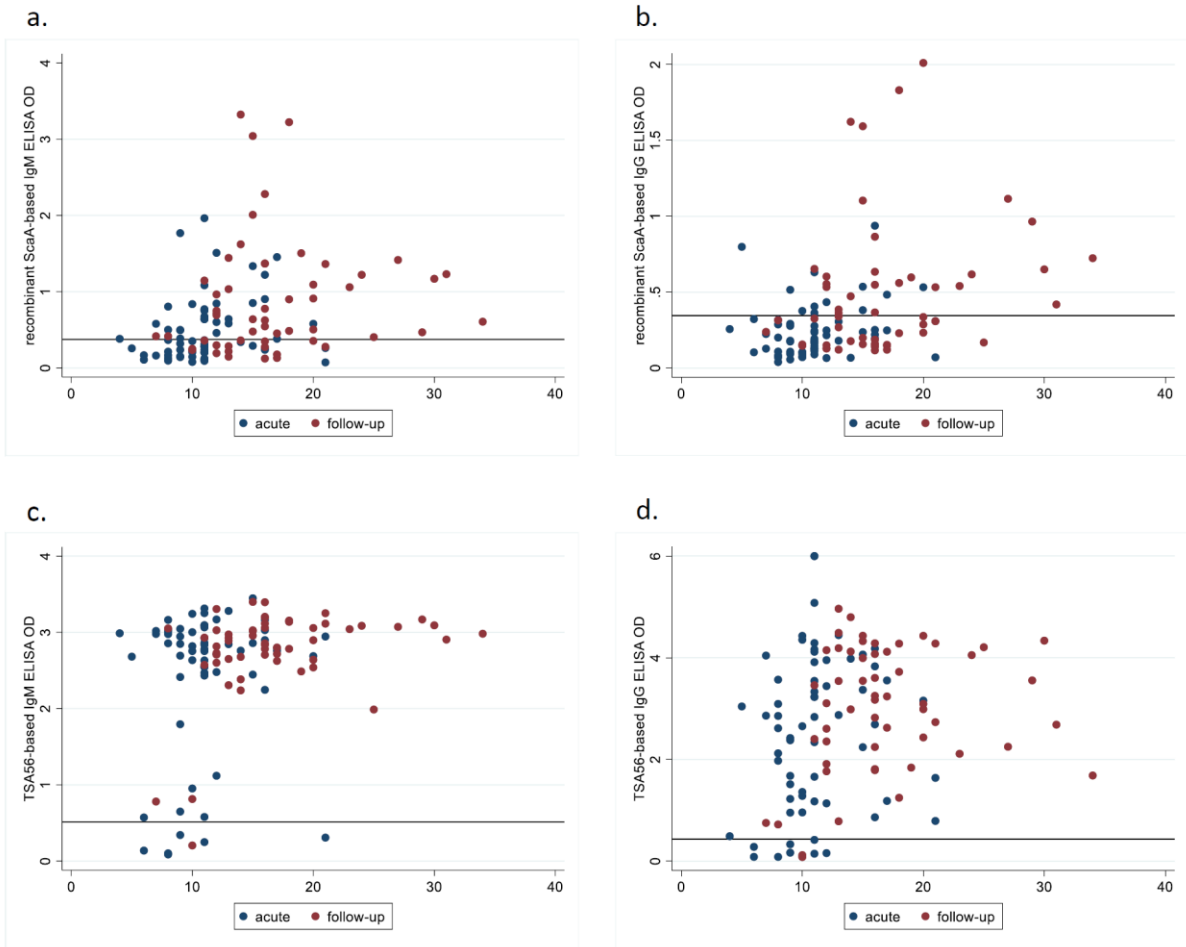


Figure 25. Scatter plots of ELISA OD values over day of illness.

a. recombinant ScaA-based IgM ELISA, b. recombinant ScaA-based IgG ELISA, c. TSA56-based IgM ELISA, d. TSA56-based IgG ELISA. The black line is the cut-off value for each ELISA.

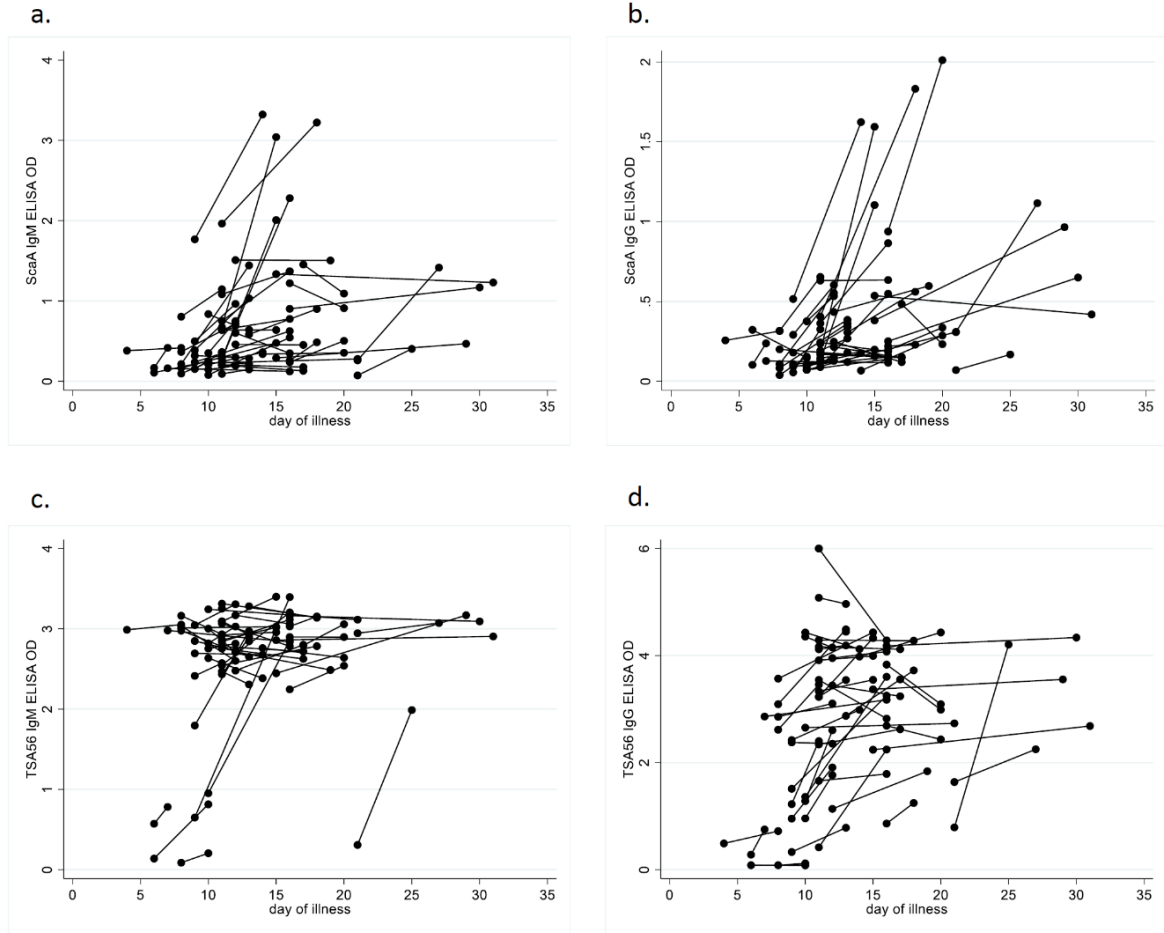


Figure 26. Changes in antibody responses to ScaA and TSA56 from admission to follow-up.
a. recombinant ScaA-based IgM ELISA, b. recombinant ScaA-based IgG ELISA, c. TSA56-based IgM ELISA, d. TSA56-based IgG ELISA.

When only admission samples (n=189) were included in the analysis, the sensitivity of a ScaA-based IgM ELISA dropped to 46.0% (33.4-59.1) from 71.0% (58.8-81.3) (Table 18). The specificities were 99.2% (95.7-100.0) at admission and 96.4% (91.7-98.8) when admission and follow-up samples were combined. The sensitivity and specificity of TSA56-based IgM and IgG ELISAs were high from admission. The sensitivity of a recombinant ScaA-based IgG ELISA dropped to 19.0% (10.2-30.9) at admission, and specificity was 100.0% (97.1-100.0). As expected, the ScaA-based IgG test had a lower sensitivity and higher specificity than the ScaA-based IgM test.

Table 18. Prevalence of scrub typhus, test sensitivities, and test specificities at admission and follow-up in the rickettsiosis cohort.

	Admission (n=189) (95% CI)	Follow-up (n=152) (95% CI)	Admission and follow-up combined (n=207) (95% CI)
Prevalence	-	-	33.3% (27.0-40.2)
IgM IFA			
Sensitivity	-	-	100%
Specificity	-	-	100%
Recombinant ScaA-based IgM ELISA			
Sensitivity	46.0% (33.4-59.1)	71.2% (57.8-82.7)	71.0% (58.8-81.3)
Specificity	99.2% (95.7-100.0)	95.8% (89.7-98.9)	96.4% (91.7-98.8)
Recombinant ScaA-based IgG ELISA			
Sensitivity	19.0% (10.2-30.9)	48.2% (34.7-62.0)	43.5% (31.6-56.0)
Specificity	100.0% (97.1-100.0)	95.8% (89.7-98.9)	97.1% (92.7-99.2)
TSA56-based IgM ELISA			
Sensitivity	90.5% (80.4-96.4)	98.2% (90.4-100.0)	94.2% (85.8-98.4)
Specificity	97.6% (93.2-99.5)	95.8% (89.7-98.9)	96.4% (91.7-98.8)
TSA56-based IgG ELISA			
Sensitivity	87.3% (76.5-94.4)	96.4% (87.7-99.6)	92.8% (83.9-97.6)
Specificity	96.0% (91.0-98.7)	84.4% (75.5-91.0)	89.1% (82.7-93.8)
Eschar			
Sensitivity	75.4% (63.5-85.0)	-	-
Specificity	99.3% (96.0-100.0)	-	-

Disease severity

As differences in the distribution of disease severity (case mix) can affect test sensitivity, sensitivities of the recombinant ScaA-based and TSA56-based tests in severe and non-severe scrub typhus patients were examined (Table 19). The sensitivities of the ScaA-based test were 84.6% in patients with a qSOFA score ≥ 2 and 67.9% in those with a qSOFA score < 2 . The test had higher sensitivity in patients with severe disease than non-severe patients although the difference was not statistically significant ($p=0.198$). The median absolute OD values were 0.851 (0.639-1.221) in patients with a qSOFA score ≥ 2 and 0.502 (0.291-0.937) in patients a qSOFA score < 2 ($p=0.149$).

When COIs were used, sensitivities of the ScaA-based test were 76.9% (46.2-95.0) in patients with a qSOFA score ≥ 2 and 71.4% (57.8-82.7) in patients a qSOFA score < 2 ($p=0.492$). The median COIs were 0.740 (0.504-0.999) and 0.387 (0.187-0.806), respectively ($p=0.149$).

Table 19. Sensitivities of a recombinant ScaA-based and TSA56-based IgM ELISA in patients with severe and non-severe scrub typhus.

	Recombinant ScaA-based IgM ELISA		TSA56-based IgM ELISA	
	Sensitivity (95% CI)	<i>P</i> -value*	Sensitivity (95% CI)	<i>P</i> -value*
qSOFA score ≥ 2 (n=13)	84.6% (54.6-98.1)	0.198	100.0% (75.3-100.0)	0.425
qSOFA score < 2 (n=56)	67.9% (54.0-79.7)		92.9% (82.7-98.0)	

*Fisher's exact test.

The positivity rate of a recombinant ScaA-based IgM ELISA in patients in whom other bacteria were isolated

The prevalence of scrub typhus should not affect test sensitivity and specificity but the background of the diseases in non-scrub typhus patients, for example gram-negative bacteraemia, may affect test specificity (183). This was a concern because autotransporter proteins are a secretion system of gram-negative bacteria, although they are diverse in amino acid sequences. Four categories of infectious diseases, common among patients with acute fever in the area, were chosen to examine whether positive rates of three serological tests differed in these diseases (Table 20). It was impossible to conclude from these data whether the positive test results were a true positivity, a significant cross-reactivity with the pathogens, or non-specific reactions inherent in IgM serological tests. There were 52 cases with gram-negative bacteraemia detected at or during hospital admission. The causal pathogen of bacteraemia was confirmed by blood culture. Three of them had a positive result on at least one of three serological tests. The first case was a patient with *Kingella denitrificans* bacteraemia, and all three serological tests were positive (a scrub typhus IgM IFA titre of 1:10,240, a TSA56-based IgM ELISA OD value of 4.0275, and a recombinant ScaA-based IgM ELISA OD value of 1.0569). This case could have been truly infected with *O. tsutsugamushi*. The second case was a patient of *Pseudomonas aeruginosa* bacteraemia with positive results of an IgM IFA (a titre of 1:320) and a TSA56-based IgM ELISA (an OD value of 0.7993). The third case was a patient with *Klebsiella pneumoniae* bacteraemia who had a positive result of a ScaA-based IgM ELISA (an OD value of 0.4215). The latter two cases had IgM titres and/or OD values that were close to each cut-off value. Bacteraemia with *Staphylococcus aureus* or *Streptococcus suis* was also examined as they are common gram-positive bacteria pathogens in the area (197). Of 18 patients with bacteraemia with either pathogen, two patients had a positive result for a ScaA-based test, and one had a positive result for a TSA56-based test. All three of them had an OD value slightly higher than the cut-off values. There were 32 tuberculosis patients without HIV infection. One patient had a positive IgM IFA result with a titre of 1:160. This case had a fever for 60 days and was bacteriologically confirmed as tuberculosis meningitis.

In the rickettsiosis cohort, 84 patients were diagnosed with murine typhus by an in-house IgG IFA although their diagnosis was not considered microbiologically confirmed. Four (4.8%) patients had a positive result for a ScaA-based test, five (6.0%) had a positive TSA56-based test result, and one (1.2%) had a positive IFA result.

Table 20. Positivity rates for a recombinant ScaA-based IgM ELISA, TSA56-based IgM ELISA, and IgM IFA in patients in whom other bacteria were isolated.

Diagnosis	Total number of patients with the diagnosis	Number of patients with a positive result for a ScaA IgM ELISA (%)	Number of patients with a positive result for a TSA56 IgM ELISA (%)	Number of patients with a positive result for an IgM IFA
Gram-negative bacteraemia*	52	2 (3.9)	2 (3.9)	2 (3.9)
<i>Staphylococcus aureus</i> bacteraemia	9	1 (11.1)	1 (11.1)	0 (0.0)
<i>Streptococcus suis</i> bacteraemia/meningitis	9	1 (11.1)	0 (0.0)	0 (0.0)
Tuberculosis without HIV infection	32	0 (0.0)	0 (0.0)	1 (3.1)

*Gram negative bacteraemia included bacteraemia of *Escherichia coli* (7), *Serratia marcescens* (7), *Klebsiella oxytoca* (5), *Klebsiella pneumoniae* (5), *Salmonella enterica* subsp. (4), *Burkholderia cepacia* (3), *Burkholderia pseudomallei* (2), and others (19).

13.6. Estimation of the sensitivity and specificity of a recombinant ScaA-based IgM ELISA by Bayesian latent class model

13.6.1. Model description

The Bayesian latent class model analysis protocol was developed based on the work by Lim et al. (132). In these models, a latent class is a true disease status (infected or non-infected with *O. tsutsugamushi*) (Figure 27). This figure was modified from the work by Limmathurotsakul et al. (170). The prevalence of scrub typhus in the cohort, test sensitivities, and test specificities could not be directly measured (dotted lines). An LCM estimates these properties based on the observed frequency of the test result combinations.

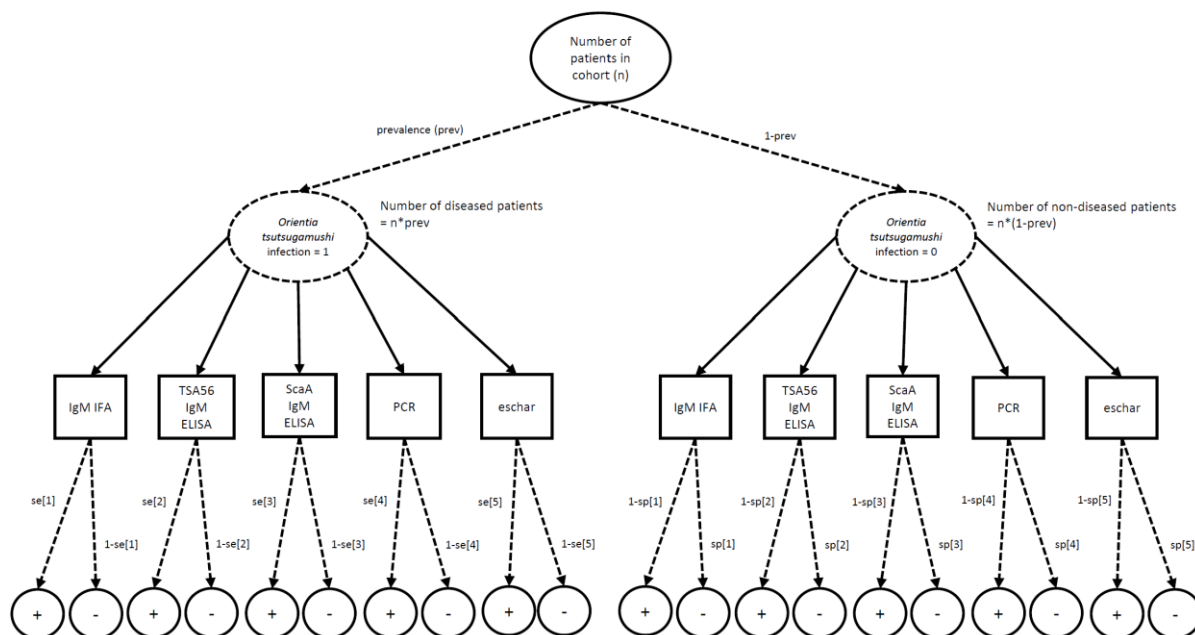


Figure 27. Diagram representation of a latent class model for *Orientia tsutsugamushi* tests and sign. Solid lines represent the application of four tests and the presence of eschar. Dotted lines represent unknown variables. se[1]: sensitivity of an IgM IFA, se[2]: sensitivity of a TSA56-based IgM ELISA, se[3]: sensitivity of a recombinant ScaA-based IgM ELISA, se[4]: sensitivity of a 47kDa-based real-time PCR assay, se[5]: sensitivity of the presence of eschar, sp[1]: specificity of an IgM IFA, sp[2]: specificity of a TSA56-based IgM ELISA, sp[3]: specificity of a recombinant ScaA-based IgM ELISA, sp[4]: specificity of a 47kDa-based real-time PCR assay, sp[5]: specificity of the presence of eschar, +: positive test result, -: negative test result.

Three models were constructed: one with conditional independence and two with conditional dependence. The model with conditional independence (model 1) assumed no correlations between all test results and the presence of eschar. This means that in a given patient, the result of any given test is not associated with the result of any other test. Two models incorporated conditional dependence between a scrub typhus IgM IFA and a TSA56-based IgM ELISA (model 2) or between all three serological tests (model 3). The rationale for selecting serological tests to be modelled for conditional dependence is as follows.

First, conditional dependence between serological tests is scientifically plausible because all tests detect IgM antibody responses against *O. tsutsugamushi*. As demonstrated in Chapter 13.5, IgM IFA titres were correlated with TSA56-based and recombinant ScaA-based ELISA OD values. Second, the *Orientia* antigens used in three tests were considered (Figure 28). An IFA uses the whole cell lysates of three *Orientia* strains. These whole cells (large circles in Figure 28) include both TSA56 (small circles) and ScaA proteins. The recombinant ScaA antigens (red triangles) are a fragment of the ScaA proteins. TSA56 proteins are abundant on the surface of the bacteria. Many small circles and some red triangles cover the surface of the large circles. The recombinant ScaA antigen is a small protein fragment of 101 amino acids, while TSA56 proteins of 500-540 amino acids have three antigen domains of approximately 100 amino acids (56). The correlation between IFA titres and TSA56 ELISA OD values was stronger than that of the correlation between IFA titres and ScaA ELISA OD values (Figure 24). Considering these facts and findings in this Ph.D. study, the correlation between an IgM IFA and a TSA56-based IgM ELISA was expected to be more substantial than that between an IFA and a ScaA-based test or that between a TSA56-based test and a ScaA-based test. Therefore, the former correlation was included in both models 2 and 3.

Conditional dependence between multiple tests was modelled using random effects. In these models, sensitivities and specificities of the tests are functions of a latent, patient-specific random variable (168). This random variable can be interpreted as a summary measure of the overall level of serological responses to *O. tsutsugamushi* in a given patient affecting the ease of detection (168). All correlations between serological tests were in infected patients. Correlations between a 47kDa-based real-time PCR assay and serological tests and between eschar and serological tests were not expected.

The checklist the STARD-BLCM is shown in Table 21. OpenBUGS code for the model assuming conditional dependence between an IgM IFA and a TSA56-based IgM ELISA and the dataset of the rickettsiosis cohort is in Appendix 7 as one example.

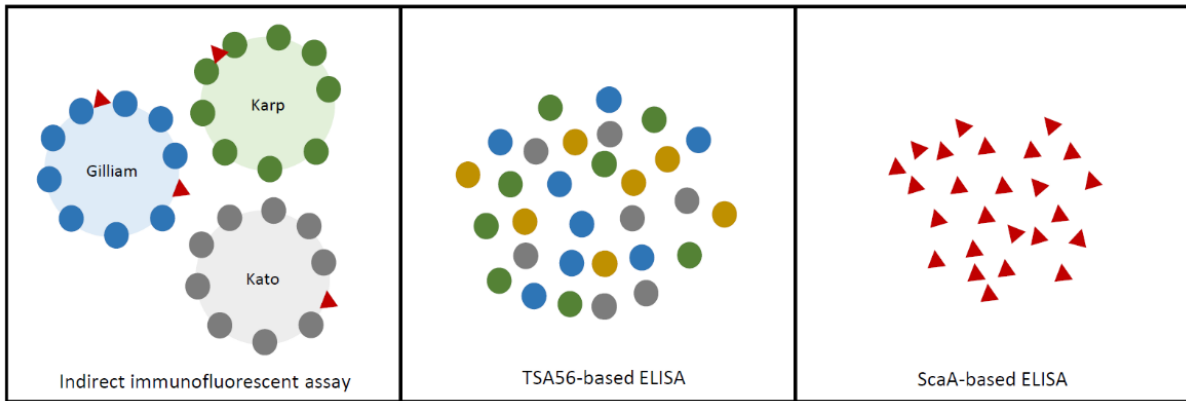


Figure 28. Schematic representation of *Orientia tsutsugamushi* antigens used in an IFA, TSA56-based ELISA, and ScaA-based ELISA.

FA Slide R. Tsutsugamushi Seiken (Denka Company Ltd, Japan) uses whole cell lysates of the Karp, Kato, and Gilliam strains as antigens. Scrub Typhus Detect ELISA System (InBios International, Seattle, WA) uses recombinant TSA56 proteins of the Karp, Kato, Gilliam, and TSA716 strains. A recombinant ScaA-based ELISA uses a conserved fragment of the ScaA autotransporter domain. Large circles represent whole cells of *O. tsutsugamushi* (green: Karp, grey: Kato, and blue: Gilliam); small circles represent TSA56 proteins (green: Karp, grey: Kato, blue: Gilliam, and yellow: TA716); and red triangles represent the fragment of the ScaA protein used as a recombinant antigen.

Table 21. Checklist of items for the Standards for the Reporting of Diagnostic Accuracy studies by the use of Bayesian Latent Class Models (STARD-BLCM) for a recombinant ScaA-based IgM ELISA.

Section & Topic	No	Item	Reported on
TITLE OR ABSTRACT			
	1	Identification as a study of diagnostic accuracy, using at least one measure of accuracy (such as sensitivity, specificity, predictive values, or AUC) and Bayesian latent class models	Abstract page 3
ABSTRACT			
	2	Structured summary of study design, methods, results, and conclusions (for specific guidance, see STARD for Abstracts)	Abstract page 3
INTRODUCTION			
	3	Scientific and clinical background, including the intended use and clinical role of the tests under evaluation	Chapters 1 and 4.
	4	Study objectives and hypotheses, such as estimation of diagnostic accuracy of the tests for a defined purpose through BLCM	Chapter 4.
METHODS			
<i>Study design</i>	5	Whether data collection was planned before the tests were performed (prospective study) or after (retrospective study)	Chapter 6.
<i>Participants</i>	6	Eligibility criteria and description of the source population	Chapter 6.
	7	On what basis potentially eligible participants were identified (such as symptoms, results from previous tests, inclusion in registry)	Chapter 6.
	8	Where and when potentially eligible participants were identified (setting, location, and dates)	Chapter 6.
	9	Whether participants formed a consecutive, random or convenience series	Chapter 6.
<i>Test methods</i>	10	Description of the tests under evaluation , in sufficient detail to allow replication, and/or cite references	Chapters 12.3. and 12.4.
	11	Rationale for choosing the tests under evaluation in relation to their purpose	Chapter 7.
	12	Definition of and rationale for test positivity cut-offs or result categories of the tests under evaluation , distinguishing pre-specified from exploratory	Chapter 13.2.
	13	Whether clinical information was available to the performers or readers of the tests under evaluation	Chapter 13.1.
<i>Analysis</i>	14a	BLCM model for estimating measures of diagnostic accuracy	Chapter 13.6.1.
	14b	Definition and rationale of prior information and sensitivity analysis	Chapter 13.6.2.
	15	How indeterminate results of the tests under evaluation were handled	Not applicable
	16	How missing data of the tests under evaluation were handled	Chapter 13.1.
	17	Any analyses of variability in diagnostic accuracy, distinguishing pre-specified from exploratory	Chapter 13.5.
	18	Intended sample size and how it was determined	Chapter 6.
RESULTS			
<i>Participants</i>	19	Flow of participants, using a diagram	Chapter 13.1.
	20	Baseline demographic and clinical characteristics of participants	Chapter 13.1.
	21	Not applicable: the distribution of the targeted conditions is unknown, hence the use of BLCM	
	22	Time interval and any clinical interventions between the tests under evaluation	Chapter 13.1.
<i>Test results</i>	23	Cross tabulation of the tests' results (or for continuous tests results their distribution by infection stage)	Tables 13 and 14
	24	Estimates of diagnostic accuracy under alternative prior specification and their precision (such as 95% credible/probability intervals)	Tables 24 and 25

	25	Any adverse events from performing the tests under evaluation	Not applicable.
DISCUSSION			
	26	Study limitations, including sources of potential bias, statistical uncertainty, and generalisability	Chapter 13.7.
	27	Implications for practice, including the intended use and clinical role of the tests under evaluation in relevant settings (clinical, research, surveillance etc.)	Chapter 13.7.
OTHER INFORMATION			
	28	Registration number and name of registry	-
	29	Where the full study protocol can be accessed	This thesis
	30	Sources of funding and other support; role of funders	Chapter 11.

The table was obtained from the EQUATOR network website (198).

13.6.2. Definition and rationale of prior information

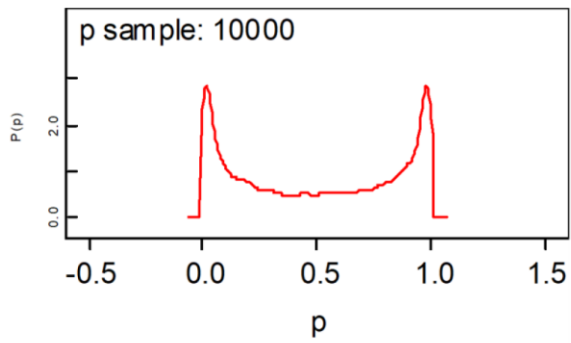
Non-informative and informative priors were used to assess the impact of alternative prior information on the final result (174). For the model comparison, non-informative priors were used. A non-informative prior was the inverse logit transformation of normal distribution (0.0, 0.1). This prior assumes that no prior information was available for sensitivity and specificity (132). After selecting the best-fitting model for each cohort, informative priors were inputted into the selected model.

The assumptions on sensitivities and specificities of four diagnostic tests and one clinical sign were based on the currently available information described in Chapter 1.5 (Table 22). The prior information for the sensitivity and specificity of the recombinant ScaA-based IgM ELISA is based on the results calculated against a scrub typhus IgM IFA as a gold standard test (Table 18). A ‘moderate’ prior was the inverse logit transformation of normal distribution (0.0, 1.0) (Figure 29). This prior believes that the sensitivity and specificity of each test is around 50% and there is less than a 1% chance that a sensitivity or specificity of a test is more than 95% or less than 5%. A ‘high’ prior was the inverse logit transformation of normal distribution (1.8, 0.8). This prior believes that a sensitivity or specificity of a test is around 85% and there is less than a 3% chance that it is more than 98% or less than 40%. A ‘very high’ prior was the inverse logit transformation of normal distribution (3, 0.4). This prior strongly believes that the sensitivity or specificity of a test is around 95% and there is less than a 3% chance that it is less than 50%.

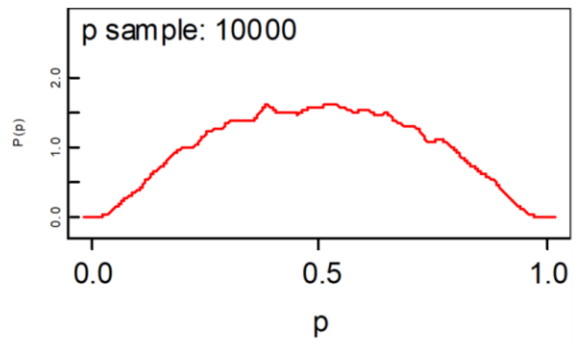
Table 22. Assumptions on sensitivities and specificities of the four diagnostic tests and the presence of eschar.

	Sensitivity	Specificity	References
IgM IFA	High	High	(110, 118, 126, 132)
ScaA-based IgM ELISA	High	Very high	-
InBios Scrub Typhus Detect™ IgM ELISA (TSA56-based)	High	Very high	(123-127)
47kDa-based real-time PCR assay	Moderate	Very high	(106, 132, 133)
Presence of eschar	Moderate	Very high	(132)

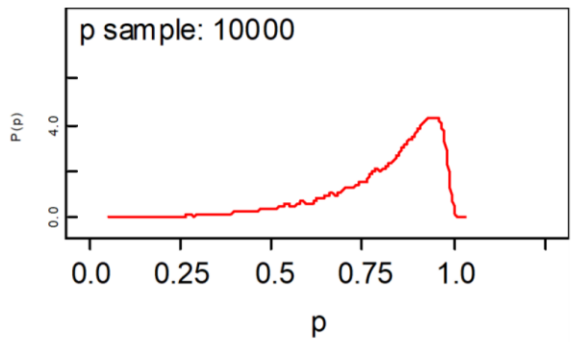
Inverse logit transformation of normal distribution (0,0.1)



Inverse logit transformation of normal distribution (0,1.0)



Inverse logit transformation of normal distribution (1.8,0.8)



Inverse logit transformation of normal distribution (3.0,0.4)

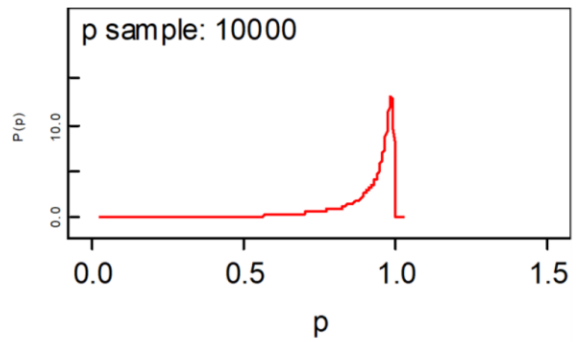


Figure 29. Prior distributions for sensitivities and specificities of the four diagnostic tests and the presence of eschar.

