The epidemiology of rickettsial infections and Q fever in Bhutan

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DECLARATIONS

Statement of originality

I hereby certify that the work embodied in this thesis is my own work, conducted under normal supervision. The thesis contains no material which has been accepted, or is being examined, for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due references has been made.

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Tshokey, PhD student

18th October 2018

Thesis by publication

I hereby certify that this thesis is presented in the form of a series of research papers published in peer reviewed journals. I have included as part of the thesis, a written statement from each co-author, endorsed in writing by the Faculty Assistant Dean (Research Training), attesting to my contribution to any jointly authored papers.

Tshokey, PhD student

18th October 2018

Co-author statements

The following statements provided by all co-authors of the five research publications included in this thesis **(as chapters 6 to 10)** confirm that I have taken the lead role and contributed significantly in all the work leading to the respective publications.

Chapter 6: Scrub typhus in Bhutan: a synthesis of data from 2009 to 2014

By signing below, I confirm that **Tshokey** was the primary contributor to the publication entitled **'Scrub typhus in Bhutan: a synthesis of data from 2009 to 2014"** published as a review article in the WHO South-East Asia Journal of Public Health (WHO/SEAJPH) in September 2016. He conceived and designed the study, wrote the study protocols and conducted the literature and online data search. He solely analysed the data, drafted the manuscript and took care of submission and publication in the journal.

Tashi Choden **Ragunath Sharma**

Date: 20/08/2018 Date: 29/08/2019

Chapter 7: Scrub Typhus Outbreak in a Remote Primary School, Bhutan, 2014

By signing below, I confirm that **Tshokey** was the primary contributor to the publication entitled **'Scrub Typhus Outbreak in a Remote Primary School, Bhutan, 2014"** published as a research letter in the CDC, Emerging Infectious Diseases (EID) journal in August 2017. He conceived and designed the study, wrote the study protocols and collected outbreak investigation reports, communicated with relevant officials and conducted the literature review. He carried out the laboratory analysis of the blood samples, analysed the data, drafted the manuscript and took care of submission and publication in the journal.

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Date: 15/08/2018	Date: 14/09/2018

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By signing below, I confirm that **Tshokey** was the primary contributor to the publication entitled **'Rickettsial Infections and Q Fever Amongst Febrile Patients in Bhutan"** published as an original research article in the Tropical Medicine and Infectious Diseases (TMID) journal in January 2018 He took the major responsibility of research design and methodology, literature review, subject selection and sample collection, laboratory investigation, data analysis and interpretation, drafting and finalization of manuscript, submission and publication in the journal.

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Keith Eastwood

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Chapter 10: Serological evidence of *Rickettsia, Orientia* and *Coxiella* in domestic animals from Bhutan: preliminary findings

By signing below, I confirm that **Tshokey** was the primary contributor to the publication entitled **"Serological evidence of *Rickettsia, Orientia* and *Coxiella* in domestic animals from Bhutan: preliminary findings"** published as an original research article in the Vector-Borne and Zoonotic Diseases (VBZ) journal in September 2018. He took the major responsibility of research design and methodology, literature review, subject selection and sample collection, laboratory investigation, data analysis and interpretation, drafting and finalization of manuscript, submission and publication in the journal.

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LIST OF ABBREVIATIONS

ACCM2	Acidified citrate cysteine medium 2
AES	Acute Encephalitis Syndrome
AHB	Annual Health Bulletin
AKI	Acute kidney injury
APC	Antigen presenting cell
ARRL	Australian Rickettsial Reference Laboratory
ATP	Adenosine triphosphate
BHU	Basic Health Unit
BSF	Brazilian spotted fever
BSL	Biosafety level
CAM	Cellular adhesion molecules
CD	Cluster of differentiation
CDC	Centre for Disease Control, Atlanta, USA
CFS	Chronic fatigue syndrome
CKD	Chronic kidney disease
CNS	Central nervous system
CoRRB	Council of RNR Research of Bhutan
CRP	C-reactive protein
CRRH	Central Regional Referral Hospital
CSF	Cerebrospinal fluid
Ct	Cycling threshold
DC	Dendritic cell
DH	District Hospitals
DNA	Deoxyribonucleic acid

EA	Enumeration area
EC	Endothelial cell
EDTA	Ethylene diamine tetra-acetic acid
ELISA/EIA	Enzyme linked Immune assay/Enzyme immune assay
ERRH	Eastern Regional Referral Hospital
FAK	Focal adhesion kinase
FBS	Foetal bovine serum
FISF	Flinders Island Spotted Fever
FITC	Fluorescein isothiocyanate
GC	guanine-cytosine
GH	General Hospitals
Hb	Haemoglobin
HREC	Human Research Ethics Committee
ID	Identification number
IFA	Micro-immunofluorescence assay
IFN-γ	gamma interferons
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
JDWNRH	Jigme Dorji Wangchuck National Referral Hospital
JEV	Japanese encephalitis virus
kDa/kD	Kilodalton
LAI	Laboratory acquired infection
LCV	Large Cell Variant

LPS	Lipoploysaccharide
MIC	Minimum inhibition concentration
MODS	Multi-organ dysfunction
MoH	Ministry of Health, Bhutan
MTA	Material transfer agreement
NAAT	Nucleic acid amplification test
NCD	Non-communicable diseases
NRH	National Referral Hospital
NSB	National Statistics Bureau, Bhutan
OD	Optical density
ORC	Outreach clinics
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PGH	Phuentsholing General Hospital
PHL	Public Health Laboratory, Bhutan (now RCDC)
PI	Principle investigator
POCT	Point of care testing
PPS	Probability proportionate to size
PSU	Primary sampling unit
QF	Q fever
QFS	Q-fever fatigue syndrome
RCDC	Royal Centre for Disease Control, Bhutan (previously PHL)
RHDV	Rabbit haemorrhagic disease virus
RMSF	Rocky Mountain spotted fever
RNA	Ribonucleic acid

ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
RRH	Regional Referral Hospitals
RTPCR (qPCR)	Real-time Polymerase chain reaction
SCID	Severe combined immune deficiency
SCV	Small Cell Variant
SFG	Spotted fever group
SOP	Standard operating procedure
ST	Scrub typhus
STG	Scrub typhus group
TCA	Tricarboxylic acid
TG	Typhus group
TGF	Transforming Growth Factor
TLR	Toll-like receptors
TNF	Tumur necrosis factor
UV	Ultraviolet
VCAM	Vascular cell adhesion molecules
VDCP	Vector-borne Disease Control Program
WBC	White blood cells
WHO	World Health Organization
WHO/SEAR	WHO South-East Asia Region

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THESIS ABSTRACT

At the commencement of this project in 2014, some data were available on scrub typhus (ST) in Bhutan but none on the Spotted Fever Group (SFG) and Typhus Group (TG) *Rickettsia* and Q fever (QF).

Following an ST outbreak in 2009, heightened awareness increased case notifications from 91(no deaths) in 2010 to 753 (3 deaths) in 2017. Another outbreak in 2014 saw two deaths from meningoencephalitis. These events inspired this doctoral thesis to explore the epidemiology of rickettsioses in Bhutan. Clinico-demographic information, human and animal samples were collected from Bhutan and analyzed at the Australian Rickettsial Reference Laboratory. Findings suggests that approximately 15% (159/1044) of patients with acute undifferentiated fevers attending 14 Bhutanese hospitals were due to rickettsioses, ST being the commonest (6.7%). About 49% of 864-healthy Bhutanese showed evidence of past exposure to ST (22.6%), SFG (15.7%), QF (6.9%) and TG (3.5%). ST and SFG exposure significantly increased with age and farming activities. Trongsa district residents had the highest exposure to ST but residents at altitudes >2000 meters were relatively protected. In animals, an overall seropositivity of 46% (106/294) with SFG (36%), ST (21%), TG (15%), and QF (4%) was determined. Seropositivity differences between animal species appeared to have been significant and warrant confirmation.

A few inferences should be interpreted with caution, but findings in general constitute baseline data for Bhutan and serve to prompt further research. Rickettsial outbreaks and the high incidence and prevalence rates established the endemicity of rickettsioses in Bhutan. Health authorities should ensure that services are equipped to manage these infections by developing diagnostic and clinical guidelines. Increased human-livestock sector collaborations in research, diagnostics and therapeutics through a ‘One Health’ approach is recommended. Future studies

should consider vector profiles, geospatial, biosocial and environmental risk assessment for strategic prevention and control of rickettsioses.

Key words: Bhutan, Epidemiology, Q fever, *Rickettsia*, Scrub typhus, Seroprevalence

Chapter 1: The basic biology and pathophysiology of *Rickettsia*, *Orientia*, and *Coxiella*

1.1 Rickettsiae

1.1.1 Introduction

Rocky Mountain spotted fever (RMSF), the prototype member of the genus *Rickettsia*, was the first rickettsial infection described and was first found in the northwestern United States of America. Its aetiology was unknown until the discovery of "a microorganism which apparently had a specific relationship to RMSF" by Ricketts in 1909[1]. Ricketts found a similar organism when he went to Mexico to investigate an epidemic of typhus in 1910. More than this causal association he was impressed by the agglutination that occurred between the sera of infected guinea-pigs and the bacterial emulsions made from crushed tick eggs[1]. Unfortunately, before he made any further progress in the identification of this organism he was infected and died of typhus within two weeks. The final identification of the causative agent of RMSF as *Rickettsia rickettsii* was made by Wolbach a decade later[2]. At about the same time, a Brazilian pathologist, da Rocha Lima discovered the agent of epidemic typhus and named it after his friend von Prowazek and American bacteriologist Ricketts as *Rickettsia prowazekii.* Following this significant discovery, several other rickettsial pathogens were described and others are still being described. Hence rickettsioses are "emerging infections".

1.1.2 Bacteriology and classification

Rickettsiae are obligate intracellular bacteria belonging to the alpha-proteobacteria. They are short rods measuring about 0.3 to 0.5 x 0.8 to 2.0 μm. These bacteria have ribosomes and DNA strands in their cytoplasm and have a typical Gram-negative structure made of lipopolysaccharide, a peptidoglycan layer, lipoprotein and an auto-transporter outer membrane protein[3]. In the host cells, they reside free in the cytoplasm obtaining amino acids and other

molecules by their active transporter systems[3]. Although they have a Gram-negative bacterial structure, rickettsiae are not stained by the Gram staining method. However, they retain basic fuchsin when stained using the Gimenez method appearing bright red with a pale blue background with malachite green counterstain.

The genus *Rickettsia* (order *Rickettsiales*, family *Rickettsiaceae*) has traditionally been classified into three biotype groups; the Spotted Fever Group (SFG), the Typhus Group (TG) and the Scrub Typhus Group (STG) based on their vector host, morphologic and antigenic cross-reactivity. With the separation of *Rickettsia tsutsugamushi* into a separate genus, "*Orientia*", in 1995[4] the genus *Rickettsia* was constituted of the other two biotypes. Around the same time, Stothard and colleagues described the ancestral divergence of *R. bellii* from the SFG and TG biotypes thereby classifying genus *Rickettsia* into three groups; the SFG, TG and the ancestral group (AG)[5, 6]. Using whole-genome sequencing, a new classification of *Rickettsia* with the addition of a fourth group, the transitional group, which is unique from SFG and TG but with possible gene exchange with the AG consisting of *R. felis* and *R. akari*, was proposed[7]. This four-group classification is, however, not accepted widely[8]. Currently, there are 30 *Rickettsia* species listed in the prokaryotic names with standing in nomenclature (http://www.bacterio.net/rickettsia.html) and at least 20 of these are known to be pathogenic[9]. The SFG *Rickettsia* include numerous species such as *R. rickettsii, R. australis, R. conorii, R. honei, R. africae, R. akari, R. felis, R. japonica* etc. The TG *Rickettsia* has only two species; *R. prowazekii* and *R. typhi* and the AG consist of two species, *R. bellii* and *R. canadensis*. The SFG *Rickettsia* are mainly associated with ticks, except *R. felis* and *R. akari* which are associated with fleas and mites respectively. Of the two TG *Rickettsia*, *R. prowazekii* is associated with human body lice and *R. typhi* is associated with fleas.

1.1.3 Growth, survival, and metabolism

Rickettsiae are obligate intracellular parasites that cannot be propagated on cell-free media. They can be grown in-vitro in the yolk sac of a developing chicken embryo, but preferably cultured on primary or established cell culture monolayers, such as chicken embryo fibroblasts, mouse L cells, and golden hamster cells. Rickettsiae proliferate by binary fission and grow in either the nucleus or cytoplasm of host cells. When inside host cells, they reside in the cytosol or nucleus rather than being surrounded by a host cell membrane.

The SFG *Rickettsia* have an optimal growth temperature of 32ºC and has a guanine-cytosine (GC) content between 32 and 33. They can polymerize actin and move into and within the host cell nuclei by actin-based motility triggered by specific rickettsial proteins[3, 10]. The TG *Rickettsia* grow best at a temperature of 35ºC and have a GC content of 29. They are unable to polymerize actin and cannot enter host cell nuclei, thus restrict themselves to the host cell cytoplasm[10]. They replicate intracellularly until the host cells burst to release the organism[3]. Rickettsiae can spread from cell to cell by traversing cell membranes without causing obvious damage. Individual organisms exit from infected cells via host cell filopodia and rarely accumulate in large numbers inside individual cells[11]. *R. rickettsii* moves between cells at astonishing speeds (up to 4.8 micrometres per second) by recruiting and polymerizing host cell actin filaments[12].

Rickettsiae mostly depend on the host cell for its nutritional needs. They lack enzymes for sugar and amino acid metabolism, and lipid and nucleotide synthesis. They have numerous specialized adaptations to survive as an intracellular parasite, including the ability to acquire host adenosine triphosphate (ATP) using a *Rickettsia*-derived ATP translocator protein and the ability to utilize host-derived glutamine as an energy source.

1.1.4 Phylogeny and taxonomy

Earlier, the phylogenetic study of rickettsiae compared morphological, antigenic and metabolic characters as for other prokaryotes. Thus, the genus *Rickettsia* historically consisted of small, rod-shaped, Gram-negative organisms stained by Gimenez method, divide by binary fission, cultivatable in living tissues and pathogenic to vertebrates[13]. However, phylogenetic classification based on these characters was highly unreliable and molecular techniques have revolutionized classification. Phylogenic studies using the 16S rRNA gene sequence have discovered that several of the bacteria previously classified under the order *Rickettsiales* did not belong to the α-subclass of the Proteobacteria phylum. This finding resulted in the reclassification of *Coxiella burnetii* and *Rickettsiella grylli* within the Legionellaceae of the γ-subclass of the Proteobacteria[14, 15]. Phylogenic classifications developed further and became more definite with the use of other genes in addition to 16S rRNA, including the use of more divergent genes like the gltA, the citrate synthase gene that encodes for the 17-kDa protein[16] and other genes like ompA, ompB, sca4, sca1 and sca2[17, 18].

Phenotypic methods to classify bacteria could rarely be applied to intracellular bacteria. Most obligate intracellular bacteria associated with eukaryotic cells were classified under the order *Rickettsiales.* The order *Rickettsiales* originally contained families *Rickettsiaceae, Bartonellaceae* and *Anaplasmataceae.* In the family *Rickettsiaceae,* the tribe *Rickettsiae* comprised the genera *Rickettsia, Coxiella, and Rochalimaea*. With the development of nucleic acid amplification techniques and nucleotide sequencing, the taxonomic classification has undergone a major change, especially in intracellular bacteria that were poorly classified with the phenotypic methods. The sequencing method makes use of the 16S rRNA or rDNA. With the use of the newer techniques, the order *Rickettsiales* was reclassified into genera *Anaplasma, Ehrlichia, Neorickettsia, Orientia, Rickettsia* and *Wolbachia*[4, 19].

1.1.5 Pathogenesis of rickettsial infections

Pathogenesis involves a sequence of events as described below.

1.1.5.1 Transmission and spread

Rickettsioses are vector-borne diseases and all human rickettsial infections involve exposure to infected arthropod vectors. In the case of *Rickettsia* species, these vectors include ticks, cat fleas, rat fleas, mites, and human body lice. The SFG *Rickettsia* are transmitted through ticks while the transitional group and TG *Rickettsia* are primarily transmitted through non-tick vectors[20]. In ticks, the transovarial and trans-stadial passage of the SFG rickettsiae ensures their survival and continuity without the requirement for multiple hosts[21]. Humans primarily serve as the dead-end host except in epidemic typhus caused by *R. prowazekii*, which can be transmitted among close communities through body lice that feed on an infected person and pass the infection to others [21]. Ticks inoculate the organisms into the skin of the hosts from their saliva when they attach to the host and during their blood meal. Uninfected or *Rickettsia*-free ticks become infected when they take a blood meal from a rickettsemic host[22], **(Fig 1.1)**.

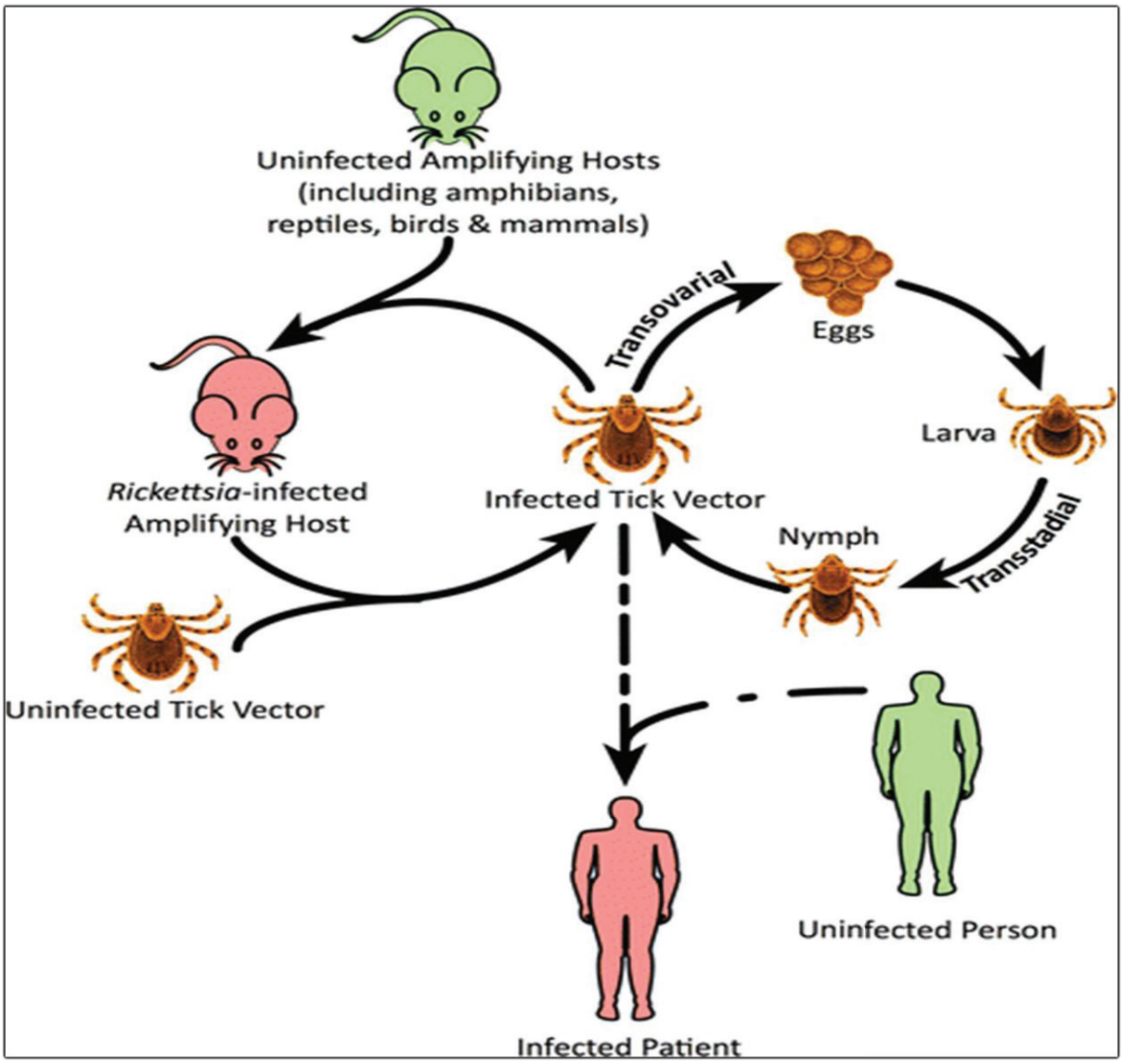


Figure 1.1: Transmission cycle for the natural maintenance of SFG *Rickettsia* [20]

The TG *Rickettsia* are mainly transmitted by contaminated faeces of human body lice (*Pediculus humanus corporis*) in case of epidemic typhus **(Fig 1.2)** and fleas (*Xenopsylla cheopis*) for endemic/murine typhus. The faeces are auto-inoculated into the skin of the patients during scratching at the itchy bite areas. Because *R. prowazekii* in louse faeces and *R. typhi* in flea faeces can remain infectious for months, the possibilities of infection via rubbing the mucous membranes or inhalation remain. Tick vectors suffer from a range of harm from the

SFG *Rickettsia* they harbour but lice harbouring *R. prowazekii* do not survive the infection and ultimately die within a couple of weeks[21].