13.6.3. Assessment of model fitness

The model fitness was compared using the Akaike Information Criterion (AIC). Models with a smaller AIC are better supported by the data (199). The number of parameters for model 1 in the rickettsiosis cohort is a prevalence of scrub typhus (1) plus sensitivities and specificities of three diagnostic tests and one clinical sign (2x4). The distributions of patients observed and predicted by three LCMs in the two cohorts are in Appendix 8.

Although model 1 had a slightly lower AIC than model 3 in the rickettsiosis cohort data (Table 23), based on the discussion in Chapter 13.6.1, conditional dependence was considered necessary to be incorporated reflecting the biological perspective and this study's findings. Therefore, model 3 was selected for the rickettsiosis cohort. Model 2 was selected for the fever cohort data based on the same reason.

Table 23. Comparison of three latent class models in the rickettsiosis and fever cohorts.

Model	Correlation between:	Rickettsiosis cohort		Fever cohort	
		Number of parameters	Akaike information criterion	Number of parameters	Akaike information criterion
1	None	9	71.6	11	117.1
2	IgM IFA and TSA56-based IgM ELISA	10	74.1	12	117.3
3	IgM IFA, TSA56-based IgM ELISA, and recombinant ScaA-based IgM ELISA	10	72.8	12	125.6

13.6.4. Results

The convergence was assessed by the visual inspection of the first 1,000-3,000 iterations of the disease prevalence, test sensitivities, and test specificities (Appendix 9). Autocorrelation was also examined. Three chains were used for the rickettsiosis cohort data, and 30,000 iterations were run per chain, with 1,000 iterations discarded as burn-in. For the fever cohort data, three chains were used, and 60,000 iterations per chain were run for non-informative priors and 40,000 iterations per chain for informative priors. In both priors, 3,000 iterations were discarded as burn-in. The results are shown in Tables 24 and 25. The density plots are in Appendix 10. The distributions of patients predicted by the selected model with informative priors are in Appendix 8. The sensitivity and specificity of a recombinant ScaA-based IgM ELISA estimated with informative priors were 73.9% (63.4-83.0) and 96.9% (93.1-98.9) in the rickettsiosis cohort and 68.6% (51.5-82.8) and 94.8% (92.7-96.4) in the fever cohort. The sensitivity and specificity of a TSA56-based IgM ELISA were 95.5% (89.2-98.7) and 97.1% (93.3-99.1) in the rickettsiosis cohort and 94.4% (79.2-99.0) and 97.3% (95.6-98.6) in the fever cohort.

Table 24. A scrub typhus prevalence, test sensitivities, and test specificities estimated using an IgM IFA as a reference standard test or using Bayesian latent class models in the rickettsiosis cohort.

	IgM IFA titre $\geq 1:160$ as a reference standard (95% CrI)	Bayesian LCM with non-informative priors (95% CrI)	Bayesian LCM with informative priors (95% CrI)
Prevalence	33.3% (27.0-40.2)	32.7% (26.4-39.4)	32.8% (26.5-39.6)
IgM IFA			
Sensitivity	100%	98.7% (92.9-100.0)	96.6% (90.4-99.2)
Specificity	100%	98.4% (95.0-99.9)	97.5% (94.1-99.2)
Recombinant ScaA-based IgM ELISA			
Sensitivity	71.0% (58.8-81.3)	72.5% (62.4-81.7)	73.9% (63.4-83.0)
Specificity	96.4% (91.7-98.8)	96.4% (92.4-98.8)	96.9% (93.1-98.9)
TSA56-based IgM ELISA			
Sensitivity	94.2% (85.8-98.4)	96.7% (89.9-99.7)	95.5% (89.2-98.7)
Specificity	96.4% (91.7-98.8)	96.7% (92.6-99.0)	97.1% (93.3-99.1)
Presence of eschar			
Sensitivity	75.4% (63.5-85.0)	77.2% (66.0-86.4)	75.2% (64.2-84.4)
Specificity	99.3% (96.0-100.0)	99.2% (96.7-100.0)	99.0% (96.6-99.9)

CrI: credible interval.

Table 25. A scrub typhus prevalence, test sensitivities, and test specificities estimated using an IgM IFA and a PCR assay as reference standard tests or using Bayesian latent class models in the fever cohort.

	IgM IFA titre \geq 1:160 and/or 47kDa-based real-time PCR as a reference standard (95% CI)	Bayesian LCM with non-informative priors (95% CrI)	Bayesian LCM with informative priors (95% CrI)
Prevalence	4.2% (2.8-6.0)	5.3% (3.5-7.7)	5.1% (3.4-7.3)
IgM IFA			
Sensitivity	100% (87.7-100.0)	74.3% (54.4-94.2)	77.9% (59.3-93.8)
Specificity	100%	99.7% (99.0-100.0)	99.3% (98.5-99.8)
Recombinant ScaA-based IgM ELISA			
Sensitivity	57.1% (37.2-75.5)	64.7% (46.6-80.5)	68.6% (51.5-82.8)
Specificity	93.8% (91.6-95.5)	94.8% (92.7-96.4)	94.8% (92.7-96.4)
TSA56-based IgM ELISA			
Sensitivity	92.9% (76.5-99.1)	97.5% (80.1-100.0)	94.4% (79.2-99.0)
Specificity	96.3% (94.5-97.6)	97.5% (95.8-98.9)	97.3% (95.6-98.6)
47kDa-based real-time PCR assay			
Sensitivity	32.1% (15.9-52.4)	25.5% (12.7-43.2)	28.9% (15.8-45.9)
Specificity	100%	99.9% (99.5-100.0)	99.8% (99.4-100.0)
Presence of eschar			
Sensitivity	14.3% (4.0-32.7)	11.4% (3.7-25.2)	16.3% (7.3-30.4)
Specificity	99.1% (98.0-100.0)	99.0% (98.1-99.6)	99.0% (98.1-99.6)

Sensitivities estimated by Bayesian LCMs were minimally different when different priors were used, and there was no substantial difference in the prevalence and specificities. There were no substantial differences in the sensitivities and specificities estimated by Bayesian LCMs in the two cohorts except for sensitivities of an IgM IFA and the presence of eschar. The potential explanations for these differences are discussed in Chapter 13.7.

13.7. Summary and discussion

A recombinant ScaA-based IgM ELISA was developed using a 101-amino acid fragment of ScaA protein. The test detected specific antibody responses against *O. tsutsugamushi*. This is the first study to demonstrate the human antibody responses to the ScaA ATD. The estimated sensitivity and specificity of the test were 73.9% and 96.9% in the rickettsiosis cohort and 68.6% and 94.8% in the fever cohort, respectively. The assay did not achieve the pre-set target: minimal sensitivity of 85% and specificity of 95%, which are converted to an LR+ of 17 and LR- of 0.16. However, considering that the new test used a small, conserved antigen of 101 amino acids of only one strain, it may be acceptable for use under some circumstances.

Interpretation of the results

As the targeted use of the test was the diagnosis of scrub typhus among patients with acute fever in endemic areas, the estimated sensitivity and specificity were interpreted in a clinical scenario. The pre-test probability of scrub typhus was assumed to be 3-30% based on the reviews that reported the prevalence of major infectious causes, including scrub typhus, among patients with acute fever in Asia (14, 19).

The recombinant ScaA-based IgM ELISA had an LR+ of 23.8 and LR- of 0.27, and the TSA56-based IgM ELISA had an LR+ of 32.9 and an LR- of 0.05 (200). Pre- and post-test probabilities of scrub typhus before and after applying the two tests are shown in Figure 30. Due to the suboptimal sensitivity of the ScaA-based test, it is not good at ruling-out the disease. There remains a significant probability that the patient has scrub typhus when the test result is negative. However, some clinicians may think this is acceptable under the circumstances. Being aware of the test weakness, they may still consider starting treatment if there are no other likely differential diagnoses, especially when the patient's condition is severe, because the disease can be fatal. Doxycycline therapy is oral, effective, safe, and affordable. The ScaA-based ELISA had a high specificity, and is good at ruling-in.

The TSA56-based test had a higher sensitivity and equivalent specificity; therefore, the probability of the disease drops more significantly compared to the ScaA-based test when the TSA56-based test result is negative (Figure 30). The test also has a shorter turn-around time because the ELISA plates are pre-coated, and the incubation times are shorter. Therefore, the TSA56-based test is better and more practical at ruling out the disease. However, the cost of the ELISA kit is a concern.

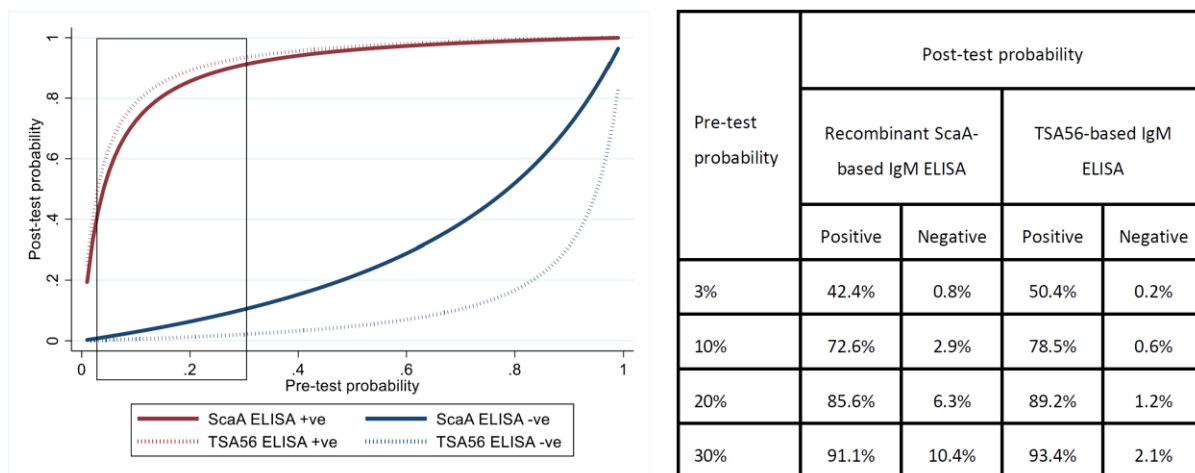


Figure 30. Pre- and post-test probabilities of scrub typhus before and after applying a recombinant ScaA-based IgM ELISA and TSA56-based IgM ELISA.

A black square on the left panel indicates an area of a pre-test probability of 3-30%.

Sensitivity and specificity of a recombinant ScaA-based IgM ELISA

When a weak antibody response is detected, it is either that the antibody response was truly weak or that an assay failed to detect the existing antibody response. In other words, it is either due to the quantity of the antibody response or the detection method flaw or both.

There are several possibilities for why IgM antibody responses against the recombinant ScaA antigen were weak. First, the ScaA ATD may not be highly antigenic. This was an expected challenge from the beginning. A conserved amino acid sequence was intentionally chosen so we did not need to combine the locally circulating strains. However, immunodominant epitopes are expected to be under immune selection pressure and display substantial variations (42). The results of a recombinant ScaA-based ELISA were similar to the work by Chen et al., which showed that IgM and IgG antibody responses against the conserved TSA47 were weaker and slower than the responses against the variable TSA56 (88). IgM and IgG responses to the recombinant ScaA antigen were slower, too (Figures 25 and 26). The mechanism behind this distinct antibody response dynamics to ScaA and TSA56 is unknown. It could potentially be attributed to the lower quantity on the cell surface and the lower antigenicity of ScaA, similar to what Chen et al. speculated for TSA47 (88). It might also be due to the different expression pattern of each protein in the course of infection, as described below. Although the amino acid sequence identity of the ScaA antigen region was >90% across different *Orientia* strains, this difference may have hampered the heterologous antibody binding.

Second, ScaA proteins may not be expressed on the outer membrane in enough quantities at the right location and at the right time in the course of infection. As stated in Chapter 1.3.2, Atwal et al. recently demonstrated that ScaA proteins were more expressed in intracellular *O. tsutsugamushi* compared to extracellular *O. tsutsugamushi* (74). To be recognised by B-cells, an antigen needs to be expressed on the surface of the pathogen outside the host cell. However, the authors also noted that the correlation between the outer membrane expression and the bacterial location was still to be elucidated. The different level of the ScaA protein expression relative to the phase of infection is a very new finding. Its implication on the suitability of the protein for an antigen of diagnostic tests and vaccines needs further research.

There are three potential flaws in the test. The first is the antigen's denatured condition which could have compromised the detection of antibody responses. The 101-amino acid antigen was a fraction of the ScaA ATD and may not have had the same conformational structure as the whole ScaA protein, even if the protein was successfully refolded. In the denatured condition, the recombinant ScaA antigen may have been linear or had some conformational structure, but that is unknown. The majority of epitopes are conformational; therefore some potential epitopes may have been lost. The second concern is the non-specific bindings of IgM antibodies to the ELISA plate and the antigen. From the measured OD values of the BGN reactions (median 0.01 in both cohorts), the BGN reactions were assessed as controlled in this indirect ELISA protocol. However, the use of antigen-non-coated wells for measuring the BGN reaction is still under discussion (201). The third issue is the dilution of serum to 1:500 to minimise the BGN reactions. This approach may have hindered the detection of positive samples with a low antibody titre and could have resulted in missing the cases in the early phase of illness.

Factors that may have affected test sensitivities and specificities

Changes in prevalence should not affect test sensitivity and specificity. However, the setting where a test is performed, differences in case mix, and background of the diseases occurring in those who do not have scrub typhus can affect test sensitivity and specificity (183). In this study, Bayesian LCMs were applied to the two cohorts with different prevalence of scrub typhus. Sensitivities and specificities estimated by the Bayesian LCMs were fundamentally not too different except for sensitivities of an IgM IFA and the presence of eschar. There were three factors that could have affected the study results.

The first was sensitivities of a scrub typhus IgM IFA. The estimated sensitivities of an IgM IFA were 96.6% and 77.9% in the rickettsiosis and fever cohorts. Those of a recombinant ScaA-based IgM ELISA were 73.9% and 68.6%. The difference in case mix in two cohorts may have affected these results. As shown in Table 19, the sensitivity of the recombinant ScaA-based IgM ELISA was higher in severe scrub typhus patients than in non-severe patients. However, the difference was not statistically significant, likely due to the small sample size. There were no scrub typhus patients with a qSOFA score of ≥ 2 in the fever cohort, which may have led to lower sensitivity. The difference in case mix could have resulted in differences in sensitivities of serological tests.

The second was the sensitivity of the presence of eschar. The sensitivities were relatively high (75.2%) in the rickettsiosis cohort and very low (16.3%) in the fever cohort. This difference in sensitivity may have been due to the following reasons. As reported in the original study by Katoh et al. (184), Bach Mai Hospital published local guidelines on the diagnosis and treatment of common illnesses, including scrub typhus, and provided clinical and diagnostic training for local healthcare workers around this time. Typical cases of scrub typhus may have been diagnosed and treated subsequently at community hospitals, and thus, less likely to be referred to Bach Mai Hospital. As described by Haynes et al. (183), “the value of the test can get used up as one moves from primary to secondary care settings.” This is particularly the case when the test is information obtained from clinical history or physical examination. Hanoi is also known to be experiencing one of the most intensive urbanisations in the world (202), so the number of scrub typhus cases in Hanoi and its outskirts may have truly decreased.

In my personal communication with the doctors at the hospital in 2019, they rarely saw scrub typhus cases at the hospital. The rickettsiosis cohort was enrolled in 2002-2003, and the fever cohort was enrolled in 2012-2013. Although both studies were performed at the same hospital, the healthcare workers at the hospital may have become less familiar with this disease over time. This unfamiliarity with scrub typhus might have limited the detection of eschar. Eschar tends to be found in covered areas such as the axilla, under-breast, groin, and genitalia (91, 93). It does not cause pain or itching. Healthcare workers need to be aware of the distribution of eschar and actively look for it. In addition, the rickettsiosis cohort enrolled patients in the study specifically targeted this disease, and eschar was in the inclusion criteria. Healthcare workers may have been more thorough to search for eschar in the rickettsiosis study.

The third is the specificity of the presence of eschar. It was high in both cohorts in this study but could change by the spectrum of competing conditions, especially other rickettsial infections (183). In the rickettsiosis cohort, approximately 60% of non-scrub typhus patients had murine typhus that

does not form eschars (195, 196). Test specificity may decrease in areas where spotted fever group rickettsiae associated with eschar, such as *R. japonica* and *R. africae*, coexists. However, clinicians may not find this problematic since the treatment is the same.

Potential solutions to improve a recombinant ScaA-based IgM ELISA

There are two technical areas to explore for an improved recombinant ScaA-based IgM ELISA. The first involves the recombinant protein production method. In this Ph.D. study, the recombinant ScaA antigen was expressed in *E. coli* BL21 (DE3) with a pET28.1a(+) vector. Due to the hydrophobicity of the antigen, it needed to be purified from inclusion bodies and collected in a denatured condition. There definitely have been a number of options in terms of the *E. coli* expression system to be changed: the choice of expression vectors with different affinity tags and *E. coli* expression strains and the combinations of many conditions of an expression and purification system (203). Aside from the *E. coli* platform, other protein expression platforms could have been explored. Although the recombinant ScaA antigen could not be produced in sufficient protein yield either using a wheat-germ cell-free expression method, there are more options available, including in vivo protein expression technology with yeast, insect, and mammalian cells and in vitro (cell-free) technology with *E. coli* extract, rabbit reticulocyte lysates, and insect and human cell extracts (204). With a more suitable method, the recombinant ScaA antigen may be produced with the correct conformation and the test with the antigen may yield improved sensitivity and specificity.

The second area of improvement concerns the ELISA format. This Ph.D. study used the most traditional format of an indirect ELISA. As discussed in Chapter 2.2, various ELISA formats are available that may yield higher sensitivity and specificity. An IgM-capture ELISA was specifically designed to detect IgM antibodies and has been used for serological diagnosis of human arbovirus infections (205). IgM antibodies in test sera are first captured by anti-human IgM antibodies immobilised on the solid surface. Viral antigens of the target disease are added and bound to the antigen-specific IgM antibodies among the captured IgM antibodies. The antigens are subsequently detected by broadly reacting antiviral monoclonal/polyclonal antibodies conjugated with enzymes. The use of capture antibodies minimises the BGN reactions, thereby improving specificity, and less competition between IgG and IgM antibodies for antigen epitopes results in improved sensitivity (205). Although it involves additional technical difficulties in optimising this complex ELISA process, an IgM-capture ELISA might have been valuable endeavour.

Implications

The clinical implication has been discussed in detail at the beginning of Chapter 13.7. Although the test's sensitivity was not high enough as a ruling-in test, a single conserved antigen of 101 amino acids detected approximately 70% of the cases. This exhibited a possibility of using conserved antigens to diagnose scrub typhus. Implications of the results in research are subject to the further understanding of ScaA proteins. More research on *Orientia* antigens and their expression and components may lead to alternative diagnostic possibilities.

A trend was observed suggesting that severe scrub typhus patients may be more likely to exhibit an immune response to the recombinant ScaA antigen than non-severe patients even though the difference was not statistically significant. The OD values and COIs of the ScaA IgM ELISA tended to be higher in severe patients than in non-severe patients although the difference was not statistically significant. Due to the small number of severe patients in the rickettsiosis cohort who were enrolled in the early phases of illness, it was not possible to determine whether these differences were seen from the early phase of infection. This potential association between the ScaA test positivity and disease severity may warrant further exploration with a more suitable sample set. Quantitative tests offer clinicians valuable additional information compared to qualitative tests. Clinicians may evaluate the likelihood of their patients having a target disease based on an antibody titres (Table 12). Moreover, if there is a significant association between antibody titres and disease severity, the test can still support clinicians making decisions regarding the treatment and follow-up of scrub typhus patients, even with a suboptimal sensitivity as a qualitative test.

Limitations

There are several limitations to this evaluation of the new test. First, no PCR assay was performed in the rickettsiosis cohort due to the unavailability of DNA samples. However, it is preferred to include tests that detect all forms of a disease in Bayesian LCMs (174). PCR assays are especially useful in the early phase of illness and are essentially completely specific (101). This study may have missed the cases with *O. tsutsugamushi* bacteraemia but without detectable IgM responses resulting in an overestimation of sensitivities of the serological tests. Although a 47kDa-based real-time PCR assay was performed for all admission samples in the fever cohort, the prevalence of scrub typhus in this cohort was low, and no sample was diagnosed only by the PCR assay.

Second, an IgM IFA was applied to a single sample per patient. The assay was applied to follow-up samples when available and admission samples otherwise. This was primarily due to financial

constraints and convalescent samples were unavailable. As stated in Chapter 13.1, the median day of sample collection of acute and follow-up samples was day 11 and day 15.5 in the rickettsiosis cohort. The median interval between sample collections was five days. In the fever cohort, acute and follow-up samples were collected on day 8 and day 14 with a median interval of five days. These follow-up samples were not convalescent. Collecting convalescent samples may be difficult in real-life practice and does not guide clinicians' decision-making process. However, to assess a new diagnostic test, it was preferable to include an IFA of paired samples that has been considered the gold standard.

Third, IgG serological tests were not included in Bayesian LCMs. The application of IgG serological tests to repeated samples is expected to increase the chance to correctly diagnose or exclude the disease. Although the information does not assist the acute clinical management, it could have improved the estimation of the sensitivity and specificity of the new test. However, as discussed in the previous paragraph, convalescent samples were not available in this Ph.D. study. The two cohorts were not the ideal dataset to investigate the diagnostic performance of IgG serological tests. Moreover, the intended use of the new test was the early diagnosis of patients with acute fever.

Fourth, in the fever cohort, only patients who provided both admission and follow-up samples were included because many of the blood samples of patients who provided only admission samples were used up before this Ph.D. study. This selection could have skewed the characteristics of the cohort compared to the overall cohort enrolled in the original study. Patients may have provided follow-up samples if they had a severer disease or complications during the hospitalisation, had baseline health comorbidities to be followed, or could afford the cost and time.

Fifth, there were not enough patients with confirmed *Orientia* serotypes. One-third of patients with positive IgM IFA results (32%, 31/97) had responses to more than two strains. However, cross-reactivity is an inherent problem of scrub typhus serological test (206, 207).

Sixth, neither rickettsiosis nor fever cohorts included a paediatric population.

Seventh, patients' blood samples of the rickettsiosis cohort had been stored at -80 °C for approximately 20 years and samples of the fever cohort for 10 years. Although the frequent freezing and thawing had been avoided, the long-term storage, freezing, and thawing of the samples could have influenced the results of the study.

Next step

Although the sensitivity and specificity of the recombinant ScaA-based IgM ELISA did not achieve the pre-set goal, a single recombinant antigen of 101 amino acids in a denatured condition detected a significant proportion of patients with scrub typhus. The denatured antigen was coated to the plates in 8 M urea coating buffer. The conformation of the recombinant antigen, when coated on the ELISA plate, is unknown, but the recombinant protein may contain B-cell linear epitope(s). The production and use of oligopeptide antigens are more affordable, stable, and less laborious than recombinant proteins. Sensitivity may decrease, but the specificity of a peptide-based ELISA is expected to be enhanced (208). If a higher specificity is achieved, the test may be useful as a confirmation test. This may still be a reasonable trade-off between a suboptimal sensitivity and other desirable characteristics such as affordability and simplicity. Therefore, an overlapping peptide screening was attempted next. The aim of the next step was not a detailed B-cell epitope mapping. It was to narrow down the antigen to a size of oligopeptides that can be commercially synthesised at a reasonable cost.

14. Development of a ScaA peptide ELISA

14.1. Overlapping peptide synthesis

Nine overlapping peptides (P1-9) were commercially synthesised by Cosmo Bio Co., LTD, Japan, with >50% purity (Figure 31). The essential characteristics of the peptides are shown in Table 26. The peptides were diluted in the buffers recommended by the manufacturer to the concentrations of 10 mg/ml and 1 mg/ml and stored in aliquots at -80 °C until used.

P1 FIRGMVNVAKFSDDSKNSSL
 P2 FSDDSKNSSLTLWQGHSHYG
 P3 TLWQGHSHYHGLTAGHYFYP
 P4 SLTAGHYFYPLKNNKCLTLV
 P5 LKNNKCLTLVPTVGIRHSYF
 P6 PTVGIRHSYFNTSGNNSVDS
 P7 NTSGNNSVDSSSNKTIGDRS
 P8 SSNKTIGDRSHKALEGIIGI
 P9 HKALEGIIGISLEQLVANNAN

Figure 31. Amino acid sequences of nine overlapping peptides 1-9.

Ten amino acids of each peptide were overlapped with the neighbouring peptides.

Table 26. Basic characteristics of nine ScaA peptides.

Peptide	Number of amino acids	Molecular weight	Purity (%)	Hydrophobic amino acids (%)	Isoelectric point	Net charge at pH 7	Dilution buffer
P1	20	2215.3	74.0	45.0	9.7	1.0	0.1% acetic acid
P2	20	2267.6	78.1	30.0	6	-0.8	
P3	20	2323.5	78.7	45.0	7.7	0.3	
P4	20	2309.0	75.5	45.0	10.2	3.1	
P5	20	2330.3	74.9	45.0	10.9	4.1	
P6	20	2153.6	85.3	35.0	8	0.1	
P7	20	2041.2	65.6	20.0	6.5	0.0	
P8	20	2096.4	80.3	45.0	9.7	1.1	dimethyl sulfoxide
P9	21	2204.6	58.8	57.1	5.3	-0.9	

Homology analysis of the amino acid sequences of nine ScaA peptides was performed in September 2021 on the NCBI BLASTp website to examine whether any of the peptides had significant identities with amino acid sequences of important causative pathogens of acute febrile illnesses. Two cases needed further investigation. The first was the sequences of peptide 2 and type VI secretion system tip protein VgrG of *Salmonella enterica* (MBH0566585.1) (Figure 32 upper row) (209). Comparing the sequences, the similarity was deemed insignificant because of the gaps. It was also unknown whether the matched sequence functioned as a linear B-cell epitope in VgrG. The second was the sequences of peptide 7 and envelope glycoprotein of HIV-1C (AHF52739.1) (Figure 32 lower row) (210). This sequence was from a study in Botswana that analysed HIV-1C *env* sequences (the V1-C5 regions of gp120) and studied transmission networks in the community. This sequence of 14 amino acids is located in a gp120 V4 loop that showed a high sequence variation; only this variant had a similar sequence.

Score	Expect	Identities	Positives	Gaps
33.7 bits(72)	19	12/19(63%)	12/19(63%)	4/19(21%)
Query 6	KNSSLTLWQG---HSY-HG	20		
	KN LTLWOG H Y HG			
Sbjct 56	KNATLTLWOGDIPHYRLHG	74		

Score	Expect	Identities	Positives	Gaps
33.7 bits(72)	20	12/14(86%)	12/14(85%)	0/14(0%)
Query 3	SGNNSVDSSSNKTI	16		
	SGNNS DS SNKTI			
Sbjct 281	SGNNSNDSNDSNKTI	294		

Figure 32. Results of amino acid sequence homology analysis of the ScaA peptides on the NCBI BLASTp website.

Upper row: peptide 2 (Query) and type IV secretion system tip protein VgrG of *Salmonella enterica* (MBH0566585.1) (Sbjct), lower row: peptide 7 (Query) and envelope glycoprotein of HIV-1C (AHF52739.1) (Sbjct).

14.2. Prediction of the three-dimensional structure and B-cell linear epitopes

To investigate potential B-cell linear epitopes in the ScaA antigen region, 1) the three-dimensional protein structure was predicted using the I-TASSER program, 2) amino acid sequences of the ScaA antigen region were compared between *Orientia* strains, and 3) B-cell linear epitopes were predicted using the IEDB program.

ScaA three-dimensional structure prediction

The model predicted with the highest confidence by I-TASSER is shown in Figure 33. As expected, it was a β -barrel consisting of β -pleated sheets and coils. This model had a C-score of 0.09, a TM-score of 0.73 ± 0.11 , and a root mean square deviation of $5.9 \pm 3.7 \text{ \AA}$. The confidence of models is quantitatively measured by a C-score that is calculated based on the significance of threading template alignments and the convergence parameters of the structure assembly simulations. A C-score is in the range between -5 and 2 ; a high-value C-score signifies a model with high confidence. A TM-score is defined to assess the topological similarity of two protein structures in the range between 0 and 1 (211). A model with a C-score > -1.5 and a TM-score of > 0.5 is expected to have a correct fold (211). Therefore, this predicted model of the ScaA ATD was expected to be of good quality.

EstA, an autotransporter protein of *Pseudomonas aeruginosa*, was identified as the most structurally close protein to the ScaA ATD with an estimated TM-score of 0.953 . EstA is an autotransporter esterase required for rhamnolipid production, cell motility, and biofilm formation (212). The crystal structure of full-length EstA was previously confirmed by X-ray diffraction (PDB DOI: [10.2210/pdb3KVN/pdb](https://doi.org/10.2210/pdb3KVN/pdb)) (213). From the structural similarity of ScaA and EstA, it was expected that the ScaA ATD would be buried in the outer membrane in the same direction as EstA (Appendix 11). Then the topology of nine peptides in the ScaA ATD was displayed in Figure 34. Peptides 1-2 and 6-8 regions were predicted to be partially or fully exposed to the extracellular space. Peptides 3-5 region was predicted to be buried in the outer membrane.

The same ScaA ATD amino acid sequence was also submitted to SWISS-MODEL. However, no template proteins had an amino acid sequence similarity of $\geq 30\%$. The program identified the top five templates, EstA, three serine protease autotransporters (EspP of Enterobacteriaceae), and NaIP from *Neisseria meningitidis*. All were transporter proteins. The sequence similarities were 11.45% between ScaA and EstA, 12.11% between ScaA and EspP, and 11.20% between ScaA and NaIP.

The amino acid sequence of TSA56 of the Karp strain (AAA26391.1) was also submitted to I-TASSER. There was no model with a sufficient C-score and TM-score. The first model had a C-score of -3.23 and a TM-score of 0.35 ± 0.12 ; other models had lower C-scores.

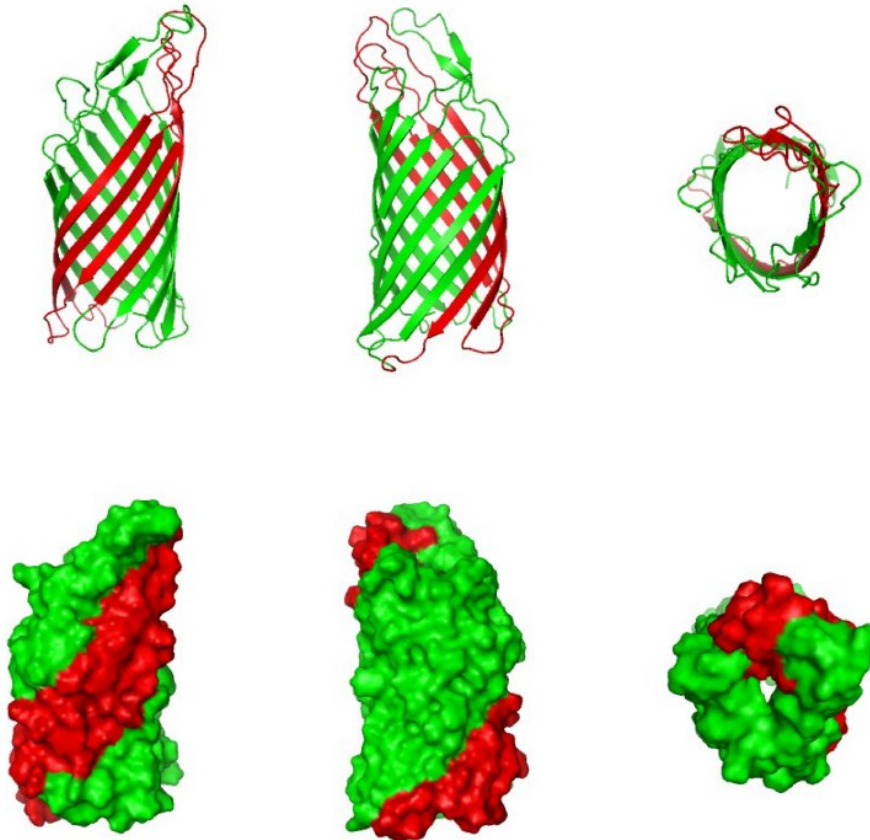


Figure 33. A predicted three-dimensional structure of the ScaA autotransporter domain.

The modelled structures on the middle columns are a 180° rotated view of the ones on the left. The right column is the view from the extracellular space. The ScaA antigen region is highlighted in red.

Amino acid sequence conservation across *Orientia tsutsugamushi* strains

Alignment of 14 amino acid sequences of the ScaA antigen region, including the one used for the assay antigen (BDP28846.1), is shown in Figure 35. The Shimokoshi strain was not included this time. The sequence variation between strains was mainly due to amino acids at 48, 55, 66, 70, 92, and 97. Peptides 6 and 7 region predicted to be exposed to extracellular space included three of these variations.

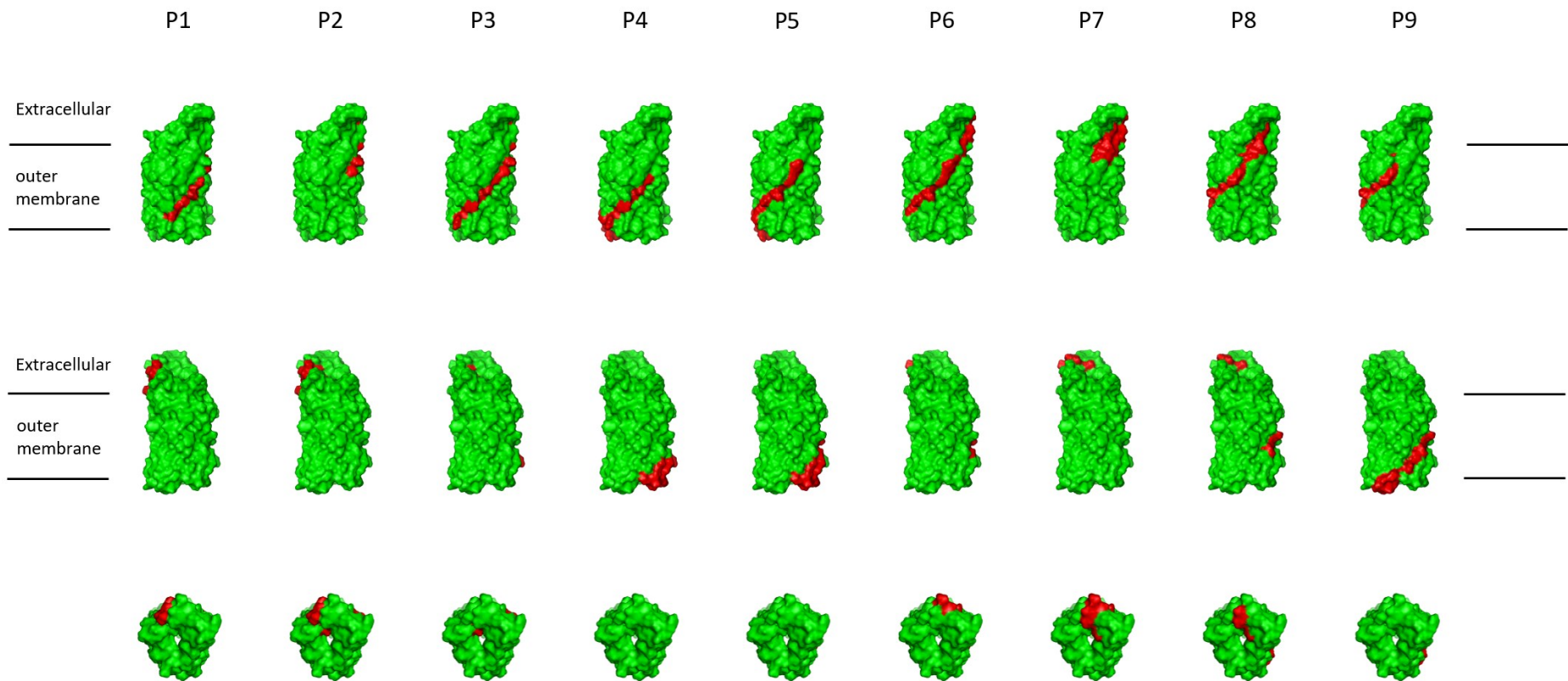


Figure 34. Topology of overlapping peptides P1-P9 in the ScaA autotransporter domain.

The modelled structures on the middle row are a 180° rotated view of those on the top. Modelled structures on the bottom are the view from the extracellular space. The corresponding area is highlighted in red.

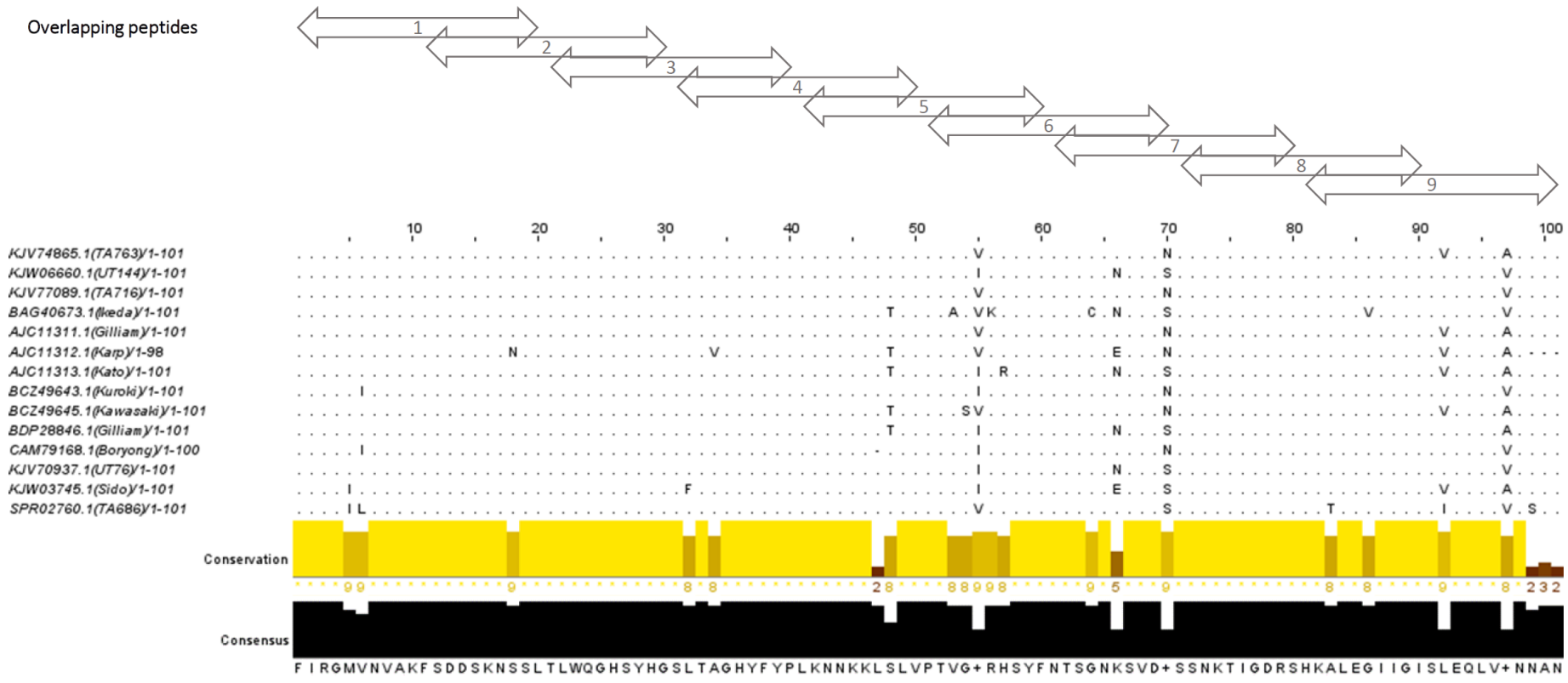


Figure 35. Amino acid sequences of the ScaA antigen region of 14 *Orientia tsutsugamushi* isolates and nine overlapping peptides. A ClustalW alignment of the amino acid sequences of 14 *Orientia tsutsugamushi* isolates, except for the Shimokoshi strain, is presented. Only amino acid differences relative to the consensus sequence are shown. BDP28846.1 was used as an antigen for recombinant ScaA-based ELISA. Nine grey arrows represent the nine overlapping peptides.

B-cell linear epitope prediction

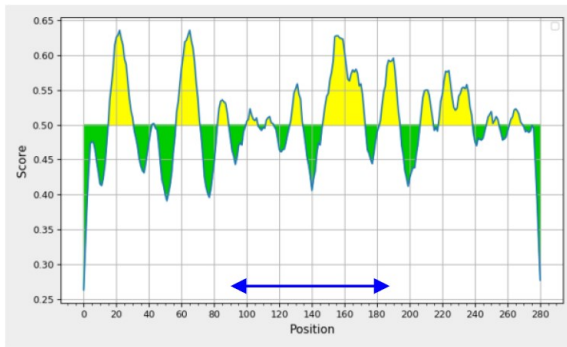
B-cell linear epitope prediction was performed in May 2021 on the IEDB program. An amino acid sequence of the entire ScaA ATD (BDP28846.1) was used (Figure 36). Amino acids 91-191 were the ScaA antigen region. In most prediction methods, the regions of amino acids 100-110 (an overlapped region of peptides 1 and 2), 120-130 (an overlapped region of peptides 3 and 4), 150-170 (a region of peptides 6-8) were predicted to include B-cell linear epitopes. As one example, a list of B-cell linear epitopes predicted by Bepipred Linear Epitope Prediction 2.0 is shown in Table 27.

Table 27. List of B-cell linear epitopes in the ScaA antigen region predicted by Bepipred 2.0.

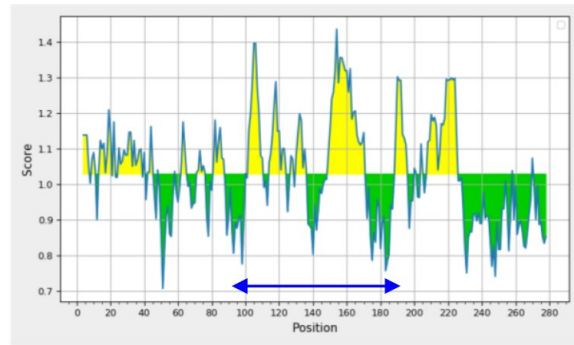
	Start	End	Amino acid sequence	Length	Corresponding overlapping peptide
1	101	107	FSDDSKN	7	P1-2
2	113	117	WQGHS	5	P2-3
3	128	135	FYPLKNNK	8	P3-5
4	148	173	SYFNTSGNKSVDNSSNKTIGDRSHKA	26	P6-7
5	183	195	EQLVANNANNNLN	8	P9-

In summary, the ScaA ATD was predicted to be a β -barrel. In the ScaA antigen region, sequences predicted to be exposed to extracellular space (peptides 1-2 and peptides 6-8 regions) were predicted to include B-cell linear epitopes as expected. However, sequences predicted to be buried in the outer membrane (peptides 3-5 and peptide 9 regions) were also predicted to include some B-cell linear epitopes

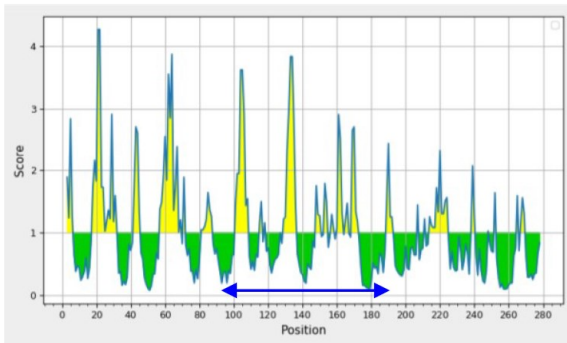
Bepipred Linear Epitope Prediction



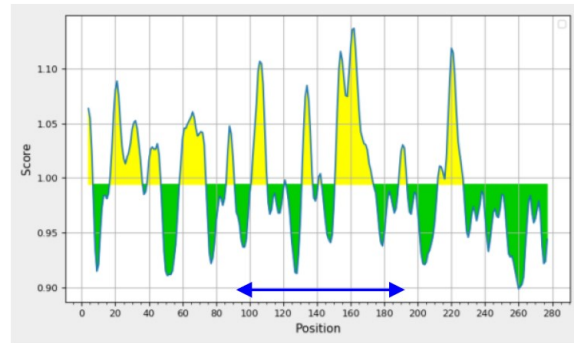
Chou & Fasman Beta-Turn Prediction



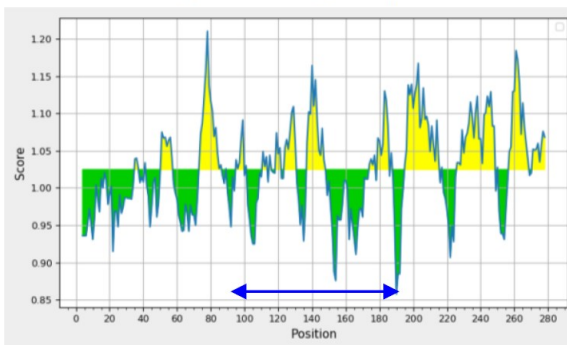
Emini Surface Accessibility Prediction



Karplus & Schulz Flexibility Prediction



Kolaskar & Tongaonkar Antigenicity



Parker Hydrophilicity Prediction

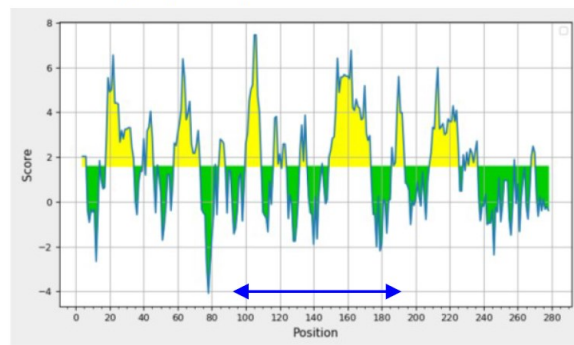


Figure 36. Results of the B-cell linear epitope prediction of the ScaA autotransporter domain on the IEDB program.

A blue arrow shows the ScaA antigen region. The cut-off scores were 0.5 for Bepipred 2.0, 1.027 for Chou and Fasman Beta-Turn prediction, 1.0 for Emini Surface Accessibility Prediction, 0.993 for Karplus and Schulz Flexibility prediction, 1.027 for Kolaskar and Tongaonkar Antigenicity, and 1.62 for Parker Hydrophilicity Prediction.

14.3. ScaA peptide dot-blot

A dot-blot assay was performed first to screen whether scrub typhus samples reacted to any of the nine peptides. Fifteen samples of patients in the assay development cohort were used. All patients except for patients 57 and 63 had positive test results for both a TSA56-based nested PCR assay and an IgM IFA, and the two patients had only positive PCR test results. The IgM dot-blot protocol is in Appendix 12. The protocol was a modification of the work by Chen et al. (214).

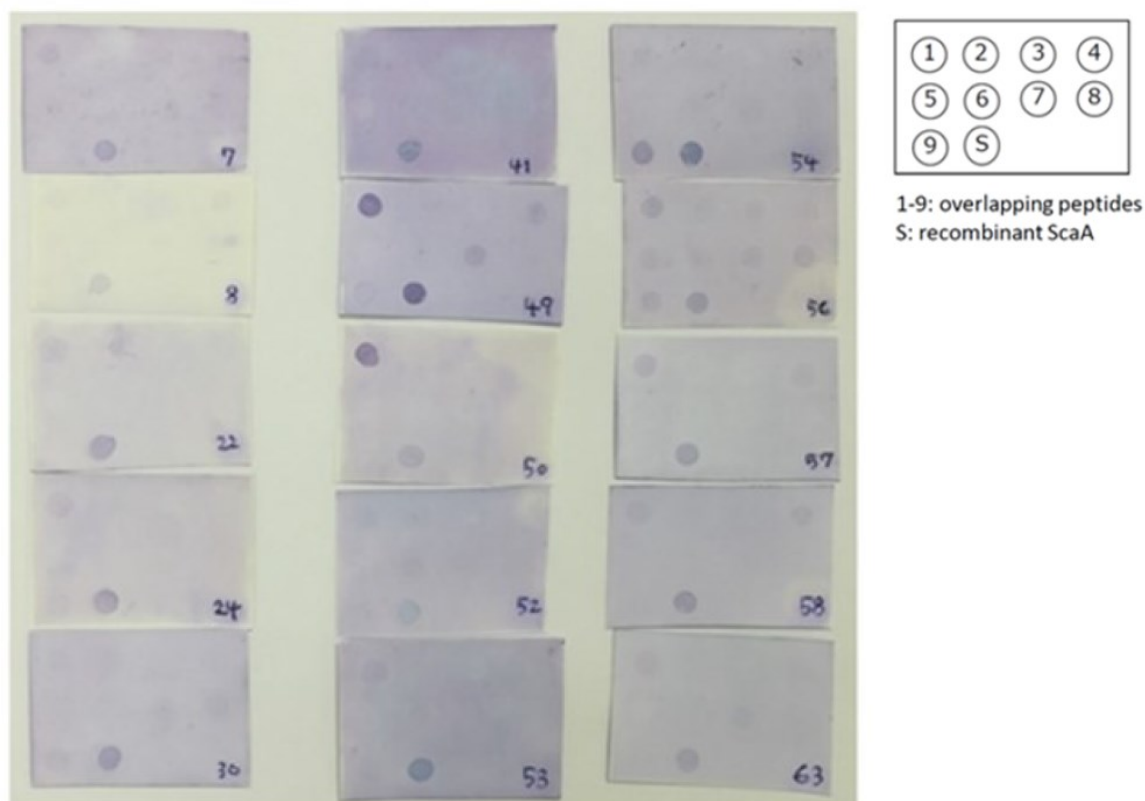


Figure 37. ScaA peptides IgM dot-blot results.

Serum samples of 15 scrub typhus patients were applied to peptides 1-9 and the recombinant ScaA protein.

As shown in Figure 37, all samples had positive signals to the recombinant ScaA protein. Although some samples had positive signals to the ScaA peptides, it was difficult to determine the test positivity due to the strong background signal on the PVDF membrane. It was also uncertain whether the oligopeptides were firmly fixed on the PVDF membrane. Therefore, all peptides were tested as antigens in an IgM ELISA.

14.4. ScaA peptide IgM ELISA protocol

The protocol development was done similarly to a recombinant ScaA-based IgM ELISA. PolySorp plates did not yield positive signals in the peptide ELISA, and MaxiSorp plates were selected. It must have been because oligopeptides of 20-21 amino acids could not be coated on the hydrophobic surface with lower binding capacity. The concentrations of the peptide antigen (1, 2, 4, and 8 $\mu\text{g/ml}$) were set by a serial dilution (Figure 38). The IgM antibody responses to peptide antigens plateaued or peaked at 4 $\mu\text{g/ml}$. The blocking agent and the concentrations of a serum sample and a secondary antibody were the same as those for a recombinant ScaA-based ELISA. The final ScaA peptide IgM ELISA protocol is in Table 28.

A peptide IgG ELISA was also performed on the assay development cohort following the same protocol except for using a goat anti-human IgG-HRP conjugate (Bethyl Laboratories, Inc) diluted to 1:10,000 as a secondary antibody (Appendix 13). As shown in Table 18, only 43.5% of patients with positive IgM IFA results in the rickettsiosis cohort had a positive result for a recombinant ScaA-based IgG ELISA. Therefore, a peptide IgG ELISA was not applied to the rickettsiosis cohort.

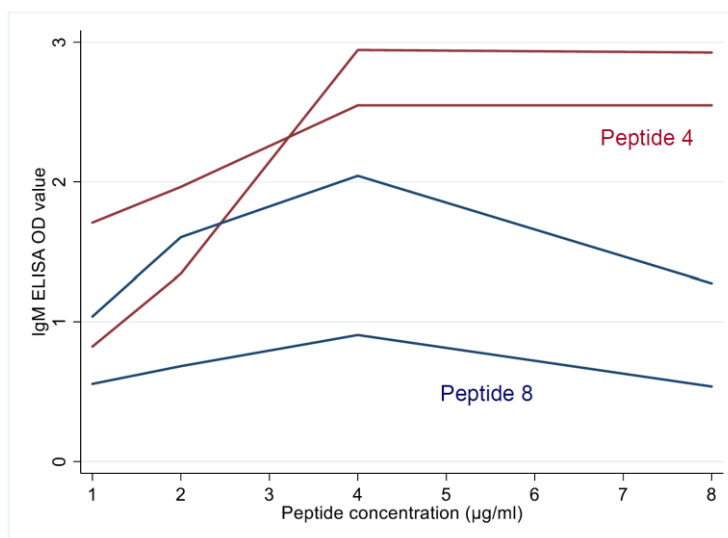


Figure 38. IgM antibody responses to the ScaA peptides of different concentrations.

A ScaA peptide IgM ELISA with either peptides 4 or 8 was applied to two samples of scrub typhus patients. 4 $\mu\text{g/ml}$ was chosen as the antigen concentration of the peptides.

Table 28. ScaA peptide IgM ELISA protocol.

1. Coat an immunoassay plate (MaxiSorp 96-well flat-bottom plates; Nunc) in duplicate with 100 µl/well of
 - a ScaA peptide antigen at a concentration of 4 µg/ml in bicarbonate buffer (0.05 M, pH 9.6)
 - bicarbonate buffer (= non-coated wells for measurement of the BGN reaction)
2. Incubate at 4 °C overnight.
3. Wash the plate with a washing buffer (PBS-T: 0.05% Tween-20 in PBS) three times.
4. Block with a blocking buffer (3% BSA in PBS-T) at room temperature for 1 hour.
5. Wash the plate with the washing buffer three times.
6. Apply 100 µl/well of sample serum diluted to 1:500 in a dilution buffer (3% BSA in PBS-T) to non-coated and antigen-coated wells.
7. Incubate at room temperature for 1 hour.
8. Wash the plate with the washing buffer five times.
9. Apply 100 µl/well goat anti-human IgM-HRP conjugate (QED Bioscience, Inc.) diluted to 1:20,000 in the dilution buffer.
10. Incubate at room temperature for 1 hour.
11. Wash the plate with the washing buffer five times.
12. Apply 200 µl/well of SIGMAFAST OPD (Sigma-Aldrich) and allow the colour to develop at room temperature for 30 minutes.
13. Apply 50 µl/well of 3 M hydrochloric acid and stop the reaction.
14. Measure the OD values at 492 nm with a plate reader (Thermo Scientific Multiskan FC).
15. Subtract the mean OD value of two blank wells from the mean OD values of non-coated and antigen-coated wells.
16. Subtract the mean OD value in the non-coated wells from the mean OD value in the antigen-coated wells of each test sample.

Cut-off values for a ScaA peptide IgM ELISA

A ScaA peptide IgM ELISA was applied to healthy controls and 207 patients in the rickettsiosis cohort. The cut-off values for peptides 1-9 are shown in Table 29. The cut-off values obtained from the healthy controls and the ROC curve of the rickettsiosis cohort were compatible in the recombinant ScaA-based IgM ELISA. However, the cut-off values derived from the two methods were different in peptide IgM ELISAs. The cut-off values derived from ROC curves were around 0.0 for all peptides (Table 29).

Table 29. Cut-off values for ScaA peptide IgM ELISA defined as the mean plus three standard deviations of healthy controls and by the receiver operating characteristic curve analysis.

	P1	P2	P3	P4	P5	P6	P7	P8	P9
Mean + 3 SDs	0.4074	0.1572	0.1791	0.2579	0.1569	0.1062	0.1008	0.2627	0.1859
ROC curve	-0.0074	-0.0096 – -0.0022	0.0254 – 0.0644	0.0332	-0.0252	0.0163 – 0.0205	-0.0062	0.0013	0.0168 – 0.0183

This discrepancy in the cut-off values derived from the two methods could have been due to the poor blocking of the BGN reaction in peptide ELISAs. As shown in Figure 39, in the recombinant ScaA-based ELISA that used Nunc PolySorp microplates, the BGN reaction was well blocked in both the rickettsiosis cohort and healthy controls (y-axis of Figure 39). The median OD values of the BGN reaction were 0.01 (IQR 0.01-0.02) in the rickettsiosis cohort and 0.02 (IQR 0.01-0.03) in healthy controls. In the peptide ELISA that used Nunc MaxiSorp plates, the BGN reaction was stronger, and its range was wider (x-axis of Figure 39) (rickettsiosis cohort, 0.23 [IQR 0.14-0.40]; healthy control, 0.19 [IQR 0.12-0.29]). 4.3% (3/69) of healthy controls had a BGN OD value of >0.5, while 18.4% (38/207) of patients in the rickettsiosis cohort did. In antigen-non-coated wells of both ELISAs, the protocols were identical except for the choice of the plate and the number of washing after the coating step.

The distributions of OD values of the BGN reaction and signals to peptide 6 in the healthy controls and the rickettsiosis cohort are shown in Figure 40. This net negative signal was previously reported in the study by Moritz et al. that measured IgG antibody responses against fibroblast growth factor receptor 3 using MaxiSorp plates by the same subtraction method (163). It may have been particularly significant in this peptide IgM ELISA because an IgM is known to be 'sticky' causing

strong non-specific reactions. An influence of heterophile antibodies to BSA was thought unlikely as non-specific reactions were well controlled in the recombinant ScaA-based ELISA that also used BSA buffers.

As expected, when a cut-off value of 0.0 was used for all peptide antigens, sensitivities of peptide ELISAs with all peptides increased, and specificities significantly decreased (Appendix 14). Stringent values were chosen as cut-off values to maintain the high specificity that is a general strength of a peptide-based test.

Attempts to improve the sensitivity of a ScaA peptide IgM ELISA

The following methods were attempted, using the assay development cohort, to improve the sensitivity of a ScaA peptide IgM ELISA: 1) a combination of multiple peptides, 2) the use of a 31mer peptide, and 3) conjugation of the peptides to BSA. This process followed the work by Dubois et al. that described how to optimise a peptide-based ELISA for human mpox infection (215). First, different combinations with different concentrations of two or three 20mer peptides were used as an ELISA antigen. The signals were lower or lost in most samples (Appendix 13). The peptides may have had different properties that affected the likeliness to bind to the solid surfaces, and multiple peptides may not have been coated simultaneously (215). Second, the same company synthesised a 31mer peptide (peptide 8/9). A 30mer peptide of peptides 3 and 4 was not selected because the region was predicted to be buried in the outer membrane. The 31mer peptide did not improve test sensitivity compared to either peptides 8 or 9 alone, and in some cases, positive signals were lost. Third, BSA conjugates of peptides 1, 4, and 8 were synthesised. These peptide-BSA conjugates did not yield positive signals in positive samples. Small peptides may not have been able to display accessible epitopes being folded by the conjugate proteins (216).

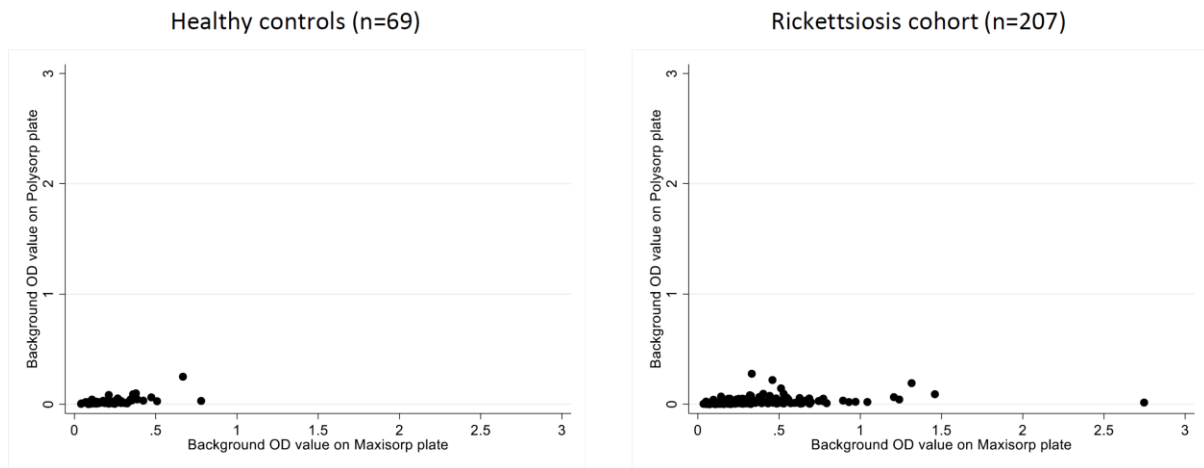


Figure 39. Background noise reactions in the recombinant ScaA-based IgM ELISA and ScaA peptide IgM ELISA in healthy controls and the rickettsiosis cohort.

X-axis: the BGN reaction OD values in the ScaA peptide IgM ELISA with Nunc MaxiSorp microplates, y-axis: the BGN reaction OD values in the recombinant ScaA-based IgM ELISA with Nunc PolySorp microplates.

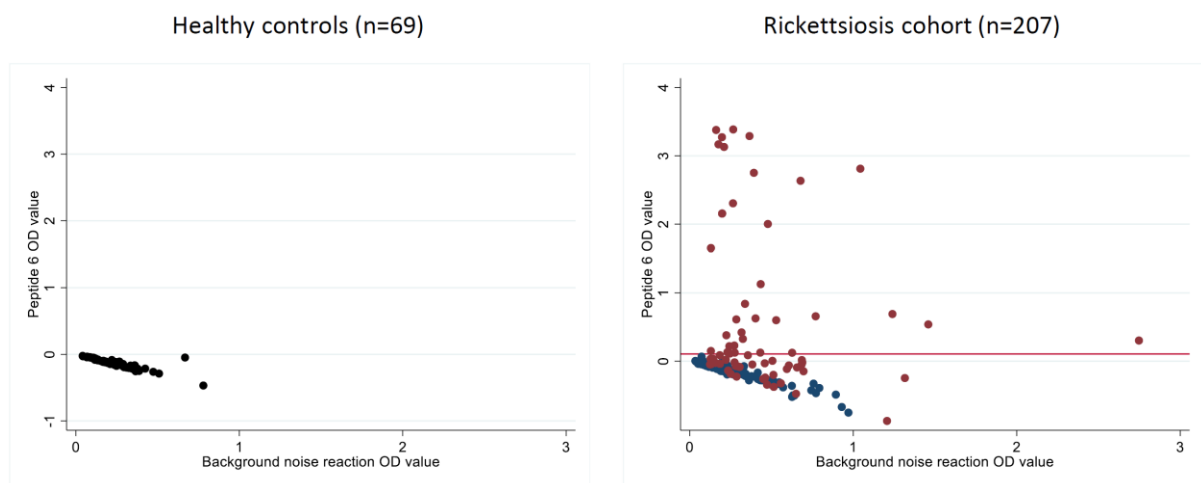


Figure 40. IgM antibody responses to ScaA peptide 6 and background noise reactions in the ScaA-based peptide ELISA in healthy controls and patients in the rickettsiosis cohort.