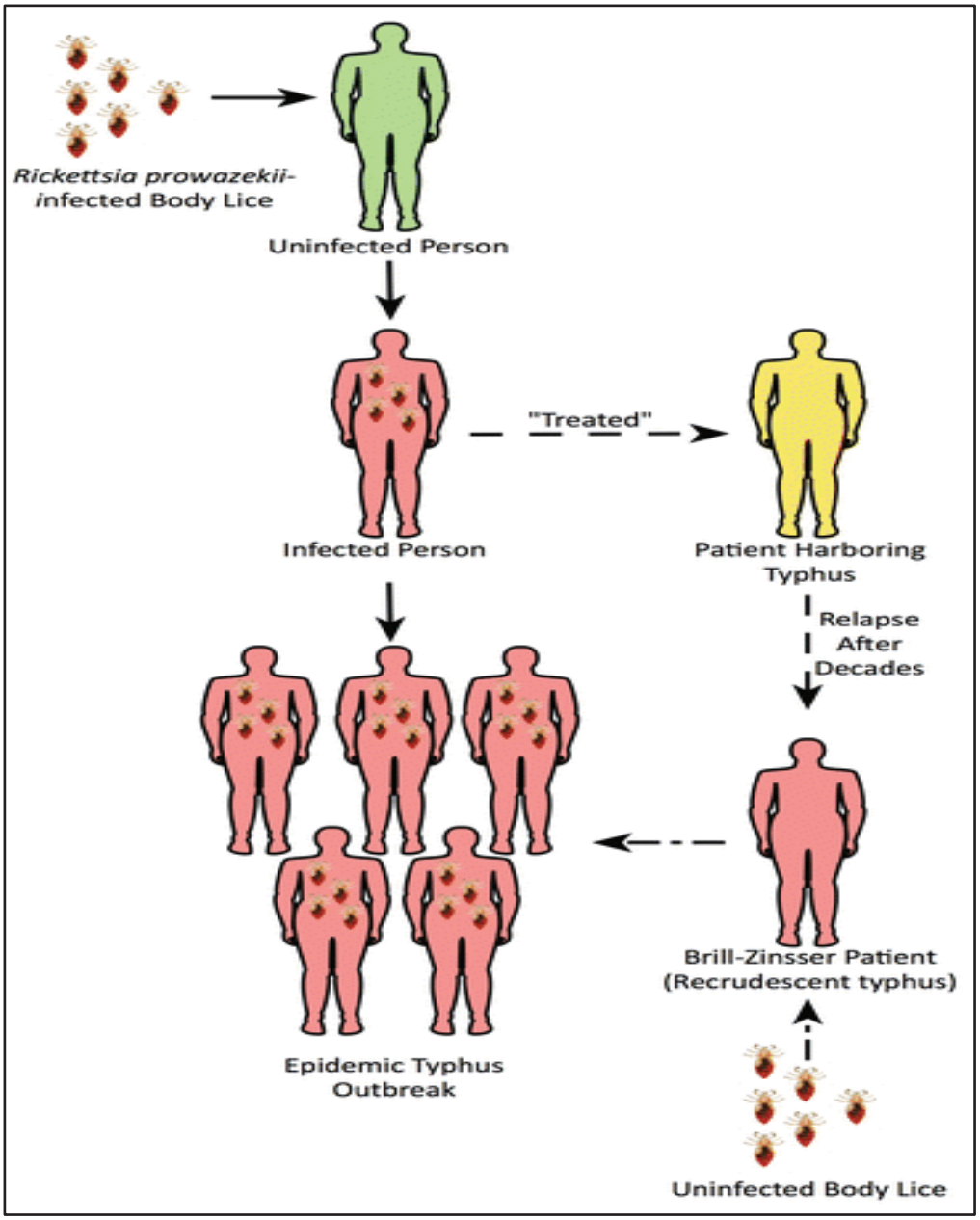


Figure 1.2: Transmission cycle of *Rickettsia prowazekii*[20]

A list of SFG and TG *Rickettsia* with their vectors, reservoirs and the disease they cause is presented in **Table 1.1**, as summarized by Diop et al[23].

Table 1.1. Classification, vectors, reservoirs and diseases of *Rickettsia* species with known pathogenicity to humans[23]

Antigenic group and species	Strain name	Rickettsiosis	Vector	Reservoirs
Spotted fever group				
R. aeschlimannii	MC16	Rickettsiosis	Ticks: *Hyalomma* sp.	Unknown
R. africae	ESF-5	African tick-bite fever	Ticks: *Amblyomma variegatun*	Ruminants
R. akari	Hartford	Rickettsialpox	Mites: *Allodermanyssus sanguinus*	Mice, rodents
R. australis	Cutlack	Queensland tick typhus	Ticks: *Ixodes* sp	Rodents
R. conorii	Malish 7	Mediterranean spotted fever	Ticks: *Rhipicephalus* sp	Dogs, rodents
R. felis	URRWXCa12	Flea-spotted fever	Flea: *Ctenocephalides* sp	Cats, rodents, opossums
R. heilongjiangensis	O54	Far Eastern tick-borne rickettsioses	Ticks: *Dermacentor sivarum*	Rodents
R. helvetica	C9P9	Aneruptive fever/ unnamed rickettsiosis	Ticks: *Ixodes ricinus*	Rodents
R. honei	RB	Flinders Island Spotted fever, Thai tick typhus	Ticks: *Aponomma hydrosauri*	Rodents, reptiles
R. japonica	YH	Japanese spotted fever/Oriental spotted fever	Ticks: *Haemaphysalis* sp.	Rodents
R. massiliae	MTU5	Mediterranean spotted fever	Ticks: *Rhipicephalus turanicus*	Unknown
R. parkeri	Portsmouth	Unnamed rickettsiosis	Ticks	Rodents
R. raoultii	Khabarovsk	Scalp eschar & neck lymphadenopathy (SENLAT)	Ticks: *Dermacentor sivarum*	Unknown
R. rickettsii	Sheila Smith	Rocky Mountain Spotted Fever	Ticks: *Dermacentor* sp.	Rodents
R. sibirica	246	North Asian tick typhus, Siberian tick typhus	Ticks: *Dermacentor* sp.	Rodents
R. sibirica	HA-91	Lymphangitis-associated rickettsiosis	Ticks: *Dermacentor* sp.	Rodents
R. slovaca	13-B	SENLAT	Ticks: *Dermacentor* sp.	Lagomorphes, rodents
Typhus group				
R. prowazekii	Brein1	Epidemic typhus/ Brill-Zinsser disease	Louse: *Pediculus humanus*	Humans, flying squirrels
R. prowazekii	Rp22	Epidemic typhus	Louse: *Pediculus humanus*	Humans, flying squirrels
R. typhi	Wilmington	Murine typhus	Fleas: *Xenopsylla cheopis*	Rodents

1.1.5.2 Invasion, adhesion to and entry into host cells

Pathogenic rickettsiae can infect and replicate in a numerous number of different cell types *in vitro* but during *in vivo* infections, both in humans and experimental animal models, the pathogens invade and proliferate within the vascular endothelial cells (ECs) that line the small and medium-sized blood vessels. These ECs form the major target cells of rickettsial infections. Rickettsiae also invade, to a lesser extent, the perivascular cells; the monocytes and macrophages and the hepatocytes, destroying them and spreading the infections to the endothelia of the vascular tree[24]. After inoculation into the skin through the bites or auto-inoculation from faeces, *Rickettsia* need to adhere to and invade host cells to be able to survive, multiply and establish the infection. Because intracellular uptake of rickettsiae is determined by the viability of the invading bacteria and metabolic activity of the host cell, the process was known as 'induced phagocytosis'[25] and rickettsial entry into human ECs occur through this induced phagocytosis, indicating that adherence of a viable bacterium to the cell surface triggers intracellular uptake by a metabolically active host cell[24]. Although some signalling pathways of rickettsial entry into the ECs such as; activation of phosphoinositide 3-kinase, Cdc42 (a small GTPase), src-family tyrosine kinase and tyrosine phosphorylation of focal adhesion kinase (FAK) and cortactin have been documented, all of the effector host proteins mediating entry are not fully described[26].

The SFG rickettsiae possess two well characterized, surface-exposed proteins, the OmpA and OmpB but only OmpB is found in TG rickettsiae. Of these proteins, OmpA was initially identified as the protein for adhesion of *R. rickettsii* to host cells[27]. Other putative adhesins encoded by gene Adr1 (RC1281) in *R. conorii* and Adr2 (RP828) in *R. prowazekii* were subsequently identified and are proposed to be involved in rickettsial invasion[28]. Modern bioinformatics analysis has unveiled 17 surface cell antigens (Sca) which encode proteins similar to auto-transporter proteins involved in rickettsial adhesion to host cell receptors. Four

of these proteins identified are Sca0 (OmpA), Sca1, Sca2 and Sca5 (OmpB) which have been shown to play important roles in rickettsial adhesion and/or invasion[29]. It has been demonstrated that Ku70, a subunit of nuclear DNA-dependent protein kinase localized in the cytoplasm and plasma membrane of mammalian cells serve as a receptor for rickettsial OmpB thereby playing an important role in *R. conorii* internalization[30]. A recent finding documents the participation of clathrin and caveolin-2-dependent endocytosis in the internalization of rickettsiae[31]. OmpA-mediated rickettsial adherence to endothelium was shown to be dependent upon its interaction with $\alpha_2\beta_1$ integrins on the surface of host cells[32]. This same study also showed that rickettsial adhesion is dependent on a discontinuous Arg-Gly-Asp (RGD)-motif of OmpA. Overall, there seem to be multiple interactions between rickettsial ligands on the outer membrane and host cell surface entities that serve as the specific receptor counterparts leading to activation of downstream signalling mechanisms that are critical for the process of entry and acquisition of an intracellular host niche.

The internalization of rickettsiae into host cells occurs through a quick and efficient process occurring within few minutes after the initial contact. Rickettsiae probably utilize the membranolytic proteins, hemolysin C and phospholipase D respectively to disrupt the phagosomal membranes and gain access to host cytosol[33]. A phospholipase A2 (PLA2)-like activity is also predicted to be involved in the entry of rickettsiae into host cells[34]. Rickettsial infection of the ECs, through the release of early-response cytokines (IL-1α and IL-6) and other components of the acute phase reactions, enhances the expression of cellular adhesion molecules (CAM); E-selectin, vascular cell adhesion molecules-1(VCAM-1) and intercellular adhesion molecules-1 (ICAM-1) on the surface of ECs and polymorphonuclear leukocytes. The enhanced rolling and adherence of mononuclear cells to infected ECs involve mechanisms dependent on these molecules[35]. Other adhesion molecules and chemokines are also involved.

1.1.5.3 Actin-based motility: intracellular movements and intercellular dissemination

After entry into cells, SFG *Rickettsia* forms a polar actin tail which facilitates their movements within and from cell-to-cell and also plays a crucial role in the establishment, dissemination, and pathogenesis of the resulting diseases. On the other hand, TG rickettsiae either do not display actin-based motility (in the case of *R. prowazekii*) or have erratic motility patterns (in the case of *R. typhi*)[12]. Therefore, primary spread from cell-to-cell in *R. prowazekii* occur by necrotic lysis of cells that have become heavily infected as a consequence of intracellular replication by binary fission leading to high accumulation compared to SFG rickettsiae[36]. In *R. typhi* cell-to-cell spread is greatly reduced because of shorter actin tail lengths but the overall rate of movement remains similar when compared with *R. rickettsii*[12].

1.1.5.4 Routes of spread in the body, target cells and organs

It has been established that pathogenic rickettsiae preferentially infect vascular endothelial cells that line the small and medium-sized vessels of their mammalian hosts. Rickettsiae are capable of infecting any nucleated cells and they possibly infect the fibroblasts, macrophages, dermal dendritic cells and lymphatic endothelia but main target cells of the rickettsiae are the vascular endothelial cells. This preference is due to the vascular location and hematogenous spread rather than the presence of any *Rickettsia*-specific receptors on endothelial cells[13]. In the skin, there is massive seeding of the dermal vascular endothelia causing endothelial cell activation and dysfunction. These lead to compromised vascular integrity and loss of barrier function resulting in non-cardiogenic pulmonary oedema, acute respiratory distress, complications of the CNS and multi-organ failure[21]. In addition, vasodilatation results in a macular rash; peri-vascular oedema causes maculopapular rash and disruption of vascular integrity leads to a petechial maculopapular rash.

Mononuclear phagocytes represent minor target cells in disseminated infections but are the major cells infected in the eschar where the rickettsiae are inoculated into the dermis by the

arthropods [21]. Rickettsiae are also engulfed by fixed mononuclear phagocytes, including those in the liver and spleen, during their spread in the bloodstream. The occurrence of prominent lymphadenopathy in the regional lymph nodes draining from the bite sites supports the assumption that *Rickettsia* spread from the bite site to the regional lymph nodes through the lymphatic vessels. From the lymph nodes, spread into the other parts of the body occurs via the bloodstream involving every organ. Although rickettsiae infect all organs, the lungs and the brain are the critical targets that determine the lethality of the infection[13].

1.1.5.5 Rickettsial infections and cell injury

Rickettsial infections occur in contiguous cells and results in organ and tissue damage. SFG *Rickettsia* spread from cell to cell by host actin-based mobility. They rarely accumulate in endothelial cells but damage the cell membrane and injure the cells. Infection of the endothelial cells stimulates them to produce reactive oxygen products that cause lipid peroxidation of the cell membrane injuring them[37, 38]. The reactive oxygen species (ROS) produced include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-) induced by the combination of tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) in rickettsial pathogenesis both as host defence and infection-induced injury mechanisms. Both *in vitro* and *in vivo* experiments have demonstrated the induction of oxidative stress mechanisms as shown by accumulation of ROS, altered level of antioxidant enzymes (superoxide dismutase, glutathione peroxidase, glutathione reductase, glucose-6-dehydrogenase (G6PD) and catalase), alterations in mRNA expression of selected antioxidant enzymes and depleted levels of intracellular reduced thiols[24]. Injured cell membranes lead to leakage of water into the cell which is sequestered in the endoplasmic reticulum. These injured endothelial cells may die and/or detach and get swept away in the bloodstream leading to hemorrhage. The endothelium may also be activated by the immune system to kill the intracellular rickettsiae[39]. Rickettsial

infection of the endothelial cells in vitro results in the development of gaps between endothelial cells[40]. The most important pathophysiologic event in rickettsial infections is due to increased permeability in the microcirculation due to gaps between infected endothelial cells[40].

1.1.6 Immunity against rickettsial infections

As in any infectious processes, immunity against rickettsial infections involves both innate and adaptive immunity. The study of host defence and immunity against rickettsial infections has been possible with disseminated infection of the endothelium in mice that established the effectors of protective immunity.

Primary immunity is characterised by the rapid action of host effector molecules and leukocytes aimed at limiting the multiplication of invading microbial organisms and destroying them before establishing an infection. It involves a cascade of events described below:

1.1.6.1 Pathogen recognition: the role of Toll-like receptors and dendritic cells

The innate immune system senses the invasion of the pathogen through pattern-recognition receptors (PRRs) including transmembrane Toll-like receptors (TLRs) and cytosolic nucleotide-binding oligomerisation-domain-(NOD)-like receptor (NLR) family proteins[41]. Recognition of pathogen-associated molecular patterns by TLRs, either alone or as heterodimers with other TLR or non-TLR receptors, triggers signals which are responsible for activation of genes required for effective host defence, particularly the inflammatory cytokines[42]. At least ten TLRs have been known in humans. TLR signalling is mediated via interactions with adaptor proteins including MyD88 and Toll-receptor-associated activator of interferon (TRIF)[43]. Toll-like-receptor 4 (TLR4) has a critical role in inflammation and immunity and it is known to be expressed in most tissues of the body[24].

Dendritic cells (DCs) as antigen presenting cells (APCs) and initiators of immune response have an important role in rickettsial infections. TLR4 signalling by ligating to rickettsial LPS

is important in activating APCs like the DCs towards the production of proinflammatory cytokines (IL-2, IL-6, IL-12, and IL-13), initiation of innate immunity (expansion of NK cell population and subsequent production of IFN-γ) and production of adaptive T_H1 type or T_H17 response. The role of DCs in recognition of rickettsiae through TLR4 and induction of vigorous proinflammatory response associated with protective immunity to rickettsiae are very important[24].

1.1.6.2 Intracellular signalling

As the primary target of rickettsial infections, the interaction between invading organism and host endothelial cells constitute one of the most important events underlying the onset and progression of infection, replication within an intracytoplasmic niche, dissemination through host and pathogenesis of the disease thereof[24]. Pathophysiological situations such as rickettsial invasion affecting the endothelial cells lead to the expression of genes dependent on the NF-κB family of transcription factors. Most of the early-response genes upregulated in response to the rickettsial invasion, like the IL-8 and Monocyte-Chemoattractant-Protein-(MCP-) 1, contain NF-κB binding sites in their promoter regions, indicating that infection-induced alterations in gene expression may be governed, at least in part, by activation of NF-κB[24]. Human endothelial cells infected with rickettsiae display nuclear translocation of NF-κB. This is due to degradation by proteasomes, of masking regulatory proteins, termed inhibitors of NF-κB, or IκB, after their phosphorylation mediated by IκB kinase (IKK) complex. However, *R. rickettsii* is also capable of directly interacting with NF-κB in its inactive form in the endothelial cell cytoplasm by an unidentified bacterial protease activity [44]. Several studies have also suggested a role for protein kinase C (PKC), in the activation of NF-κB by infectious agents, such as rickettsiae, and other stimuli.

Mitogen-activated protein kinases (MAPKs) also play a key role in signal transduction events. Three major MAPK cascades (extracellular signal-regulated kinases, c-Jun-N-terminal kinases,

and p38 MAPK) have been described. They can be activated simultaneously or independently to constitute a central regulatory mechanism that coordinates signals originating from a variety of extracellular and intracellular mediators. The enzymes in the MAPKs module are activated by pro-inflammatory cytokines, reactive oxygen species (ROS) and stress of infections and their activation lead to phosphorylation of many proteins with substantial regulatory functions throughout the cell, the NF- κB. *R. rickettsii* infection selectively induces activation of p38 MAPK in endothelial cells[45].

Human umbilical vein endothelial cells (HUVECs) infected with *R. conorii* secret large amounts of IL-6 and IL-8 via an IL-1α-dependent pathway. Il-6 and IL-8 might play a role in the development of vasculitis induced by rickettsial infection. Il-6 may also mediate acute phase protein production and also be involved in local differentiation and proliferation of T lymphocytes by stimulating IL-2 production and IL-2 receptor (IL-2R) expression on T cells and B lymphocyte stimulation[46]. IL-8 is a powerful chemotactic agent for polymorphonuclear leukocytes, stimulates polymorphonuclear leukocyte transendothelial migration and activates their function, but seems to have no effect on endothelial cells[47].

1.1.6.3 Acute phase response

In MSF, rickettsial invasion and proliferation in the ECs destroy the vessels and activate the acute-phase response. During this response, some events appear to be involved in the promotion of inflammatory events (IL-1α, IL-8, IFN-γ, complement proteins, C-reactive protein, and fibrinogen) while other responses appear to diminish inflammation (ceruloplasmin and α1-antitrypsin). When the infection resolves, all these mediators return to normal range, acute phase response subsides and homeostatic balance is restored, as depicted in **Fig 1.3**[24].

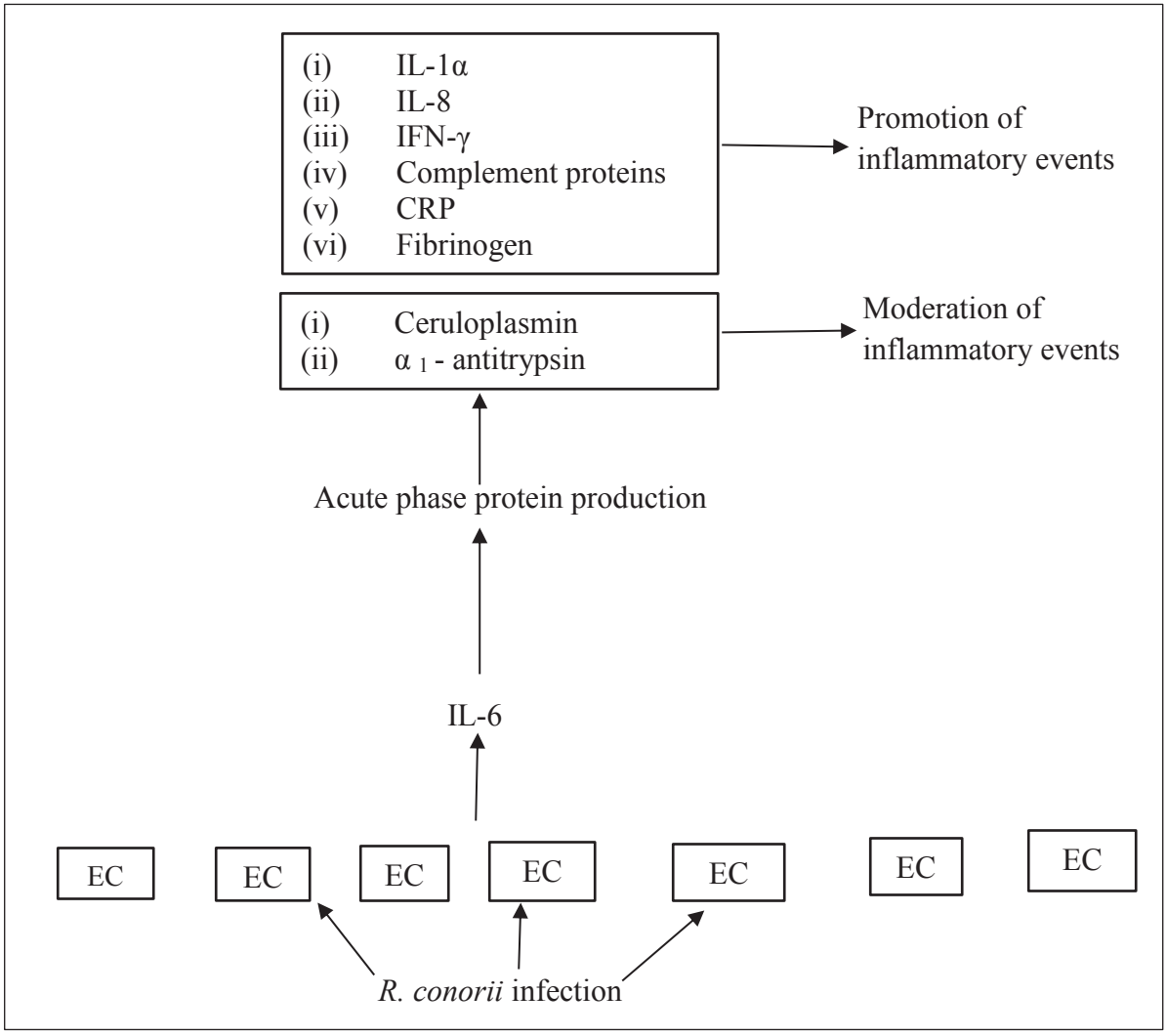


Figure 1.3. Acute phase response in rickettsial diseases[24]

1.1.6.4 Intracellular killing

Perivascular infiltration of CD4+ and CD8+ T lymphocytes, NK cells, macrophages, marginated blood elements and infected endothelial cells themselves secrete cytokines and chemokines through the paracrine and autocrine stimulation to kill the intracellular pathogen[28]. Human cells might be able to control rickettsial infections intracellularly by a cytokine-chemokine (RANTES) and a nitric oxide-dependent mechanism and in particular one or more of the three possibilities involving nitric oxide synthesis, hydrogen peroxide production and tryptophan degradation[48]. AKN-1 cells (human hepatocytes) stimulated by IFN-γ, TNF-α, and

RANTES kill intracellular rickettsiae by inducible nitric oxide synthase (iNOS) expression and nitric oxide-dependent mechanism[24].