An IgM antibody response to an antigen peptide (y-axis) was obtained by subtracting a mean OD value in peptide-non-coated wells (x-axis) from a mean OD value in peptide-coated wells. The red line indicates the cut-off value calculated from the mean plus 3 standard deviations of healthy controls. Red dots represent scrub typhus patients, and blue dots represent non-scrub typhus patients.

14.5. ScaA peptide IgM ELISA results

The ScaA peptide ELISA detected specific IgM antibody responses against *O. tsutsugamushi*. IgM antibody responses to nine overlapping peptides of 207 patients in the rickettsiosis cohort are shown in Figure 41. Most non-scrub typhus patients had OD values around 0.0, except for a few outliers. However, some scrub typhus patients had increased OD values to peptides 3, 4, 6, 7, 8, and 9.

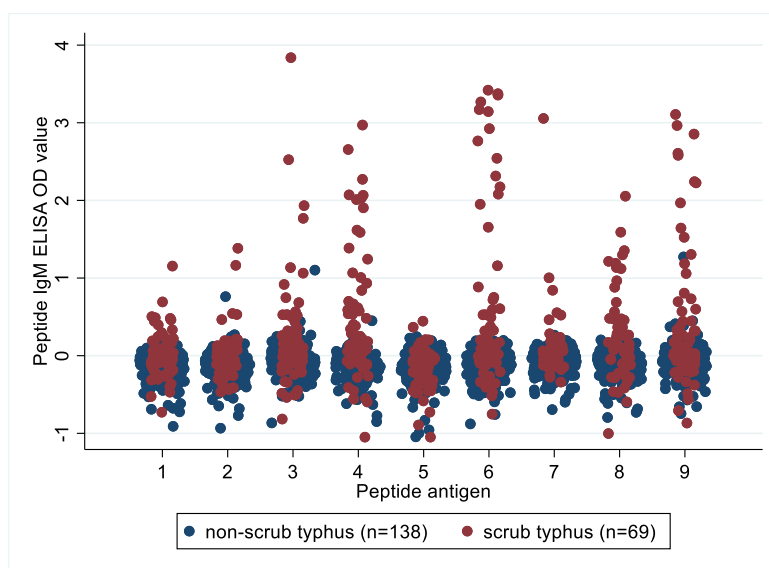
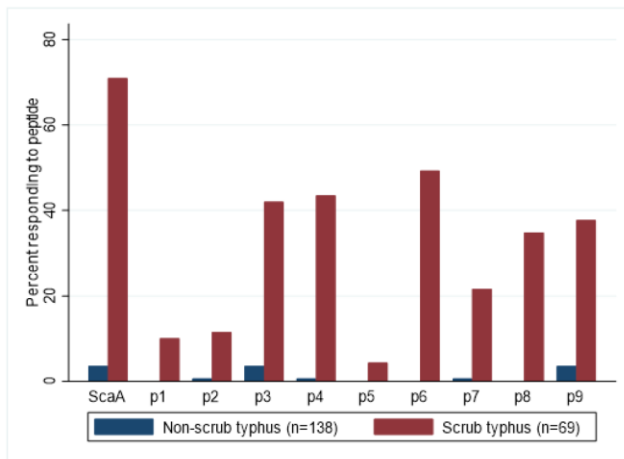


Figure 41. IgM antibody responses to ScaA peptides 1-9 in scrub typhus and non-scrub typhus patients in the rickettsiosis cohort.

The proportion of patients with an OD value over the cut-off value for each peptide is shown in Figure 42. Half (49.3%) of scrub typhus patients had a positive result for a ScaA peptide 6 IgM ELISA. Approximately 40% responded to peptides 3 (42.0%) and 4 (43.5%), although the region was predicted to be buried in the outer membrane. One-third of the patients responded to peptides 8 (34.8%) and 9 (37.7%), and one-fifth to peptide 7 (21.7%). Approximately 10% responded to peptides 1 (10.1%) and 2 (11.6%). None of a ScaA peptide IgM ELISA with any one of the nine peptides could achieve the sensitivity of a recombinant ScaA-based IgM ELISA. Specificities were generally high. In the non-scrub typhus group, none responded to four peptides (1, 5, 6, and 8), 0.7% to peptides 2, 4, and 7, and 3.6% to peptides 3 and 9.



Peptide	Sensitivity (95% CI)	Specificity (95% CI)
P1	10.1% (4.2-19.8)	100.0% (97.4-100.0)
P2	11.6% (5.1-21.6)	99.3% (96.0-100.0)
P3	42.0% (30.2-54.5)	96.4% (91.7-98.8)
P4	43.5% (31.6-56.0)	99.3% (96.0-100.0)
P5	4.3% (0.9-12.2)	100.0% (97.4-100.0)
P6	49.3% (37.0-61.6)	100.0% (97.4-100.0)
P7	21.7% (12.7-33.3)	99.3% (96.0-100.0)
P8	34.8% (23.7-47.2)	100.0% (97.4-100.0)
P9	37.7% (26.3-50.2)	96.4% (91.7-98.8)

Figure 42. Proportions of patients responding to peptides 1-9 and the recombinant ScaA protein in scrub typhus and non-scrub typhus patients in the rickettsiosis cohort.

It was a concern that the ELISA result may not have been reliable in cases with high BGN reactions. A high BGN reaction could have masked the true signal after the subtraction leading to reduced test sensitivity. Sensitivities of peptide ELISAs were compared in scrub typhus patients with a BGN OD value of >0.5 or ≤0.5 (Table 30). BGN OD values were higher in scrub typhus patients than in non-scrub typhus patients (scrub typhus, 0.338 [IQR 0.2304-0.5575]; non-scrub typhus, 0.1994 [IQR 0.1191-0.3250]; $p < 0.001$). There was no statistically significant difference in proportions of scrub typhus patients with a positive result for a recombinant ScaA-based IgM ELISA in the high and low BGN groups (high BGN, 16/22 [72.7%]; low BGN, 33/47 [70.2%]; $p = 0.534$).

Sensitivities of a ScaA peptide 6 IgM ELISA were 36.4% in the high BGN group and 55.3% in the low BGN group, although the difference was not statistically significant ($p = 0.113$). The same trend was observed for the tests using peptides 4, 7, 8, and 9. The subtraction of poorly controlled BGN reactions may have lowered the sensitivities of ScaA peptide ELISAs.

Table 30. Sensitivities of ScaA peptide ELISAs in scrub typhus patients with low and high background noise reactions.

Peptide	All scrub typhus patients (n=69)	Scrub typhus patients with BGN ≤0.5 (95% CI) (n=47)	Scrub typhus patients with BGN >0.5 (95% CI) (n=22)	P-value*
P3	42.0% (30.2-54.5)	40.4% (23.4-55.7)	45.5% (24.4-67.8)	0.445
P4	43.5% (31.6-56.0)	46.8% (32.1-61.9)	36.4% (17.2-59.3)	0.291
P6	49.3% (37.0-61.6)	55.3% (40.1-69.8)	36.4% (17.2-59.3)	0.113
P7	21.7% (12.7-33.3)	23.4% (12.3-38.0)	18.2% (5.2-40.3)	0.439
P8	34.8% (23.7-47.2)	38.3% (24.5-53.6)	27.3% (10.7-50.2)	0.268
P9	37.7% (26.3-50.2)	42.6% (28.3-57.8)	27.3% (10.7-50.2)	0.170

* Fisher's exact test. High BGN reaction was defined as an OD value of >0.5 in antigen-non-coated wells.

The pattern of signals to nine overlapping peptides in 69 patients with a positive scrub typhus IgM IFA result is shown in Table 31. The level of IgM ELISA OD values was expressed in the colour gradients. The pattern varied by patient. Fifty-eight out of 69 patients had a positive result for a ScaA peptide ELISA using any one of nine peptides: 46 of 49 patients with a positive recombinant ScaA-based IgM ELISA result (highlighted in blue), and 12 of 20 patients with a negative test result. Some had positive signals to both peptides 3 and 4 and a region across peptides 6-9. The former could have been antibody responses to the overlapped sequence between the two peptides (SLTAGHYFYFYP). A few patients had a positive signal to peptide 5; therefore, the overlapped sequence between peptide 6 and 7 is another epitope candidate (NTSGNNSVDS). These results were consistent with the prediction results discussed in Chapter 14.2.

Table 31. IgM ELISA OD values to the recombinant ScaA antigen and ScaA peptides 1-9 of 69 scrub typhus patients with a positive IgM IFA result in the rickettsiosis cohort.

Patients are listed in the order of lower recombinant ScaA-based IgM ELISA OD values, and those with positive results are highlighted in blue. ScaA peptide IgM ELISA OD values were highlighted in a yellow gradient from 0.0 to 4.0.

The sensitivity and specificity of a ScaA peptide 6 ELISA were evaluated since peptide 6 yielded the highest sensitivity among the nine ScaA peptides. Out of 32 (=2⁵) possible combinations of test results with four diagnostic tests and one clinical sign, 16 patterns were observed (Table 32). 'All tests negative' (61.8%) was the most common pattern, followed by 'all test positive' (11.6%).

Table 32. Number of patients by combinations of test results including a ScaA peptide 6 IgM ELISA in the rickettsiosis cohort.

IgM IFA	TSA5-based IgM ELISA	Recombinant ScaA-based IgM ELISA	ScaA peptide 6 IgM ELISA	Presence of eschar	Observed number of patients (%)
-	-	-	-	-	128 (61.8)
-	-	-	-	+	1 (0.5)
-	-	+	-	-	4 (2.0)
-	+	-	-	-	4 (2.0)
-	+	+	-	-	1 (0.5)
+	-	-	-	-	2 (1.0)
+	-	-	-	+	1 (0.5)
+	-	+	-	-	1 (0.5)
+	+	-	-	-	4 (2.0)
+	+	-	-	+	7 (3.4)
+	+	-	+	-	1 (0.5)
+	+	-	+	+	5 (2.4)
+	+	+	-	-	5 (2.4)
+	+	+	-	+	15 (7.2)
+	+	+	+	-	4 (2.0)
+	+	+	+	+	24 (11.6)

Grey rows show patients confirmed as scrub typhus when an IgM IFA was considered a gold standard.

As peptide 6 included amino acid sequence variations between the *Orientia* strains (Figure 35), sensitivities of the three ELISAs by serotypes were examined (Table 33). Serotypes were determined between the IgM IFA titres of 1:160 and 1:20,480 this time. There was no statistically significant difference in test sensitivities, but the sample sizes of patients with the Karp or Gilliam strains were small.

Table 33. Sensitivities of a TSA56-based IgM ELISA, recombinant ScaA-based IgM ELISA, and ScaA peptide 6 IgM ELISA by IgM IFA serotypes.

Serotype	Number of patients	TSA56 ELISA positive (%)	<i>P</i> -value*	ScaA ELISA positive (%)	<i>P</i> -value*	Peptide 6 ELISA positive (%)	<i>P</i> -value*
Karp	8	7 (87.5)	0.618	5 (62.5)	0.631	4 (50.0)	0.673
Kato	42	39 (92.9)		29 (69.1)		18 (42.9)	
Gilliam	3	3 (100.0)		3 (100.0)		2 (66.7)	
Karp and Kato	3	3 (100.0)		2 (66.7)		2 (66.7)	
Karp and Gilliam	3	3 (100.0)		1 (33.3)		1 (33.3)	
Kato and Gilliam	5	5 (100.0)		5 (100.0)		4 (80.0)	
Karp, Kato and Gilliam	5	5 (100.0)		4 (80.0)		3 (60.0)	
Total	69	65 (94.2)		49 (71.0)		34 (49.3)	

* Fisher's exact test.

The sensitivity and specificity of a ScaA peptide 6 IgM ELISA were 49.3% (37.0-61.6) and 100.0% (97.4-100.0) when absolute OD values were used. COIs were calculated and the ROC analysis was performed to determine the COI cut-off value (Figure 43 and Table 34). As showed in Figure 41, many samples had a negative OD value after the subtraction of the insufficiently blocked BGN reaction, which resulted in negative COIs. Approximately 40% of the patients in the rickettsiosis cohort had COIs between -0.1 and 0.1. The sensitivity and specificity of the ScaA peptide test changed considerably within this small range (Table 34). With a cut-off COI of >0.0, the sensitivity and specificity of a ScaA peptide 6 IgM ELISA were 60.9% (48.4-72.4) and 98.6% (94.9-99.8). When the COI cut-off value of 0.1 was used, the sensitivity and specificity of the test were 43.5% (31.6-56.0) and 100.0% (97.4-100.0).

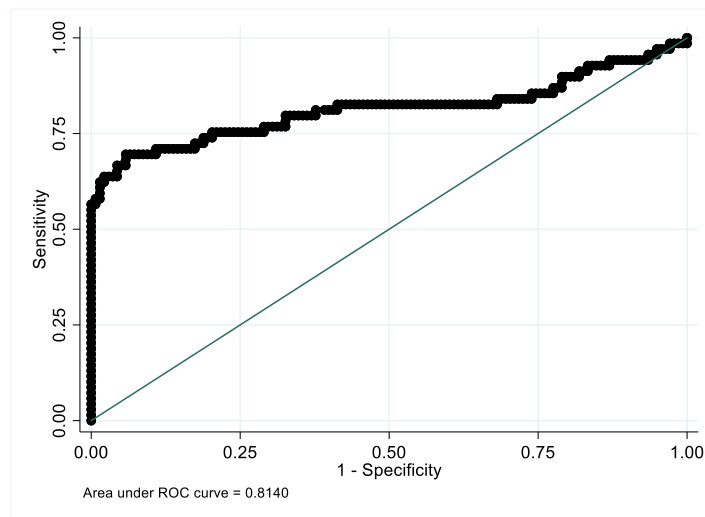


Figure 43. Receiver operating characteristics curve of the ScaA peptide 6 IgM ELISA in the rickettsiosis cohort when cut-off indices were used.

Table 34. Sensitivity, specificity, and Youden index of three cut-off values for the cut-off index of the ScaA peptide 6 IgM ELISA in the rickettsiosis cohort.

Cut-off COI	Sensitivity (95% CI)	Specificity (95% CI)	Youden index
-0.1	82.6% (71.6-90.7)	58.0% (49.3-66.3)	0.406
0.0	60.9% (48.4-72.4)	98.6% (94.9-99.8)	0.595
0.1	43.5% (31.6-56.0)	100.0% (97.4-100.0)	0.435

14.6. Estimation of the sensitivity and specificity of a ScaA peptide IgM ELISA by Bayesian latent class model

The sensitivity and specificity of a ScaA peptide 6 IgM ELISA were estimated by Bayesian LCMs. The LCM models were constructed similarly, as discussed in Chapter 13.6.1. Conditional dependence between serological tests, at least between a scrub typhus IgM IFA and TSA56-based IgM ELISA, were thought necessary to incorporate into the models. Model 4, which assumed conditional dependence between all four serological tests, was added. The model comparison was performed using non-informative priors. The distribution of patients by test results observed and predicted by the three LCMs is in Appendix 15. Based on differences in AIC, model 4 was selected (Table 35).

Table 35. Comparison of three latent class models in the rickettsiosis cohorts.

Model	Correlation between:	Fever cohort	
		Number of parameters	Akaike information criterion
2	IgM IFA and TSA56-based IgM ELISA	12	138.6
3	IgM IFA, TSA56-based IgM ELISA, and recombinant ScaA-based IgM ELISA	12	138.3
4	IgM IFA, TSA56-based IgM ELISA, recombinant ScaA-based IgM ELISA, and ScaA peptide 6 IgM ELISA	12	116.5

A ‘moderate’ and ‘very high’ priors were used as informative priors for sensitivity and specificity of a ScaA peptide 6 IgM ELISA, respectively, based on the results assuming an IgM IFA as a gold standard test. Prior information for other parameters were the same as described in Table 22. The assessment of the convergence and autocorrelation was performed similarly (Appendix 16). Three chains were used, and 40,000 iterations per chain for non-informative priors and 20,000 iterations per chain for informative priors were run. In both priors, 1,000 iterations were discarded as burn-in. The density plots of the prevalence, sensitivities, and specificities are in Appendix 17. The distribution of patients by test results predicted by model 4 with informative priors is in Appendix 15. The estimated sensitivity and specificity of a ScaA peptide 6 IgM ELISA were 50.4% (39.9-61.2) and 99.5% (97.8-99.9) (Table 36). Estimated sensitivities and specificities of an IgM IFA, TSA56-based and recombinant ScaA-based IgM ELISAs, and the presence of eschar were similar to the results estimated without a ScaA peptide 6 IgM ELISA (Table 24). There was no substantial difference in the prevalence, sensitivities, and specificities when non-informative and informative priors were used.

Table 36. A scrub typhus prevalence, test sensitivities, and test specificities estimated using an IFA as a reference standard test or using Bayesian latent class models in the rickettsiosis cohort.

	IgM IFA titre \geq 1:160 as a reference standard (95% CI)	Bayesian LCM with non-informative priors (95% CrI)	Bayesian LCM with informative priors (95% CrI)
Prevalence	33.3% (27.0-40.2)	32.5% (26.2-39.2)	32.7% (26.4-39.4)
IgM IFA			
Sensitivity	100%	98.9% (93.5-100.0)	96.4% (90.4-99.2)
Specificity	100%	98.2% (94.7-99.8)	97.4% (93.9-99.2)
ScaA peptide 6 IgM ELISA			
Sensitivity	49.3% (37.0-61.6)	50.7% (40.3-61.4)	50.4% (39.9-61.2)
Specificity	100.0% (97.4-100.0)	99.7% (98.1-100.0)	99.5% (97.8-99.9)
Recombinant ScaA-based IgM ELISA			
Sensitivity	71.0% (58.8-81.3)	72.6% (62.6-81.7)	73.6% (63.6-82.7)
Specificity	96.4% (91.7-98.8)	96.3% (92.2-98.7)	96.7% (92.8-98.9)
TSA56-based IgM EISA			
Sensitivity	94.2% (85.8-98.4)	97.0% (90.4-99.8)	95.4% (89.1-98.7)
Specificity	96.4% (91.7-98.8)	96.5% (92.5-98.9)	96.9% (93.0-99.1)
Presence of eschar			
Sensitivity	75.4% (63.5-85.0)	77.7% (66.4-86.8)	75.6% (64.5-84.7)
Specificity	99.3% (96.0-100.0)	99.2% (96.7-100.0)	99.1% (96.7-99.9)

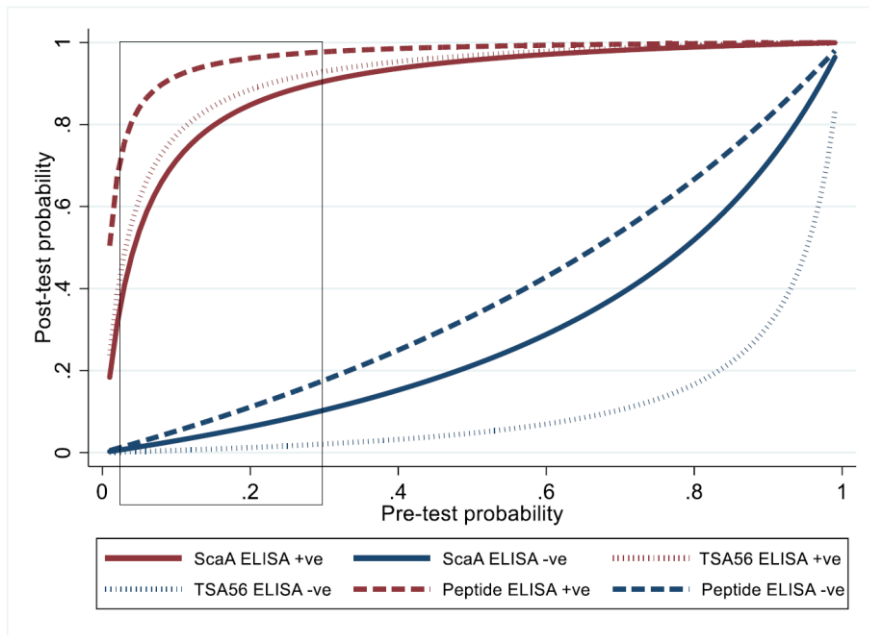
14.7. Summary and discussion

A ScaA IgM peptide ELISA was developed using nine overlapping peptides of the ScaA antigen region. The test detected specific IgM antibody responses against *O. tsutsugamushi*, but none of the peptides achieved the sensitivity of the recombinant ScaA antigen. Half of scrub typhus patients had a positive signal to peptide 6. Approximately 40% of the patients had a positive signal to peptides 3-4, and 30 % had to a region of peptides 8-9. Although there have been reports that showed promising results in the use of peptide antigens for the diagnosis of infectious diseases, including Lyme disease, mpox, visceral leishmaniasis, and Chagas disease (215, 217-219), ScaA peptide IgM ELISAs for scrub typhus did not achieve high sensitivity, although specificities were generally very high.

Interpretation of the result

An LR+ and LR- of a ScaA peptide 6 IgM ELISA were 100.8 and 0.5. The results were interpreted in the same scenario of a disease prevalence of 3-30%. Figure 44 shows how pre- and post-test probabilities change before and after applying three ELISAs. Among the three ELISAs, the ScaA peptide test gives the highest post-test probability after a positive result reflecting its very high specificity. Post-test probabilities after a negative result considerably vary by the tests due to the differences in tests' sensitivities. A ScaA peptide 6 IgM ELISA is very good at ruling-in the disease but not good at ruling-out. The test does not reduce the post-test probability as substantially as a TSA56-based IgM ELISA or recombinant ScaA-based IgM ELISA. When the prevalence of scrub typhus is 30%, the post-test probability with a negative test result for a ScaA peptide 6 IgM ELISA is still 17.6%.

Even though the use of oligopeptides is simple and inexpensive compared to the purification of recombinant proteins, the lack of power to rule-out the disease may not be a reasonable trade-off. Clinicians would become confident about giving antibiotics treatment with a positive result of a ScaA peptide IgM ELISA, but they need to be aware that the test is not good at ruling-out the disease.



Pre-test probability	Post-test probability					
	ScaA peptide 6 IgM ELISA		Recombinant ScaA-based IgM ELISA		TSA56-based IgM ELISA	
	Positive	Negative	Positive	Negative	Positive	Negative
3%	75.7%	1.5%	40.8%	0.8%	48.8%	0.2%
10%	91.8%	5.3%	71.2%	2.9%	77.4%	0.6%
20%	96.2%	11.1%	84.8%	6.3%	88.5%	1.2%
30%	97.7%	17.6%	90.5%	10.4%	93.0%	2.1%

Figure 44. Pre- and post-test probabilities of scrub typhus before and after applying a ScaA peptide 6 IgM ELISA, recombinant ScaA-based IgM ELISA, and TSA56-based IgM ELISA.

A black square on the figure indicates an area of a pre-test probability of 3-30%.

Challenges of using oligopeptide antigens

In addition to the antigenicity and quantity of ScaA proteins and the timing and location of the protein's expression, other challenges made it difficult to improve the peptide-based test sensitivity.

Firstly, an oligopeptide antigen of 20-21 amino acids is not expected to include many epitopes due to its size. Compared to a TSA56-based ELISA with antigens of 500-540 amino acids of four strains or an IFA with whole cell lysate of three strains, the number of epitopes included in one oligopeptide

antigen is expected to be fewer. Secondly, the peptide antigens may not have had the right structure. The ScaA antigen region had been narrowed down stepwise, as shown in Figure 45. However, at each stage, the structure of the antigen protein was unknown. The oligopeptide antigens may have had different conformations when coated on the microplates compared to when diluted in an optimal buffer. They may also have been unable to display accessible epitopes to antibodies on solid surfaces (215). These factors could not be ascertained. Thirdly, because of their small molecular sizes, oligopeptide antigens may not have been fixed well on ELISA plates.

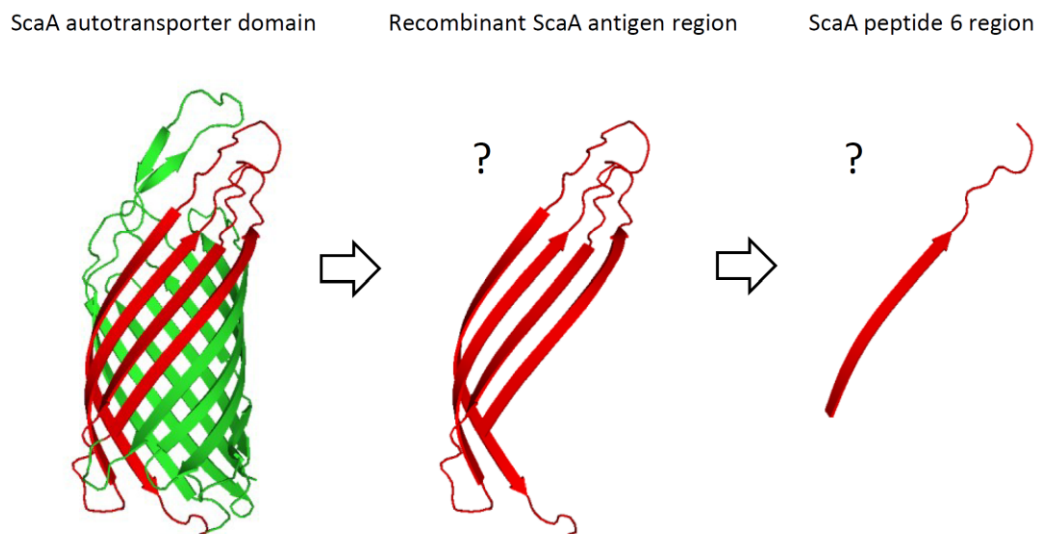


Figure 45. Schematic representation of the stepwise process to narrow down the ScaA antigen region.

ScaA was narrowed down from the autotransporter domain of 281 amino acids (left) to the ScaA antigen of 101 amino acids (centre), then to ScaA peptide 6 of 20 amino acids (right). The three-dimensional structures of the recombinant ScaA antigen and ScaA peptides were unknown.

Control of the background noise reaction

The control of BGN reactions is a fundamental problem in an indirect ELISA system (116). This was particularly important in a peptide-based IgM ELISA. ELISA microplates with high capacity to absorb proteins were necessary to capture oligopeptides, instead strong BGN reactions were inevitable. Using an IgM format may have exacerbated the problem. This strong BGN reaction in samples of patients with acute fever was also demonstrated as the strong background signals on the PVDF membrane in a ScaA peptide dot-blot assay (Chapter 14.3). As shown in Table 30, there was a trend

that sensitivities of ScaA peptide ELISAs were lower in patients with a high BGN OD value than in patients with a low BGN OD value. The poorly controlled BGN reaction may have masked true signals to peptide antigens. It is of note that scrub typhus patients had stronger BGN reactions than non-scrub typhus patients. As shown in Table 10, a higher proportion of patients in the scrub typhus group defined by an IgM IFA had a fever for >7 days, a qSOFA score >2, and white blood cell count >10,000 / μ L than in the non-scrub typhus group. A longer duration of fever, severer medical conditions, and stronger inflammation may have contributed to the higher BGN reaction, as suggested by Guven et al. (162). The result of this Ph.D. study poses a question of whether subtracting the signals in antigen-non-coated wells from the signals in antigen-coated wells was the right approach to correct the BGN reactions. As Haberland et al. proposed, this problem needs further discussion and investigation (201).

False-positive results of serological tests due to the BGN reaction have been reported in febrile patients in the tropics. Ghosh et al. reported that a significant proportion of patients with acute malaria had a transient test positivity for an ELISA for HIV-1 and -2 (220). In the study in Sudan by Elshafie et al., many patients with visceral leishmaniasis had antibody reactivity to joint-specific protein collagen type II in an ELISA. Most of these patients had similarly high reactivity in antigen-non-coated BSA-blocked wells (221). They argued that the false reactivity was due to the ELISA system per se and that measurement and subtraction of the signals in antigen-non-coated wells were necessary.

Careful attention must be paid to the BGN reaction in an indirect ELISA, especially when applied to patients with inflammation. ELISA conditions that can affect the measured intensity of the reaction, such as an ELISA plate, blocking agent, and serum dilution, need to be optimised. In this Ph.D. study, the choice of the ELISA plate from MaxiSorp and PolySorp microplates substantially impacted the control of the BGN reaction (Figure 39). Choosing the right solid surface material is key in developing an accurate ELISA system (222).

Implications

An oligopeptide antigen detected IgM antibody responses against *O. tsutsugamushi* in half of the patients in an ELISA system. The peptide IgM ELISA was a good ruling-in test but not a good ruling-out test due to its suboptimal sensitivity. Lachumanan et al. reported the potential of linear oligopeptides as antigens for scrub typhus diagnosis in the early 1990s (223). They performed an epitope mapping with overlapping octapeptides of heat shock protein (Sta58). They identified

human antibody responses against the hydrophilic region of the protein in a small number of patient samples. In this Ph.D. study, the peptide-based test was applied to a clinical sample set of >200 patients and the sensitivity and specificity were evaluated. The test definitely needs further improvement, but the mixture of multiple conserved peptide antigens may be worth trying. The control of the BGN reaction was a challenge. Improving the control has the potential to improve the test sensitivity.

Among the nine peptide antigens, a ScaA peptide 6 IgM ELISA yielded the highest sensitivity of approximately 50%. Peptide IgM ELISAs with peptides 3, 4, and 9 also had a sensitivity of around 40%. The unanswered question is why antibody responses were detected to these peptides predicted to be buried in the outer membrane by I-TASSER with high confidence. This study could not answer the question.

Limitations

There are four limitations besides those discussed for a recombinant ScaA-based IgM ELISA in Chapter 13.7. The first is the control of the BGN reaction. The second is the choice of cut-off values. This would potentially be solved when the BGN reaction is well controlled. The third is the use of anti-IgM antibodies. It may be argued that a ScaA peptide ELISA should have been developed using anti-IgG antibodies because IgG has a higher affinity than IgM. However, the aim of the overlapping peptide library screening was to develop a simplified IgM ELISA with peptide antigens for scrub typhus diagnosis. The final limitation pertains to the choice of a positive control. In the rickettsiosis cohort, it was observed that most positive samples had low antibody titres to a ScaA peptide 6 (Figure 41). This was an expected challenge for a peptide-based serological test with a conserved oligopeptide antigen that likely includes a small number of epitopes. Among 34 scrub typhus patients with a positive ScaA peptide 6 IgM ELISA result, 8 (23.5%) had OD values between 0.1 and 0.3, 11 (32.4%) had OD values between 0.3 and 1.0, and 15 (44.1%) had OD values of >1.0. Low OD values were naturally converted to low COIs, especially when corrected using a positive control with a high OD value. Measuring a small signal is technically difficult and is vulnerable to inter-plate variations. Even with the standardization of inter-plate variations with COIs, the choice of a positive control in this protocol may have exacerbated the difficulty of measuring small, subtle signals. This limitation may be overcome by improving the blocking method of the BGN reaction, utilising a more sensitive ELISA format, and selecting a more appropriate positive control.