1.1.6.5 Role of lymphocytes

CD4+T lymphocytes and related cytokines

In patients with acute MSF, a reduction of circulating T cells, particularly the CD4+ (helper/inducer T cells), CD4+/CD45RO+ (memory T cells) and CD4+/CD45+ (naïve cells) subsets was demonstrated. This could have been due to adhesion of cells to the vascular endothelium followed by their entrance into the site of inflammation. In addition, spontaneous and/or activation-induced apoptosis could have a role in this reduction[49]. Other lymphocyte subsets like the CD8+ (Cytotoxic/suppressor T cells), CD16+ (NK cells) and CD20+ (B cells) also decrease but not significantly while monocytes (CD14+/HLA-DR+) increase significantly. After successful treatment of the infection, all cell subsets return to their normal levels but monocytes persist at a higher level for longer after recovery. Inflammatory and immunologic responses mediated by an increased level of T_H1-type (TNG-α and TNF-γ) and T_H2-type cytokines (IL-10 and IL-6), appear to be important for recovery from the infection. Serum levels of TNF-α, TNF-γ, IL-10, and IL-6 are increased significantly compared to control healthy subjects[24].

CD8+ T cells

CD8+ T lymphocytes, probably activated by antigen-presenting ECs in association with antigens on class I major histocompatibility complex (MHC-I), contribute to protective immunity during rickettsial infections by both MHC-I restricted cytotoxic T-lymphocyte (CTL) activity and production of IFN-γ. CTL activity might, however, be more critical in recovery from rickettsial infections than the effects of IFN-γ production by CD8+ T cells as

shown in **Fig 1.4.** Thus, CD4+ and CD8+ T lymphocytes are both a potentially rich source of IFN-γ, which could activate endothelial rickettsicidal activity and tip the balance in favor of survival [24].

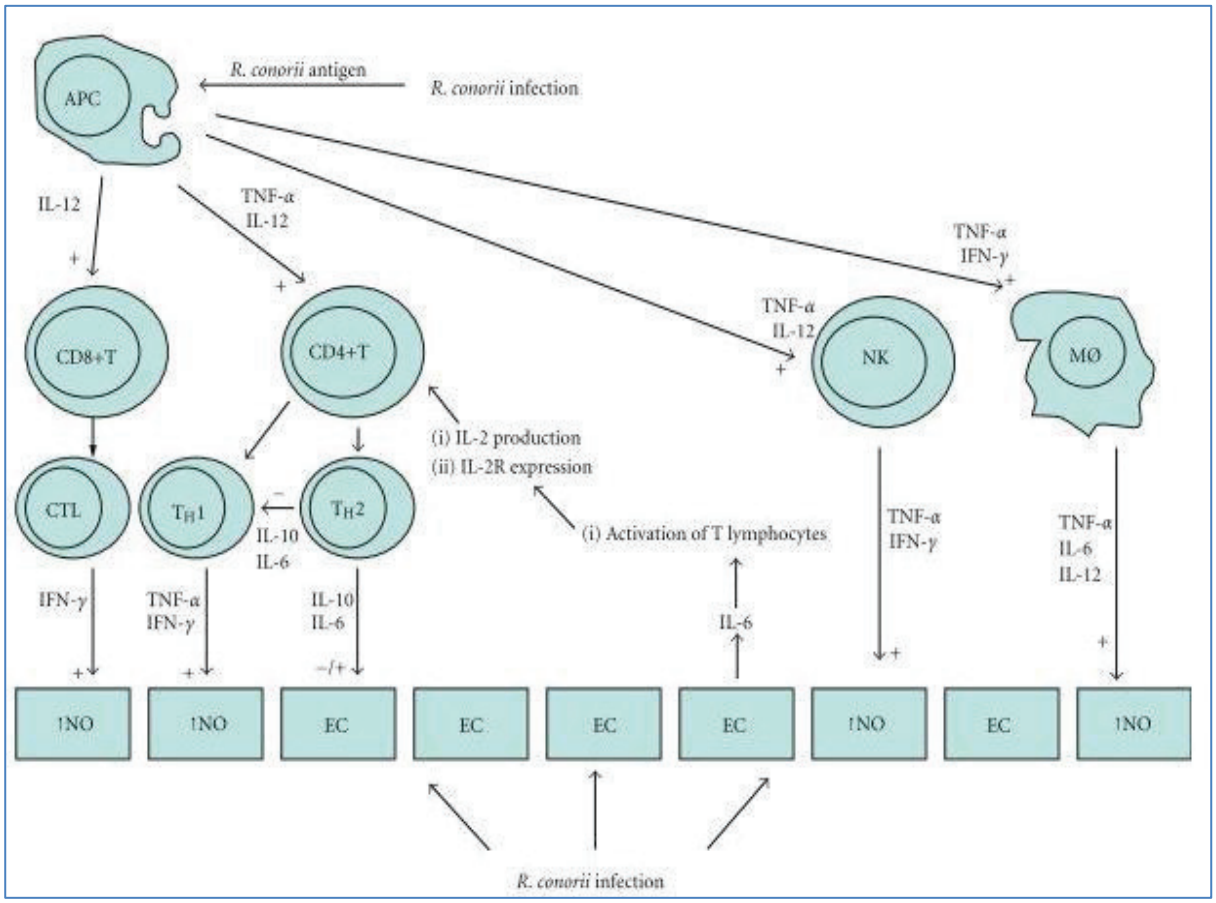


Figure 1.4. Immunologic alterations in rickettsial diseases[24]

1.1.6.6 Role of antibodies

Conventionally, since the rickettsiae are obligate intracellular organisms, the role of antibody was not thought to be important and the focus was mainly on cellular immunity. The availability of a well-characterized mouse model of SFG rickettsiosis enabled the potential role of antibodies to *R. conorii* and its surface antigens in protective immunity to be studied [50]. Feng and colleagues (2004) found that not only did polyclonal antibodies to *R. conorii* and monoclonal antibodies to major outer membrane protein A (OmpA) and OmpB protect severe

combined immunodeficiency (SCID) mice challenged with a lethal dose of *R. conorii*, but also antibodies administered to SCID mice 4 or 5 days after establishment of infection significantly reduced the infectious rickettsial contents in the spleen, lungs, and liver 24 and 48 h later and prolonged the life of the recipients by an average of 12 days. This exhibited that antibody response also forms an important and powerful tool in immunity against rickettsial infections. In addition, antibodies act as opsonins rendering microorganisms susceptible to destruction by the macrophages though antibodies themselves are not bactericidal.

1.2 *Orientia*

1.2.1 Introduction

Orientia tsutsugamushi is the causal organism of scrub typhus in humans. It was previously known as *Rickettsia tsutsugamushi*. Although *R. tsutsugamushi* appeared like all other *Rickettsia* in many aspects there were marked differences in the organization of its outer envelope. This was found in the comparative study on the ultrastructural components of the outer cell envelopes of *R. prowazekii, R. rickettsii*, and *R. tsutsugamushi* [51]. This study, using electron microscopy revealed that the outer leaflet of the cell wall of *R. tsutsugamushi* was comparatively thicker than the inner leaflet which was the opposite in the case of other rickettsia species. Other remarkable differences included lack of peptidoglycan and lipopolysaccharide in the cell wall of this organism. In addition, there was a large difference between the protein compositions of this organism and other rickettsiae. During the time when the genus *Rickettsia* was undergoing major taxonomic changes with gene sequencing techniques, *R. tsutsugamushi* was found to be very distinct from other *Rickettsia* by 16S rRNA gene sequencing. Analysis of 16S rRNA gene sequence indicated 98.5% to 99.9% homology among the different species of scrub typhus organisms but only 90.2% to 90.6% homology

with other rickettsiae [52]. These findings warranted a new genus for *R. tsutsugamushi* and hence it was separated as *Orientia tsutsugamushi* [4].

1.2.2 Bacteriology

The causative organism of scrub typhus was demonstrated inside human and animal macrophages in 1924 and six years later passaged in Descemet's membrane by Nagayo and colleagues[53]. *O. tsutsugamushi* is a rod-shaped organism measuring approximately 0.5 μm in width and 1.2 to 3.0 μm in length, slightly larger than typhus and spotted fever group rickettsiae. They are obligate intracellular organism like other rickettsiae. When stained by Giemsa stain, they stain deep purple and are characteristically grouped in perinuclear clusters. They multiply slowly in tissue culture and are less stable than other rickettsiae in the environment when extracellular.

O. tsutsugamushi display immense antigenic variations among different strains and this is greater than that observed among the different strains of the other groups of rickettsiae. Gilliam, Karp, and Kato are the three classic strains used as prototype strains in most studies. Making up 10-15% of its total protein, the variable 56-kDa protein is the major surface protein of *O. tsutsugamushi*[54]. This is an immune-dominant protein antigen that is reactive with the group and strain-specific monoclonal antibodies. It is recognized by sera from most scrub typhus patients. Among the various strains, there is 59-82% amino acid homology[55]. There are other four major surface proteins at the 110, 47 and 25kDa and they also display substantial antigenic variability[54, 56]. It is the multiple components that exhibit variability in a background of strong homology that is responsible for the strain heterogeneity among scrub typhus group.

1.2.3 Pathology and pathogenesis

The pathology of scrub typhus is unclear in many ways and there is a poor correlation between clinical findings and pathological observations. There is immense geographical variation in the severity of the disease, but the determinants of severity are not well defined. The most notable pathological finding in scrub typhus is disseminated focal vasculitis and peri-vasculitis, especially involving the vessels of the skin, heart, lungs, and brain. Focal hemorrhages and endo-vasculitis are seen but are usually not so prominent as in RMSF and epidemic typhus[57]. Vasculitic lesions in the kidney and heart have been reported consistently[57]. The most important lesions are interstitial pneumonia with alveolar oedema, hemorrhage, interlobular oedema and meningoencephalitis[57, 58].

O. tsutsugamushi predominantly infects ECs but are found in several other cells including the dendritic cells (DCs), macrophages, PMNs, and lymphocytes. The organism has been demonstrated in a variety of human cells like the monocytes, macrophages, Kuppfer cells, cardiac myocytes, hepatocytes and endothelial cells[57-59]. The bacterium invades host cells by induced phagocytosis and then is taken into phagosomes[24]. The definite mechanism of spread after deposition in the skin is not known in humans. But the presence of the organism in the bloodstream can be inferred since the standard method of isolating *O. tsutsugamushi* is inoculation of the patient's peripheral blood into mice. In an experimental study of transmitting scrub typhus to human volunteers with laboratory-reared chiggers, the participants suffered from febrile illness with eschar and regional lymphadenopathy. This occurred after 8 to 10 days of the incubation period and during this time, the organisms inoculated from the salivary gland of the chiggers into the dermis apparently spread to the regional lymph nodes and bloodstream[60]. Bacteraemia occurred one to three days before onset of fever.

1.2.3.1 Invasion of ECs, DCs, and macrophages

Initially, specific receptors for attachment to cells were not identified, but it was found that heparin sulphate proteoglycans contribute to the attachment of *O. tsutsugamushi* to the cells[61]. Recent findings have added more insights into this. Invasion of the host cells by the organism is mainly mediated by interactions between bacterial surface components and complementary host receptors, which stimulate host signal transduction pathways required for bacterial access. *O. tsutsugamushi* uses host fibronectin interactions with one of its outer membrane proteins, the 56-kDa, type-specific antigen (TSA56), for internalization. Fibronectin then facilitates bacterial entry into the host cells via interactions with integrins, α5β1. Once integrins are engaged, signalling molecules at the inner surface of the plasma membrane that acts as integrators of responses to extracellular stimuli are activated. Focal adhesion kinase (FAK), Src kinase and RhoA GTPase are activated by *O. tsutsugamushi* invasion and the signalling adaptors talin and paxillin are recruited to the site of infection. In addition, extensive actin reorganization and membrane ruffling in the region surrounding the *O. tsutsugamushi* cells are induced within ten minutes after attachment. These findings have demonstrated that *O. tsutsugamushi* exhibit integrin-mediated signalling and rearrangements of the actin cytoskeleton for the invasion of eukaryotic host cells[62, 63]. Many other membrane proteins, as well as the TSA56, have been known to interact with other receptors to trigger these signalling events for entry of the pathogen.

1.2.3.2 Activation of ECs, DCs, and macrophage

After the invasion of the dermis by the organism, inflammation is initiated. Proinflammatory cytokines and chemokines secreted by activated DCs (TNF-α, IL-13β, IL-6, macrophage inflammatory proteins (MIP)-1 α/β, MIP-2 and MCP-1) are mainly responsible for recruitment of leukocytes into the inflammatory tissues. Members of the CC chemokine subfamily,

including MIP-1 α/β (CCL3/CCL4), MCP-1 (CCL2) and RANTES (CCL5) preferably attract monocytes and lymphocytes while those of the CXC chemokine subfamily, IL-8, and MIP-2 (CCL8), attract neutrophils. The CC chemokines (MIP-α/β and RANTES) also attracted T_H1 cells but not T_H2 cells. Stimulation of T cells in the presence of MIP-1α enhances production of IFN-γ by T_H1 cells, while stimulation of T cells in presence of MCP-1 leads to an increase in IL-4 production (a T_H2 cytokine)[24]. Altogether, T-cell differentiation by a subset of chemokines produced by activated DCs might be a crucial factor in the induction of a resistant versus susceptible immune response during infections by *O. tsutsugamushi.*

ECs are also an important part of the inflammation process. Activation of ECs by proinflammatory cytokines including TNF-α and IL-1 (released by infected cells) result in the upregulation of cell adhesion molecules (P-selectin, E-selectin, ICAM-1 and VCAM-1) to promote cellular influx via transendothelial migration, as well as production of cytokines and chemokines like IL-1α, IL-6, IL-8 (CXCL8), IL-10, IL-15, TNF-α, CXCL1 to 3, MCP-1 (CCL2), CCL5 and CCL17, to initiate and propagate local inflammatory responses[24].

1.2.4 Immunity against *Orientia tsutsugamushi*

Scrub typhus is an acute but self-limiting illness. Many studies have clearly documented the role of host immune responses during infection. Both the cellular and humoral immunity has been demonstrated to be important in the killing of *O. tsutsugamushi* by the mouse macrophage [64]. However, the process by which the organisms are eliminated during the infection is not clearly demonstrated. B-lymphocyte depleted mice are more susceptible to infections and antibodies enhance the clearance of *O. tsutsugamushi* from the blood [65]. The T-cell-mediated cytotoxic immune mechanism plays an important role [66]. *O. tsutsugamushi* is a potent inducer of chemokines and cytokines as noted in experimental inoculations.

Immunity towards scrub typhus is incomplete and there can be repeated reinfections[67]. Human studies demonstrated that heterologous immunity lasts for one to three months and homologous immunity lasts for about one to three years [68]. However, reinfection with heterologous strains results in milder disease [69] and first infections are often more severe than the subsequent infections [70].

1.2.4.1 Innate immunity

Nod-like receptor (NLR) family proteins are involved in innate immunity in *Orientia* infections like the TLRs are important in innate immunity to rickettsiae. The NLR family is composed of 22 intracellular molecular-pattern-recognition molecules. One subfamily that includes NOD1 and NOD2 can sense peptidoglycan polymers from cell wall components. NOD1 and NOD2 activation trigger recruitment of adaptor protein-receptor-interacting serine-threonine kinase 2 (RIPK2 or RIP2) followed by activation of NF-κβ and MAPKs. Another family that includes NLRP proteins are essential for regulation of caspase-1 activation via the N-terminal pyrin domain by inflammasome formation, consisting of NALP3, caspase recruitment domain (ASC), and caspase-1. The NALP-3 inflammasome then cleaves pro-IL-1β, pro-IL-18 and pro-IL-33 to mature IL-1β, IL-18, and IL-33 respectively. During infection with *O. tsutsugamushi,* NOD1 senses an *Orientia* component in ECs and activates the downstream pathway of NF-κβ leading to the production of IL-32, an IL-18-induced cytokine. Increased IL-32 levels affect secretion of proinflammatory cytokines, IL-1β, IL-6 and IL-8 as well as ICAM-1 expression in ECs[24], **Fig 1.5.**

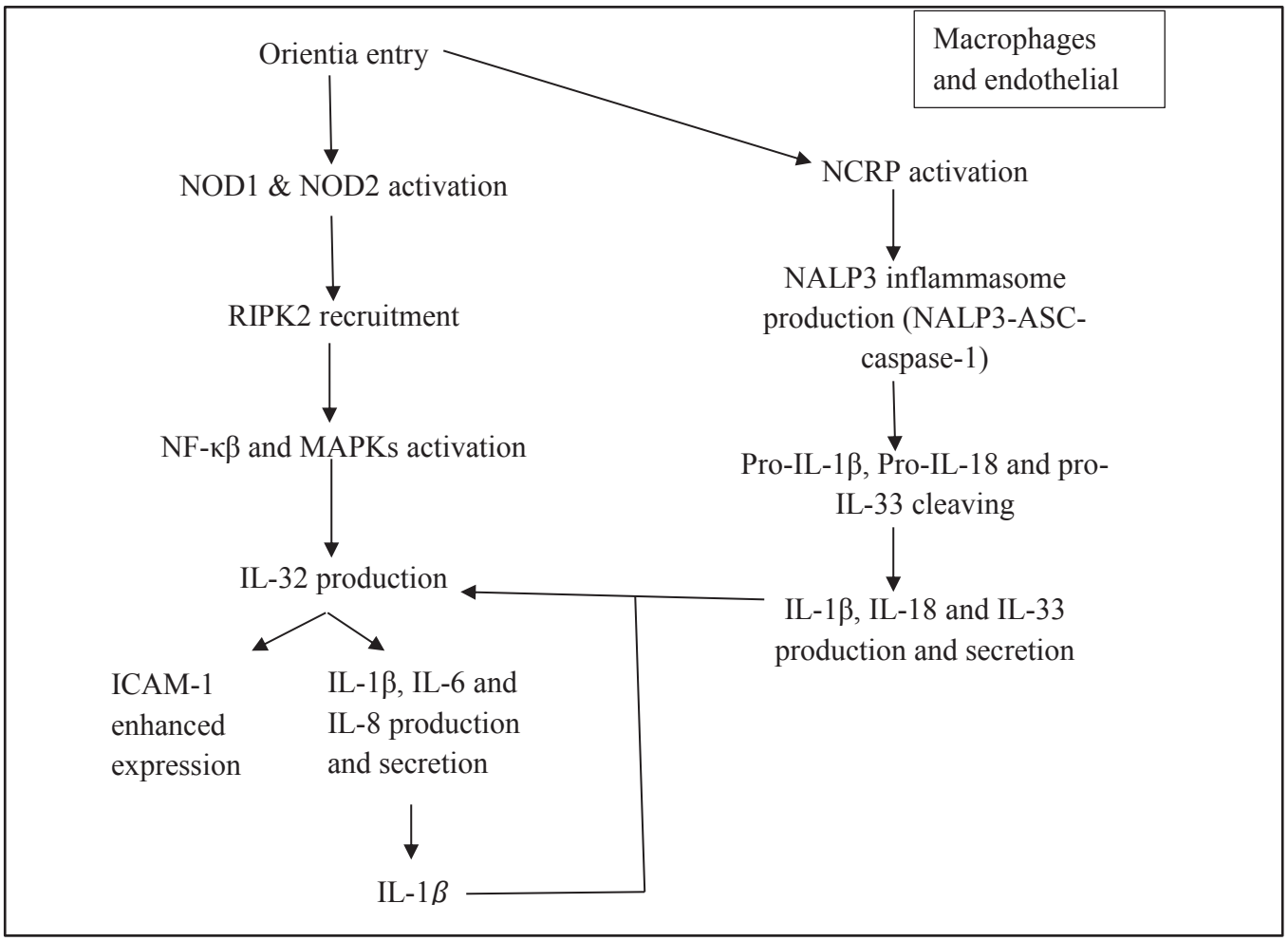


Figure 1.5: Innate immune system in *Orientia* infection[24]

1.2.4.2 Acquired immunity

CD4+ lymphocytes and related cytokines

Type -1 cell-mediated immunity and IFN-γ production of T cells in response to *Orientia tsutsugamushi* antigen is essential for immune protection against infection, whereas the opposite T_H2 subset is considered to be detrimental. In animal experiments, T_H1 and T_H2 type responses are not clearly polarised since both activating (IL-12, IFN-γ) and suppressive (IL-10) cytokines are detected simultaneously, **Fig. 1.6**[24].

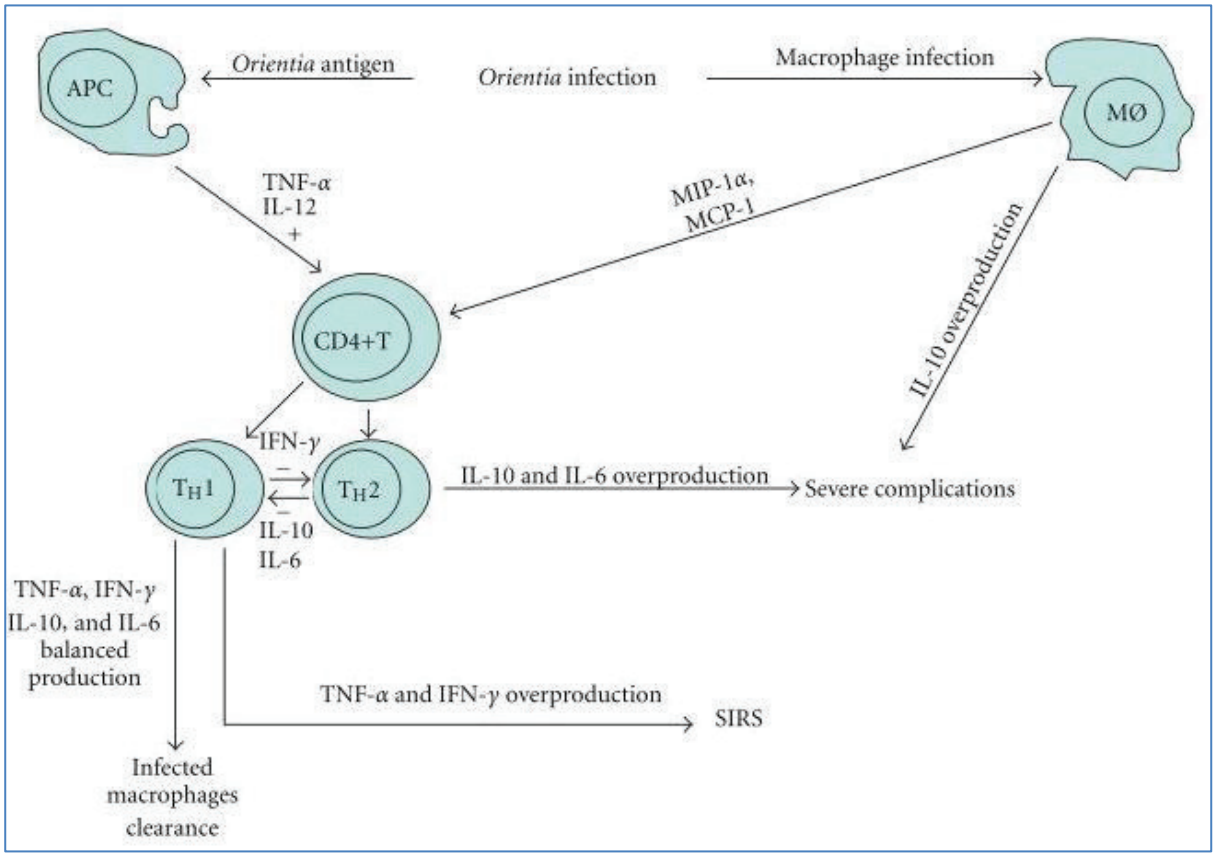


Figure 1.6: T lymphocytes and related cytokines *Orientia* infection[24]

Humoral immunity

The effect of antibodies that appear early during the infection on the course of the infection is not clearly understood. But humoral immunity might play a role protective immunity against *O. tsutsugamushi* by inhibiting the events required in attachment, entry and/or trafficking and replication in the cytoplasm[71]. Antibodies have been demonstrated to enhance opso-phagocytosis of *O. tsutsugamushi* by professional phagocytes like macrophages and polymorphonuclear cells. Antibodies also inhibit invasion of the bacterium into non-professional phagocytes such as the ECs, epithelial cells and fibroblasts[71]. Never the less, neither cytokines nor antibodies alone enable macrophage cultures to completely suppress infection by Orientia, indicating that both cellular and humoral immunity play a role in clearing

O. tsutsugamushi by facilitating uptake and ensuing intracytoplasmic destruction of the bacterium.

There is wide antigenic heterogeneity among the *O. tsutsugamushi* strains that a whole cell vaccine prepared against one strain generally fail to protect against infection from others. Orientia lacks both peptidoglycan and LPS but contains major strain variable 56-kDa protein with the antigenically variable 110-, 47-, and 25-kDa proteins. The 56-kDa protein, among all antigens, is often recognized by both human and animal host immune systems during infections. The 56-kDa protein is closely identical, structurally and functionally, to the eukaryotes' protein, the Heat Shock Protein (Hsp) 60. There is enough evidence to indicate that heat shock proteins mainly serve as target molecules in the anti-infectious or auto-aggressive immune responses. Therefore, the 56-kDa protein has been considered as a recombinant vaccine candidate[71]. In a follow-up of patients following documented infections 1 month and 46 months, IgM levels declined gradually but remained elevated above the diagnostic cut-off for up to 12 months post-infection. However, IgG levels continued to rise to reach a peak at 10 months, followed by a slow decline over several months. In most cases, the IgG levels remained above the cut-off threshold for more than 36 months[72].

1.3 *Coxiella*

1.3.1 Introduction

The name "Q" fever (QF) (Q for query) was first given by Derrick in 1937 while investigating an outbreak of unknown fever cases in Brisbane, Australia, among abattoir workers [73]. The causative organism of QF, *Coxiella burnetii* was first isolated from the ticks of Nine Mile Creek in Montana, USA [74]. After Cox first cultivated the organism in the USA and Burnett recovered them from Derrick's outbreak samples sent to him, the organism was named after them as *C. burnetii*, although initially, it was named as *Rickettsia burnetii*.

1.3.2 Bacteriology

C. burnetii is a non-motile pleomorphic coccobacillus measuring about 0.3-1.0μm. Despite having a Gram-negative cell wall, they are not usually stainable by the Gram staining technique[75] and are stained by the Gimenez method to identify isolates from culture or clinical samples[76, 77]. *C. burnetii* was considered a strict intracellular parasite until its successful growth in a complex nutrient axenic media[78] and its first isolation from clinical material in this cell-free medium called acidified citrate cysteine medium 2 (ACCM2)[79]. They replicate to high numbers in eukaryotic host cells with an estimated doubling time of 20 to 45 hours[80]. Although significant differences exist amongst different isolates of *C. burnetii*, these differences were not sufficiently great to permit a division into different species [81]. Therefore, *C. burnetii* is the sole species in its genus and the differences only lead to different strains of the organism.

Previously, *Coxiella* was placed with the orders *Rickettsiales* and *Chlamydiales* but separated after the 16s RNA sequencing revealed that the *Rickettsiales* and *Chlamydiales* were different and that *Coxiella* was not related to either of them[81]. *Coxiella* was considered a different genus in 1948 due to its culture and biochemical characteristics.

1.3.3 Metabolism and transport

Coxiella is an intracellular parasite like *Rickettsiae*, but unlike *Rickettsiae* (that live in the cytoplasm), *Coxiella* resides within a parasitophorous vacuole (PV)[82]. The PV has characters of a phagolysosome acquires an acid pH, acid hydrolysates and cationic peptides[83]. Within the PV, the organisms are highly activated by low pH and metabolic processes like nutrient transport and substrate metabolism are initiated [84]. *C. burnetii* display a broad spectrum of biosynthetic capabilities and utilise sugar via the Entner-Doudoroff(glycolysis), the Embden-Meyerhof-Parnas(gluconeogenesis), the pentose-phosphate pathway, and the tricarboxylic acid (TCA) cycle. *C. burnetii* depend on exogenously acquired amino acids since they lack genes

for glyoxylate bypass of TCA. Several other complete amino acid biosynthetic pathways or key enzymes are also absent. Energy from ATP is generated through oxidative phosphorylation and electron transport chain reaction.

Intracellular organisms like *Coxiella* need transport mechanism to facilitate nutrient and precursor uptake as they depend on host factors. *C. burnetii* possesses two proton-driven transport systems for glucose and xylose, 15 amino acid transporters and three peptide transporters [85]. Although *C. burnetii* have an intact synthetic pathways for purines and pyrimidines, there is active uptake and incorporation of purine nucleotides but the pyrimidine uptake is passive and relies on diffusion [86]. Several cellular transporter genes possessed by *C. burnetii* are for drug-efflux systems and they probably mediate resistance to host-produced defensins and antibiotics.

1.3.4 Developmental life cycle and phase variation

C. burnetii has an unusually high tolerance to chemical and physical factors enabling the organism to remain viable in the environment for a long time and remain infectious. This is the result of expressing different cell types in its biphasic life cycle. *C. burnetii* can be seen in two distinct cell forms within the phagolysosomal compartment of eukaryotic host cells[87]. A developmental life cycle for *C. burnetii* which included different vegetative cells and sporogenesis was formulated by McCaul and William[88]. They described two forms of *C. burnetii* and described them as Small Cell Variant (SCV) and Large Cell Variant (LCV) as different stages of developmental life cycle. The LCV is an exponentially replicating vegetative form of the organism found in infected cells and the SCV is metabolically inactive, non-replicating, extracellular and presumably the infectious form[75, 89]. These two forms can be separated by density-gradient centrifugation and differentiated upon morphological markers. The SCVs are 0.2 to 0.5 μm in diameter, small rod-shaped with condensed chromatin, a thick

envelope and close network of the intracytoplasmic membrane system. The SCV is typical of stationary phase and is observed after prolonged culture (21 days) in Vero cells and ACCM2 medium[90]. The SCV is resistant to heat shock, pressure, sonication, osmotic shock, and oxidative stress. Transcriptome analysis of SCV has depicted upregulation genes involved in oxidative stress response, cell wall remodelling and arginine acquisition. There is also an unusually high number of cross-links in their cell wall peptidoglycan probably involved in environmental resistance[89]The LCVs are larger (>0.5 μm) and more pleomorphic. They have dispersed filamentous chromatin. They show typical Gram-negative bacterial characteristics when growing in exponential phase with cytoplasmic membrane, periplasmic space and outer membrane[91, 92].

C. burnetii exhibit antigenic variation which is dependent on the host. After several passages in embryonated hen eggs or cell culture, the bacterial population transforms from a wild virulent (phase I) to a mutant avirulent (phase II) form [93, 94]. This is similar to the smooth to rough variation in many enterobacteriaceae. Phase I form exhibit a smooth, full-length LPS, are highly infective, and naturally found in infected animals, human, and ticks. The phase II form has a truncated, rough LPS and may differ in surface protein composition, surface charge, and cell density. Phase variation is not a single-step process since there are intermediate-phase or semi-rough LPS molecules[95]. In the Nine Mile strain, this phase shift is caused by a large chromosomal deletion[96].

1.3.5 Pathogenesis

1.3.5.1 Sources and routes of transmission

C. burnetii infects various hosts, including humans, ruminants (cattle, sheep, goats), dogs, cats, birds, rodents, reptiles, and ticks. Identifying infections in an animal are challenging and most infections can remain asymptomatic. The organism is excreted in urine, milk, faeces, and birth

products. These products, especially the latter, contain large numbers of bacteria that become aerosolized after drying [97]. Human infections occur mainly through aerosols but infection by ingestion of infected dairy products is possible. The portal of entry is through the oropharynx even in animals. The hypothetical risk of transmission through the digestive tract by consuming products from *C. burnetii*-infected animals has been controversial and the role of ticks in the transmission of *C. burnetii* to humans is limited. In a rare case, a kangaroo tick, *Amblyomma triguttatum* was implicated as the route of transmission of *C. burnetii* in a case of QF pericarditis in Western Australia[98]. In addition, reports on the human-to-human transmission of *C. burnetii* remains anecdotal. With an infectious dose as low as one organism, *C. burnetii* is a highly infectious organism[99]. The extreme infectivity, the possibility of aerosolization and its high environmental stability makes *C. burnetii* a potential biological weapon and the Centre for Disease Control and Prevention, USA, classified *C. burnetii* under category B bioterrosism agent[100, 101].

1.3.5.2 Entry into and proliferation in the host cells

C. burnetii infects a large number of cell types including monocytes, macrophages and a variety of transformed cells[80]. One of the key virulence factors of the organism is the ability to efficiently invade and subsequently grow within eukaryotic cells. *C. burnetii* was known to reach its internal niche by phagocytosis and is known to enter passively[80, 102]. The phase II organisms are more readily internalized by the host cells than are the phase I variants. Opsonization by specific Ig enhances phagocytosis of both phase I and II. Upon contact between the organism and the host cell membrane, protrusions and polarized projections are induced over an extensive region of the cell surface.

1.3.5.3 Virulence factors of *C. burnetii*

Differences in virulence of different strains of *C. burnetii* were compared in several animal models such as mice, guinea pigs, rabbits, and non-human primates. The pathogenicity and virulence of *C. burnetii* depend on the animal species, the route of infection (such as intraperitoneal, intranasal injection, aerosolization), the strain of the organism (example Nine Mile Phase I vs Priscilla Phase I strains) and the inoculum size used[103]. For example, the Nine Mile strain was proven to be more virulent than the Priscilla strain in BALB/c mice and guinea pigs when infected via the intraperitoneal route[104]. The genetic diversity and virulence potential of different strains of *C. burnetii* are also related to the expression of LPS. The phase I (infective form) and phase II (avirulent form) forms of *C. burnetii* are a result of irreversible modification of the LPS resulting from a genomic deletion[75]. Phase I organisms are more virulent than phase II variants. The major difference between the LPS of the virulent and avirulent strains are seen in the core sugar, the LPS of the avirulent strains lacking the O antigen. The LPS of the virulent strains also contain sugars such as verinose, dihydrohydroxystreptose and galactosamine uronyl-glucosamine[105]. The genome size of *C. burnetii* ranges from 1.5 to 2.4 Mb among different strains[106]. *C. burnetii* can harbour one of four plasmids; QpH1, QpRS, QpDG, QpDV or QpRS-like plasmid[107]. These plasmid types are associated with specific genomic groups. Isolates within genomic groups I, II and III are derived from patients with acute Q fever whereas those in genomic groups IV and V are derived from patients with chronic disease[108]. These findings have tempted the possible association of specific plasmids with virulence among strains. Other virulence factors in the organism include genes that encode for adhesion, invasion, detoxification and for a peptidyl-poly-cis-trans-isomerase. Adhesion genes encode for RGD motif or proteins containing ankyrin[85], detoxification genes encode for superoxide dismutase, catalase, and acid phosphatase enzymes

that allow escape of the organism from the microbicidal activity of macrophages by detoxification of reactive oxygen intermediates produced by the host cells[109].

1.3.5.4 Role of the host and immunity to *C. burnetii*

Host immunological responses also contribute to the pathophysiology in *C. burnetii* infection. QF is usually asymptomatic in about 60% of the infections[110, 111] and primary infections almost always resolve without antibiotic treatment. This fact suggests that host immune response is sufficient to control the infection[112], which depends on the cell-mediated immunity involving innate and adaptive partners of the immune response. There are certain host factors which make them susceptible to *C. burnetii* infection. Age is a risk factor for *C.burnetii* infection and symptomatic QF is more common in individuals 15 years and older[113]. Although the mechanism remains poorly understood, pregnancy has been found to a risk factor for *C. burnetii* infection. This has been partially explained by the trophoblastic tissue of pregnancy being target cells to *C. burnetii* and the silencing of immune responses. It has also been shown that the interaction of *C. burnetii* with the trophoblastic tissues does not induce an inflammatory reaction which accounts to the relative immune silence during QF in pregnancy[114]. In addition, *C. burnetii* has been shown to be unable to activate decidual DCs[115]. Other host factors like preexisting valvulopathy enhance the risk of developing endocarditis during QF infection.

In vertebrates, infection with *C. burnetii* results in granuloma formation in infected organs under the control of gamma interferons (IFN-γ). This possibly results from the migration of monocytes through the vascular endothelium. A typical QF granuloma has a lipid vacuole in the center surrounded by a fibroid ring[75]. During acute infections, only very few bacteria can be found in the granuloma[111]. Granuloma formation is partly dependent on TLR4 with TLR4 deficient mice infected with *C. burnetii* having fewer numbers of granuloma[116]. The presence of neutrophils in these granulomas suggest the involvement of these white cells in defence against *C. burnetii* infection[117]. Human Dendritic cells (DCs) are specialized antigen presenting

cells (APCs) and are involved in presenting antigens to T cells. DCs have been demonstrated to be a target cell of *C. burnetii*, with phase I, but not phase II forms of *C. burnetii* infection blocking the maturation of DCs.

Immunity to *C. burnetii* is T-cell dependent but it does not lead to the eradication of the organism and *C. burnetii* can be found in apparently cured patients as well as in dental pulp of experimentally infected and apparently cured guinea pigs[116]. *C. burnetii* nucleic acids have been found in monocytes and bone marrow of individuals infected months or years earlier[111]. It was reported that CD8-T cells are more efficient than CD4-T cells in controlling the infection. The critical step in the protective response in QF infection is the polarization of the T-cell response towards the Th1 phenotype (the ability to produce IFN-γ) as in any other intracellular pathogen. In acute infections, specific antibodies are produced with IgG directed mainly against phase II antigens and IgM against both phases I and phase II antigens[75]. Chronic QF, on the other hand, is characterized by the inefficient immune response[111]. In chronic QF, *C. burnetii* continue to replicate despite the presence of high levels of specific antibodies (IgG, IgM, and IgA) directed against both phase I and phase II organisms. There is also a decrease in lymphocyte count and the CD4/CD8 ratio[118]. It has been found that the polarization of macrophages dictates their microbicidal activity against *C. burnetii*[119]. *C. burnetii* mainly induces the expression of M2 polarization-related genes, like those encoding Transforming Growth Factor (TGF)-β1, IL-1 receptor antagonists (IL-1ra), CCL18, the mannose receptor, and arginase-1 but also induce the expression of genes encoding two cytokines associated with M1 (IL-6 and CXCL8). On the other hand, *C. burnetii* inhibit the expression of M1-associated genes like those encoding for TNF, CD80, CCR7, and TLR2[119]. *C. burnetii*-stimulated macrophages secrete high levels of TGF- β1 and CCL18 and express the mannose receptor and arginase-1, the latter being associated with the absence of nitric oxide release by macrophages. There is poor release of IL-6 and CXCL8 by *C. burnetii*-stimulated macrophages[119]. It has

been suggested that this atypical M2 activation program is responsible for the persistence of *C. burnetii* in macrophages[120].

The role of antibodies, the second arm of adaptive immunity, in protection against *C. burnetii* infection was considered dispensable[101]. Although initial studies showed that passive transfer of antibodies protected guinea pigs from subsequent challenge with *C. burnetii* and passive immunization of naïve mice with antibodies extracted from vaccinated mice provided full protection[121], immune sera or B cells from C. burnetii-challenged mice did not confer protection when transferred to severe combined immune deficiency (SCID) mice[121]. This concept is also supported by the presence of high antibody titers directed against phase I and II antigens in persistent *C. burnetii* infection which are unable to clear the bacteria. These high antibody titers have a deleterious effect on the host due to the formation of immune complexes leading to tissue damage[122].

Chapter 2: Clinical features, laboratory diagnosis, treatment, prevention and control

2.1 Clinical features

Members of the *Rickettsia* and *Orientia* genus cause a wide variety of infections known together as rickettsioses with an outwardly similar clinical presentation but of varying severity[21]. Rickettsial infections included in this study are the Spotted Fever Group (SFG) and Typhus Group (TG) *Rickettsia.* Infections caused by *Orientia tsutsugamushi* and *Orientia chuto* together form the Scrub Typhus Group (STG). Q fever (QF), caused by *Coxiella burnetii* is frequently included with the rickettsioses.