PART IV. Overall discussion and conclusions

15. The main finding of the study

A recombinant ScaA-based IgM ELISA was developed using a 101-amino acid fragment of the ScaA ATD. This amino acid sequence had a high identity of >90% across 14 *Orientia* strains except for the Shimokoshi strain. The test detected specific antibody responses against *O. tsutsugamushi*. Bayesian LCMs estimated the sensitivity and specificity of the new test in the two cohorts previously collected in Vietnam. Both cohorts enrolled adult patients with fever who were hospitalised at Bach Mai Hospital, a referral hospital in Hanoi. The rickettsiosis cohort was enrolled in 2002-2003 aiming to describe clinical presentations of rickettsiosis in the area. Approximately 30% of the patients had scrub typhus, and 40% had murine typhus. The fever cohort was enrolled in 2012-2013. The study included a wider range of febrile causes, and the prevalence of scrub typhus was 4-5%.

The estimated sensitivity and specificity of a recombinant ScaA-based IgM ELISA were 73.9% and 96.9% in the rickettsiosis cohort and 68.6% and 94.8% in the fever cohort. The pre-set target sensitivity and specificity could not be achieved. The sensitivity and specificity of 73.9% and 96.9% are being converted to an LR+ of 23.8 and LR- of 0.27. The results were interpreted in a setting where the prevalence of scrub typhus is between 3% and 30% among patients with acute fever. The recombinant ScaA-based IgM ELISA was a good ruling-in test, but not good at ruling-out the disease due to its insufficient sensitivity. The difference in the severity of scrub typhus in the two cohorts may have resulted in the differences in the sensitivities of the ScaA-based test. The sensitivity of the test was higher in severe patients than in non-severe patients, although the difference was not statistically significant, likely due to the small sample size. Although the sensitivity was insufficient as a ruling-out test, if the test is simpler and less expensive, this may still be a reasonable trade-off. Therefore, an overlapping peptide screening was attempted to narrow down the antigen to a size of oligopeptides that can be commercially synthesised at a reasonable cost.

To develop a simplified peptide ELISA, overlapping peptides of the ScaA antigen region were investigated as antigens. Nine overlapping peptides of 20-21 amino acids were commercially synthesised. Potential B-cell linear epitopes in the ScaA antigen region of 101 amino acids were predicted by the IEDB program. Among the potentially epitopic regions, two regions (peptides 1-2 and peptides 6-8) were predicted to be exposed to the extracellular space, and the remaining two were predicted to be buried in the outer membrane (peptides 3-5 and peptide 9 regions) by I-

TASSER. An exposed region (peptide 6-8) included most of the amino acid sequence variations between *Orientia* strains.

A ScaA peptide IgM ELISA was developed. The test detected specific antibody responses against *O. tsutsugamushi*, but sensitivities of peptide IgM ELISAs were lower than that of a recombinant ScaA-based IgM ELISA. Approximately 50% of scrub typhus patients responded to peptide 6, 40% each to regions of peptides 3-4 and peptides 8-9. The pattern of the positivity to the overlapping peptides was consistent with the prediction result. The sensitivity and specificity of a ScaA peptide 6 IgM ELISA estimated by Bayesian LCM were 50.4% and 99.5%. An LR+ and LR- of the test were 100.8 and 0.5. An LR+ was higher than that of a TSA56-based IgM ELISA reflecting the improved specificity but an LR- also became higher. The peptide test is better at ruling-in the disease and worse at ruling-out compared to the recombinant ScaA-based test. Although the loss of sensitivity and the gain of specificity in a peptide ELISA were expected, this did not seem a reasonable trade-off between a suboptimal sensitivity and merits of using peptide antigens.

The possible explanations for the suboptimal sensitivity of a recombinant ScaA-based IgM ELISA were the antigenicity of ScaA proteins; the quantity, timing and location of the protein expression; and a denatured condition of the synthesised ScaA antigens. The additional challenges of a peptide ELISA were the control of the BGN reaction and the coating of oligopeptides to the solid surfaces.

The contributions of the study were the detection of specific antibody responses to the ScaA ATD and the exhibition of conserved oligopeptides as potential antigens to detect human antibody responses against *O. tsutsugamushi*. The previous studies used the ScaA passenger domain (54, 64). Although one conserved peptide of 20 amino acids did not yield enough sensitivity, the sensitivity may increase by combining it with similarly conserved antigen regions of other outer membrane proteins, instead of combining a diverse antigen of multiple *Orientia* strains.

16. Discussion and future directions

The diagnosis of scrub typhus is difficult due to five reasons: 1) acute fever in Southeast Asia, South Asia, and elsewhere in the tropics has a wide range of differential diagnoses, 2) the clinical presentation of scrub typhus in those settings is non-specific, 3) *O. tsutsugamushi* is an intracellular bacterium, 4) the bacteria shows significant antigenic and geographical diversity, and 5) currently available diagnostic techniques for the infection require expertise and resources. The first three are unalterable. The fourth may possibly be overcome by combining antigens of multiple strains or employing conserved antigen(s). The fifth can be solved by utilising simpler techniques if promising antigens can be identified.

Serological tests for scrub typhus diagnosis

The challenge in developing serological tests for scrub typhus diagnosis is balancing the two properties of *Orientia* antigens: antigenicity vs conservation. This study tried to develop a serological test with a single conserved antigen to diagnose the infection of this diverse organism. An IgM ELISA with a recombinant antigen of 101 amino acids detected 70% of scrub typhus patients, and an IgM ELISA with a peptide of 20 amino acids detected half of them. The results demonstrated the potential of oligopeptides as antigens for diagnostic tests for scrub typhus. The combination of similarly antigenic and conserved peptides may yield improved sensitivity. One restraint of this study is the sole focus on one domain of one membrane protein. In order to identify multiple conserved antigenic regions, it would have been more efficient to screen many outer membrane proteins.

The combination of the conserved antigens has also been recommended and attempted for vaccine and diagnostic test development (143). *Orientia* outer membrane proteins that have been reported to contain antigenic domains include TSA56, TSA47, TSA22, heat shock protein, ScaA-E, and a newly identified 27kDa protein (69, 224). Even though TSA56 is known to be antigenically diverse, this most immunodominant protein occupying one-fifth of the organism's proteasome would need to be incorporated into the combination. Kim HI et al. demonstrated that the immunisation with the recombinant antigen of concatenated conserved blocks of TSA56 provided protective immunity against not only the homologous but also heterologous infections (144). Seong et al. previously reported human IgM antibody binding to the AD I and AD III and IgG antibody binding to the AD I (56). The AD I overlaps with the conserved block 1 defined by Kim HI et al. and the AD III partly with conserved blocks 3 and 4. The conserved blocks of TSA56 may become a promising candidate for

scrub typhus serological tests as well as for vaccines. It is up to the future understanding of the differences in the gene and protein expression between intracellular and extracellular *O. tsutsugamushi* populations (74); the timing and location of the proteins' expression might become necessary to be considered.

Here, one approach to develop a serological tests with conserved peptide antigens is suggested. Firstly, an overlapping peptide screening will investigate amino acid sequences of the conserved regions in the candidate proteins. Bioinformatics technology has been used for the B-cell linear epitope prediction of infectious diseases, for instance, malaria and COVID-19 (225, 226). However, it is of note that the currently available predictors are still developing, and the sensitivity and specificity of the prediction are suboptimal (146, 227). The screening process may be done with a dot-blot or an ELISA technique.

Then, identified peptide antigens could be considered used in combination. It is often difficult to simultaneously fix multiple oligopeptides on solid surfaces displaying their epitopes (215). To circumvent this problem, peptides may be conjugated to carrier proteins. However, these carrier proteins are considerably larger than the peptides and might fold the conjugated peptide altering the peptide's function (216). In fact, the performance of a ScaA peptide ELISA was worse when the peptides were mixed or conjugated to BSA in this Ph.D. study. Instead of handling multiple oligopeptides, there have been attempts to use chimeric antigens. Kim YJ et al. produced a chimeric recombinant TSA56 protein derived from the Karp, Kato, and Gilliam strains and developed an LFA (228). The assay reportedly had very high sensitivity and specificity. Namsa et al. recently published a computational analysis of multi-epitope chimeric antigens for vaccines and diagnostic tests (229). They bioinformatically predicted linear and conformational B-cell epitopes and T-cell epitopes of TSA56 protein and the ScaA passenger domain, constructed multi-epitope chimeric antigens, and successfully cloned the antigens into a pET-15b vector, and expressed in *E. coli* BL21 (DE3). Their recombinant chimeric antigens are yet to be experimentally validated.

If promising antigens are validated, it might be possible to produce POCTs with simpler serological techniques such as an LFA. An ELISA format is still too time-consuming, laborious, and expensive in many parts of the world where laboratory testing capacity is lacking (230, 231). The turn-around time of an ELISA is at least a few hours, even with a pre-coated and blocked plate. Most scrub typhus case is considerably cheaper to treat than to diagnose unless it is causing severe disease.

Orientia tsutsugamushi is an intracellular bacterium; therefore, some may argue that serological tests may not be the best option to detect the infection with it. Diagnostic tests that detect cell-

mediated immunity are useful for some infectious diseases such as an IFN- γ release assay for tuberculosis. However, they are unlikely to be suitable for diagnosing acute fever in the tropics. Diagnostic tests need to be accurate, simple, affordable, and timely. The WHO has advocated the necessary standards for diagnostic tests named ASSURED (affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free and deliverable to end-users) and the revised REASSURED with addition of real-time connectivity and ease of specimen collection (232). As discussed in Chapter 2.3. and illustrated by the ScaA-based tests in this study, serological tests are subject to inherent limitations: the inability to detect an infection in the early phase of illness, the necessity of paired samples, background immunity, and non-specific reactions. Nevertheless, serological tests are and will be essential tools in clinical practices.

Evaluation of a new diagnostic test for febrile illnesses in the tropics

To evaluate a new diagnostic test, we need to choose the right population, apply necessary diagnostic tests, perform appropriate statistical analysis, and interpret the results correctly.

The first step is choosing the right population. In the case of a diagnostic test for scrub typhus, the day of illness, the case mix, and the availability of paired samples need to be considered. Day of illness is always a key information in diagnosing patients with febrile illnesses. The sensitivity of cultures, NAATs, and antibody detection tests changes as the disease progresses. Cultures and NAATs are useful in the early phase of illness, and antibody detection tests are in the later phase. Disease severity also evolves, and test sensitivity is higher in severe cases than in mild cases. A use of paired samples is widely recommended for serological tests. The availability of convalescent samples may improve the possibility to diagnose or exclude scrub typhus in patients who could not be diagnosed on a single sample. Ideally, the study population needs to reflect the full spectrum of all aspects of the disease. However, choosing the right population is not straightforward. The factors discussed are intertwined with each other, and the study setting plays a key role (Figure 46).

Many clinical studies are implemented in a referral hospital setting where human and material resources are available, and the prevalence of the disease is measurable. Patients may be referred from the primary healthcare setting because their condition is severe, and the referral process takes time. Patients with a severe condition are more likely to stay in a hospital for a long time and come back for a follow-up visit, therefore providing a convalescent sample. As a result, the sensitivity of diagnostic tests may be estimated to be higher in a referral setting (183). On the other hand, cultures and NAATs can have false-negative results outside the window of bacteraemia or viraemia

and/or due to an exposure to antimicrobials before the test. Antimicrobials are sold without prescription or healthcare workers' supervision in many parts of the world (233). In a referral setting where more patients may be pre-exposed to antimicrobials, sensitivities of such tests can be underestimated. In addition, patients with typical clinical signs are likely to be diagnosed in a primary setting, and patients without those signs are more likely to be referred to higher-level care. The sensitivity of the presence of a pathognomonic symptom can be therefore underestimated in referral settings (183).

In this study, the patients were from two cohorts enrolled at the identical tertiary hospital in Hanoi at different times. Interestingly, sensitivities of an IgM IFA, a recombinant ScaA-based IgM ELISA and the presence of eschar were different in the two cohorts, possibly reflecting the improvement of awareness of scrub typhus among local healthcare workers over time in the area. Improved awareness at the primary healthcare level must have resulted in early diagnosis of the disease and a reduced number of severe cases and case referrals to the hospital. The information on how patients were seen and referred in the study setting was vital to interpret the result.

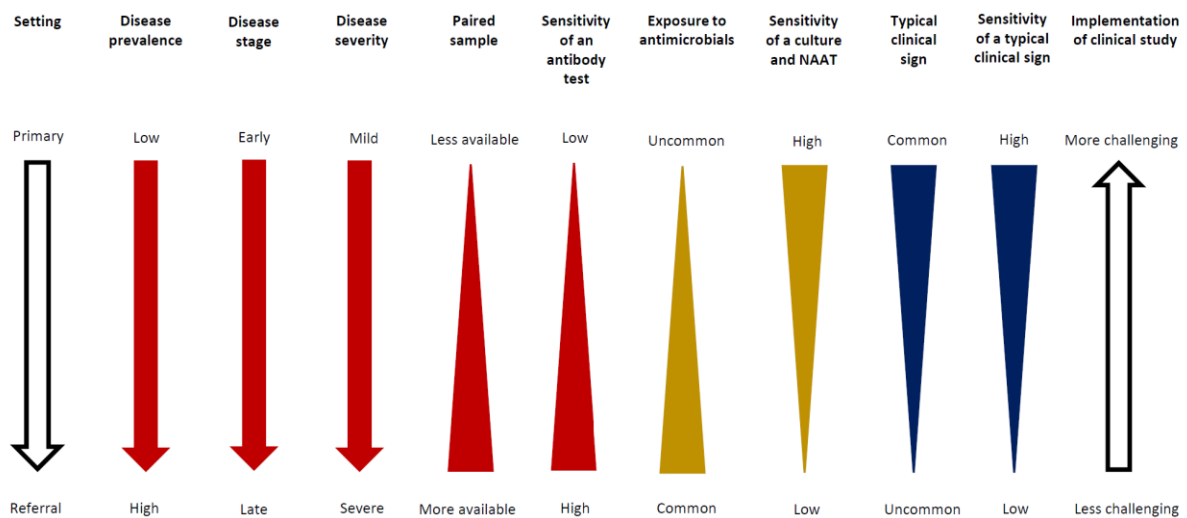


Figure 46. Schematic representation of the factors that affect sensitivities of diagnostic tests for acute febrile illnesses.

The second step is applying necessary diagnostic tests, but what are the 'necessary' diagnostic tests? Preferably, we need to apply diagnostic tests detecting all aspects of the target disease (174). In infectious diseases, cultures, NAATs, antigen and antibody detection tests, and pathognomonic symptoms are the main ones. Biomarkers, such as a cerebrospinal fluid cell count and glucose level

for bacterial and viral meningitis, might also be included. The limited availability of PCR assay results in this study may have significantly impacted the final results. DNA samples were not available for the rickettsiosis cohort, and no patient was diagnosed as scrub typhus only by a PCR assay in the fever cohort, although the test was applied. Due to the limited information on the infecting genotypes, there was insufficient data on the extent to which the conserved ScaA antigen was able to detect *O. tsutsugamushi* infection regardless of the infecting strains. Paired samples were unavailable in many cases in this study and an IgM IFA was applied to a single sample per patient. The composite reference standard should have been as robust as possible to evaluate a new test.

The third step is the appropriate statistical analysis. In this study, the sensitivity and specificity of a new test were estimated by Bayesian LCMs. The model assumed that none of the diagnostic tests was perfect. Informative priors were obtained from the available literature, including the knowledge that the sensitivity and specificity of an IgM IFA of a single sample might not be very high. However, the estimated results were not very different from the results assuming an IgM IFA and/or a PCR assay as gold standard tests, especially in the rickettsiosis cohort. The sensitivity and specificity of an IgM IFA estimated with informative priors were high. This may have been because all necessary diagnostic tests could not be applied as discussed. The study by Lim et al. that showed the sensitivity and specificity of an IgM IFA were lower than previously thought included some cases only detected by PCR assays (132). Appropriate statistical analysis is essential, but still unable to compensate for biases introduced at the stage of choosing the right population and applying all necessary tests.

The last step is the interpretation of the result. Test properties need to be interpreted in clinical scenarios where the test is expected to be used. This study used an LR+ and LR- linking sensitivity, specificity, and the expected scrub typhus prevalence among patients with acute fever. The interpretation of an LR+ and LR- is still highly multifactorial and contextual. In the clinical management of scrub typhus, having a safe, affordable, and effective therapy, clinicians might start empirical treatment with a relatively high degree of uncertainty. It is ultimately up to the question each clinician tries to answer with the test, together with other information. Multiple LR+ can be offered to multiple slices of test results to aid the clinicians' complex decision-making process. This is what clinicians often intuitively do. The additional information on how sure they can be with a positive test result will affect how rigorously they will still look for other differential diagnoses. A diagnostic test with a high LR+ and low LR- is naturally assessed as a good test. However, it does not directly translate into improved clinical practices and patients' outcomes (234). Evaluation of the clinical utility and cost-effectiveness is vital to understand how useful a new diagnostic test is in real-life practices. The tests need to be used to help patients and healthcare workers

Diagnosis of febrile illnesses in the tropics

This Ph.D. study challenged the development and evaluation of a diagnostic test for scrub typhus. Similar challenges lie ahead for other major causes of fever in the tropics, such as leptospirosis, typhoid fever, and Japanese encephalitis. Leptospirosis especially shares many diagnostic challenges in common: its clinical presentation is non-specific; *Leptospira* spp. has many serovars and serogroups; and the so-called gold standard microscopic agglutination test (MAT) is imperfect (170, 235). The MAT needs to include locally circulating serovars as antigens, which requires information on circulating serovars in a given area and maintenance of live leptospires of those serovars in the laboratory. The MAT cut-off titre also needs to be determined locally.

For comprehensive management of acute febrile illnesses, host biomarkers that might differentiate bacterial from non-bacterial causes have been evaluated, for example, white blood cell count, neutrophil count, CRP, and procalcitonin (236). The expert panel proposed a TPP for such tests, including target sensitivity and specificity of 90% and 80% (237). Lubell et al. evaluated the performance of CRP and procalcitonin in detecting bacterial infections among patients with acute fever in Cambodia, Laos, and Thailand (238). The sensitivity and specificity of CRP >10 mg/L to detect bacterial infections were 95% and 49%. The NIDIAG network reported that the sensitivity and specificity of CRP >10 mg/L to detect bacterial infections were 76.3% and 47.8% among patients with persistent fever for seven days or longer in Cambodia, the Democratic Republic of Congo, Nepal, and Sudan (239). These studies demonstrated that CRP had a low LR+ to differentiate bacterial from non-bacterial causes: an LR+ of <2.0 with a cut-off value of >10 mg/L and an LR+ of <3.0 with a cut-off value of >20 mg/L. Due to the low specificities of these biomarkers, they are not good at ruling-in bacterial infections but may be useful to guide antimicrobial therapy. However these biomarker tests will not replace pathogen-specific diagnostic tests. Some of the causative pathogens, for example rickettsiosis, Q fever, and brucellosis, require specific clinical management (7, 240).

When clinicians examine febrile patients, the question is not just whether a patient has one particular disease but why he/she is sick and how he/she can be treated. Answering whether a patient has scrub typhus is not enough. If it is not scrub typhus, then what is it? To cover the wide range of differential diagnoses of acute febrile illnesses in a cost-effectively and logistically feasible manner, multiplex platforms have been considered (240). A recent international modified Delphi survey identified priority analytes for multiplex POCTs to diagnose acute non-malarial fever in rural Southeast and South Asia stratified by age and care setting (17). Dengue non-structural protein 1 and *Salmonella typhi* antigens became the top two priorities in both primary and secondary care settings and for paediatric and adult patients across all regions. An *Orientia* antigen also ranked in

the top seven analytes in mainland Southeast Asia. Although currently available POCTs are mostly based on serological techniques, simplified multiplex PCR assays have been developed, too. The BioFire Global Fever Panel is an automated multiplex PCR assay with the FilmArray system and is able to detect *Leptospira* spp., chikungunya virus, dengue virus, and *Plasmodium* spp. in whole blood in one hour (241).

A diagnostic test is seldom used in isolation. These tests would be used in combination and/or incorporated into the local management algorithm. Clinicians bring together all available information on clinical history, physical examination findings, and laboratory results and make the best diagnosis possible in a given setting. In practice, it is often impossible to confirm diagnoses within a limited time frame. This process is almost like craftsmanship, especially when there is a wide range of differential diagnoses and diagnostic resources are scarce. Diagnostic tests need to be evaluated in such clinical pathways. The evaluation of real-life clinical utility and cost-effectiveness is therefore essential. However, it is not normally reflected in TPPs for diagnostic tests, and only the analytical performance of a new test is reported (177). This is understandable because the assessment of clinical utility is extremely challenging, and moreover maybe different in different environments. The impact of diagnostic tests on patients' health outcomes is often indirect, unlike that of therapeutic interventions (242). The process of evaluating diagnostic tests for acute febrile illnesses in a variety of circumstances remains challenging and interesting.

17. Impact of the COVID-19 pandemic on the Ph.D. study

The COVID-19 pandemic significantly impacted this Ph.D. study. Firstly, I took an interruption of studies during February to May 2020 and worked as a full-time clinician. I engaged in the following activities: 1) the infection prevention and control (IPC) services at the quarantine centre in Chiba for the Japanese returnees from Wuhan between 8 and 10 February upon request from NU; 2) the IPC services for the COVID-19 outbreak on the cruise ship Diamond Princess at Yokohama harbour on 22, 24, and 25 February, upon request from the National Center for Global Health and Medicine (NCGM); 3) clinical services at the NCGM outpatient clinic to take care of COVID-19 patients from 4 March to 19 April; and 4) the IPC services for the COVID-19 outbreak on the cruise ship Costa Atlantica at Nagasaki harbour between 6 and 31 May, upon request from Medecins Sans Frontieres Japan. After this period, I continued working part-time in a clinic in Yokohama city providing the COVID-19 vaccine and travel-related vaccines until December 2021. In addition, from September 2021 to April 2023, I was asked to provide a technical and operational assistance on the COVID-19 clinical management and health system strengthening at the WHO Regional Office for the Western Pacific.

Secondly, I needed to adjust my research plan. I had to use the blood samples that were already stored at NU. Therefore, the number of samples was short of the calculated sample size. I also had to give up on projects to apply the new ScaA-based test to sample sets of 1) patients enrolled in an observational study of community acquired-bacteraemia study at San Lazaro Hospital, Manila, the Philippines and 2) febrile patients during and/or after international travel seen at NCGM, Tokyo, Japan.

18. Conclusions

A recombinant ScaA-based IgM ELISA was developed with a recombinant 101-amino acid fragment of the ScaA ATD that had a high amino acid sequence identity of >90% across *Orientia* strains except for the Shimokoshi strain. The estimated sensitivity and specificity of a recombinant ScaA-based IgM ELISA were approximately 70% and 95%. The antibody response against the recombinant ScaA protein was slower to develop than that against TSA56. Narrowing down the epitope region reduced the test sensitivity but increased the specificity. A ScaA peptide IgM ELISA with one of the overlapping peptides had a sensitivity and specificity of 50% and 99%. The use of oligopeptide antigens posed additional challenges to optimise ELISA conditions. Overall, the sensitivities of the ScaA serological tests were not sufficient. This lack of sensitivity may have been due to the low antigenicity of ScaA and the quantity, timing, and location of its expression in the course of infection. Despite the insufficient sensitivities, the potential of the use of conserved peptide antigens was demonstrated. The sensitivity of the peptide-based test may improve by combining it with other conserved antigens. Through the evaluation of a new diagnostic test for scrub typhus in two cohorts previously collected in Vietnam, it was highlighted that the setting where a new test was evaluated could affect the sensitivity and specificity of the test.

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APPENDICES

Appendix 1. Experiment results with ScaA ATD antigens. All data was provided by Dr Miura K (178).

1.1 Amino acid sequences of the ScaA-ATD-A, B, and C proteins.

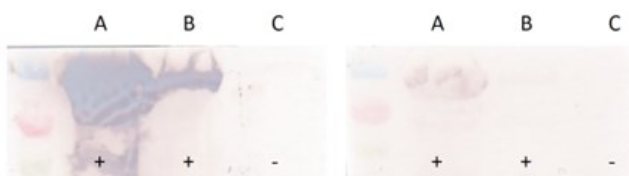
Amino acid sequences highlighted in grey were overlapped with the neighbouring antigens.

```
A NINKQSWNIWADGFFSNVNQQDHENIQGYKTDISGIPIGADKHLKNNAIIGAAISYAKFDTKHTDSRIEKINSNVYLLSLYGQYSFQSTTFIRGMVNVAKF
B FIRGMVNVAKESDDSKNSSLTWQGHSHYHGSITAGHYFYPLKNNKLTLPVPTVGIRHSYFNTSGNNSVDSSSNKTIGDRSHKALEGIIIGISLEQLVANNAN
C SLEQLVANNANNNLNVLSTVYGYVHHNLYDCQDSAQLPNSNSTFNPDVITAHEQCLHKTIFYQLGVKLAIKRNIMDIGIACDVYLAEKYISHTGIIYAKASF
```

1.2. Western blotting analysis of the ScaA-ATD-A and B proteins.

Left panel: ScaA-ATD-A and B proteins were recognised by anti-6xHistidine tag monoclonal antibody (1:1000 dilution; 015-23094, Wako Inc).

Right panel: ScaA-ATD-A and B proteins were recognised by hyperimmunised mouse serum (1:500 dilution).



1.3. Amino acid sequences of the ScaA-ATD-A1, A2, B1, and B2 proteins.

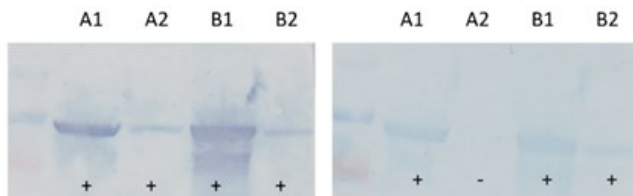
Amino acid sequences highlighted in grey were overlapped.

```
A1 NINKQSWNIWADGFFSNVNQQDHENIQGYKTDISGIPIGADKHLKNNAIIGAAISY
A2 NNAIIGAAISYAKFDTKHTDSRIEKINSNVYLLSLYGQYSFQSTTFIRGMVNVAKF
B1 FIRGMVNVAKESDDSKNSSLTWQGHSHYHGSITAGHYFYPLKNNKLTLPVPTVGIR
B2 KLTLPVPTVGIRHSYFNTSGNNSVDSSSNKTIGDRSHKALEGIIIGISLEQLVANNAN
```

1.4. Western blotting analysis of the Sca-ATD-A1, A2, B1, and B2 proteins.

Left panel: ScaA-ATD-A1, B1, and B2 proteins were recognised by an anti-6xHistidine tag monoclonal antibody (1:1000 dilution; 015-23094, Wako Inc).

Right panel: ScaA-ATD-A1, B1, and B2 proteins were recognised by hyperimmunised mouse serum (1:500 dilution).

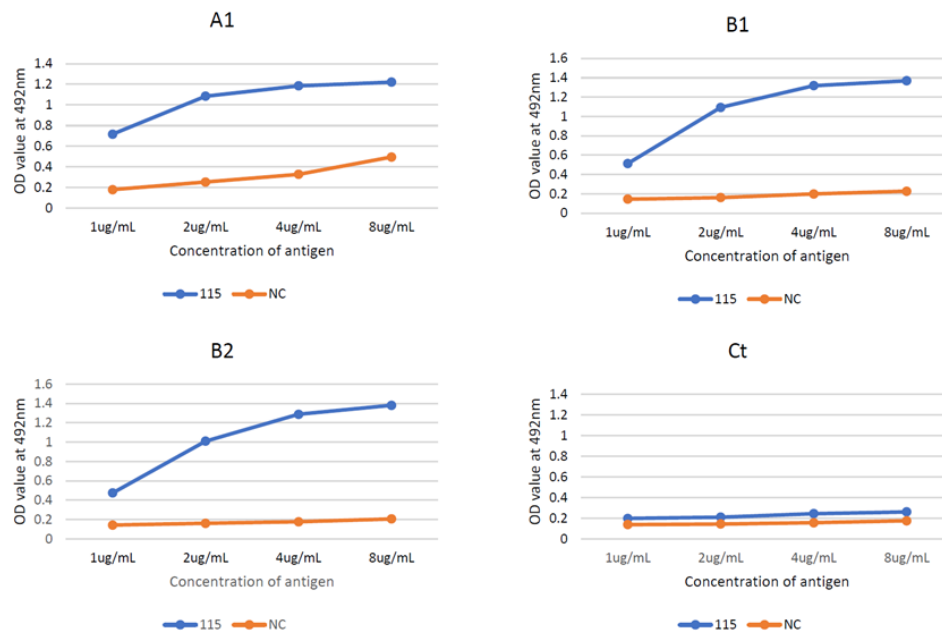


1.5. A brief description of the protocol of an IgG ELISA with recombinant ScaA-ATD-A1, -B1, and -B2 proteins.

Nunc MaxiSorp microplates were coated with 100 μ l/well of the purified antigen (ScaA-ATD-A1, B1, and B2) and the control antigen in bicarbonate buffer. A control antigen was synthesised using an empty pET43.1a(+) vector. A washing buffer was 0.05% Tween-20 in PBS, the blocking buffer was 3% skim milk in PBS, and the dilution buffer was 1% skim milk in PBS. Serum samples were diluted to a concentration of 1:100. Secondary antibodies were a goat anti-human IgG-HRP conjugate for human samples and a goat anti-rat IgG-HRP conjugate for rat serum samples. 200 μ l/well of SIGMAFAST OPD (Sigma-Aldrich) was applied to each well, and after 30 minutes, 50 μ l/well of 3 M hydrochloric acid was added to stop the reaction. ELISA OD values were measured with a plate reader at 492nm.

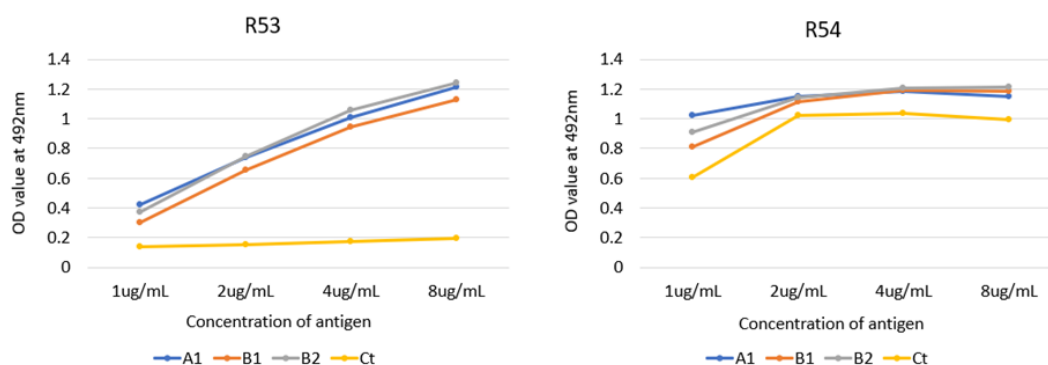
1.6. Rat IgG antibody responses against ScaA ATD antigens and control antigen.