2.1.1 Clinical features of Rickettsial infections

Rickettsial infections are known by different names depending on the rickettsial species, vector host or the region/country of occurrence. Some common infections (and their causal species) in the SFG *Rickettsia* include Rocky Mountain Spotted Fever (RMSF) (*R. rickettsii*), Rickettsialpox (*R. akari*), Indian tick typhus/Boutonneuse fever/Mediterranean spotted fever (MSF) (*R. conorii*), Flea-borne spotted fever or Cat-flea typhus (*R. felis*), African tick bite fever (*R. africae*), North Asian tick typhus/Siberian tick typhus (*R. sibirica*), Queensland tick typhus (*R. australis*), Finders Island spotted fever (*R. honei*), Japanese spotted fever/Oriental spotted fever (*R. japonica*), Tick-borne lymphadenopathy (TIBOLA)/scalp eschar and neck lymphadenopathy (SENLAT)/Dermacentor-borne necrosis, erythema and lymphadenopathy (DEBONEL) (*R. raoultii and R. slovaca*) etc[23]**.** The two infections constituting the TG *Rickettsia* are Endemic/Murine flea-borne typhus (*R. typhi)* and Epidemic louse-borne typhus (*R. prowazekii*). Some common features that suggest an early diagnosis of rickettsial diseases include a history of tick bite or exposure, recent travel history to an endemic area and a similar illness in family members or co-workers. However, as many as 40% of patients may be

unaware of the tick bite since the bites are usually painless and may go unnoticed or be easily forgotten[123].

Clinically, the classic form of RMSF, regarded as the prototype rickettsial infection usually present with an abrupt onset after an incubation period of about 7 days (average 2-14 days)[124]. The main clinical features include fever, headache (usually persistent, intense, and intractable), rash, confusion, and myalgia. Patients may appear toxic and usually progresses to mental confusion and delirium. Gastrointestinal (GI) symptoms (such as abdominal pain and diarrhoea) were common during the early stages of illness and conjunctival injection may be seen [123]. Clinical complications such as psychiatric problems, renal failure, intracranial bleeding, gangrene, neuropathy, syndrome of inappropriate secretion of antidiuretic hormone (SIADH), and respiratory failure occurred in 9% of cases. Disease manifestations ranged from asymptomatic to severe, with a mortality rate approaching 25% in untreated cases [124]. Clinical manifestations of rickettsial diseases due to other species are frequently compared with that of RMSF in terms of severity and complications.

R. australis causes Queensland tick typhus (QTT), an increasingly recognized important cause of community-acquired acute febrile illness in eastern Australia[125]. Acute QTT is probably underestimated although there have been several reports of it from different locations in Australia[126] including a fatal case of a previously healthy 68-year-old man from progressive organ faulure[127]. Compared to the incubation period of most other rickettsial infections (1-2 weeks), QTT has a shorter incubation period of 5 days (3-6 days) on average. Acute infection often begins with a high-grade fever (up to 41^{o}C), headache, malaise, myalgia, and a rash[125]. In this review of QTT, other features included tender lymphadenopathy (70%) (localized to the draining lymph nodes near the tick bite), eschar (up to 50-65%), arthralgia, splenomegaly, abdominal pain, dry cough, sore throat, conjunctivitis, and photosensitivity[125]. Fever usually responds to treatment within 48 hrs and if this doesn't happen and fever is prolonged, end-

organ dysfunction, and intensive care admissions are likely[126]. In a 15 years retrospective study, 36 cases of laboratory-confirmed QTT cases had fever (≈100%), rash (83%), eschar (17%), lymphadenopathy (28%), headache (67%), arthralgia (56%), respiratory symptoms (cough and dyspnoea) (28%), abdominal pain (28%) and confusion and drowsiness (3%). The median temperature was 39.1ºC (37.7-40.5ºC) and fever lasted for an average of 1.4 days. Rash was mostly maculopapular (70%) and distributed widely[128].

R. conorii is the causal organism of Mediterranean Spotted Fever (MSF) also known as Boutonneuse fever (BF). It is referred to as Indian tick typhus in the Indian subcontinent. It usually presents as a benign self-limiting disease characterized by high fever, a skin rash and, sometimes, a characteristic ulcer at the tick bite site called *tache noir*. It is usually a benign infection, although severe manifestations have been previously described, mainly in adults[129]. Neurological manifestations are uncommon, but a case of *R. conorii* encephalitis was described with a typical presentation of high fever, headache, rash, *tache noir* lesion, myalgia, nausea and vomiting with acute and convalescent serological confirmation. It was complicated by neurological manifestations, acute renal failure, acute hepatic failure, and thrombocytopenia[130]. *R. conorii* was also documented to affect the eyes causing visual loss with focal retinitis and macular oedema, scotoma and bilateral anterior uveitis. However, response to oral antibiotics and steroids was good with progressive functional and anatomic recovery[131]. In an Indian eye institute, 10 patients with rickettsial retinitis probably due to Indian tick typhus and epidemic typhus were seen from March 2006 to October 2014. All patients had a fever for 10-30 days and presented with sudden onset of visual loss with mild to moderate redness and pain[132]. In the Uttar Pradesh state of India, 200 (46%) of the 432 patient samples tested were positive for a rickettsial infection by the non-specific Weil Felix test. Of those positive, only 115 (27%) samples were found to be positive by enzyme-linked immunosorbent assay (ELISA) and immunofluorescent assay (IFA) against *R. conorii*. Fever, hepatomegaly, thrombocytopenia,

lymphadenopathy and rashes, nausea followed by icterus, cyanosis, headache, oedema and abdominal pain were the common symptoms reported. Eschar was found in only four (3.4%) patients[133]. In a Sri Lankan study, 134 of the 210 clinical cases met the cut-off antibody titre and were confirmed to be due to *R. conorii*. All these patients presented with fever and skin rash. Rash was discrete maculopapular in 132 (98%) of the cases and fern-leaf type skin necrosis in eight (6%). Some patients reported oedema of the face and legs, particularly in the elderly[134].

R. honei is a SFG *Rickettsia* that has been detected in three continents; Thailand in Asia (first isolated in 1962 and confirmed in 2001), in Australia (1993) and in Texas, USA (1998)[135]. It causes Thai Tick typhus in Thailand and Flinders Island spotted fever in Australia but not yet associated with any clinical syndrome in the USA. A SFG *Rickettsia* which caused several cases of rickettsioses in eastern Australia was found to be due to a *Rickettsia* that was genetically similar to *R. honei* and was named *R. honei* strain marmionii[136]. Patients in these cases presented with fever (100%), headache (71%), arthralgia (43%), myalgia (43%), cough (43%), rash (maculopapular/petechial) (43%), nausea (29%), pharyngitis (29%), and lymphadenopathy (29%). Eschar was seen in two of the seven patients described. In 2016, a middle-aged woman presented with a two-week history of a mild cough, myalgia, fever, and lethargy and was admitted to a regional hospital in Queensland, Australia. Her condition deteriorated, and she was suspected of having meningococcal septicaemia and managed in the intensive care unit (ICU). Despite treatment with inotropes and several antimicrobial drugs, the patient succumbed to the illness. *R. honei* was identified from the blood of the patient through molecular and next-generation sequencing techniques[137]. A case of *R. honei* rickettsiosis in Nepal presented with a 5-day history of fever (up to 40.3°C), headache, diarrhoea, and severe arthralgias. The patient's condition worsened within 48 hours and she developed photosensitivity, tinnitus, frontal headache, insomnia, confusion, cough, distress, hypotension,

tachycardia, hypoxia, disorientation, bilateral deafness, conjunctivitis, multiple lymphadenopathies, tender hepatosplenomegaly, bilateral rales, and a purpuric rash but no eschar[138].

R. japonica belongs to the SFG *Rickettsia* and causes Japanese spotted fever (JSF) also known as Oriental spotted fever. It occurs widely in Japan[139] and has also been reported from South Korea[140]. In Japan, JSF is a reportable disease and about 300-1000 cases of JSF occur every year with a gradual increase in endemic areas. A classical triad of JSF symptoms of high fever, erythema with no pain or itching, and tick bite eschar have been described to occur in JSF[141]. In a review of 53 cases of JSF diagnosed at the Mahara hospital, Tokushima prefecture in Japan, patients presented with fever (100%), rash (100%), eschar (94%), malaise (89%), chills (87%), headache (81%) and confusion (11%). Forty-three (81%) of the patients required hospital admission and there was one (1.9%) death[139]. Disease onset was abrupt after 2-10 days of working in the fields. In a retrospective review of 55 cases that occurred in another Japanese hospital (Minami-Ise Municipal Hospital) between 2007-2015, patients complaint of fever (95%), erythema (93%), eschar (62%), malaise (40%), GI symptoms (38%), headache (16%), arthralgia (12%), sore throat (11%), myalgia (6%) and 11% of the patients reported being aware of tick bites[142]. Amongst these cases, only two had severe complications; one with disseminated intravascular coagulation (DIC) and the second with cardiomyopathy died due to the complication.

R. sibirica is a member of the SFG *Rickettsia* that causes North Asian tick typhus/Siberian tick typhus. It occurs mainly in north Asia including Russia, China, Mongolia and Kazakhstan. Common clinical features of *R. sibirica* rickettsiosis include fever, headache, weakness, myalgia, rash, eschar, and lymphadenopathy. The disease is usually mild and non-fatal with rare severe complications. Two severe cases of *R. sibirica* rickettsiosis were reported in China, leading to the one death due to severe complications[143]. Recently, *R. sibirica*

mongolitimona was detected as the cause of a high-grade fever, rash, axillary lymphadenitis and an eschar in a 30-year-old French woman who had just returned from travel to a jungle in Sri Lanka. The organism was identified through a positive skin biopsy by qPCR, PCR amplification and sequencing targeting the ompA gene[144]. In a French study of 465 patients with rickettsioses (positive in culture or in molecular assays), 20 (22%) were due to *R. sibirica mongolitimonae*. Amongst these patients, the symptoms at disease onset included fever (100%), generalized maculopapular rash (100%), inoculation eschar (100%), myalgia (55%) and headache (15%). A rope-like lymphangitis from the eschar to the draining lymph node was detected in 7 (35%) of the patients and one patient required intensive care[145]. Acute myocarditis due to *R. sibirica* has been increasingly reported and it is strongly recommended to include rickettsial infections in the differential diagnosis with adequate epidemiology linkage[146].

Reports on another SFG *Rickettsia, R. felis* as a cause of undifferentiated fever has been increasing especially in the Americas, Europe, Africa, Australia and East Asia but there are limited reports from South Asia, including Bhutan. *R felis* is also described as an emerging human pathogen with several human cases reported[147]. A cross-sectional study (July 2012- Jan 2014) amongst 150 febrile patients in the Mymensingh Medical College Hospital, Bangladesh confirmed (by qPCR and sequencing) *R. felis* as the cause of the illness in 69 (46%). In this study, in addition to fever, headache was reported by 29 (42%), myalgia by 17 (25%) and rash was seen in only 2 (3%) patients[148]. A case series of *R. felis* infections from Laos reported patients with fever, severe headache, myalgia and eschar[149]. All the three cases reported contact with cats and/or dogs before presenting with the illness. An Italian traveller, who returned from a two-week trip to Nepal, presented to a hospital emergency department with a week of fever, retro-orbital headache, nausea and vomiting. She was diagnosed as a case of *R. felis* rickettsiosis as per the serology results in Western blot test which was positive for *R. felis* only[150]. In Thailand, 2,225 febrile patients from two community hospitals were tested for *R.*

felis between 2002-2005. Seven (0.8%) of these patients tested positive by indirect IFA defined as IgG titre ≥1:128 or IgM titre ≥ 1:64 or a more than four-fold rise in antibody titre. Only two patients (a 25-year-old lady and a 45-year-old man) from Chiang Rai province tested positive by *R. felis* specific qPCR. Both the patients had a fever, myalgia, arthralgia, headache, abdominal pain, cough, and chest pain but no rashes, eschars, or lymphadenopathies. In addition, the male patient had photophobia, had vomited, and reported contact with cats[151]. *R. felis* has been reported to be present in humans, animals (cats, dogs and mice) and parasites (lice, fleas and ticks) in China[152]. Recent studies suggested that *R. felis* may be contributing significantly to febrile illnesses throughout Australia and medical professionals need to be vigilant and laboratories need to be equipped with appropriate diagnostics[153-155].

In *R. parkeri* rickettsiosis, first described in the Gulf Coast of the USA[156], the first recognized symptoms included a 'sore' or 'a pimple' which occurred about five days (2–10 days) after the tick bite[157]. Many clinical features such as fever, myalgia, malaise, headache, and a maculopapular eruption were similar to RMSF[158] with 50% of the *R. parkeri* rickettsiosis patients receiving a diagnosis of RMSF[157]. However, *R. parkeri* rickettsiosis appears to be a milder illness than classical RMSF; with lesser hospitalization rates, temperature peaks <40°C, the absence of severe neurological involvement or organ failure and no mortality. A remarkable difference between *R. parkeri* and RMSF was the occurrence of an eschar in >90%, a vesicular rash in 40% and no gastrointestinal symptoms in patients with *R. parkeri* compared to classic RMSF where eschars are rarely seen, vesicular or pustular exanthems are seldom documented and gastrointestinal manifestations occur in >60% of patients[159, 160].

Murine/endemic typhus caused by flea-borne *R. typhi* is one of the commonest rickettsial infections in the world[161]. The disease has a relatively benign clinical course with the classic triad of fever, rash, and headache although the infection may lead to neurological complications[162]. Murine typhus has similar presenting features as that of *R. felis* infections,

except that while eschar occurs commonly in *R. felis*, it has been rarely seen in Murine typhus. Murine typhus is often accompanied by GI symptoms (such as anorexia, nausea, vomiting, and abdominal pain), and other complications of the respiratory, renal, and neurologic system[163]. A mortality rate of 4% has been reported in hospitalized patients[164]. In the first report from South Korea, *R. typhi* was found to be the cause of a brain abscess mimicking a brain tumour resulting into delayed diagnosis and appropriate management of the disease[165]. The case was a previously healthy 52-year-old man presenting with an intractable, ongoing (5 months), moderate headache in the vertex area following a visit to a rural province in South Korea. The only other symptom was a skin rash that covered his whole body and lasted for a month, disappearing spontaneously. Brain abscess was diagnosed by a lesion in magnetic resonance imaging (MRI) and positive serology.

The louse-borne *R. prowazekii* causes epidemic typhus, one of the oldest infectious diseases of humankind[166]. Human beings are the principal reservoir of this infection and infections in humans is lifelong. The lifelong infection results from the bacteria remaining in a latent form for a long period and recrudescence, in the form of Brill-Zinsser disease[167]. Brill-Zinsser disease is much milder than epidemic typhus and usually develops when an individual is immunosuppressed resulting from severe stress or malnutrition[168]. In an outbreak in Burundi, 102 cases presented with *sutuma* (crouching position) (100%), fever (100%), headache (100%), delirium (81%), cough (70%), vomiting (57%), rash (25%), conjunctivitis (15%), diarrhoea (13%), splenomegaly (8%) and coma (4%)[167]. Epidemic typhus has an incubation period of 10-14 days[169]. Patients usually experience 1–3 days of malaise before the abrupt onset of severe headaches and fever[170]. Mortality rates of up to 60% were reported in the pre-antibiotic era but now stands at about 4% with correct diagnosis and treatment[170].

2.1.2 Clinical features of Scrub typhus

Scrub typhus (ST) is a mild to life-threatening disease with a fatality rate as high as 50% in untreated cases. ST presents with abrupt onset of high fever, severe headache, lymphadenopathy, generalized myalgia, eschar and rash. The eschar, a painless lesion at the site of the bite of an infected *Leptotrombidium* chigger mite is seen a few days after the chigger bite before the disease presentation. It is an important early sign associated with ST and a pathognomonic feature when present[171].

In Bhutan (work included in this project), the commonest complaints of patients attending hospitals with ST were fever (100%), headache (77%), arthralgia (60%), myalgia (29%), rash (25%), lymphadenitis (3%), and eschar (4%). The presence of an eschar ($p < 0.001$), myalgia ($p = 0.003$), and lymphadenopathy ($p = 0.049$) were significantly associated with the laboratory diagnosis of ST[172]. In an outbreak of ST in a remote Bhutanese school, children presented with fever, headache, cough, myalgia, rash and eschar. Complications leading to meningoencephalitis was seen and caused the death of two students[173]. A study on clinical profile of 228 ST patients in a tertiary hospital in north India between July 2013 to December 2014, recorded high-grade fever (85%), breathlessness (42%), jaundice (32%), abdominal pain (28%), renal failure (11%), diarrhoea (10%), rashes (9%) and seizures (7%)[174]. In another similar study in north India of 90 ST cases, predominant clinical features included fever (83%), myalgia (62%), abdominal pain (27 %), headache (27%), nausea/vomiting (23%), dry cough (23%), hepatomegaly (27 %), splenomegaly (24%), lymphadenopathy (22%) and eschar (11%)[175]. In south India, 623 patients mostly presented with fever (100%), nausea/vomiting (54%), shortness of breath (49%), headache (46%), cough (38%), and altered sensorium (26%)[176]. An eschar was present in 43.5% of patients compared to 11% in the north Indian patients. A recent Indian study reported clinical manifestations of fever (100%), myalgia (65%), cough (51%) and vomiting (46%) with major complications of meningitis/

meningoencephalitis (13%) and multi-organ dysfunction syndrome (MODS) (5%) and pneumonia (5%)[177]. At the Christian Medical College in south India, children (< 15 years of age) admitted with ST with or without meningitis were reviewed over a period of five years (January 2010 to February 2015). Of the 427 children with ST, 63 (15%) had meningitis. Of those with meningitis, 24 (38%) had seizures, 17 (27%) had altered sensorium and 37 (59%) had neck stiffness. Children with meningitis had significantly (p = 0.028) shorter duration (median 7 days) of fever at presentation compared to those without meningitis (median 10 days). A headache and vomiting were significantly more common in those with meningitis. Finding an eschar, being a male, presenting with breathing difficulty and hepatomegaly were significantly more common in those without meningitis. Duration of hospitalization was significantly longer in those with meningitis, whereas acute respiratory distress syndrome (ARDS) was significantly more common in those without meningitis. Neurological deficit was not seen in both groups. Surprisingly, no mortality was seen in the meningitis group compared to 3.6% mortality in the non-meningitis group although the observation was not significant (p= 0.213)[178]. In a Vietnamese study of 279 acute febrile patients admitted in the national hospital in Hanoi, the presence of an eschar (OR=46.6), dyspnea (OR=10.9), hypotension (OR=9.0), and lymphadenopathy (OR=7.2) was found to be significantly associated with a diagnosis of ST. In contrast, patients with murine typhus were more likely to have myalgia (OR=1.6), rash (OR=1.6), and relative bradycardia (OR=1.45)[179].

ST can present as a life-threatening illness or develop multi-organ complications during illness. Amongst the 228 north Indian ST patients, Sharma and colleagues (2016) recorded acute kidney injury in 32%, acute respiratory distress syndrome (ARDS) in 25%, disseminated intravascular coagulation (DIC) in 16%, a hepatorenal syndrome in 38% and MODS in 20%[174]. Similar complications including MODS (34%) and ARDS (34%) were also noted in south Indian patients[176]. In the south Indian study, shock requiring vasoactive agents, central nervous

system (CNS) dysfunction and renal failure independently predicted mortality. Involvement of the CNS is well known in cases of ST. Recently ST has emerged as the commonest cause of Acute Encephalitis Syndrome (AES) in children when compared to Japanese encephalitis (JE), dengue, chikungunya and West Nile viruses in a pilot AES surveillance[180]. In a retrospective study (2013-2015) of AES patients in the Indian Assam state where Japanese encephalitis virus (JEV) is considered prevalent 104 of the 511 patients tested positive for ST. The main clinical features recorded were fever (100%), altered sensorium (100%), headache (67%), unconsciousness (56%), nausea (40%), and neck rigidity (1%). No patients had any eschar. Of the 104 patients, 53 could be followed up and 26 (49%) had died after discharge from the hospital[181]. Between the year 2001-2013, a retrospective analysis of 510 patients with ST revealed a ST related acute kidney injury (AKI) of 36% of which 26% (132) were at risk, 7% (37) with injury and 3% (14) with kidney failure. Compared to those without AKI, the AKI group was significantly older (74 vs 63 years, $p<0.001$) and had more comorbidities such as hypertension, diabetes mellitus and chronic kidney disease (CKD). AKI frequently occurred in hypertensive patients on angiotensin receptor blockers or ACE inhibitors ($p=0.002$), and in patients with diabetes with higher glycated haemoglobin levels ($p=0.033$). These results suggested underlying CKD, older age, lower serum albumin level and time to hospital presentation after symptom onset were important risk factors to determine the occurrence of AKI[182].

Reported fatality estimates for ST are likely to be inaccurate due to non-uniform and unclear record keeping, lack of proper surveillance and reporting systems and poor data quality. Mortality estimations are challenging and dependent on regional strains of the bacteria, infectious doses, patient age and comorbidities. A review reported median mortality remains at 6.0% ranging widely between 0-70% and concluded that mortality rates from untreated ST appeared to be lower than previously reported estimates[183]. This review found that many

studies depended on clinical diagnosis alone and data on secondary outcomes were lacking. In addition, mortality was mostly explained by location, the age of the patient and in patients with myocarditis, delirium, pneumonitis, or signs of haemorrhage, but not according to sex or the presence of an eschar or meningitis[183]. Another recent systematic review reported that mortality from ST varied between 1.4% in treated cases to 6.0% in untreated cases, but this can increase to 14% in CNS involvement and up to 24% during MODS[184]. The overall case-fatality rate in Indian studies varied from 5%[175] to 9.0%[176] to 14%[174] which escalated as high as 49% when the CNS was involved[181]. In a retrospective study to explore predictors of mortality in ST patients admitted in the intensive care unit (ICU) in Nepal, the overall mortality rate was 20% (24/120) and factors associated with mortality were; heart rate >100/minute ($p < 0.001$), systolic blood pressure <90 mmHg ($p = 0.025$), diastolic blood pressure <60 mmHg ($p = 0.032$), serum creatinine >1.4 mg/dl ($p < 0.001$), AKI requiring dialysis ($p = 0.029$), ARDS ($p < 0.001$), and shock requiring vasopressor ($p < 0.001$). In regression analysis, older age (OR= 1.06; 95% CI, 1.01-1.12; $p = 0.019$) and high serum creatinine (OR = 1.06; 95% CI, 1.01-1.12; $p = 0.019$) were found to be significant independent predictors of poor outcome in ICU admitted ST patients[185].

Reinfection with *Orientia* can occur especially in the endemic areas due to high antigenic heterogenicity of the bacteria[186]. In early vaccine trials, it was demonstrated that resistance to reinfection with homologous strains may occur up to three years and immunity to heterologous strains seem to dissipate within two years[68, 69]. In a case report of a reinfection of ST in a 58-year-old male agriculturalist in India, confirmed by serology (IFA and ELISA) and qPCR, the reinfection occurred within 14 months of the first episode and presenting symptoms differed to a certain extent in the two episodes[67]. More importantly, an eschar which is considered a pathognomonic feature of ST (when present) was seen in the first episode but absent in the reinfection. This case report supported the concept of waning immunity as early as one year

after the infection, especially if the infecting strain was of heterologous type. The absence of eschar in primary infection and absence in reinfection probably shows that immunity from the primary infection may only be sufficient to prevent the formation of an eschar but not avoid active infection[67].

2.1.3 Clinical features of Q fever

Q fever (QF), the clinical infection in humans caused by *Coxiella* is frequently included with the rickettsioses and causes a similar non-specific febrile illness. The incubation period in QF of 20 days (2-6 weeks) is longer than other rickettsial infections[110]. Primary infection with *Coxiella* is usually asymptomatic[110] in about 60% of the infections[111] and in an outbreak of QF, between 10% to 85% of the infections can be symptomatic[187]. QF can occur in any age group but it is more prevalent between the ages of 30 and 70 years[188]. More adults than children and more men than women have symptomatic infections[110, 189]. Symptoms of QF may vary between individual patients and symptomatic illness was traditionally divided into acute (lasting less than 6 months) and chronic (lasting for 6 months or more)[75]. However, there is a lack of consensus on the definite distinction of acute from chronic QF and a need to redefine different clinical forms of the disease[190]. In addition, an entity of presentation, 'post-QF fatigue syndrome' is now recognized.

2.1.3.1. Acute Q fever

Clinical manifestations of acute QF appeared to vary with the geographical location of the infection[75, 101]. Acute QF commonly present in three forms such as a self-limiting flu-like illness, atypical pneumonia or hepatitis[75]. The non-specific flu-like illness with an abrupt onset is the commonest clinical presentation. Frequent symptoms include a high-grade fever (39-40^{o}C), fatigue, headache, and myalgias. QF pneumonia is mostly mild with a non-productive cough, fever, and minimal clinical findings although some may present with acute respiratory

distress. Pleural effusion may also be seen although chest radiograph can be normal or not specific and resemble viral pneumonia[110]. QF hepatitis presents with fever and raised serum transaminases with no obvious clinical findings on examination[75]. Other presentations of acute QF include a maculopapular or purpuric rash (10%), pericarditis or myocarditis (1%), severe headache, aseptic meningitis, and encephalitis (1%)[191]. Unlike many other rickettsial infections, the rash is absent in over 90% of cases[110]. Usually, 2-5% of the acute cases require hospitalization [111] but varied from as high as 50% during active case finding to about 20% when cases stabilized in the Netherlands outbreak[192]. Symptoms of acute QF can last from over a week to three months with a mortality rate ranging from 0.5% to 1.5%[75].

In the Bhutanese patients (work included in this thesis) attending 14 hospitals between October 2014 to June 2015, presenting symptoms of QF patients included fever (100%), headache (76%), arthralgia (66%), rash (28%), myalgia (17%), lymphadenopathy (3%) and others (cough, anorexia, backache, abdominal pain) (3%)[172].

2.1.3.2 Chronic QF

Chronic QF infection is the most serious form of the disease and occurs in about 1-5% of the infections[75]. It can ensue months to years after the initial acute infection even without a history of past infections. During the infection with *C. burnetii*, the commonly affected organs include the heart, arteries, bones, and the liver[110], Chronic QF can manifest in forms of carditis (endocarditis, pericarditis or myocarditis), chronic osteomyelitis, chronic hepatitis or chronic vascular infection[111, 193].

2.1.3.3 Q fever fatigue syndrome

In general, Chronic fatigue syndrome (CFS) is defined as fatigue for six months or more together with at least four of the following symptoms: lack of concentration or/and memory

that interferes with normal activities, sore throat, tender cervical or axillary lymph nodes, joint pain without swelling, muscle pain, headache, no refreshing sleep, and malaise lasting longer than 24 hours after exertion[194]. CFS has been mostly explained by three factors such as post-infection, immunological or depression.

CFS seen in patients following exposure (symptomatic or asymptomatic) to *C. burnetii* has been known with several names but Q-fever fatigue syndrome (QFS) is most customary. A recent review reported that although most patients recover from fatigue within 6–12 months after acute QF, approximately 20% remain chronically fatigued[195]. Cases of QFS has been described worldwide[195, 196] including 20% of Australian QF patients[197]. In QFS, other complaints besides fatigue included musculoskeletal complaints (myalgia and arthralgia), neurocognitive, sleep problems, headache, blurred vision, increased (night) sweating, respiratory tract problems, mood disorders and other symptoms (gastrointestinal disorders, severe malaise, poor appetite, dizziness, loss of libido, loss of taste etc). The rate of occurrence of each symptom varied over time lapsed after the acute QF episode, with a slow decrease over years[195]. QFS studies have reported major health and work-related consequences which were frequently accompanied by nonspecific complaints. However, the definition of this syndrome has varied over the years[101] and there is no consensus in regard to the aetiology, prevention, treatment, and prognosis of QFS.

2.1.3.4 The current controversy over the definition and diagnosis of chronic Q fever

QF is an old disease described since the late 1930s and gained importance as a zoonosis worldwide. The clinical manifestation of QF has been traditionally described conveniently as acute (lasting less than 6 months) and chronic (lasting 6 months or more)[75]. Medical literature including diagnostic and treatment guidelines on QF has revolved around this definition until now. However, there have been recent proposals for a redefinition of chronic QF through

reviews and consensus of working groups and expert opinions which are unfortunately contradicting each other.

Following a large outbreak of QF in the Netherlands between 2007 to 2010 and beyond, a Dutch working group proposed a new definition of chronic QF through the review of existing literature (reflecting wide variations in diagnostic criteria) and their clinical experience from the extensive outbreak. The proposal based on the consensus in Netherland and the Dutch guideline categorized chronic QF into proven, possible or probable chronic infection considering serology, PCR, clinical symptoms, risk factors and diagnostic imaging[198] as outlined in **Table 2.1.**

Table 2.1. Dutch consensus guideline on chronic Q fever diagnostics[198]

Proven chronic Q fever	Probable chronic Q fever	Possible chronic Q fever
1. Positive *C. burnetii* in blood or tissue[a] OR 2. IFA ≥1:1024 for C. burnetii phase I IgG AND - definite endocarditis according to the modified Duke criteria OR - Proven large vessel or prosthetic infection by imaging studies (FDG-PET, CT, MRI or AUS)	IFA ≥1:1024 for *C. burnetii* phase I IgG AND one or more of the following criteria: - Valvulopathy not meeting the major criteria of the modified Duke criteria - Known aneurysm and/or vascular or cardiac valve prosthesis without signs of infection by means of TEE/TTE, FDG-PET, CT, MRI or abdominal doppler ultrasound - Suspected osteomyelitis or hepatitis as manifestation of chronic Q fever - Pregnancy - Symptoms and signs of chronic infection, such as fever, weight loss and night sweats, hepato-splenomegaly, persistent raised ESR and CRP - Granulomatous tissue inflammation, proven by histological examination - Immunocompromised state	IFA ≥1:1024 for *C. burnetii* phase I IgG without manifestations meeting the criteria for proven or probable chronic Q fever
Abbreviations: CT, computed tomography; IFA, Immunofluorescence assay; TEE, transesophageal echocardiography; TTE, transthoracic echocardiography. [a]In absence of acute infection		

A French expert and a QF specialist (Didier Raoult) from a World Health Organization (WHO) and national reference centre on Q fever for over 30 years agreed that the Dutch consensus was timely and the old concept of chronic QF would benefit from a 'readjustment'. However, he rejected the proposed diagnostic guideline of the Dutch group calling it solely based on the experience and consensus in the Netherlands and set forth his own proposal. He suggested that the old definition of chronic QF be abandoned and a new score-based diagnosis for QF endocarditis and vascular infection be used[199]. He reasoned that all acute QF infections,

including asymptomatic infections, can subsequently result in an endocarditis or a vascular infection. According to him, the old definition of chronic QF (presence of the disease for more than 6 months), was basically endocarditis and vascular infections as well as a few other localised diseases such as pericarditis, osteomyelitis etc. He suggested the collective term of "persistent focalized QF" for all clinical entities of chronic QF. His proposed definition of QF endocarditis and QF vascular infections are detailed in **Tables 2.2** and **2.3.**

Table 2.2. Definition of Q fever endocarditis (Didier Raoult's proposal)[199]

A. Definite criterion
Positive culture, PCR, or immunochemistry of a cardia valve.

B. Major criteria
Microbiology: positive culture or PCR of the blood or an emboli or serology with IgGI antibodies ≥6400
Evidence of endocardial involvement:
Echocardiogram positive for IE: oscillating intra-cardiac mass on valve or supporting structures, in the path of regurgitant jets, or on implanted material in the absence of an alternative anatomic explanation; or abscess; or new partial dehiscence of prosthetic valve; or new valvular regurgitation (worsening or changing of pre-existing murmur not sufficient)
Pet-scan showing a specific valve fixation and mycotic aneurysm

C. Minor criteria
Predisposing heart condition (known or found on echography)
Fever, temperature >38ºC
Vascular phenomenon: major arterial emboli, septic pulmonary infarcts, mycotic aneurysm (see pet-scan), intracranial haemorrhage, conjunctival haemorrhage, and Janeway's lesions
Immunologic phenomena: glomerulonephritis, Osler's node, Roth's spots, or rheumatoid factor
Serologic evidence: IgGI antibodies ≥800 <6400

Diagnosis definite
1) 1A criterion
2) 2B criteria
3) 1B criterion and 3C criteria (including 1 microbiology evidence and cardiac predisposition)

Diagnosis possible
1) 1B criterion, 2C criteria (including 1 microbiology evidence, and cardiac predisposition)
2) 3C criteria (including positive serology, and cardiac predisposition)

Table 2.3. Criteria for diagnosis of QF vascular infection[199]

A. Definite

Positive culture, PCR or immunochemistry of an arterial samples (prosthesis or aneurysm) or a periarterial abscess or a spondylodiscitis linked to aorta.

B. Major criteria

Microbiology: Positive culture, PCR of the blood or emboli, or serology with IgGI antibodies ≥6400

Evidence of vascular involvement:

CT-scan: aneurysm or vascular prosthesis + periarterial abscess, fistula, or spondylodiscitis.

Pet-scan: specific fixation on an aneurysm or vascular prosthesis

C. Minor criteria

Serological IGI ≥800 <6400

Fever, temperature ≥ 38ºC

Emboli

Underlying vascular predisposition (aneurysm or vascular prosthesis)

Diagnosis definite

1) A criterion
2) 2B criteria
3) 1B criterion and 2C criteria (including microbiology and vascular predisposition)

Diagnosis possible

Vascular predisposition, serological evidence and fever or emboli.

Following these proposals and controversies, a study was carried out in the Netherlands subjecting 284 chronic QF patients to the Dutch guideline and the diagnostic criteria proposed by Raoult. In this study, of the patients who had proven chronic Q fever by the Dutch guideline, only 46 (30.5%) would not have received a diagnosis by Raoult's criteria, and 14 (4.9%) would have been considered to have possible chronic QF[200]. The most critical difference between the Dutch guideline and Raoult's criteria is the consideration of a positive *C. burnetii* PCR as a

marker of proven chronic Q fever in the absence of acute Q fever by the Dutch guideline. Kampschreur and colleagues (2015) thought that patients without endocarditis or vascular infection on imaging studies but with a positive PCR in blood should be treated for chronic Q fever as they may suffer from (not yet clinically visible) endocarditis or vascular infection (as observed in the post-mortem of two patients in their study)[200]. A single positive *C. burnetii* PCR of blood was highly suggestive for chronic Q fever when acute Q fever is excluded, and these patients wouldn't be followed up if they were not categorized as possible or probable chronic QF in their initial assessment. In this study, the Dutch consensus guideline was found to be easy to use, more sensitive, provided added treatment advice and applicable to patients with chronic Q fever manifestations that were rarer than endocarditis and vascular infection[200]. In animals, *C. burnetii* causes coxiellosis. Coxiellosis is often asymptomatic[97] but infertility and abortions have been reported in numerous mammalian species[201]. Several outbreaks of Coxiellosis have been reported in Europe[202], Netherlands[192] and Australia[203].

2.2 Laboratory diagnosis

Rickettsioses (including Q fever) are difficult to diagnose both clinically and in the laboratory. Diagnostic work-up should generally include a detailed history, clinical examination and laboratory testing, making use of a local diagnostic algorithm. In one of the first largest study on RMSF, it was concluded that early clinical diagnosis of RMSF is difficult because the illness may have a gradual or an abrupt onset, the symptoms and signs may be unusual in timing or frequency, and the clinical appearance may vary depending on age and location of the patient[124]. It takes a combination of clinical judgement, a high index of suspicion in an endemic situation to suspect a diagnosis of rickettsial diseases and reliable laboratory support to confirm that diagnosis. Current microbiological tests include serological techniques, nucleic acid amplification test (NAAT) and cell culture, of which only the first method is widely available

in different assays. In addition to microbiological tests, other laboratory parameters in the blood may be helpful in suspecting a diagnosis of rickettsial infections.

2.2.1 Serological methods

Serological methods remain the main diagnostic tool for rickettsial diseases and antibody detection with IFA is the current methods of choice (gold standard). One of the most important drawbacks of serology is that it usually takes 10-14 days to become positive and examination of both acute and convalescent sera are usually required to confirm an acute infection[204]. Serological tests when conducted early in infection (before antibodies develop) can be negative and follow-up samples (when antibody develops) are usually unavailable[205]. Following a rickettsial infection, IgM antibodies can be detectable for months and IgG for years[204, 206]. There are several serological techniques as described below.

2.2.1.1 Weil-Felix agglutination test

This is the oldest serological method for the detection of antibodies against rickettsial diseases described as early as 1916 by Weil and Felix[207]. The Weil-Felix test is based on a cross-reaction between antibodies to *Rickettsia* (such as *R. prowazekii, O. tsutsugamushi*, and *R. rickettsii*) with the OX strain (OX2, OX19 and OXK) of the bacteria *Proteus*[207, 208]. The basis of the test was on the observation that serum of patients who recovered from typhus (epidemic typhus or scrub typhus) agglutinated some strains of *Proteus* genus. *R. prowazekii* (epidemic typhus) and *R. typhi* (murine typhus) agglutinated OX19, *R. rickettsii* (RMSF) agglutinated both OX19 and OX2 and *O. tsutsugamushi* agglutinated OXK. However, *Rickettsia* and *Proteus* do not have a close phylogenetic relation and this observation is a typical example of antigen cross-reaction between unrelated microbes.

The reactions that occur are non-specific and cannot different a *Proteus* infection from a Rickettsial infection. The reliability of the Weil-Felix test has been questioned by several authorities since its use. Low sensitivity and specificity of the Weil-Felix test was demonstrated with wide discrepancy in results compared to a micro-IFA[209], an indirect immune-peroxidase assay[210], an IgM ELISA[211] and other serological tests[212]. Problems with the Weil-Felix tests rest not only with the non-specificity of the antigen but also with the non-uniform antibody cut-off titers (ranging between 1:20 to 1:320)[210, 213, 214] in making diagnostic decisions. This test is now considered obsolete and is not recommended to be used as better tests are widely available. Unfortunately, it may still be used in certain developing countries such as India[215], owing to the unavailability and cost implications with newer diagnostics.

2.2.1.2 Micro-immunofluorescence assay

The indirect immunofluorescence technique was developed as early as 1959 but its utility in the diagnosis of RMSF and epidemic typhus was described only in 1976. It is the current diagnostic tool of choice (gold standard) for rickettsial diagnosis[216, 217] and other diagnostic assays are compared to it[218, 219]. Despite being regarded as the current gold standard for the diagnosis of rickettsial diseases, various IFA diagnostic cut off titers have been used. Diagnostic titers varied from 1:10 to 1:400 between countries as well as between geographical regions. There is a lack of consensus leading to confusion and non-comparability of results amongst users[217]. Studies comparing other serological tests with the IFA have also used different cut-off points for IFA, which might have resulted into inconsistent results. A Chinese study[220] used an IFA titer (against both IgG an IgM) of 1:80 as positive to compare a rapid diagnostic test (RDT) with IFA in ST diagnosis, . Such a practice is not only unscientific but can easily encourage manipulation of results leading into questionable scientific and epidemiological validity.

The IFA method uses the whole rickettsial cell fixed onto a glass slide as the antigen to detect the desired antibody. In the case of QF, the phase I (virulent phase grown in animals) and phase II (avirulent phase grown in embryonated chicken eggs or tissue culture) *C. burnetii* cells are used as the antigen. This enables the detection of early antibody response (antibodies to phase II, protein antigens) in acute QF and the later antibody response (antibodies to phase I, LPS antigen) seen in chronic QF[205]. Although phase I antibodies are also seen in acute QF they fade much quicker in acute infection but remain longer in chronic QF. Antibodies to the specific rickettsial diseases or Coxiella, if present, react with the rickettsial cell fixed on the glass slide and the positive reaction is visualised as a fluorescence using a UV microscopy. The IFA technique can distinguish different classes of immunoglobulins (IgA, IgM or IgG) with the use of specific anti-human immunoglobulins (anti-IgA, anti-IgM and anti-IgG) or specific anti-animal immunoglobulins (anti-goat, anti-bovine, anti-horse, anti-dog etc). This assay also can give antibody titers through the testing of serial doubling dilutions of the sample until an endpoint (highest titre) is reached[205, 217, 221].

2.2.1.3 Enzyme immunoassay

Enzyme immunoassay (EIA) is a serological method based on the detection of a coloured product of an enzymatic reaction which depicts the extent of antigen-antibody reaction that is dependent on the concentration of antibody in the patient's serum sample. The intensity of the coloured end-product measured as optical density (OD) (at the recommended wavelength) is compared (as a ratio) with the OD of the known negative control sera [205]. This serological modality is widely used and mostly automated with numerous commercial kits for diagnosis of rickettsial infection, ST and QF. A positive result in an EIA indicates the level of the antibody present in the patient serum but does not give antibody titers. For example, EIA gives an indication that a patient with a reading of 2.6 has more antibody than the one with 1.7, but

there is no definite antibody titre [205]. Available commercial EIA kits have variable sensitivity and specificity mostly depending on the antigen(s) (either a native molecule, recombinant molecule or a lipopolysaccharide) used in the assay. If an EIA kit is to be used as a screening test, it must have a high sensitivity of at least ≥95% so that only ≤5% of the genuine cases may be missed. A screening test can have a lower specificity since any positives (true or false) should be confirmed with a second more specific test such as an IFA[205].

After an excellent performance in a comparative study, the ELISA has been proposed as an alternative serologic diagnostic test to the IFA for scrub typhus[218]. While working out an optimal sample dilution and cut-off optical density (OD) and to estimate the accuracy of IgM ELISA, the sample dilution of 1:400 was found to be optimal for the IgM ELISA in ST diagnosis. With the optimal cut-off OD of 1.474 at a sample dilution of 1:400, the IgM ELISA exhibited a sensitivity of 85.7% and a specificity of 98.1% using paired samples[222]. The authors also concluded that the IgM ELISA for scrub typhus had high diagnostic accuracy and is less subjective than the IgM IFA and suggested the use of IgM ELISA as an alternative reference test to the IgM IFA for the serological diagnosis of scrub typhus. Further to the comparable accuracy with IFA, replacement of IFA with ELISA technologies for IgM detection in ST diagnosis was also based on a number of advantages of ELISA such as its ease of use not requiring highly trained personnel, objectivity in reading and rapid turnaround time with automation[222].

2.2.1.4 Complement fixation test

Complement fixation test is one of the oldest method of antibody detection. It is not widely used due to lack of sensitivity and it not recommended as a screening test. However, it can be a useful test to confirm those samples positive in screening tests (such as the EIA). Currently, it has been automated & adopted to test for antibodies against *C. burnetii* phase 2 antigens[205].

2.2.1.5 Other serological assays

There are other serological tests such as the latex agglutination test, indirect hemagglutination and microagglutination for diagnosis of rickettsial diseases. These are, however, not widely used.