Two rat samples were applied to an IgG ELISA with ScaA-ATD-A1, B1, and B2 proteins and a control antigen (Ct). A positive control sample (115) was confirmed to have *O. tsutsugamushi* infection by a TSA56-based nested PCR assay and an IgG IFA. A negative control (NC) sample was withdrawn from a conventional rat.



1.7. Human IgG antibody responses against indicated ScaA ATD antigens and a control antigen.

Two samples of scrub typhus patients (R53 and R54) were applied to an IgG ELISA with ScaA-ATD-A1, B1, and B2 proteins and a control antigen (Ct). These samples were from patients with a positive result for a TSA56-based nested PCR assay and an IgM IFA titre of $\geq 1:1280$. Patient R54 had high OD values for both ScaA proteins and a control antigen.



56	<10	0.1517	0.069	0.1315	0.0904	-0.1803	-0.1849	-0.1739	-0.2214	-0.236	-0.1783	-0.1119	-0.2155	-0.1976
57	<10	0.1106	0.0902	0.1616	0.1225	0.046	0.2575	0.2186	-0.1594	-0.112	-0.2144	-0.1202	-0.0761	-0.0927
58	<10	0.2127	0.1047	0.0868	0.2079	-0.029	-0.0258	-0.022	-0.0777	-0.0642	-0.0476	-0.0497	-0.0492	-0.0261
59	<10	0.0899	0.1437	0.1207	0.0904	-0.0359	-0.0402	-0.0434	-0.0645	-0.0839	-0.062	-0.0485	-0.0604	-0.0234
60	<10	0.1679	0.1092	0.1222	0.0919	-0.0889	-0.0933	-0.1046	-0.1142	-0.1274	-0.1112	-0.086	-0.1031	-0.1139
61	<10	0.0908	0.0741	0.0961	0.0894	-0.077	-0.0775	-0.0734	-0.0897	-0.0886	-0.0789	-0.0517	-0.0854	-0.0566
62	<10	0.1354	0.07	0.1066	0.0817	-0.2396	-0.2225	-0.174	-0.2055	-0.2267	-0.2497	-0.1962	-0.2391	-0.2017
63	<10	0.1566	0.0998	0.1218	0.0756	-0.2033	-0.2144	-0.2249	-0.2557	-0.2663	-0.1989	-0.0986	-0.237	-0.2327
64	<10	0.1904	0.0922	0.1619	0.1773	-0.1428	-0.1549	-0.1561	-0.1824	-0.2097	-0.1503	-0.1207	-0.1681	-0.0946
65	<10	0.1644	0.1074	0.1372	0.1354	-0.1184	-0.1442	-0.1215	-0.1776	-0.193	-0.2161	-0.137	-0.1901	-0.1459
66	<10	0.1097	0.143	0.0867	0.094	-0.1043	-0.1289	-0.1292	-0.068	-0.1639	-0.1177	-0.0866	-0.132	-0.1017
67	<10	0.1656	0.0879	0.185	0.1177	-0.2083	-0.1992	-0.1752	-0.2272	-0.2495	-0.2048	-0.1316	-0.2191	-0.0551
68	<10	0.1459	0.2054	0.1083	0.0958	-0.1152	-0.1243	-0.1029	-0.1575	-0.1541	-0.1358	-0.0951	-0.1497	-0.1418
69			0.0824		0.1246	-0.0925	-0.1093	-0.111	-0.1418	-0.1417	-0.1286	-0.0493	-0.1149	-0.1165

Appendix 3. IgM IFA results of 20 febrile patients with clinically confirmed diagnosis other than scrub typhus.

Clinically confirmed diagnosis	IgM IFA titre
HIV infection and tuberculosis	80*
HIV infection and tuberculosis	<10
HIV infection and tuberculosis	<10
Pulmonary tuberculosis	<10
Pulmonary tuberculosis	<10
Pulmonary tuberculosis	40**
Tuberculous meningitis	<10
Tuberculous meningitis	<10
<i>Streptococcus pneumoniae</i> meningitis	<10
<i>Streptococcus suis</i> meningitis/bacteraemia	<10
<i>Staphylococcus aureus</i> endocarditis	10***
<i>Staphylococcus aureus</i> bacteraemia	<10
<i>Enterococcus</i> spp. bacteraemia	<10
<i>Haemophilus influenzae</i> bacteraemia	<10
<i>Escherichia coli</i> bacteraemia	<10
<i>Klebsiella pneumoniae</i> bacteraemia	<10
Dengue	<10
Dengue	<10
Dengue	<10
Japanese encephalitis	<10

The diagnoses of 17 patients except for those with dengue and Japanese encephalitis were bacteriologically confirmed.

*A positive result for the Karp strain. ** A positive result for the Gilliam strain. *** A positive result for the Karp strain.

Appendix 4. Protocols for the expression and purification of a recombinant ScaA protein with a pET43.1a(+) vector and an IgM ELISA.

4.1. Expression of the ScaA gene fragment.

A nucleotide sequence of the ScaA gene fragment of the *O. tsutsugamushi* Gilliam strain (GenBank accession number LC720723.1) was amplified by a PCR assay using the forward and reverse primers (Table 6). The amplified fragment was digested using *EcoR* I and *Hind* III restriction enzymes and then cloned into the *EcoR* I and *Hind* III site of a pET43.1a(+) vector (Novagen, Madison, WI). The partial ScaA gene fragment insert was confirmed by DNA sequencing.

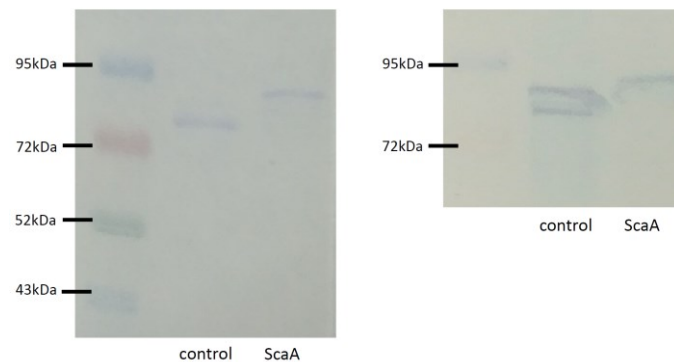
4.2. A recombinant ScaA protein purification protocol.

1. Overnight culture	<ul style="list-style-type: none"> - Autoclave an LB medium. - Add ampicillin to a concentration of 100 µg/ml into the medium. - Pick 1-2 colonies of <i>E.coli</i> BL21 (DE3) of an expressed strain and place into the medium. - Incubate the mediums with shaking at 37 °C overnight.
2. Expansion of starter culture	<ul style="list-style-type: none"> - Centrifuge the overnight culture medium (2,500 g, 10 °C, 10 minutes). - Discard the supernatant. - Add the deposit into an autoclaved LB medium until the O.D.₆₀₀ value of the medium reaches 0.2. - Incubate with shaking at 37 °C for 1-2 hours until the O.D.₆₀₀ value of the culture reaches 0.7-0.8.
3. Induction	<ul style="list-style-type: none"> - Add isopropyl β-D-thiogalactopyranoside to a final concentration of 0.8 mM to the culture. - Shake the culture overnight at 25 °C. - Centrifuge the culture (2,500 g, 10 °C, 10 minutes). - Collect the deposit and resuspend in 1/20 volume of the culture of the binding buffer (20 mM Tris, 50 mM NaCl, 40 mM imidazole, pH 8.0).
4. Sonification	<ul style="list-style-type: none"> - Sonicate the sample on ice for 5 minutes. - Centrifuge the sample (10,000 g, 4 °C, 10 minutes). - Collect the supernatant.
5. Elution	<ul style="list-style-type: none"> - Apply the supernatant to His GraviTrap (GE Healthcare). - Wash the column three times with the binding buffer. - Apply 2ml of the elution buffer (20 mM Tris, 50 mM NaCl, 500 mM imidazole, pH 8.0) and collect the elute.
6. Removal of	<ul style="list-style-type: none"> - Apply the elute to HiTrap Desalting column (GE Healthcare)

imidazole	- Apply the imidazole-free binding buffer (20 mM Tris, 50 mM NaCl, pH 8.0) to the column and collect the flow-through.
------------------	------------------------------------------------------------------------------------------------------------------------

4.3. Analysis of the purified recombinant ScaA protein.

Left panel: The recombinant ScaA protein and control protein were resolved by SDS-PAGE and stained with Coomassie Blue. The estimated molecular weights of the recombinant ScaA protein and the control protein were 74kDa and 66kDa. Right panel: The Western blot analysis showed that both proteins were recognised by anti-Histidine monoclonal antibody.



4.4. A protocol for an IgM ELISA using a recombinant ScaA protein purified with a pET43.1a(+) vector.

1. Coat an immunoassay plate (MaxiSorp 96-well flat-bottom plates; Nunc) in duplicate with 100 μ l/well of
 - the recombinant ScaA protein at a concentration of 1 μ g/ml in PBS
 - PBS (= non-coated wells for measurement of the BGN reaction)
2. Incubate the plate at 4 $^{\circ}$ C overnight.
3. Wash the plate with a washing buffer (PBS-T: 0.05% Tween-20 in PBS) five times.
4. Block the wells with a blocking buffer (3% skim milk in PBS) at room temperature (20-25 $^{\circ}$ C) for 1 hour.
5. Wash the plate with the washing buffer five times.
6. Apply 100 μ l/well of sample serum diluted to 1:500 in a dilution buffer (1% skim milk in PBS) to non-coated and antigen-coated wells.
7. Incubate at room temperature for 1 hour.
8. Wash the plate with the washing buffer five times.
9. Apply 100 μ l/well goat anti-human IgM-HRP conjugate (QED Bioscience, Inc.) diluted to 1:10,000 in the dilution buffer.
10. Incubate at room temperature for 1 hour.

11. Wash the plate with the washing buffer five times.
12. Apply 200 μ l/well of SIGMAFAST OPD (Sigma-Aldrich) and allow the colour to develop at room temperature for 30 minutes.
13. Apply 50 μ l/well of 3 M hydrochloric acid and stop the reaction.
14. Measure the OD values at 492 nm with a plate reader (Thermo Scientific Multiskan FC).
15. Subtract the mean OD value of two blank wells from the mean OD values of all wells.
16. Subtract the mean OD value in the non-coated wells from the mean OD value in the antigen-coated wells of each test sample.

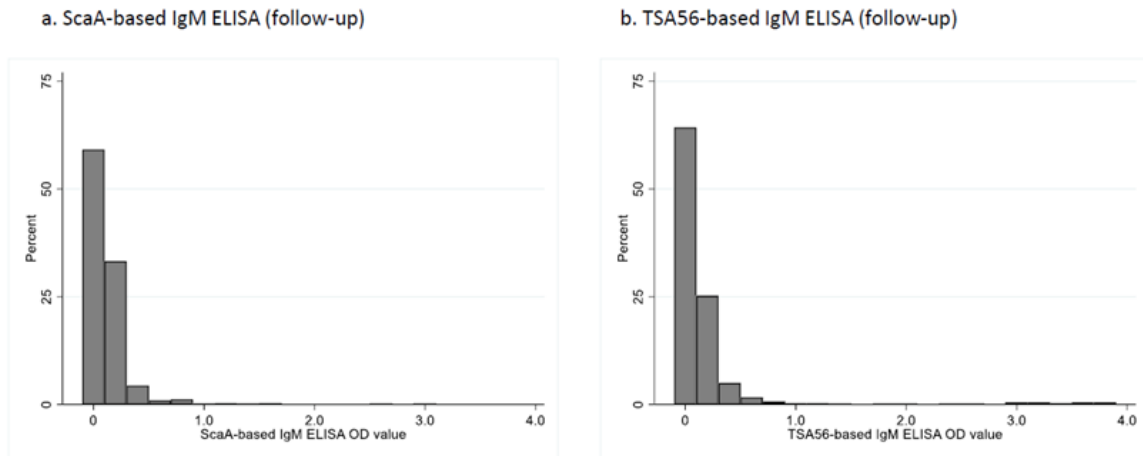
Appendix 5. The results of diagnostic tests for *O. tsutsugamushi* infection of 42 patients in the assay development cohort.

Diagnosis	Gold standard	Patient ID	Day of illness	TSA56 genotype	IgM IFA titer	Serotype	TSA56 ELISA		ScaA ELISA	
							IgM	IgG	IgM	IgG
scrub typhus	PCR + IFA -	VR02-R16 006	7	Karp	<10		0.11	0.4726	0.0755	0.1178
		VR02-R15 062	34	Karp	20		0.485	0.5322	0.0937	0.0863
		VR02-R16 057	8	Karp	40		0.541	0.5168	0.2071	0.0747
		VR02-R15 063	35	Karp	40		1.825	0.4298	0.1719	0.7065
	PCR +/- IFA +	VR02-R16 008	8	Gilliam	320	Gilliam	1.344	0.4579	0.1121	0.1062
		VR02-R16 037			160	Kato	1.644	0.2059	0.2885	0.1454
		VR02-R16 058	6	Karp	320	Karp	2.671	1.7585	0.2864	0.0798
		VR02-R16 053	17	Karp	>=1280	Kato/Gilliam	2.842	0.156	0.1482	0.0854
		VR02-R16 032	9		640	Gilliam	2.903	1.9915	0.1324	0.1092
		VR02-R16 052	11	Karp	>=1280	Gilliam	2.98	1.9065	0.3738	0.0958
		VR02-R16 030	5	TA763	320	Karp/Kato	3.016	1.9035	1.634	0.0988
		VR02-R16 054	8	Karp	>=1280	Kato	3.247	2.7348	0.5175	0.0935
		VR02-R16 050	9	Gilliam	>=1280	Gilliam	3.291	0.5067	0.9362	0.1713
		VR02-R16 055	7		640	Gilliam	3.336	0.1524	0.3603	0.0934
		VR02-R16 023	7		>=1280	Kato	3.346	4.2128	0.3661	0.1356
		VR02-R16 022	8	Karp	>=1280	Karp/Kato	3.352		1.841	0.3277
		VR02-R16 041	8	Karp	>=1280	Karp/Kato/Gilliam	3.383	4.7682	0.8412	0.1452
		VR02-R16 021	7		320	Karp/Kato	3.384	0.6805	0.161	0.0984
		VR02-R16 051	3		>=1280	Karp/Kato/Gilliam	3.386	0.1612	0.4883	0.3048
		VR02-R16 024	14	Karp	640	Karp/Kato	3.392		2.473	
		VR02-R16 056	6	Karp	>=1280	Karp/Kato	3.399	4.1662	1.875	0.1524
		VR02-R16 040	14		>=1280	Karp/Kato/Gilliam	3.404	4.2999	2.6277	0.3411
		VR02-R16 007	8	Karp	>=1280	Karp/Kato/Gilliam	3.442		2.364	
VR02-R16 039		-	>=1280	Karp/Kato	3.493	1.1353	1.0646	0.2707		
VR02-R16 049	12	Gilliam	>=1280	Gilliam	>3.5		0.602			
non scrub typhus	PCR - IFA -	VR02-R16 026	9		<10		0.074	0.3104	0.232	0.0507
		VR02-R16 018			<10		0.076	0.2939	0.0867	0.185
		VR02-R16 015			<10		0.081	0.2022	0.1288	0.0774
		VR02-R16 033			<10		0.086	0.1011	0.1517	0.0654
		VR02-R16 028	7		<10		0.088	0.0942	0.1245	0.0635
		VR02-R16 016			<10		0.099	0.1292	0.602	0.1003
		VR02-R16 014			<10		0.111	0.0997	0.0734	0.0822
		VR02-R16 048			<10		0.119	0.1935	0.383	0.0939
		VR02-R16 029			<10		0.13	0.0815	0.1487	0.0537
		VR02-R16 046			<10		0.181	0.1915	0.2198	0.1377
		VR02-R16 020			<10		0.184	0.1102	0.1138	0.0656
		VR02-R16 044			<10		0.184	0.082	0.362	0.0851
		VR02-R16 019			20		0.213	0.2428	0.2363	0.1059
		VR02-R16 045			<10		0.216	0.2666	0.2826	0.1016
		VR02-R16 017			<10		0.231	0.287	0.1118	0.1063
		VR02-R16 042			<10		0.3	0.0943	0.482	0.1056
		VR02-R16 043			10		0.356	0.1095	0.1933	0.0706

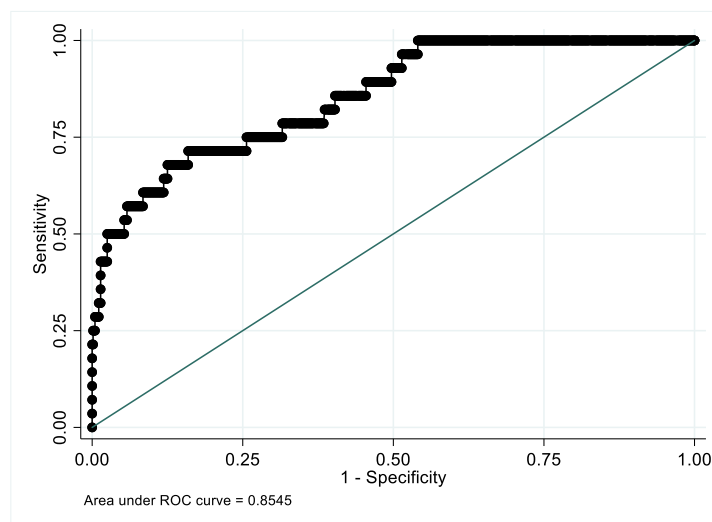
Grey: ten samples were used for choosing a blocking agent and an ELISA plate and setting the concentrations of an antigen and a secondary antibody; green: samples tested positive for a TSA56-based nested PCR assay; pink: samples tested positive for an IgM IFA; and blue: sample tested positive for a TSA56-based ELISA.

Appendix 6. The recombinant ScaA-based IgM ELISA results in the fever cohort.

6.1. Distribution of patients by ELISA OD values at follow-up in the fever cohort.



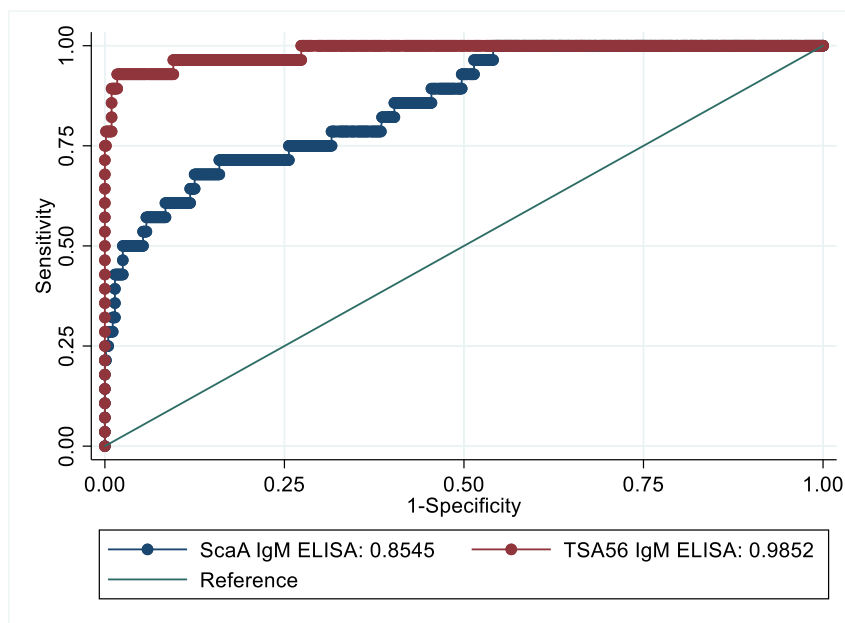
6.2. Receiver operating characteristics curve of a recombinant ScaA-based IgM ELISA in the fever cohort.



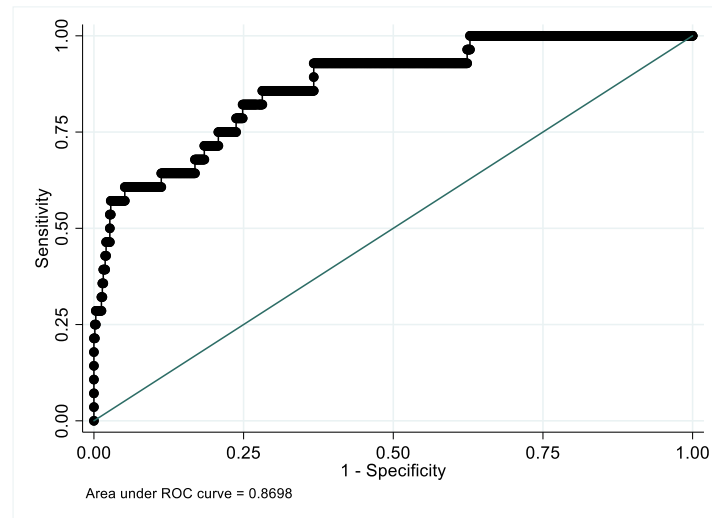
6.3. Sensitivity, specificity, and Youden index of five cut-off values for a recombinant ScaA-based IgM ELISA in the fever cohort

Cut-off value	Sensitivity (95% CI)	Specificity (95% CI)	Youden index
0.2	82.1% (63.1-93.9)	60.9% (57.0-64.7)	0.43
0.3	67.9% (47.6-84.1)	85.3% (82.3-88.0)	0.532
0.3721	57.1% (37.2-75.5)	93.8% (91.6-95.5)	0.509
0.4	53.6% (33.9-72.5)	94.4% (92.3-96.0)	0.48
0.5	50.0% (30.6-69.4)	97.3% (95.8-98.4)	0.473

6.4. Receiver operating characteristics curves of the recombinant ScaA-based and TSA56-based IgM ELISAs in the fever cohort.



6.5. Receiver operating characteristics curve of the recombinant ScaA-based IgM ELISA in the fever cohort when cut-off indices were used.



6.6. Sensitivity, specificity, and Youden index of five cut-off values for the cut-off index of a recombinant ScaA-based IgM ELISA in the fever cohort

Cut-off value	Sensitivity (95% CI)	Specificity (95% CI)	Youden index
0.1	75.0% (55.1-89.3)	79.2% (75.9-82.3)	0.542
0.2	60.7% (40.6-78.5)	93.1% (90.9-95.0)	0.538
0.25	57.1% (37.2-75.5)	95.9% (94.1-97.3)	0.53
0.3	57.1% (37.2-75.5)	96.9% (95.2-98.1)	0.54
0.4	46.4% (27.5-66.1)	98.0% (96.6-98.9)	0.444

Appendix 7. OpenBUGS code and the rickettsiosis cohort dataset for model 2 (conditional dependence between two serological tests). The code was based on the work by Lim et al. (132).

```
# Model 2: conditional dependence between two serological tests 1 & 2

model{
  for (i in 1:207){
    status[i]~dbern(prev)

    # Likelihood

    for (j in 1:4){
      y[i,j]~dbern(p[i,j])
      ypred[i,j]~dbern(p[i,j])
    }

    for (i in 1:207){
      for (j in 3:4){
        logit(p[i,j])<-status[i]*alpha[j]+(1-status[i])*beta[j]
      }
      for (j in 1:2){
        logit(p[i,j])<-status[i]*alpha[j]+(1-status[i])*beta[j]+status[i]*re[i]*Ab
        logit(pstatus[i,j])<-status[i]*alpha[j]+(1-status[i])*(-1000)+status[i]*re[i]*Ab
      }

      re[i]~dnorm(0,1)
    }

    # Prior

    prev~dbeta(0.5,0.5)
    Ab~dnorm(0.0,0.1)I(0,)

    for (j in 1:4){
      alpha[j]~dnorm(0.0,0.1)
      beta[j]~dnorm(0.0,0.1)I(,1)
      logit(s[j])<-alpha[j]
      logit(sp[j])<--beta[j]
    }

    se[3]<-s[3]
    se[4]<-s[4]

    for (j in 1:2){
      se[j]<-sum(pstatus[,j])/sum(status[])
    }

    # Prediction

    for (i in 1:207){
      for (k in 1:16){
        for (j in 1:4){
          arraymatched[i,k,j]<-equals(ypred[i,j],pattern[k,j])
        }
        nmatched[i,k]<-sum(arraymatched[i,k,])
        matchedpattern[i,k]<-equals(nmatched[i,k],4)
      }
    }

    for (k in 1:16){
      freqpred[k]<-sum(matchedpattern[,k])
    }
  }
}
```

```

#Bayesian p value

for (i in 1:207){
  for (k in 1:16){
    for (j in 1:4){
      arraymatchedobs[i,k,j]<-equals(y[i,j],pattern[k,j])
    }
    nmatchedobs[i,k]<-sum(arraymatchedobs[i,k,])
    matchedpatternobs[i,k]<-equals(nmatchedobs[i,k],4)
  }
}

for (k in 1:16){
  freqobs[k]<-sum(matchedpatternobs[,k])
}

for (k in 1:16){
  pvalue[k]<-step(freqpred[k]-freqobs[k])
}

# PPV and NPV

for (i in 1:207){
  for (j in 1:4){
    testpos[i,j]<-y[i,j]
    testneg[i,j]<-1-y[i,j]
    dispostestpos[i,j]<-status[i]*y[i,j]
    disnegtestneg[i,j]<-(1-status[i])*(1-y[i,j])
  }
}

for (j in 1:4){
  sumtestpos[j]<-sum(testpos[,j])
  sumtestneg[j]<-sum(testneg[,j])
  sumdispostestpos[j]<-sum(dispostestpos[,j])
  sumdisnegtestneg[j]<-sum(disnegtestneg[,j])
  ppv[j]<- sumdispostestpos[j]/sumtestpos[j]
  npv[j]<- sumdisnegtestneg[j]/sumtestneg[j]
}
}

```

```

# initial variables 1

list(prev=0.3, alpha = c(3,3,3,3), beta= c(0,0,0,0))

# initial variables 2

list(prev=0.3, alpha = c(1,1,1,1), beta= c(-1,-1,-1,-1))

# initial variables 3

list(prev=0.3, alpha = c(0,0,0,0), beta= c(-3,-3,-3,-3))

```

```

# Informative prior

prev~dbeta(0.5,0.5)
Ab~dnorm(0.0,0.1)I(0,)

for (j in 1:4){
  alpha[1]~dnorm(1.8,0.8)
  alpha[2]~dnorm(1.8,0.8)
  alpha[3]~dnorm(1.8,0.8)
  alpha[4]~dnorm(0.0,1.0)
  beta[1]~dnorm(-1.8,0.8)I(,1)
  beta[2]~dnorm(-3.0,0.4)I(,1)
  beta[3]~dnorm(-3.0,0.4)I(,1)
  beta[4]~dnorm(-3.0,0.4)I(,1)
  logit(s[j])<-alpha[j]
  logit(sp[j])<--beta[j]
}

se[3]<-s[3]
se[4]<-s[4]

for (j in 1:2){
  se[j]<-sum(pstatus[,j])/sum(status[])
}

```

```
#Data (1:ifa, 2:tsa, 3:sca, 4:esc)
```

```
list(y=structure(
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```
.Data=c(
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),
.Dim=c(207,4)),
pattern=structure(

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.Data=c(
0,0,0,0,
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1,1,1,0,
1,1,1,1
),
.Dim=c(16,4))

```

Appendix 8. Distribution of patients by a combination of test results observed and predicted by Bayesian latent class models.

8.1. Rickettsiosis cohort

IgM IFA	TSA56 IgM ELISA	ScaA IgM ELISA	Presence of eschar	Observed number of patients	Mean number of patients predicted by Bayesian latent class model (Bayesian p -value)			
					Non-informative priors			Informative priors
					Model 1	Model 2	Model 3	Model 3
-	-	-	-	128	125.8 (0.397)	125.8 (0.397)	125.7 (0.390)	125.2 (0.338)
-	-	-	+	1	1.444 (0.647)	1.431 (0.644)	1.422 (0.653)	1.617 (0.709)
-	-	+	-	4	4.855 (0.626)	4.827 (0.621)	4.944 (0.636)	4.368 (0.564)
-	-	+	+	0	0.073 (1.0)	0.125 (1.0)	0.077 (1.0)	0.132 (1.0)
-	+	-	-	4	4.737 (0.610)	4.701 (0.605)	4.764 (0.612)	4.301 (0.554)
-	+	-	+	0	0.250 (1.0)	0.259 (1.0)	0.453 (1.0)	0.696 (1.0)
-	+	+	-	1	0.344 (0.277)	0.348 (0.281)	0.309 (0.255)	0.527 (0.393)
-	+	+	+	0	0.536 (1.0)	0.554 (1.0)	0.412 (1.0)	1.139 (1.0)
+	-	-	-	2	2.670 (0.630)	2.655 (0.629)	2.662 (0.634)	3.769 (0.812)
+	-	-	+	1	0.473 (0.346)	0.485 (0.357)	0.934 (0.530)	0.882 (0.543)
+	-	+	-	1	0.464 (0.349)	0.474 (0.357)	0.374 (0.294)	0.613 (0.438)
+	-	+	+	0	1.199 (1.0)	1.228 (1.0)	0.917 (1.0)	1.479 (1.0)
+	+	-	-	5	4.039 (0.377)	4.08 (0.384)	3.954 (0.363)	4.058 (0.382)
+	+	-	+	12	13.320 (0.643)	13.31 (0.643)	12.88 (0.615)	11.81 (0.505)
+	+	+	-	9	10.680 (0.692)	10.72 (0.695)	10.89 (0.711)	11.63 (0.776)
+	+	+	+	39	36.070 (0.333)	35.96 (0.326)	36.26 (0.337)	34.8 (0.242)

8.2. Fever cohort

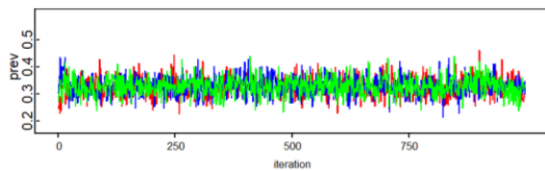
IgM IFA	TSA56 IgM ELISA	ScaA IgM ELISA	47kDa PCR	Presence of eschar	Observed number of patients	Mean number of patients predicted by Bayesian latent class model (Bayesian p -value)			
						Non-informative priors			Informative priors
						Model 1	Model 2	Model 3	Model 2
-	-	-	-	-	578	575.1 (0.423)	575.2 (0.426)	574.7 (0.410)	572.6 (0.332)
-	-	-	-	+	6	5.925 (0.495)	5.927 (0.496)	5.91 (0.493)	6.068 (0.515)
-	-	-	+	-	0	0.682 (1.0)	0.763 (1.0)	0.749 (1.0)	1.33 (1.0)
-	-	-	+	+	0	0.009 (1.0)	0.018 (1.0)	0.017 (1.0)	0.032 (1.0)
-	-	+	-	-	32	32.990 (0.551)	32.7 (0.538)	33.28 (0.565)	32.57 (0.531)
-	-	+	-	+	0	0.349 (1.0)	0.395 (1.0)	0.352 (1.0)	0.442 (1.0)
-	-	+	+	-	0	0.064 (1.0)	0.194 (1.0)	0.069 (1.0)	0.267 (1.0)
-	-	+	+	+	0	0.004 (1.0)	0.021 (1.0)	0.004 (1.0)	0.040 (1.0)
-	+	-	-	-	16	17.380 (0.594)	17.14 (0.579)	17.58 (0.603)	17.41 (0.596)
-	+	-	-	+	0	0.407 (1.0)	0.414 (1.0)	0.495 (1.0)	0.420 (1.0)
-	+	-	+	-	0	0.659 (1.0)	0.671 (1.0)	0.876 (1.0)	0.540 (1.0)
-	+	-	+	+	0	0.086 (1.0)	0.087 (1.0)	0.114 (1.0)	0.099 (1.0)
-	+	+	-	-	8	4.173 (0.105)	4.314 (0.107)	3.855 (0.085)	3.57 (0.052)
-	+	+	-	+	0	0.456 (1.0)	0.467 (1.0)	0.404 (1.0)	0.536 (1.0)
-	+	+	+	-	0	1.151 (1.0)	1.166 (1.0)	1.004 (1.0)	1.072 (1.0)
-	+	+	+	+	0	0.158 (1.0)	0.156 (1.0)	0.136 (1.0)	0.212 (1.0)
+	-	-	-	-	2	2.406 (0.594)	2.396 (0.592)	2.408 (0.593)	4.441 (0.872)
+	-	-	-	+	0	0.042 (1.0)	0.040 (1.0)	0.051 (1.0)	0.090 (1.0)
+	-	-	+	-	0	0.051 (1.0)	0.044 (1.0)	0.072 (1.0)	0.100 (1.0)
+	-	-	+	+	0	0.007 (1.0)	0.006 (1.0)	0.010 (1.0)	0.019 (1.0)

+	-	+	-	-	0	0.367 (1.0)	0.339 (1.0)	0.343 (1.0)	0.716 (1.0)
+	-	+	-	+	0	0.035 (1.0)	0.031 (1.0)	0.031 (1.0)	0.098 (1.0)
+	-	+	+	-	0	0.087 (1.0)	0.073 (1.0)	0.076 (1.0)	0.197 (1.0)
+	-	+	+	+	0	0.013 (1.0)	0.010 (1.0)	0.011 (1.0)	0.039 (1.0)
+	+	-	-	-	6	5.818 (0.508)	5.938 (0.527)	5.687 (0.489)	4.793 (0.354)
+	+	-	-	+	1	0.817 (0.523)	0.811 (0.522)	0.784 (0.510)	0.948 (0.585)
+	+	-	+	-	3	2.115 (0.351)	2.066 (0.339)	2.008 (0.325)	1.927 (0.305)
+	+	-	+	+	0	0.308 (1.0)	0.291 (1.0)	0.287 (1.0)	0.395 (1.0)
+	+	+	-	-	9	10.460 (0.704)	10.59 (0.721)	10.76 (0.738)	9.975 (0.662)
+	+	+	-	+	1	1.489 (0.726)	1.471 (0.723)	1.505 (0.731)	2.028 (0.838)
+	+	+	+	-	4	3.882 (0.519)	3.755 (0.499)	3.864 (0.519)	4.149 (0.573)
+	+	+	+	+	2	0.559 (0.119)	0.532 (0.111)	0.550 (0.116)	0.857 (0.214)

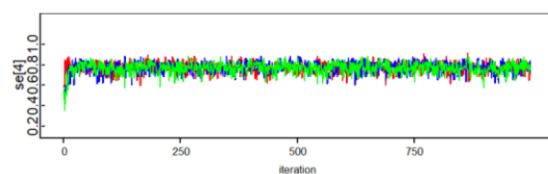
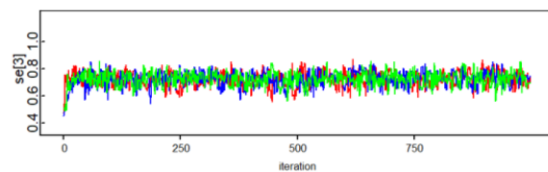
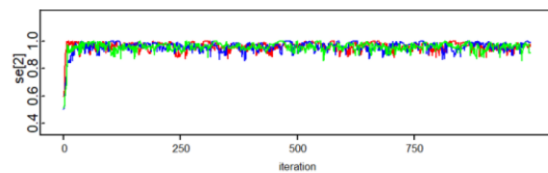
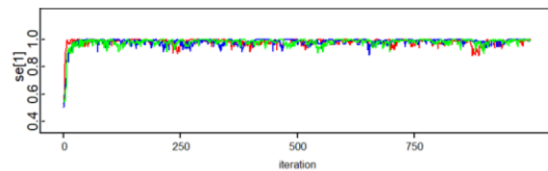
Appendix 9. History plots for a scrub typhus prevalence, test sensitivities, and test specificities on iterations 1-1,000 (rickettsiosis cohort) and on iterations 1-3,000 (fever cohort).