Other cheaper, easy to use test kits of acceptable sensitivity and specificity such as the immunochromatographic rapid test kits (SD Bioline Tsutsugamushi assay, Standard Diagnostics Inc, Republic of Korea; PanBio scrub typhus IgM and IgG rapid immunochromatographic assay, PanBio, Australia) and dip-stick tests (Integrated Diagnostics Inc, Baltimore, Maryland) for diagnosis of ST, are handy to use in developing country settings where more sophisticated assays are unavailable due to cost or lack of expertise [218]. They are more popular as a point of care testing (POCT). The test procedure of the rapid diagnostic test (RDTs) are simple and can be performed even in the field. RDTs also give results within as short as 10-15 minutes making them handy for immediate patient care. These characters can be taken advantage of in rural settings where higher and better tests are unavailable. However, RDTs have variable sensitivity and specificity depending on the manufacturer, the population studied and disease endemicity in the locality. Therefore, results of the RDTs should be used in combination with strong clinical judgement and other laboratory parameters to decide on treating a suspected patient to prevent complications from an easily treatable infection.

A comparative summary of serological tests for rickettsial diagnosis is presented in the following table (Table 2.4).

Table 2.4 Comparative summary of serological tests for Rickettsial infections

Test	Advantages	Disadvantage	Sensitivity and specificity
Weil-Felix test	• Low cost • Relatively easy procedure	• Cannot differentiate between *Proteus* and rickettsial infections • Poor sensitivity and specificity especially for acute infection • Require paired sera	1. Sensitivity 33%, specificity 46%[213] 2. Poor sensitivity. Reasonably specific (up to 93%) at higher titres (1:160 or more)[223]
Complement fixation test	• Good availability • Relatively cheaper • Useful as a confirmatory test	• Subjective • Low sensitivity • No standards between laboratories • No automation • Require paired sera to make a diagnosis of acute infection	1. Sensitivity 73%, specificity 90% against *C. burnetii* IgM[219]
Rapid diagnostic tests (RDTs)	• Comparatively cheaper • Easily available • No require complex facilities • Easy procedure • Handy for field use • Rapid resuts	• Of variable sensitivity and specificity • Cannot give antibody titres • Most detect total antibody level and cannot differentiate different classes of antibodies	1. Access Bio *CareStart* Scrub Typhus test (Somerset, NJ) (IgM) - Sensitivity 23%, specificity 81% (acute), sensitivity 33%, specificity 79% (convalescent)[224] 2. SD BIOLINE *Tsutsugamushi* test (Kyonggi-do, Republic of Korea) (IgG, IgM, or IgA) – sensitivity 21%, specificity 74% (acute), sensitivity 77%, specificity 77% (convalescent)[224] 3. Pooled sensitivity 66% and specificity 92%[225]
ELISA	• Relatively cheap • Widely available • Can be adapted to give antibody titers • Relatively simple procedure • Suitable for large sample load	• Require equipment • Some level of expertise required • Need adaptation to local setting	1. Sensitivity 87%, specificity 98% on paired sera[222] 2. PanBio *Coxiella burnetii* IgM ELISA sensitivity 99%, specificity 88%[219]
IFA	• Highly sensitive and specific • Considered the reference test • Can differentiate different immunoglobulin sub-class • Commercially available	• Require equipment and expertise • Subjective • No standards between laboratories • Cross-reaction between species & cannot differentiate species • No automation • Not friendly for large number of samples	1. Highly sensitive and specific 2. Reference test to compare other tests

2.2.2 Nucleic acid amplification test (NAAT)

The detection of nucleic acids (particularly DNA) of rickettsiae in blood or tissue provides an early and definitive diagnosis of the infection. The technique for NAAT is commonly through a polymerase chain reaction (PCR). PCR testing and immunohistochemical staining of skin specimen obtained by performing a biopsy may help confirm the clinical diagnosis in patients with a rash during the early presentation. PCR technique can not only detect but also quantify the amount of DNA which will indicate a concentration of the organism in the patient's sample[226]. For NAAT, blood samples should ideally be collected within one week of onset of the illness. A positive PCR supported by clinical findings confirms the diagnosis but a negative PCR does not exclude the possibility of a rickettsial infection since organisms may be eliminated from the blood rapidly[187]. Compared to serology which usually takes about two weeks to become positive, PCR provides the earliest assay for the diagnosis and effective treatment, if positive. Although a positive PCR does not indicate whether the detected microbe is dead or alive, in course of an acute infection, a positive PCR most likely indicates a 'viable microbe'[205]. In addition to blood, a PCR can be performed on samples such as tissues, biopsies and ectoparasites.

The PCR assays mostly developed in-house in reference laboratories, target different genes of the rickettsial and *Coxiella* genome. For example, the citrate synthase and the 17 kDa gene are usually targeted for all Rickettsiae, the ompA, ompB and Sca4 gene are targeted for spotted fever group *Rickettsia*, and the 16s rRNA and 56kDa gene are targeted for STG. A *Coxiella* PCR targets the Com 1 gene (a gene coding for a surface, outer-envelope antigen). Sensitivity of NAAT assays are usually high but specificity may depend on the genes selected and the primers used to amplify the genes. Sensitivity can be affected by various technical factors (such as too much human DNA; failure of the polymerase to work; failure to extract the microbial DNA) causing false-negative results. Similarly, the specificity can also be affected,

usually resulting from contamination of the assay mixture with extraneous DNA resulting in false-positive results[205]. The high sensitivity and specificity with a rapid turn-around-time makes the PCR an ideal test for the routine diagnostic laboratories.

2.2.3 Culture and isolation

Although culture and isolation are a definitive diagnosis of the causative organism, it is not suitable as a method for routine diagnosis. As rickettsia are obligate intracellular organisms, it requires tissue culture for its growth and this involves several technical difficulties. Recently, *C. burnetii* was cultured in axenic cultures and does not require cell culture for grown[78, 79]. Organisms can be cultured from blood samples, organs and tissue, live ticks. *C. burnetii* was also successfully isolated from serum samples of patients with acute Q fever, with isolation rates as high as 81% in PCR-positive samples[227]. Long-term (>200 days) viability of *Coxiella* in serum was established in this study. However, rickettsial isolation in culture is unnecessary for a routine diagnosis, laborious, and may be hazardous to laboratory personnel[228].

2.2.4 Other supportive diagnostic tests

In addition to the numerous pathogen-specific diagnostic tools, there are several other laboratory parameters which would be helpful in suggesting a diagnosis of rickettsial infection. These indicators may be helpful in the situation of a high index of suspicion and negative or equivocal specific microbiological tests in an endemic area. For instance, studies have recorded that during illness due to ST, common laboratory abnormalities at presentation included a deranged hepatic function in 61%, anaemia in 54%, leukopenia in 15%, and thrombocytopenia in 90% of the patients [174]. Common laboratory findings included elevated transaminases (87%), thrombocytopenia (79%), and leukocytosis (46%)[176]. A recent study revealed raised aminotransferase levels and thrombocytopenia in most of the 168 confirmed cases of ST[177]. In

a review of 427 children with ST, meningitis occurred in 15% of the cases. The mean cerebrospinal fluid (CSF) white blood cell count was 71cells/mm^3, the mean CSF protein was 67mg/dl and the mean CSF glucose was 55 mg/dl. Haemoglobin and platelet were significantly lower in those without meningitis[178]. A Thai study on the utility of biomarkers in the diagnosis of acute undifferentiated fevers found that C-reactive protein (CRP), Haemoglobin (Hb) level, White cell count (WBC), neutrophil and lymphocyte counts were significant variables. A lower CRP (OR 0.97, 95% CI 0.95–0.99, p = 0.001) was an important predictor for viral infection while presence of an eschar and a higher CRP (OR 1.03, 95% CI 1.01–1.05, p = 0.001) remained as significant predictor variables for bacterial infection on multivariate analysis. CRP also outperformed procalcitonin. It was concluded that an accurate, pathogen-specific rapid diagnostic tests coupled with biomarker point-of-care tests such as CRP, can be beneficial in guiding the correct use of antibiotics and improve antimicrobial stewardship[229].

In view of the difficulty in the diagnosis of rickettsial infections in the absence of adequate laboratory support or unavailability of test kits, Rathi and colleagues suggested a scoring system based on clinical and laboratory parameters for diagnosis of SFG *Rickettsia.* In this system consisting of a maximum of 35 points (25 clinical and 10 laboratory parameters), a clinical score of 14 or more on the proposed scoring system showed very high sensitivity and specificity for the diagnosis of SFG rickettsia[230]. A clinically useful diagnostic algorithm was also developed and tested in Sri Lanka. This algorithm concluded that the duration of illness at sampling was important in interpreting serology results in an endemic setting. A sample obtained ≤7 days of illness with an IgG titre of ≤1:128 required a follow-up sample for the diagnosis but if the sample was taken after 7 days of illness, a single titre of ≥1:256 was diagnostic for all ST and 90% of SFG cases[231].

2.3 Treatment of rickettsial infections, Scrub typhus and Q fever

2.3.1 Medical treatment

For a rickettsiosis, adequate antibiotic therapy initiated at the early stage (within the first week) of the illness is highly effective and has the most favourable outcome. With the right diagnosis and adequate treatment, the fever usually settles within 24-72 hours after starting antibiotic therapy. If this fails, the diagnosis of rickettsiosis should be re-considered, and further investigations initiated[232]. Treatments for different rickettsioses are similar but physicians should be wary of some species-specific differences that exist[233].

As per the 2016 Centre for Disease Control (CDC), USA recommendation for healthcare and public health professionals, doxycycline is the drug of choice for treatment of all tickborne rickettsial diseases in patients of all ages, including children aged <8 years and should be commenced immediately in persons with signs and symptoms suggestive of rickettsial diseases[234]. Oral or intravenous doxycycline, usually in a dose of 200 mg/day is the drug of choice for rickettsioses[235]. The duration of treatment is unclear and can vary for different clinical disease and the infecting rickettsial species. With good response to treatment, it may be stopped 2-3 days after the patient is afebrile[232]. A strong suspicion or confirmed diagnosis of a rickettsial infection is one of the clinical indications of doxycycline use in children where benefit outweighs side effects as recommended by the American Academy of Paediatrics and the CDC, Atlanta[234]. In addition to the dose of doxycycline used for rickettsial infections, staining of the teeth rarely occur[236]. Doxycycline cannot be used in pregnancy or allergic patients and alternatives are required[233]. Chloramphenicol (500 mg 6 hourly orally or intravenously for 7 days) may be used as an alternative to doxycycline, but precaution should be taken due to bone marrow toxicity. Chloramphenicol is also less effective than doxycycline. In pregnant patients, either chloramphenicol (early trimester) or doxycycline (late trimester) may be used. Ciprofloxacin and ofloxacin have also been shown to be effective against

RMSF[232]. A prospective, open, randomised trial of doxycycline versus azithromycin for uncomplicated murine typhus was conducted in Laos between 2004 and 2009. The study enrolled 216 patients (72 per arm) with evidence of uncomplicated murine typhus by rapid diagnostics kits and/or PCR. The study found that azithromycin was inferior to doxycycline for the oral therapy of uncomplicated murine typhus and there was no difference between the three days and seven days regimens of doxycycline[237]. However, concerns have been growing after the report of ST infections with poor response to doxycycline in northern Thailand, indicating the emerging resistance in *Orientia*[238].

Macrolide compounds, clarithromycin and azithromycin, due to their lack of adverse effects and good compliance, are good alternatives to tetracyclines or chloramphenicol in the treatment for children aged <8 years and pregnant women. A single dose of azithromycin 500mg was successfully used to treat ST in pregnant women[239]. The efficacy of azithromycin could be explained by its ability to penetrate polymorphonuclear leukocytes and macrophages (target cells for *Orientia*) and its post-antibiotic effect. However, there have been reports of failure of azithromycin therapy[240]. In addition, with available literature only in less than 100 pregnancies in the last 18 years, mostly resulting from under-recognition and underdiagnosis of cases, a recent cohort and case series analysis concluded that evidence supporting azithromycin, the most commonly used treatment in pregnancy, is weak. The authors called for urgent collaborative clinical trials in pregnant women to reduce the burden of adverse maternal and newborn outcomes and to determine the safety and efficacy of antimicrobial treatment[241].

Acute QF can be managed similarly to that of other rickettsial diseases with doxycycline remaining the antibiotic of choice. Emerging resistance of *C. burnetii* strains against doxycycline (MIC = 8μg/ml) in a case where the patient with endocarditis died is concerning[242] However, chronic QF requires prolonged courses of antimicrobial therapy. Because doxycycline alone does not cure Q fever endocarditis, a combination therapy has been proposed

and the efficacy of doxycycline and hydroxychloroquine was proven. In QF endocarditis, combination therapy with hydroxychloroquine and doxycycline for 18-36 months is the preferred treatment[110]. Relapse and positive valve culture have been observed even several years after the treatment of QF endocarditis. There is no test to confirm that the patient has been cured but the response to treatment can be monitored by falling antibody titers, although they take long to decrease. IgM antibodies (if present) disappear first, followed by IgA; however, IgG antibodies remain positive for years. After 3 years, treatment can be stopped when the titre of IgG antibodies against phase I antigens has fallen below 1:400[110]. Several alternative combination regimens such as a fluoroquinolone with doxycycline or rifampin with doxycycline have been proposed. A recent review has reported an optimum duration of treatment with doxycycline and hydroxychloroquine in Q fever endocarditis to be 18 months for native valves and 24 months for prosthetic valves. This duration should be extended only in the absence of favourable serological outcomes. Serological monitoring of the patients should be continued for at least 5 years because of the risk of relapse[243]. Surgery and valve replacement have played an important role in the management of endocarditis from chronic QF. Surgical requirements varied from 60%[244] to 45%[243]. Despite this high rate of surgical interventions, so far, there were no comparative studies assessing the role of surgery in Q fever endocarditis. However, clinical and serological cure were deemed possible without valve replacement, even in patients with endocarditis on prosthetic valves[243]. Definite indications for surgery include heart failure and abscesses.

2.3.2 Supportive management

In addition to specific antibiotic treatment, patients with rickettsial diseases require supportive management as simple as antipyretics in mild diseases to high-end life support in MODS. Clinical conditions of thrombocytopenia, hypoalbuminemia, hypotension, and coagulation

defects all require adequate supportive therapy. Management in the intensive care units may be required in serious respiratory and CNS involvement. Some severely ill patients, such as in RMSF, may be given glucocorticoids but efficacy has not been documented[233].

2.4 Prevention and control of *Rickettsia, Orientia* and *Coxiella* infections

The first step in preventing an infection is to understand its nature and epidemiology. However, despite being one of the oldest infections, rickettsial diseases (including ST) remain a truly neglected zoonotic infections. The knowledge of the public on the diseases is generally poor and the awareness amongst healthcare workers is weak. There is a huge need to improve the understanding of these zoonotic diseases since they are re-emerging infections with huge potential for outbreaks in endemic areas and as imported infections in non-endemic countries. With proper understanding and reliable data, travellers to endemic areas can take appropriate precautions during their visits to endemic areas. However, unlike several infections of public health importance such as malaria, tuberculosis, HIV/AIDS and dengue fever, there are no specific public health programs and systemic vector control strategies in place[184] in most countries, especially in developing countries like Bhutan.

Personal avoidance of ticks and mites by wearing proper protective clothing and footwear and use of repellents remains an integral part of protection against rickettsial infections. Chiggers reside in the grass, woodlands, and other vegetated areas. People should be encouraged to avoid such vegetations while outdoors or take preventive actions such as not to sit or lie on bare ground or grass and use sheets or other covers to sit on the ground[245]. If a bite is noted, prompt removal of the vector might prevent the infection. Attempting to control the tick and mite reservoirs are not usually feasible. Antibiotic prophylaxis following tick or mite exposure is not currently indicated to prevent rickettsial infection[193]. For epidemic typhus, delousing of louse-infested individuals and the use of insecticides to treat clothing and beddings are effective

preventive measures against the spread of louse-borne typhus. In the case of endemic typhus, prevention is primarily by controlling the flea and rat populations. Insecticides should be used before rodenticides to prevent rat fleas from seeking alternate hosts if rats are no longer available[193].

Due to the increasing cases with frequent outbreaks and reports of reduced response to antibiotic treatment, a vaccine against ST is warranted. The effort to develop a vaccine for scrub typhus has been active for over fifty years but researchers have not been successful in producing a vaccine that is capable of producing long-term protection[246]. As such, until now there is no licensed vaccine against ST and other rickettsial infections. A South Korean study attempted two (simple and complex) mathematical modelling of ordinary differential equations including human, rodent and mite groups to find their implications for prevention and control of ST. Sensitivity analyses showed that the most influential parameters for diseases transmission were rodent and mite populations, contact rate between them and trans-ovarian transmission. However, the contact rate between humans and mites was more influential than the mortality rate of rodent and mite groups. Therefore, the results of the modelling indicated that reducing the contact rate between humans and mites was the only practical method to control the incidence of ST, as the control of mites or rodents had only limited effects. The modelling also showed that the current control program was not sufficient to reduce or even maintain the incidence rate among humans when the population sizes of mites and rodents are growing. This emphasizes that more intensive control programs are needed to address the increasing vector population to control ST[247].

QF is a vaccine-preventable disease and vaccination is the most effective prevention. A vaccine is in use for both the human and livestock population but not widely available. The human vaccine called Q-vax (produced by Seqirus, Melbourne, Australia) is available for commercial use only in Australia. It is a whole-cell formalin-inactivated vaccine prepared from highly

purified phase I Henzerling strain *C. burnetii* cells[248]. It is given primarily to farmers, abattoir workers, veterinarians, clinical and laboratory workers. In the state of Victoria, Australia, the need for vaccination is assessed through a risk assessment by health, age, lifestyle and occupation (HALO). The vaccine is administered in a single dose of ≥25μg in a volume of 0.5ml subcutaneously. Due to severe adverse reaction at the injection site and sometimes systemically from re-exposure, the vaccine should not be given to people who are already immune to the bacteria due to previous vaccination or past infections. Therefore, all potential vaccinees must be screened for past exposure (by testing for *Coxiella* antibodies) and a skin test to identify possible adverse reaction before vaccination. Commonly described adverse events following vaccination include tenderness and erythema (rarely oedema) at the vaccination site and transient headache. Other severe but uncommon adverse effects observed were abscess at inoculation site and subcutaneous lumps which gradually disappear[248]. The protective efficacy of the vaccine lasted at least for five years. In addition, unlike other killed rickettsial vaccines, the Q-vax has the property of stimulating long-lasting T lymphocyte memory and this probably is responsible for its unusual protective efficacy as a killed vaccine[248]. A more recent study found that immune responses to infection or vaccination can persist for at least 10 years and that measurement of both antibody titers and in vitro T cell responses provides the most sensitive indicator of immunity[249].

There were several QF vaccines developed for use in animals, the most reliable being those developed from phase I inactivated bacteria. However, currently, QF vaccines in animals is available only in Europe. Coxevac (from CEVA, Hungary), is a vaccine prepared from a highly purified phase 1 bacterium of the Nine Mile *C. burnetii* strain. The vaccination is recommended to be given to non-infected ruminants before their first pregnancy[250]. This vaccine has been proven to effective in reducing abortion rates and bacterial load in vaginal mucus, faeces, and the milk in goats[251]. In a study to compare the efficacy of two animal vaccines (Coxevac, phase

I and Chlamyvax FQ, phase II) against *Coxiella* excretion as a risk to human health, the phase I vaccine was more effective and dramatically reduced both abortion and excretion of bacteria in the milk, vaginal mucus and faeces. In contrast, the phase II vaccine did not affect the course of the disease or excretion[251].

In addition to vaccination, QF can be prevented by preventing exposure to aerosols from birth and abortion materials of animals. During the outbreak of QF in the Netherlands, abortion waves on dairy goat farms were proven to be the primary source of infection for humans[192]. Outbreak control measures included a nationwide mandatory hygiene protocol for professional dairy (goat and sheep) farms, notification of abortion cases in herds and mandatory vaccination of small ruminants and bulk milk monitoring[192]. Screening of pregnant women and blood donors was also initiated. Mass information sharing to veterinarians, physicians and the public through targeted mailings, publications and the media helped disseminate the required information. The decisions to cull gestating animals to control the outbreak may be made considering the effectiveness of the ongoing outbreak control measures. In animals, antibiotic treatment with oxytetracycline in the last months of pregnancy was proposed to reduce abortion rates and bacterial shedding[252].

Chapter 3: The epidemiology of rickettsial diseases, Scrub typhus and Q fever in Asia

All rickettsial bacteria are zoonoses, being transmitted through infected arthropod vectors such as ticks, mites, chiggers, or fleas that also serve as their natural hosts[253]. Human infections occur through the bites of (or faecal contamination from) the vectors or aerosols generated from vertebrate host animals (in the case of *Coxiella*). Humans are the dead-end host with no role in the transmission of the bacteria. The incidences of important rickettsial diseases like scrub typhus, Rocky Mountain spotted fever (RMSF) and the Mediterranean spotted fever have fluctuated with long periods between the peaks and troughs of reported cases[254]. This may partly explain the emerging and re-emerging nature of rickettsioses at different localities around the world.