9.1. The rickettsiosis cohort with non-informative priors.

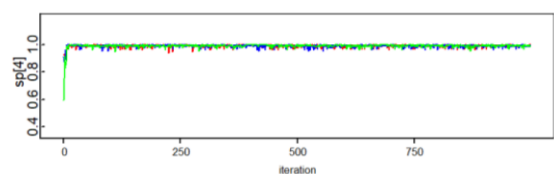
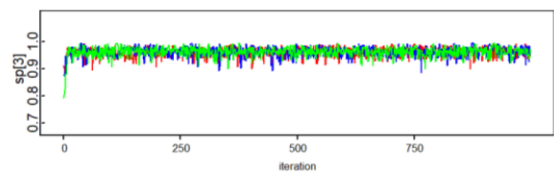
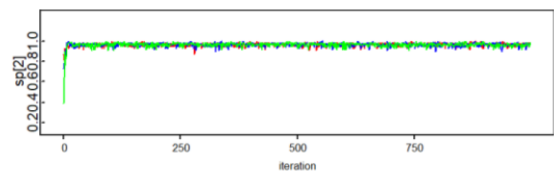
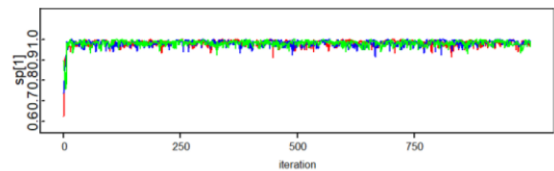
Prevalence



Sensitivity



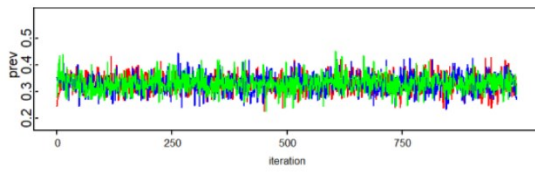
Specificity



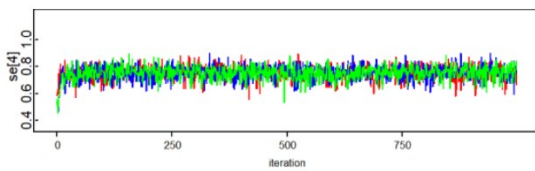
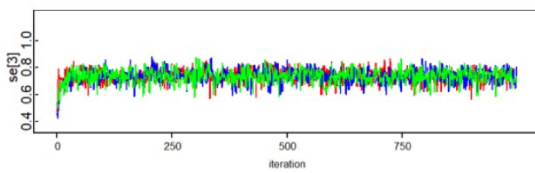
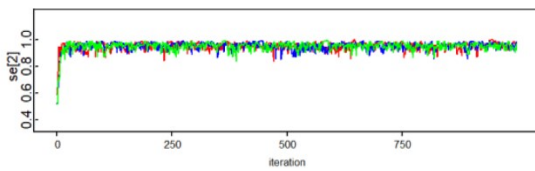
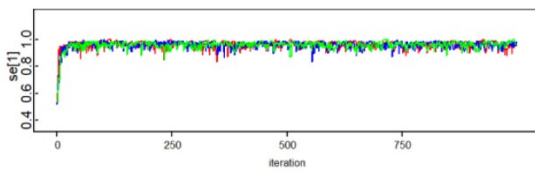
prev: prevalence of scrub typhus, se[1]: sensitivity of an IgM IFA, se[2]: sensitivity of a TSA56-based IgM ELISA, se[3]: sensitivity of a recombinant ScaA-based IgM ELISA, se[4]: sensitivity of eschar, sp[1]: specificity of an IgM IFA, sp[2]: specificity of a TSA56-based IgM ELISA, sp[3]: specificity of a recombinant ScaA-based IgM ELISA, sp[4]: specificity of eschar.

9.2. The rickettsiosis cohort with informative priors.

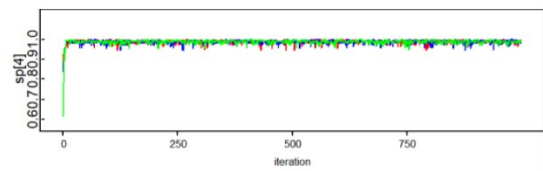
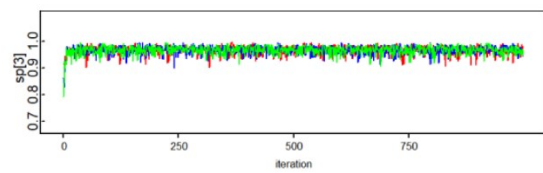
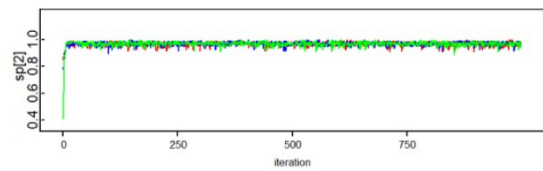
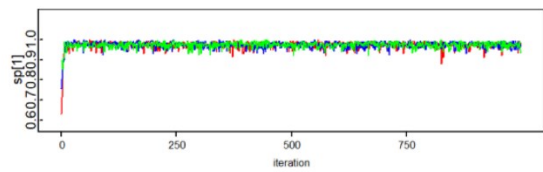
Prevalence



Sensitivity



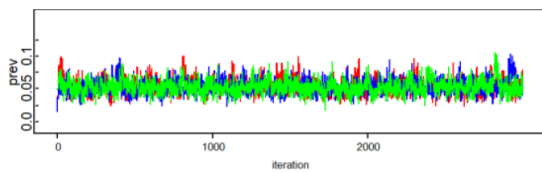
Specificity



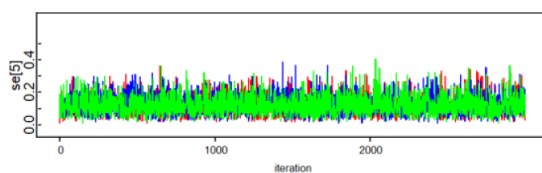
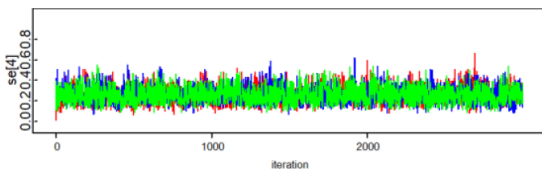
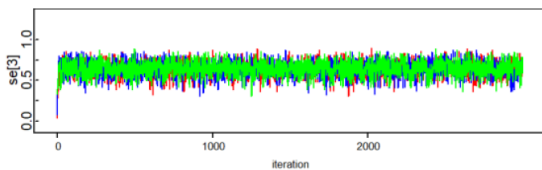
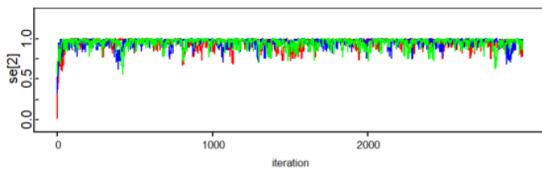
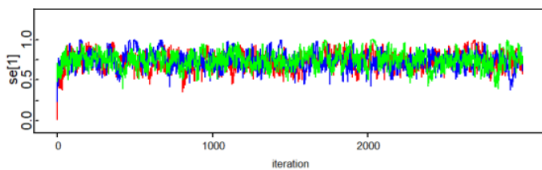
prev: prevalence of scrub typhus, se[1]: sensitivity of an IgM IFA, se[2]: sensitivity of a TSA56-based IgM ELISA, se[3]: sensitivity of a recombinant ScaA-based IgM ELISA, se[4]: sensitivity of eschar, sp[1]: specificity of an IgM IFA, sp[2]: specificity of a TSA56-based IgM ELISA, sp[3]: specificity of a recombinant ScaA-based IgM ELISA, sp[4]: specificity of eschar.

9.3. The fever cohort with non-informative priors.

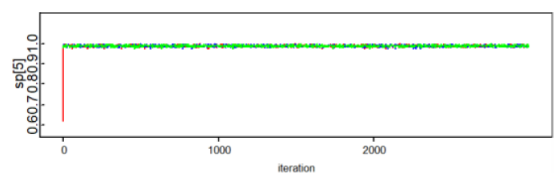
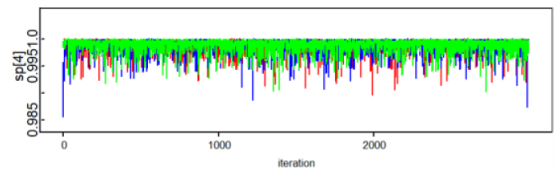
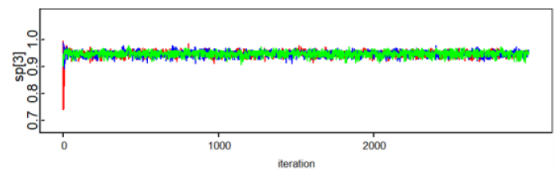
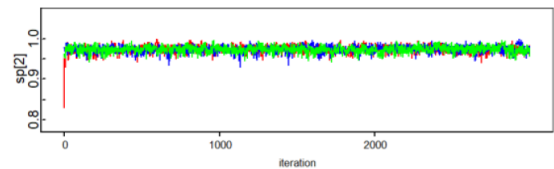
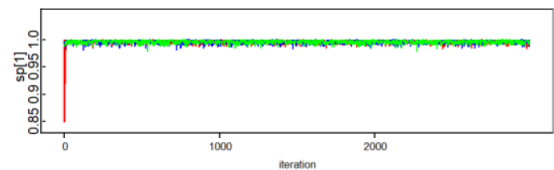
Prevalence



Sensitivity



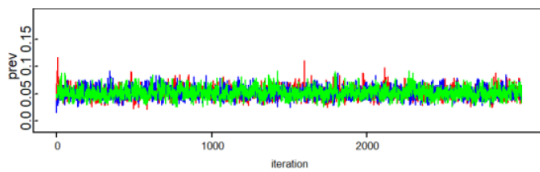
Specificity



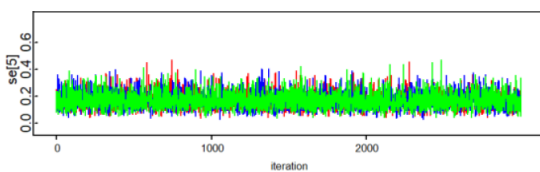
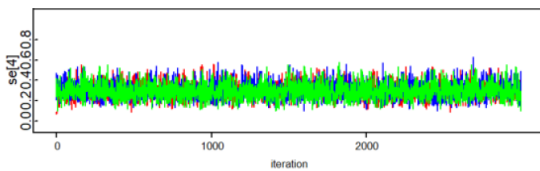
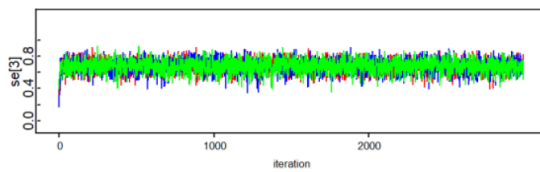
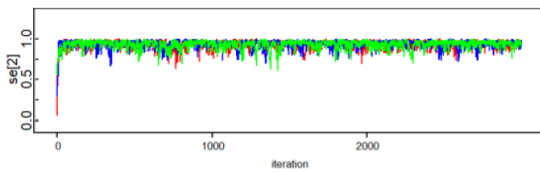
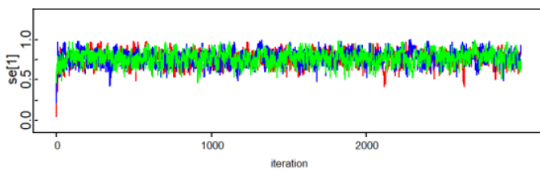
prev: prevalence of scrub typhus, se[1]: sensitivity of an IgM IFA, se[2]: sensitivity of a TSA56-based IgM ELISA, se[3]: sensitivity of a recombinant ScaA-based IgM ELISA, se[4]: sensitivity of a 47kDa-based real-time PCR assay, se[5]: sensitivity of eschar, sp[1]: specificity of an IgM IFA, sp[2]: specificity of a TSA56-based IgM ELISA, sp[3]: specificity of a recombinant ScaA-based IgM ELISA, sp[4]: specificity of a 47kDa-based real-time PCR assay, sp[5]: specificity of eschar.

9.4. The fever cohort with informative priors

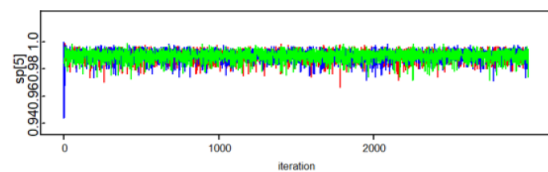
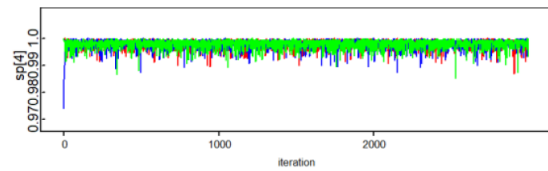
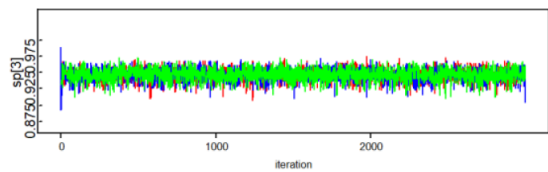
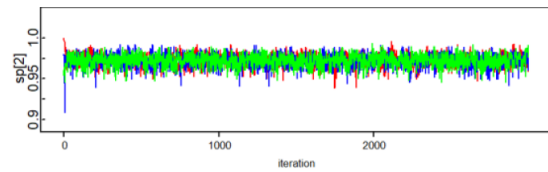
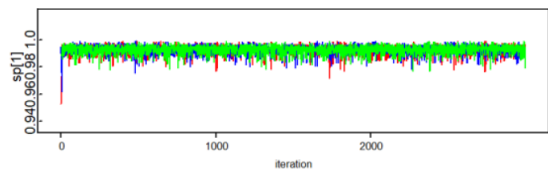
Prevalence



Sensitivity



Specificity

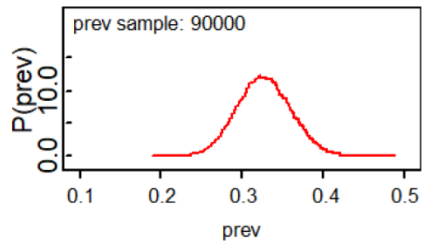


prev: prevalence of scrub typhus, se[1]: sensitivity of an IgM IFA, se[2]: sensitivity of a TSA56-based IgM ELISA, se[3]: sensitivity of a recombinant ScaA-based IgM ELISA, se[4]: sensitivity of a 47kDa-based real-time PCR assay, se[5]: sensitivity of eschar, sp[1]: specificity of an IgM IFA, sp[2]: specificity of a TSA56-based IgM ELISA, sp[3]: specificity of a recombinant ScaA-based IgM ELISA, sp[4]: specificity of a 47kDa-based real-time PCR assay, sp[5]: specificity of eschar.

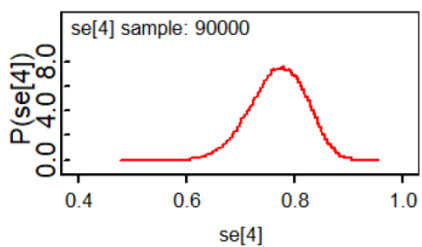
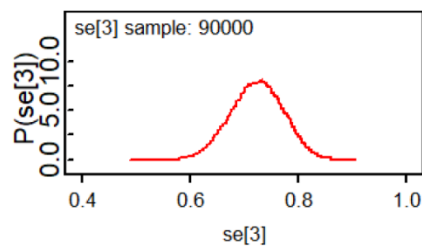
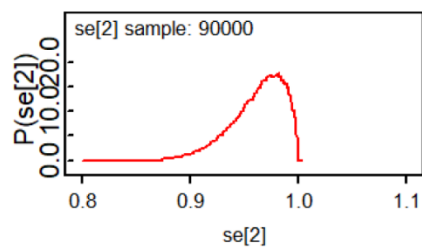
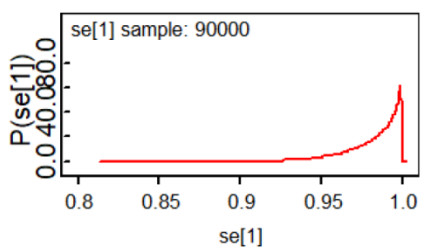
Appendix 10. Density plots for a scrub typhus prevalence, test sensitivities, and test specificities.

10.1. The rickettsiosis cohort with non-informative priors.

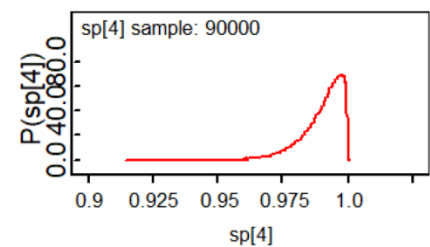
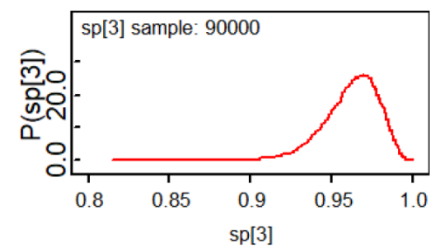
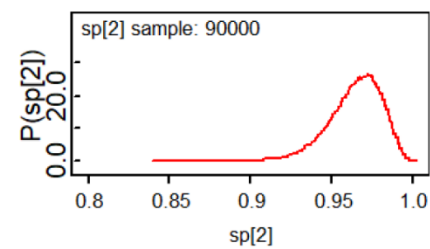
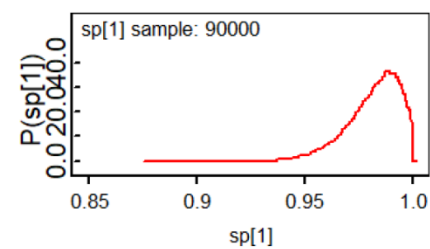
Prevalence



Sensitivity

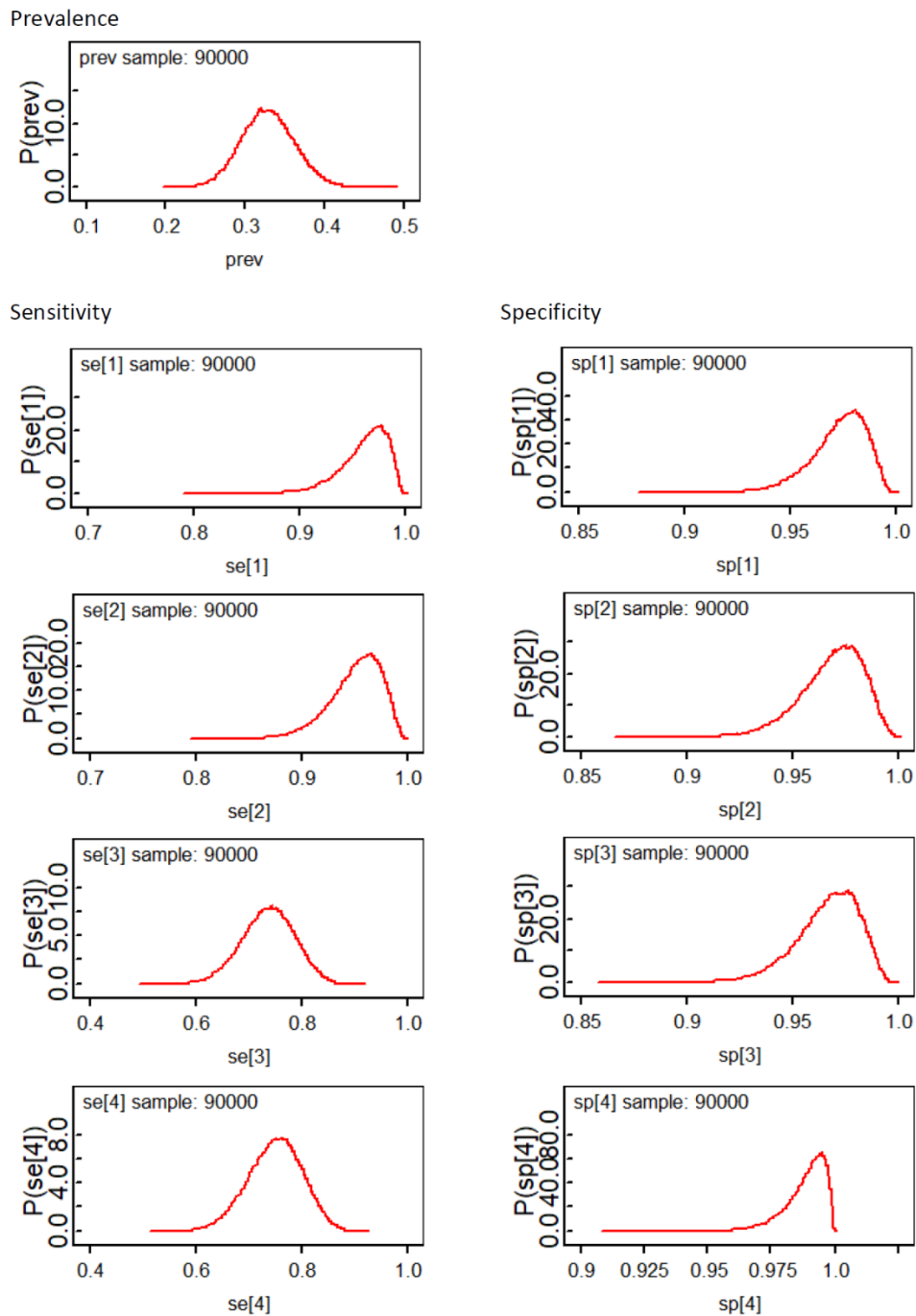


Specificity



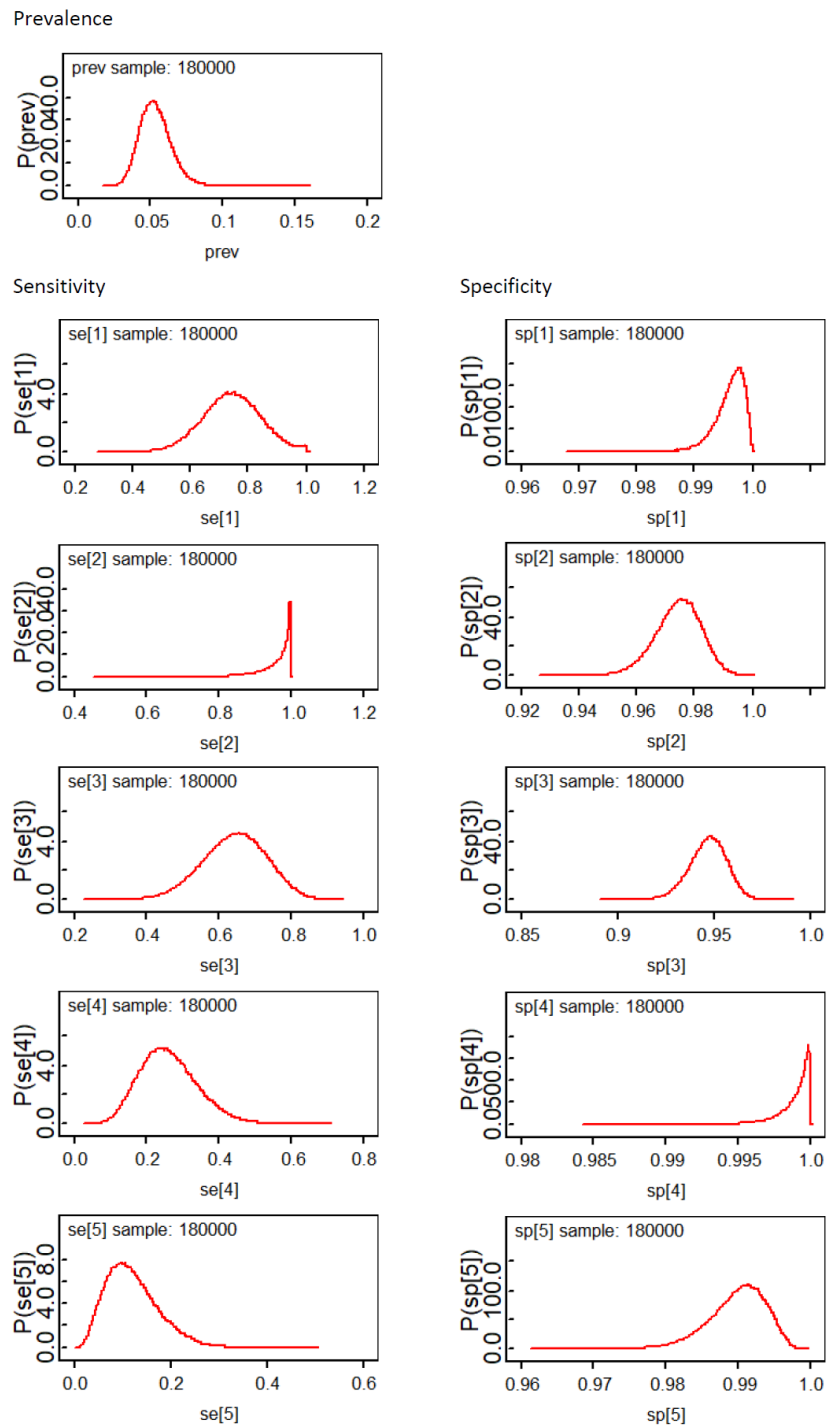
prev: prevalence of scrub typhus, se[1]: sensitivity of an IgM IFA, se[2]: sensitivity of a TSA56-based IgM ELISA, se[3]: sensitivity of a recombinant ScaA-based IgM ELISA, se[4]: sensitivity of eschar, sp[1]: specificity of an IgM IFA, sp[2]: specificity of a TSA56-based IgM ELISA, sp[3]: specificity of a recombinant ScaA-based IgM ELISA, sp[4]: specificity of eschar.

10.2. The rickettsiosis cohort with informative priors.



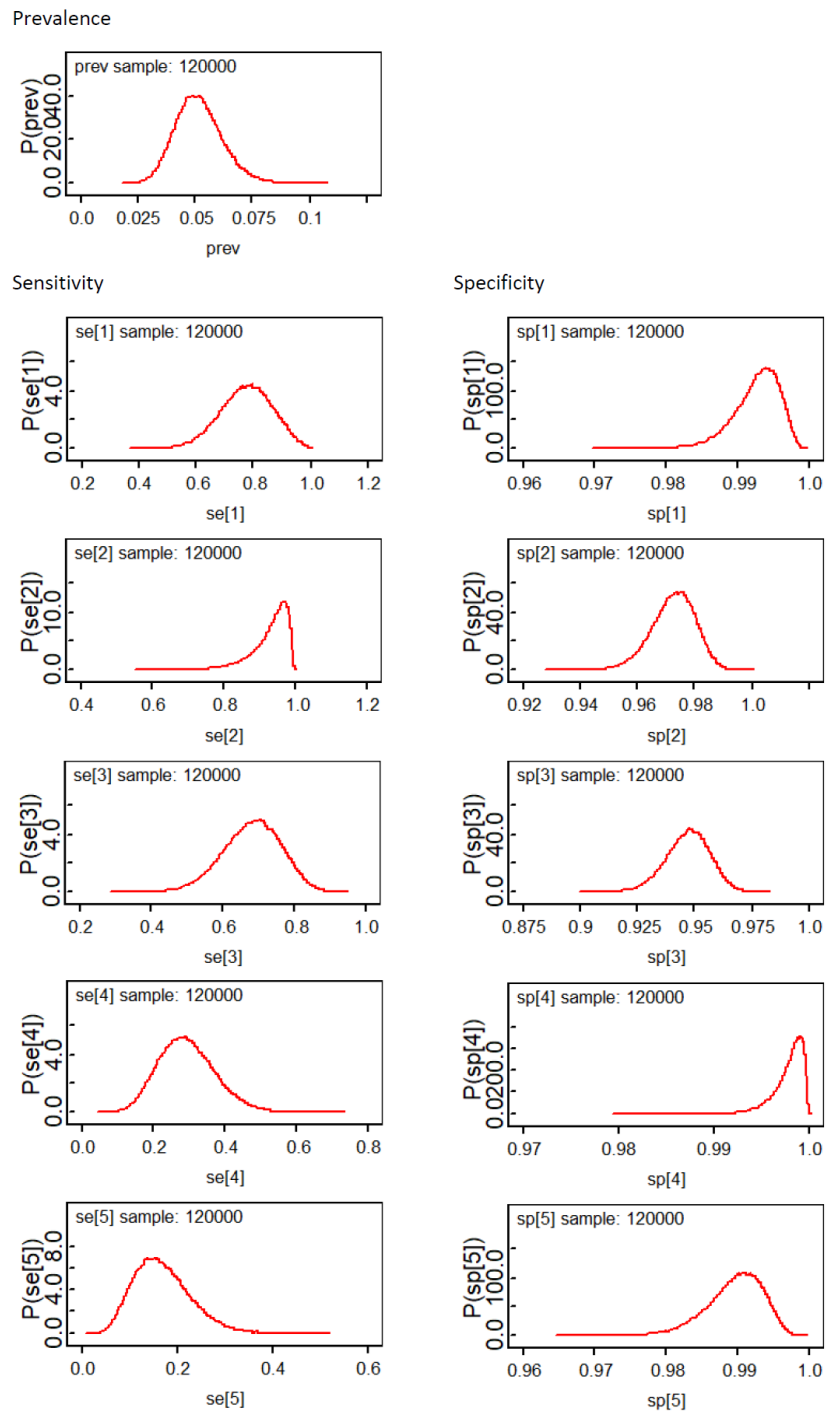
prev: prevalence of scrub typhus, se[1]: sensitivity of an IgM IFA, se[2]: sensitivity of a TSA56-based IgM ELISA, se[3]: sensitivity of a recombinant ScaA-based IgM ELISA, se[4]: sensitivity of eschar, sp[1]: specificity of an IgM IFA, sp[2]: specificity of a TSA56-based IgM ELISA, sp[3]: specificity of a recombinant ScaA-based IgM ELISA, sp[4]: specificity of eschar.

10.3. The fever cohort with non-informative priors



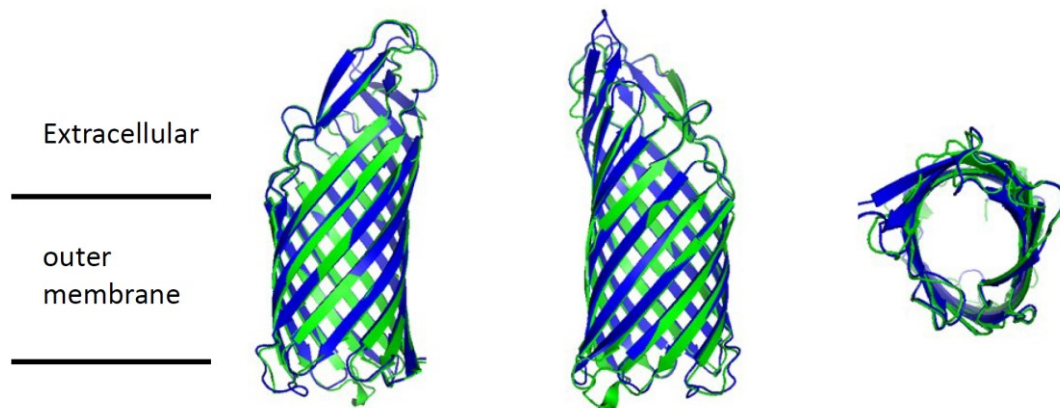
prev: prevalence of scrub typhus, se[1]: sensitivity of an IgM IFA, se[2]: sensitivity of a TSA56-based IgM ELISA, se[3]: sensitivity of a recombinant ScaA-based IgM ELISA, se[4]: sensitivity of a 47kDa-based real-time PCR assay, se[5]: sensitivity of eschar, sp[1]: specificity of an IgM IFA, sp[2]: specificity of a TSA56-based IgM ELISA, sp[3]: specificity of a recombinant ScaA-based IgM ELISA, sp[4]: specificity of a 47kDa-based real-time PCR assay, sp[5]: specificity of eschar.

10.4. The fever cohort with informative priors



prev: prevalence of scrub typhus, se[1]: sensitivity of an IgM IFA, se[2]: sensitivity of a TSA56-based IgM ELISA, se[3]: sensitivity of a recombinant ScaA-based IgM ELISA, se[4]: sensitivity of a 47kDa-based real-time PCR assay, se[5]: sensitivity of eschar, sp[1]: specificity of an IgM IFA, sp[2]: specificity of a TSA56-based IgM ELISA, sp[3]: specificity of a recombinant ScaA-based IgM ELISA, sp[4]: specificity of a 47kDa-based real-time PCR assay, sp[5]: specificity of eschar.

Appendix 11. The three-dimensional structures of the ScaA autotransporter region and the corresponding region of EstA, an autotransporter protein of *Pseudomonas aeruginosa*.



The I-TASSER program identified EstA had the closest structural similarity to the predicted model of the ScaA ATD. The three-dimensional structure of EstA was previously confirmed by X-ray diffraction (PDB DOI: 10.2210/pdb3KVN/pdb) (213).

The modelled structures on the middle columns are a 180° rotated view of the ones on the left. The right column is the view from the extracellular space. ScaA is green-coloured, and EstA is blue-coloured.

Appendix 12. A ScaA peptide dot-blot protocol.

1. Soak the PVDF membrane in methanol for 2 minutes
2. Equilibrate the PVDF membrane with a running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3) for 30 minutes
3. Place the PVDF membrane onto the chromatography paper. Wait until the running buffer has disappeared from the PVDF membrane surface.
4. Add 2 μ L (20 μ g/dot) of each peptide sample to the membrane with a 10 μ L tip.
5. Wait until the drops on the PVDF membrane have disappeared.
6. Block the membrane in PBS (-) with 5% skim milk for 30 minutes at room temperature with gentle shaking.
7. Dilute a serum sample at a final dilution of 1:100 in a dilution buffer (1% skim milk in PBS) to the membrane.
8. Incubate the membrane in the serum solution at room temperature with gentle shaking.
9. Wash the membrane in PBS for 5 minutes with gentle shaking. Repeat the wash step twice.
10. Dilute the secondary antibody (goat anti-human IgM-HRP conjugate [QED Bioscience, Inc.]) at a final dilution of 1:5000 in the dilution buffer.
11. Incubate the membrane in the secondary antibody solution at room temperature for 1 hour with gentle shaking.
12. Wash the membrane in PBS for 5 minutes with gentle shaking. Repeat the wash step twice.
13. Develop the reaction on the membrane with HRP substrate solution in the dark.
14. Stop the reaction by washing the membrane with PBS when the signal appears.
15. Air-dry the membrane.

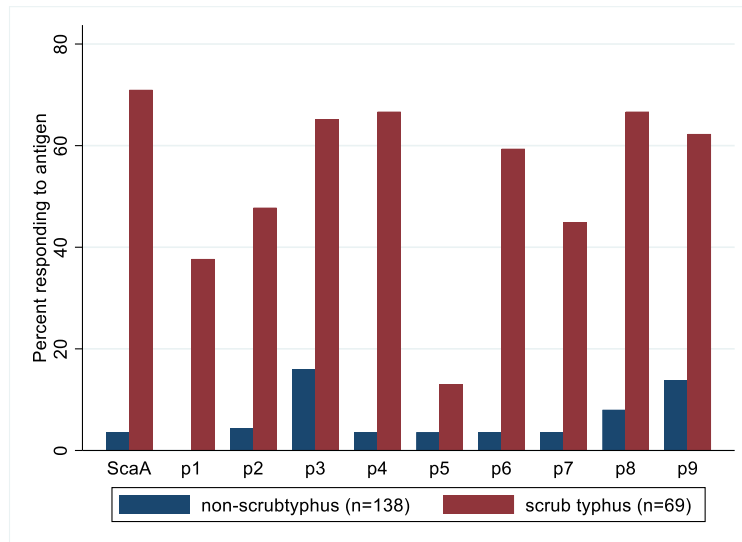
This protocol is a modification of work by Chen et al. (214). SDS: sodium dodecyl sulphate.

Appendix 13. The results of recombinant ScaA-based IgM and IgG ELISAs and ScaA peptide ELISAs of 42 patients in the assay development cohort.

Diagnosis	gold standard	ID	ScaA IgM ELISA									ScaA peptide combination (P1+P4+P8)						ScaA IgG ELISA								
			ScaA-B	P1	P2	P3	P4	P5	P6	P7	P8	P9	2+2+4	3+1+4	4+0+4	2+0+4	ScaA-B	P1	P2	P3	P4	P5	P6	P7	P8	P9
			scrub typhus	PCR + IFA -	VR02-R16 006	0.0755	-0.041	0.012	0.045	-0.005	-0.017	0.051	0.054	-0.029	0.209	-0.017	-0.016	-0.014	-0.009	0.1178	0.649	0.017	0.027	-0.047	-0.028	0.008

Patients with a positive result for a ScaA peptide IgM ELISA were highlighted in yellow. An IgM ELISA with a combination of ScaA peptides was attempted using peptides 1, 4, and 8 of different concentrations. For example, 2+2+4 was an antigen combination of peptide 1 (concentration of 2 μg/ml), peptide 4 (concentration of 2 μg/ml), and peptide 8 (concentration of 4 μg/ml).

Appendix 14. Proportions of patients responding to peptides 1-9 and the recombinant ScaA protein in scrub typhus and non-scrub typhus patients in the rickettsiosis cohort when the cut-off values of 0.0 were used for all peptide antigens.



ScaA peptide	Sensitivity (95% CI)	Specificity (95% CI)
Peptide 1	37.7% (26.3-50.2)	100.0% (97.4-100.0)
Peptide 2	47.8% (35.6-60.2)	95.6% (90.8-98.4)
Peptide 3	65.2% (52.8-76.3)	84.1% (76.9-89.7)
Peptide 4	66.7% (54.3-77.6)	96.4% (91.7-98.8)
Peptide 5	13.0% (6.1-23.3)	96.4% (91.7-98.8)
Peptide 6	59.4% (46.9-71.1)	96.4% (91.7-98.8)
Peptide 7	44.9% (32.9-57.4)	96.4% (91.7-98.8)
Peptide 8	66.7% (54.3-77.6)	92.0% (86.2-96.0)
Peptide 9	62.3% (49.8-73.7)	86.2% (79.3-91.5)

Appendix 15. Distribution of patients by a combination of test results, including a ScaA peptide 6 IgM ELISA, observed and predicted by Bayesian latent class models.

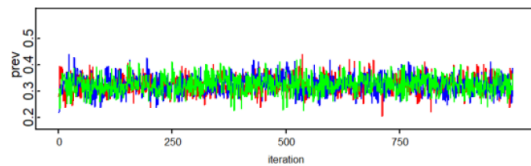
IgM IFA	TSA56 IgM ELISA	ScaA IgM ELISA	ScaA peptide 6 ELISA	Presence of eschar	Observed number of patients	Mean number of patients predicted by Bayesian latent class model (Bayesian p -value)			
						Non-informative priors			Informative priors
						Model 2	Model 3	Model 4	Model 4
-	-	-	-	-	128	124.7 (0.322)	124.7 (0.318)	124.9 (0.329)	124.1 (0.264)
-	-	-	-	+	1	1.551 (0.668)	1.479 (0.654)	1.423 (0.649)	1.607 (0.713)
-	-	-	+	-	0	0.547 (1.0)	0.554 (1.0)	0.556 (1.0)	0.856 (1.0)
-	-	-	+	+	0	0.014 (1.0)	0.037 (1.0)	0.011 (1.0)	0.027 (1.0)
-	-	+	-	-	4	5.179 (0.666)	5.270 (0.678)	5.141 (0.662)	4.59 (0.592)
-	-	+	-	+	0	0.083 (1.0)	0.068 (1.0)	0.073 (1.0)	0.116 (1.0)
-	-	+	+	-	0	0.027 (1.0)	0.024 (1.0)	0.024 (1.0)	0.037 (1.0)
-	-	+	+	+	0	0.019 (1.0)	0.008 (1.0)	0.004 (1.0)	0.022 (1.0)
-	+	-	-	-	4	4.962 (0.638)	5.003 (0.647)	4.869 (0.627)	4.418 (0.568)
-	+	-	-	+	0	0.141 (1.0)	0.198 (1.0)	0.379 (1.0)	0.642 (1.0)
-	+	-	+	-	0	0.045 (1.0)	0.062 (1.0)	0.039 (1.0)	0.088 (1.0)
-	+	-	+	+	0	0.084 (1.0)	0.145 (1.0)	0.063 (1.0)	0.180 (1.0)
-	+	+	-	-	1	0.267 (0.228)	0.259 (0.221)	0.262 (0.223)	0.361 (0.295)
-	+	+	-	+	0	0.218 (1.0)	0.162 (1.0)	0.220 (1.0)	0.607 (1.0)
-	+	+	+	-	0	0.067 (1.0)	0.049 (1.0)	0.037 (1.0)	0.130 (1.0)
-	+	+	+	+	0	0.229 (1.0)	0.166 (1.0)	0.120 (1.0)	0.393 (1.0)
+	-	-	-	-	2	3.124 (0.696)	3.080 (0.693)	2.820 (0.658)	3.869 (0.822)
+	-	-	-	+	1	0.212 (0.183)	0.359 (0.279)	0.796 (0.484)	0.790 (0.504)
+	-	-	+	-	0	0.066 (1.0)	0.109 (1.0)	0.054 (1.0)	0.104 (1.0)

+	-	-	+	+	0	0.182 (1.0)	0.335 (1.0)	0.144 (1.0)	0.233 (1.0)
+	-	+	-	-	1	0.268 (0.227)	0.235 (0.203)	0.257 (0.220)	0.388 (0.313)
+	-	+	-	+	0	0.476 (1.0)	0.366 (1.0)	0.500 (1.0)	0.777 (1.0)
+	-	+	+	-	0	0.141 (1.0)	0.108 (1.0)	0.081 (1.0)	0.169 (1.0)
+	-	+	+	+	0	0.490 (1.0)	0.379 (1.0)	0.275 (1.0)	0.516 (1.0)
+	+	-	-	-	4	1.953 (0.148)	1.907 (0.139)	2.578 (0.268)	2.433(0.238)
+	+	-	-	+	7	6.450 (0.456)	6.325 (0.442)	8.426 (0.715)	7.01 (0.541)
+	+	-	+	-	1	1.927 (0.830)	1.868 (0.822)	1.298 (0.692)	1.487 (0.743)
+	+	-	+	+	5	6.777 (0.768)	6.640 (0.761)	4.449 (0.440)	4.549 (0.460)
+	+	+	-	-	5	5.040 (0.543)	5.074 (0.548)	4.445 (0.448)	5.01 (0.54)
+	+	+	-	+	15	17.730 (0.751))	17.900 (0.768)	15.210 (0.545)	15.31 (0.554)
+	+	+	+	-	4	5.298 (0.734)	5.317 (0.738)	6.226 (0.832)	6.468 (0.855)
+	+	+	+	+	24	18.700 (0.155)	18.810 (0.156)	21.280 (0.308)	19.74 (0.197)

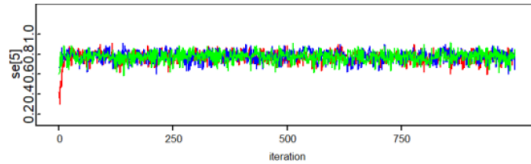
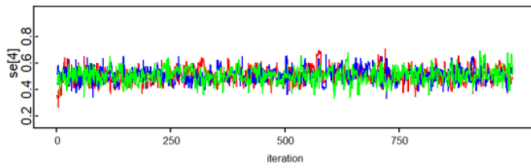
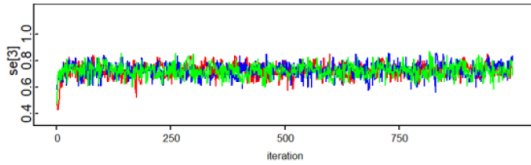
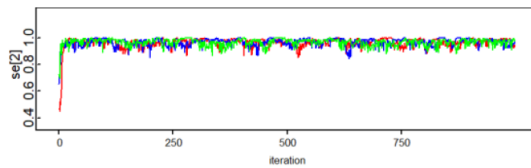
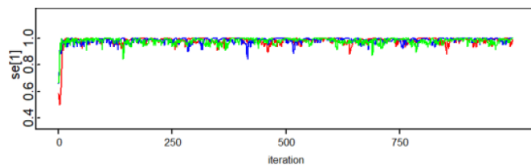
Appendix 16. History plots for a scrub typhus prevalence, test sensitivities, and test specificities on iterations 1-1,000.

16.1. Non-informative priors.

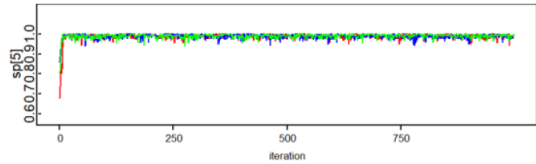
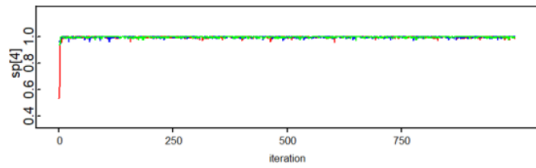
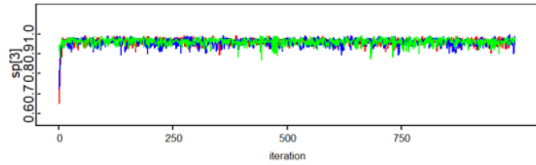
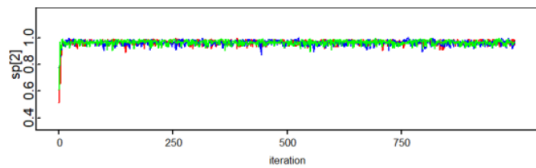
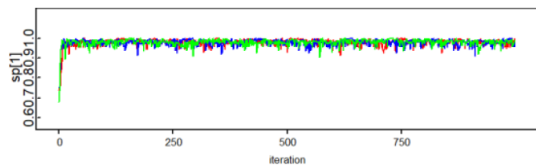
Prevalence



Sensitivity



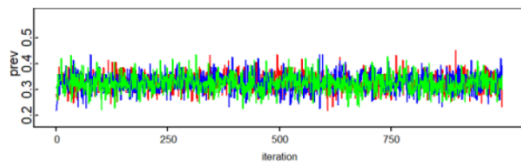
Specificity



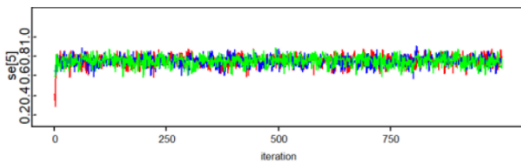
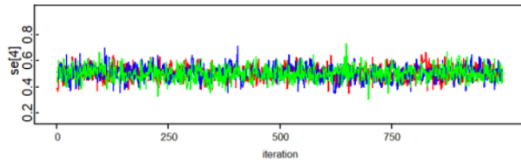
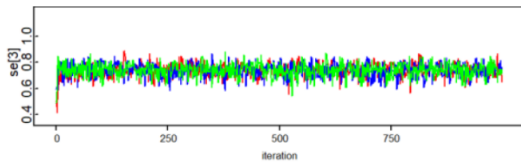
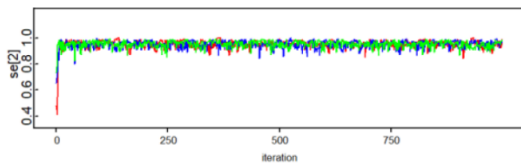
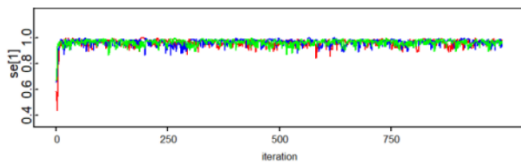
prev: prevalence of scrub typhus, se[1]: sensitivity of an IgM IFA, se[2]: sensitivity of a TSA56-based IgM ELISA, se[3]: sensitivity of a recombinant ScaA-based IgM ELISA, se[4]: sensitivity of a ScaA peptide 6 IgM ELISA, se[5]: sensitivity of eschar, sp[1]: specificity of an IgM IFA, sp[2]: specificity of a TSA56-based IgM ELISA, sp[3]: specificity of a recombinant ScaA-based IgM ELISA, sp[4]: specificity of a ScaA peptide 6 IgM ELISA, sp[5]: specificity of eschar.

16.2 Informative priors

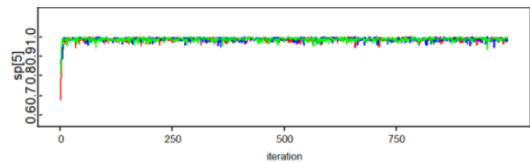
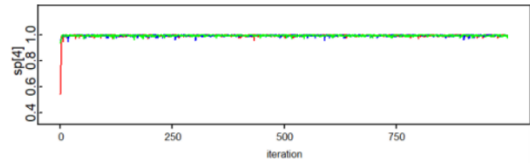
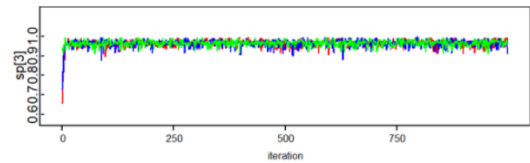
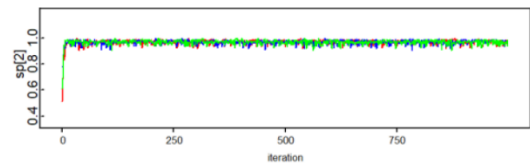
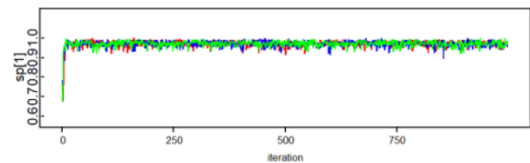
Prevalence



Sensitivity



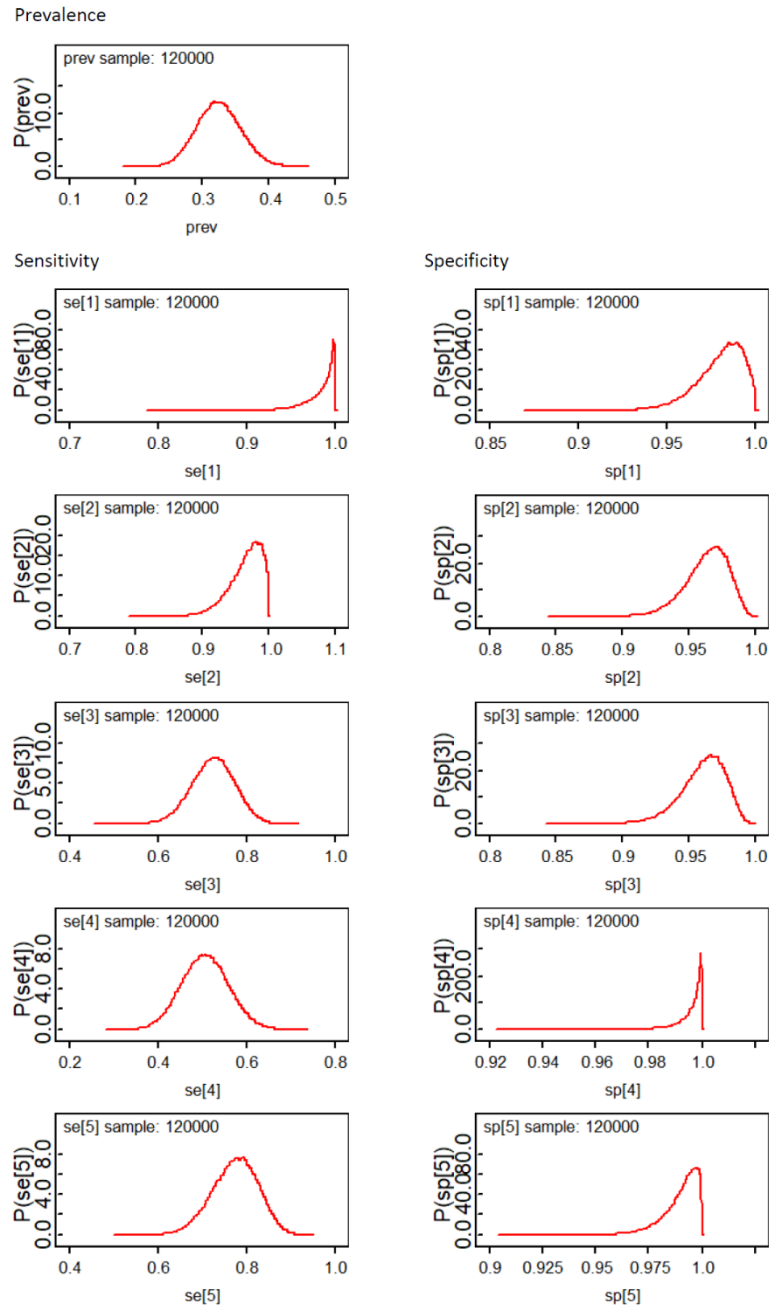
Specificity



prev: prevalence of scrub typhus, se[1]: sensitivity of an IgM IFA, se[2]: sensitivity of a TSA56-based IgM ELISA, se[3]: sensitivity of a recombinant ScaA-based IgM ELISA, se[4]: sensitivity of a ScaA peptide 6 IgM ELISA, se[5]: sensitivity of eschar, sp[1]: specificity of an IgM IFA, sp[2]: specificity of a TSA56-based IgM ELISA, sp[3]: specificity of a recombinant ScaA-based IgM ELISA, sp[4]: specificity of a ScaA peptide 6 IgM ELISA, sp[5]: specificity of eschar.

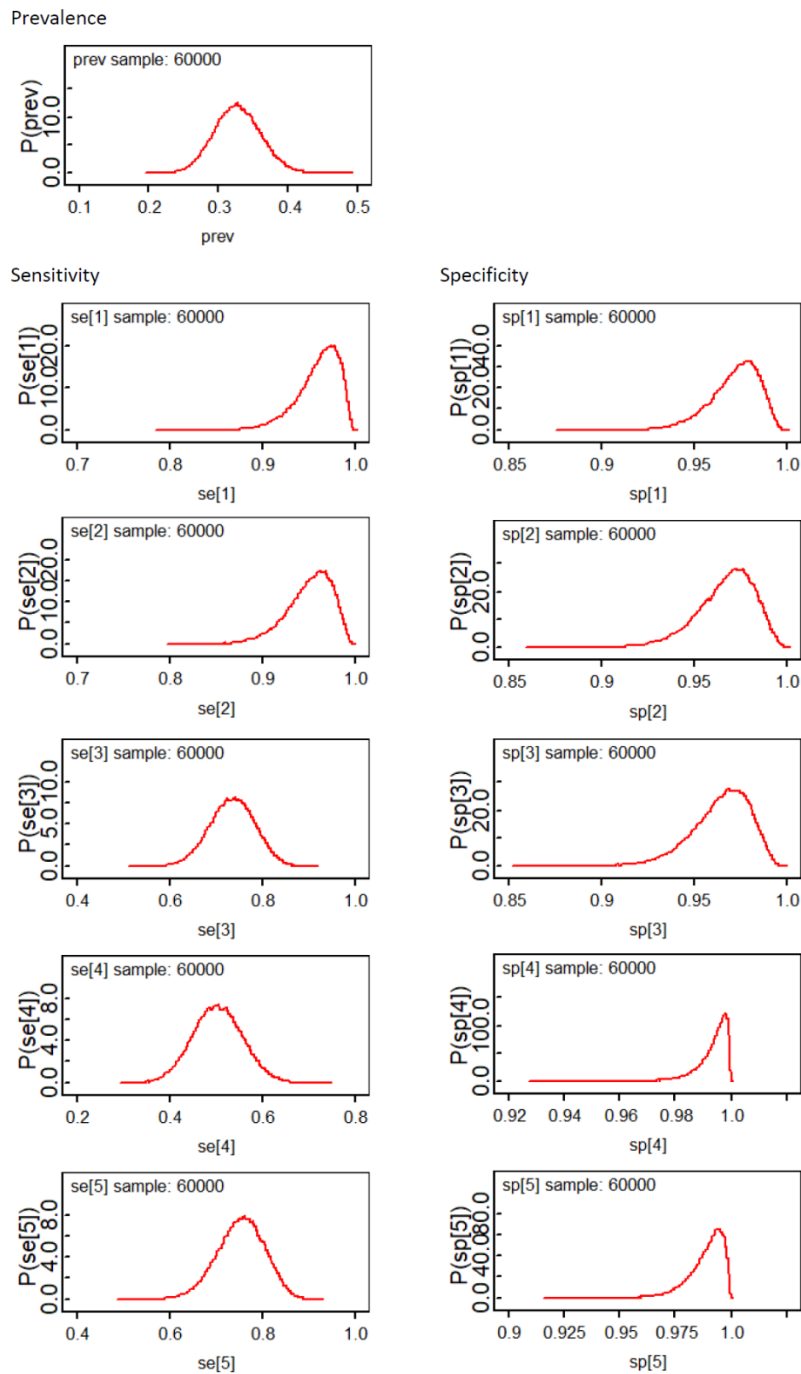
Appendix 17. Density plots for a scrub typhus prevalence, test sensitivities, and test specificities.

17.1. Non-informative priors.



prev: prevalence of scrub typhus, se[1]: sensitivity of an IgM IFA, se[2]: sensitivity of a TSA56-based IgM ELISA, se[3]: sensitivity of a recombinant ScaA-based IgM ELISA, se[4]: sensitivity of a ScaA peptide 6 IgM ELISA, se[5]: sensitivity of eschar, sp[1]: specificity of an IgM IFA, sp[2]: specificity of a TSA56-based IgM ELISA, sp[3]: specificity of a recombinant ScaA-based IgM ELISA, sp[4]: specificity of a ScaA peptide 6 IgM ELISA, sp[5]: specificity of eschar.

17.2. Informative priors.



prev: prevalence of scrub typhus, se[1]: sensitivity of an IgM IFA, se[2]: sensitivity of a TSA56-based IgM ELISA, se[3]: sensitivity of a recombinant ScaA-based IgM ELISA, se[4]: sensitivity of a ScaA peptide 6 IgM ELISA, se[5]: sensitivity of eschar, sp[1]: specificity of an IgM IFA, sp[2]: specificity of a TSA56-based IgM ELISA, sp[3]: specificity of a recombinant ScaA-based IgM ELISA, sp[4]: specificity of a ScaA peptide 6 IgM ELISA, sp[5]: specificity of eschar.