3.1 The epidemiology of rickettsial diseases

Spotted fever group (SFG) and typhus group (TG) *Rickettsiae* occur worldwide and both are a significant cause of morbidity in south-east Asia[255]. Until the current studies included in this thesis, the only report of rickettsial infections in Bhutan was from a small surveillance work carried out by the national public health laboratory (PHL) in 2009[256]. In this surveillance, serum samples were collected from patients suspected of ST in Gedu hospital in the south-western part of the country following a probable outbreak of scrub typhus (ST)[257] in the previous months. Incidentally, a few serum samples from Mongar hospital in the east were also included. The samples were tested in an external laboratory. Five of the 33 (1.5%) patients were positive for acute murine typhus as indicated by raised antibody titre of both IgM and IgG in paired serum. In the current study (work included as part of this thesis), an overall seroprevalence of 49% against rickettsioses was detected in the healthy Bhutanese population. Of these, about 15.7% and 3.5% were exposed to SFG and TG *Rickettsia* respectively[258]. Amongst the acute

febrile patients in Bhutanese hospitals, at least 15.2% (159/1044) had evidence of a concurrent rickettsial infection, of which 4.4% (46/1044) and 0.4% (4/1044) were due to SFG and TG rickettsia respectively[172].

In India, rickettsial diseases were thought to have been eradicated but now appear to be re-emerging and have been documented from all parts of the country[193]. In northeast India, bordering Bhutan, a seroprevalence of 13.8% and 4.2% against SFG and TG respectively have been reported[259]. A retrospective study of 161 children with fever in a hospital in central India found 75 (45.6%) positive against rickettsial diseases; 52 (69.3%) with SFG and 23 (30.7%) with ST. A mortality rate of 9% was reported in this study[230]. When two groups of military personnel (first group consisting of actively deployed healthy army personnel and the second group with acutely ill army personnel) in northern Sri Lanka were tested and compared for rickettsial infections, 84% (48/57) of the healthy group had serological evidence of exposure to rickettsioses and 67.3% (33/49) of the ill group were serologically positive for acute rickettsioses[260]. *R. felis*, a member of the SFG *Rickettsia* causes flea-borne spotted fever or cat-flea typhus in humans and is recognised as an emerging human pathogen[261]. *R. felis* was found to be endemic in north-central Bangladesh with a PCR positivity rate of 46% (96/150) amongst febrile patients between July 2012 to January 2014[148]. In that study, *R. felis* positivity was highest during the late rainy season (September – October) and lowest during the winter seasons (December to April) revealing seasonal differences in the infection. A study amongst febrile patients from different parts of Bangladesh revealed a seropositivity of 8.9% (64/720) against SFG and 1.4% (10/720) against TG *Rickettsia*[262], which were much lower than that reported previously. Another study detecting IgM against *R. typhi* (causing murine typhus) using ELISA in serum samples of patients who submitted samples for other tests in six Bangladeshi teaching hospitals revealed a high seropositivity rate of 66.6% (805/1209)[263]. In Nepal, amongst a sub-group of patients presenting with fever of unknown origin, serological

evidence of acute murine typhus was detected in 21 of 125 (17%) patients, with 12 of 21 (57%) positive in PCR for *R. typhi*[264]. *R. honei*, a member of the SFG *Rickettsia* found in Thailand and Texas (USA) also causes Flinders Island Spotted Fever (FISF) in Australia[135]. In the year 2009, *R. honei* was proven to be the causative agent in a 67-year-old female patient with high fever, rash and laboratory derangements as shown by positive PCR and a four-fold rise in antibody titre in Nepal[138]. The patient worked in wild dog protection and reported removal of a tick 2 weeks before admission and had contact with dogs, rats, ticks and fleas. In the national hospital of Vietnam in Hanoi, amongst 579 hospitalized patients with acute undifferentiated fever (excluding cases of malaria, dengue fever, and typhoid fever), 237 (40.9%) were serologically positive for *O. tsutsugamushi* and 193 (33.3%) for *R. typhi*[179] but SFG was not tested.

In animals, rickettsial exposures are common and occur worldwide. A seroprevalence of 42% against SFG was reported in Sri Lankan dogs in the study area[265]. In a Mongolian study, an overall seroprevalence against SFG of 20% with individual seroprevalence rate of 30%, 13%, 21%, 35%, and 2% for cattle, goats, sheep, horses and camels respectively was reported [266]. Apart from Asia, seropositivity rates as high as 93.9% (568/605) in dogs against SFG were reported in Germany[267]. In Panama, 70% (14/20) of horses and 65% (13/20) of dogs were positive for SFG antibodies[268]. In Brazil, *R. rickettsii* causes Brazilian spotted fever (BSF). In a cluster of cases in an animal shelter, 2.6% (3/115) of employees and 97.5% (114/117) dogs tested seropositive for the SFG *Rickettsia* and there were five deaths[269].

3.2 The epidemiology of Scrub typhus

ST was traditionally thought to be restricted to the Asia-Pacific region covering a triangular area known as the "Tsutsugamushi triangle" extending from Afghanistan and Pakistan in the west to China and Korea in the north-east and the islands of the southwestern Pacific and

northern Australia in the southeast. In this region, an estimated one million new cases occur annually and a billion people are at risk of the infection[270]. A recent systematic review reported that ST is a leading cause of treatable non-malarial febrile illness in Asia as indicated by prospective fever studies. In addition, seroepidemiological studies in Asia implicated that ST is common across Asia with a median seroprevalence of 22.2% (range 9.3%-27.9%)[184].

Human infections occur when they encroach on a zone of vegetation with chiggers, so people involved in the clearing of land, logging, road building, and military operations are at high risk of infections. ST cases occurred about four times higher in those people working in occupations related to contact with forest[177]. Transmission of ST to vertebrate hosts including humans is seasonal. Chigger activity is determined by temperature and humidity[270]. In one of the most extensive reviews on the geographical distribution and seasonality of ST in Japan from 1955 to 2014, the classical ST (better known as 'tsutsugamushi disease' in locally) seemed to have disappeared in the mid-1960s, with a long absence. After that, an epidemic with October-December seasonality in 1975 in southern prefectures and a May-June seasonality in 1979 in the northern prefectures of the country was seen. The current epidemic was described into three types: a large October–December epidemic with a small May–June epidemic in the southern part of Japan, a large May–June epidemic with a mid-sized October–December epidemic on the Pacific coast of northern Japan, and a large May–June epidemic with a small October–December epidemic on the Japan Sea coast of northern Japan[271].

Although data is limited (at the time of commencement of this project), ST is better known than other rickettsial diseases in Bhutan. This is primarily due to the availability of a rapid diagnostic test kit for ST (SD Bioline Tsutsugamushi Test; Standard Diagnostics, Yongin, South Korea) in the National Public Health Laboratory (PHL) and selected hospitals (mostly in the southern part of the country with a warm and humid climate) since the year 2009/2010. The first officially recognized and investigated cases of ST in Bhutan was that of a probable

outbreak in the communities surrounding Gedu hospital in the south-western part of the country in July 2009[257]. There might have been earlier cases which were not documented. During the outbreak of fever with rashes, cases were first managed as dengue fever but later thought to be ST based solely on the presence of typical eschars on a majority (5/7) of the admitted patients, although no laboratory tests were carried out. Three lives were lost in this outbreak. This incident prompted a limited surveillance of ST from the same area by the PHL with few other incidentally collected samples from Mongar hospital in the East[256]. In this surveillance, about 85% (28/33) had antibodies (both IgM and IgG) against *Orientia tsutsugamushi*, although only 2 were based on rising antibody titre and the rest from a single acute serum sample. Presence of an eschar in 21% of those with positive antibody was an indirect assurance that those with high single antibody titres could be due to current ST infections as eschars are clinically pathognomonic for ST. ST was included in the Bhutan national notifiable diseases list in 2008[272] but no active notification occurred until 2010. Since 2010, a few health centres across the country have started reporting ST cases as 'Rickettsial diseases' and cases numbers have increased consistently from 91 in 2010 to 118 in 2011, 218 in 2012, 351 in 2013, 450 in 2014, 605 in 2015, 665 in 2016, and 753 in 2017[273, 274]. However, mortality from reported rickettsial diseases has remained low with none to a maximum of two deaths in a year[274]. Most of these reported cases were probably based on clinical diagnosis since laboratory testing was available in only a few hospitals. In the current study (work included in this thesis), ST (22.6%) was the most prevalent infection amongst the rickettsial diseases in the 864 healthy Bhutanese tested[258]. ST (6.7%) was also the commonest rickettsial infections amongst the 1044 acute febrile patients tested in the 14 selected Bhutanese hospitals between October 2014 to June 2015[172]. An outbreak of ST in a remote school in Bhutan was responsible for the loss of two young lives due to complications (meningoencephalitis) resulting from delayed medical treatment[173].

ST continues to emerge in previously unknown locations and re-emerges in already known areas with increasing incidence and prevalence. While systematic data is lacking even in countries with the highest burden, at least four Asian countries (South Korea, Japan, China and Thailand) with an established passive surveillance system for ST have recorded clearly increasing trends over recent years[184]. In South Korea, an almost four-fold increase in the number of ST cases was reported in 2013 (10,485 cases) compared to 2001 (2,637 cases)[275]. South Korea also reported an almost 21 fold increase in urban cases of ST from 2.8/100,000 in 2003 to 59.7/100,000 in 2013[276]. In Japan, the annual incidence rates of ST have been around 3.5/100,000 population[277, 278]. In China, the number of cases in 2014 was almost fifteen times higher than that observed in 2006 and scrub typhus occurred more in females and peaked in the months of July and October[279]. In Guangzhou city of southern China, the incidence of ST increased from 3.3 per 100,000 in 2006 to 9.85 per 100,000 in 2012, with a peak in summer (June-July) and another peak in September-October[280]. Thailand is also an endemic country for ST with reported outbreaks [281] and reports of high ST incidence[282].

ST has been increasingly reported in recent years from countries surrounding Bhutan. In northeast India bordering southern Bhutan, a seroprevalence of 30.8% against ST[259] was reported. In this part of India, ST was recognized as the leading cause of infectious disease and an important cause of infectious fever[174]. In Darjeeling, a district of the north Indian hills near Bhutan, an overall incidence of ST in 2005 was reported to be 34 cases/100,000 population/pa, varying from 2 cases/100,000 population in July to 20/100,000 population in September and decreasing to zero in December[283]. This post-summer peak was also seen in the Uttarakhand district of India where ST cases peaked from July to October although cases occurred throughout the year[177]. In India, amongst 540 (521 children) patients with Acute Encephalitis Syndrome (AES), ST (25%, positive by serology, PCR or both) has recently emerged as the leading cause of AES compared to other causes such as Japanese encephalitis virus (8%), West

Nile viruses (7%), dengue virus (6%) and chikungunya virus (5%) in a pilot surveillance[180]. Another Indian study in the Uttar Pradesh state concluded that the evidence available on AES since 2014 indicated ST as the leading cause of AES, accounting to >60% of AES cases. In contrast, during the study period, Japanese encephalitis virus (JEV) commonly thought to be the main cause of AES, accounted for only 10% of the cases[284]. There are several other reports of ST presenting as an AES syndrome in India and other countries[173, 181, 285-288]. In Nepal, 175 of the 434 (40.3%) patients from various region of Nepal tested by the National public health of Nepal in 2015 were positive for IgM antibodies to *O. tsutsugamushi*[289]. Samples from febrile patients collected from different parts of Bangladesh revealed an antibody positivity against *Orientia* of 8.8% (63/720)[262]. Another study detecting IgM using ELISA in serum samples of patients who submitted samples for other tests in six Bangladeshi teaching hospitals revealed a seropositivity rate of 23.7% (287/1209)[263]. India and Nepal have reported several outbreaks of ST in different states all over the country in the recent years[289-293]. Such outbreaks of ST have occurred frequently in military units[294-296].

Northern Australia forms the south-eastern part of the 'Tsutsugamushi triangle' and ST has been reported widely from this region. With five serologically confirmed cases (two near-fatal with multisystem involvement) a new focus of ST was described in the remote rainforest of Northern Australia with possible recognition of more such foci due to increasing tourism in the area[297]. In 1996, a strain of *O. tsutsugamushi* different from the classic Karp, Kato and Gilliam strains were isolated from the blood of a 38-year-old man working in the Litchfield park in the Australian Northern Territory. This strain of *O. tsutsugamushi* was designated as Litchfield strain[298]. In northern Queensland (Australia), a large outbreak (45 clinical cases, comprising 36% of the potentially exposed individuals) of ST amongst military personnel occurred despite existing military protocols of providing doxycycline prophylaxis. Resistance to doxycycline was excluded using a quantitative PCR-based susceptibility assay and the

outbreak occurred probably due to the failure to adhere to adequate prophylaxis over the duration of the military exercise[296]. An outbreak of ST also occurred for the first time in the northern province of Solomon island situated to the north-east of Australia in 2014[299].

Beyond the 'Tsutsugamushi triangle', ST cases were detected in the year 2006, through two reports from entirely different locations; one in an Australian tourist returning from the Middle East that led to the isolation of a new species of *Orientia*, *O. chuto*[300] and another in a patient in Chili as demonstrated by both serology and molecular study of eschar biopsy[301]. Occurrences of ST infections in South America was further supported by recent case reports from Chile, laboratory confirmed by both serology and molecular testings carried out at multiple recognised laboratories[302]. Presence of ST in Africa was reported through a seroreactivity of 3.6% amongst febrile children (1-12 years) in a hospital in Kenya in the year 2011-2012. This finding was validated by detecting seroconversion from non-reactive in the acute phase to reactive in the convalescent phase or as a four-fold rise in antibody titre between acute- and convalescent-phase serum samples[303]. In another African study, 6% (3/49) of the abattoir workers in Djibouti tested positive against *Orientia*[304]. These reports from three separate previously unrecognized ST endemic locations have shown that ST is more widespread rather than restricted to the Asia-pacific region. This conclusion on ST occurring beyond the Asia-Pacific region was also reached in a recent review on epidemiology of ST[171].

Orientia naturally inhabits several different species of *Leptotrombidium* mites, both in the environment and on mammals, commonly rodents[305]. Human infections occur through the bite of infected chigger mites which are the larval stage of its life cycle. Prevalence of *Orientia* in domestic animals have been reported rarely in Asia and there are no data from Bhutan. The majority of the few reports on *Orientia* in animals are focused on rodents and other small animals. In Thailand, *O. tsutsugamushi* was detected in 10 of the 22 species amongst 3,498 small mammals studied that included *Rattus bukit, Rattus rattus, Rattus argentiventer, Rattus*

berdmorei, Rattus losea, Bandicota indica, Rattus koratensis, Bandicota savilei, Rattus exulans, and *Tupaia glis*[305]. Of the six species of small mammals collected at a Korean and US military camp, 56%, 67%, 25% and 100% of *Apodemus agrarius, Mus musculus, Micromys minutus* and *R. rattus* were positive for *Orientia* antibodies[306]. Dogs in Sri Lanka had an exposure rate of 24% against *O. tsutsugamushi*[265].

3.3 The epidemiology of Q fever

Q fever (QF) has a worldwide distribution[97] and have been reported almost anywhere they have been sought except New Zealand[101, 307]. Concerns on the introduction of QF into New Zealand through the potential use of infected rabbits or their organs to transport Rabbit haemorrhagic disease virus (RHDV) illegally from Australia (to control rabbits in New Zealand) was raised but found to be unlikely[308]. Cattle, sheep and goats are the main reservoirs of *C. burnetii* although increasing number of animals including domestic and marine mammals, reptiles, ticks and birds have been known to shed the organism[309]. Wildlife has also been implicated to constitute a reservoir of *Coxiella* from the evidence of infections after exposure to kangaroos and wallabies in Australia[310, 311]. Much of the organism is concentrated in the birth products but also found in urine, faeces and milk of infected animals[312]. *C. burnetii* has a very low infectious dose of about 10-15 organisms for human infection[313]. Human infections usually occur from inhalation of aerosolized bacteria in the environment following delivery or abortion in infected animals. QF is a zoonosis with no scientifically proven human-to-human transmission. Thus, the epidemiology of QF in human population always reflect the circulation of the organism in animal reservoirs[101] and outbreaks in humans could often be preceded by outbreaks in an animal population.

The heightened importance of *C. burnetii* more recently has been attributed to its increasing role in endocarditis, it's classification as a potential bioterrorism agent (thereby becoming a

reportable/notifiable infection in many countries), its emergence as a common cause of fever in intertropical area and the evidence that it could become a major public health problem (as experienced in the Netherlands outbreak)[101]. The epidemiology of QF depend on the geographic area (considering endemic and hyperendemic areas), the occurrence of large epidemic outbreaks and whether it is a reportable disease or not in that country[101]. Studies and case reports on QF, both in human and animal populations are mostly from developed countries with limited data, especially from south Asian countries. No studies have been conducted on QF in Bhutan in both the human and animal population prior to the current study and there were no data in any form. In the current study (work included in this thesis), the seroprevalence of QF (6.9%) in healthy Bhutanese population was lower than that of ST (22.6%) and SFG (15.7%)[258]. Amongst the acutely ill febrile patients attending 14 Bhutanese hospitals, QF was responsible for about 3% (29/1044) of the illness[172]. In countries neighbouring Bhutan, QF has not been studied as much as ST and other *Rickettsiae*. With only about 25 publications from both human and animal population combined to date since the first description of QF in 1954, QF is probably underreported in India and there are no recent data [314]. One of the few recent studies among febrile patients in a hospital from Karnataka state reported a QF case incidence of 4.5% (9/198) confirmed by two real-time PCRs. This finding revealed the importance of including QF in the differential diagnosis of acute febrile illness in Indian health centres[314]. Another revelation of the underestimated QF prevalence in India was through a study detecting *Coxiella* by serology, PCR and culture in women who aborted spontaneously. This study reported an overall positivity of 25.7% by one or more assays amongst the 74 women sampled[315]. *C. burnetii* was also found to be an important cause of atypical pneumonia in India[316]. A study of undifferentiated fever in Nepal detected only one seropositive case against *Coxiella* amongst 125 patients tested[264]. In Bangladesh, only 10 of the 720 (1.4%) febrile

patients from different parts of the country tested positive for *Coxiella* antibodies by an ELISA test kit[262].

QF is a notifiable disease in all Australian states. With notification rates ranging between <1.0 per 100,000 to 6.3 per 100,000 population per annum, QF is the most commonly reported zoonotic disease [187]. These notification rates are probably underestimated since many cases are undiagnosed and thereby un-notified[317]. QF is known to occur in outbreaks but such occurrences may be overlooked and missed especially in developing country setting due to lack of laboratory support. Significant outbreaks have been reported from many European countries; Spain with 42 cases in 1982[318], Switzerland with 415 cases in 1987[319], the United Kingdom with 147 cases in 1992[320], Germany with 331 cases in 2005[321] and in the Netherlands with over 2500 cases between 2007-2009[322]. In Europe, acute Q fever cases occur more frequently in spring and early summer[188].

Coxiella also infects animal species ranging from domestic and wild mammals to birds, reptiles and arthropods such as ticks[97]. Although most animal infections go unnoticed[97], infertility and abortions have been reported in numerous mammalian species[201]. In an Indian study, the overall QF positivity in 920 animals (in combined methods of PCR and serology) with reproductive disorders was found to be 14% and individually 13% in cattle, 17% in buffaloes, 11% in sheep and 6% in goats[323]. A seroprevalence study in a small animal slaughterhouse in India's Uttar Pradesh state detected a 4% (22/500) *Coxiella* IgG seropositivity in goats using a commercial ELISA kit[324]. To the north of Bhutan, QF was found to be highly prevalent in Tibetan sheep in China with an overall 14% (304/2112) positive for antibodies against *C. burnetii*. Seroprevalence in female and male sheep were 15% (95% CI, 13.18–16.78) and 13% (95% CI, 10.3–15.6) respectively[325]. A similar study in free-range yaks in China reported a seroprevalence of 14% (75/552) against *C. burnetii*, with no significant difference between female (14%) and male yaks (13%)[326]. In mainland China, a systematic review of QF between

1989-2013 found an overall prevalence of QF of 10% (1139/11,209) in the human population, 15% (288/1918) in cattle and 12% (176/1440) in goats[327]. This review found that *C. burnetii* infections occurred widely in China and had been reported in 64 cities/municipalities from 19 provinces amongst human and/or animal populations. In animals, the seroprevalence was highest in cattle and goats compared to other domestic animals and a wide variety of ticks were found to be infected[327]. In one of the first studies in Bangladesh, an overall serological evidence of *C. burnetii* infection in cattle and goats were found to be 1% (8/1149); 1% (4/620) in cattle and 1% (4/529) in goats[328]. The presence of QF in domestic animals in Bangladesh was further supported by detecting it in three species of domestic ruminants with a seroprevalence of 10%, 3% and 4% in sheep, goats and cattle, although the difference was not significant. Only one of the 23 aborted sheep foetal membranes was found to be *Coxiella* positive in RT-PCR[329]. A meta-analysis on *C. burnetii* in horses worldwide reported an overall seroprevalence of 16% but none of the 122 cases of equine abortion, stillbirth or neonatal foal death was positive for *Coxiella* DNA by PCR[201].

Chapter 4: Hypothesis, aims and objectives

4.1 Hypothesis

Spotted fever group (SFG) and typhus group (TG) rickettsiae occur worldwide and both are a significant cause of morbidity in south-east Asia[255]. In India, rickettsial diseases were thought to have been eradicated but now appear to be re-emerging and have been documented from all parts of the country[193]. Scrub typhus (ST) was traditionally thought to be restricted to the Asia-Pacific region covering a triangular area known as the "Tsutsugamushi triangle" extending from Afghanistan and Pakistan in the west to China and Korea in the north-east and the islands of the southwestern Pacific and northern Australia in the southeast **(Fig. 4.1).** Recently, ST has been reported in the Middle East[300], South America (Chile)[301, 302] and Africa[303, 304]. These reports from previously unrecognized ST endemic locations have shown that ST is more widespread rather than restricted to the Asia-pacific region.

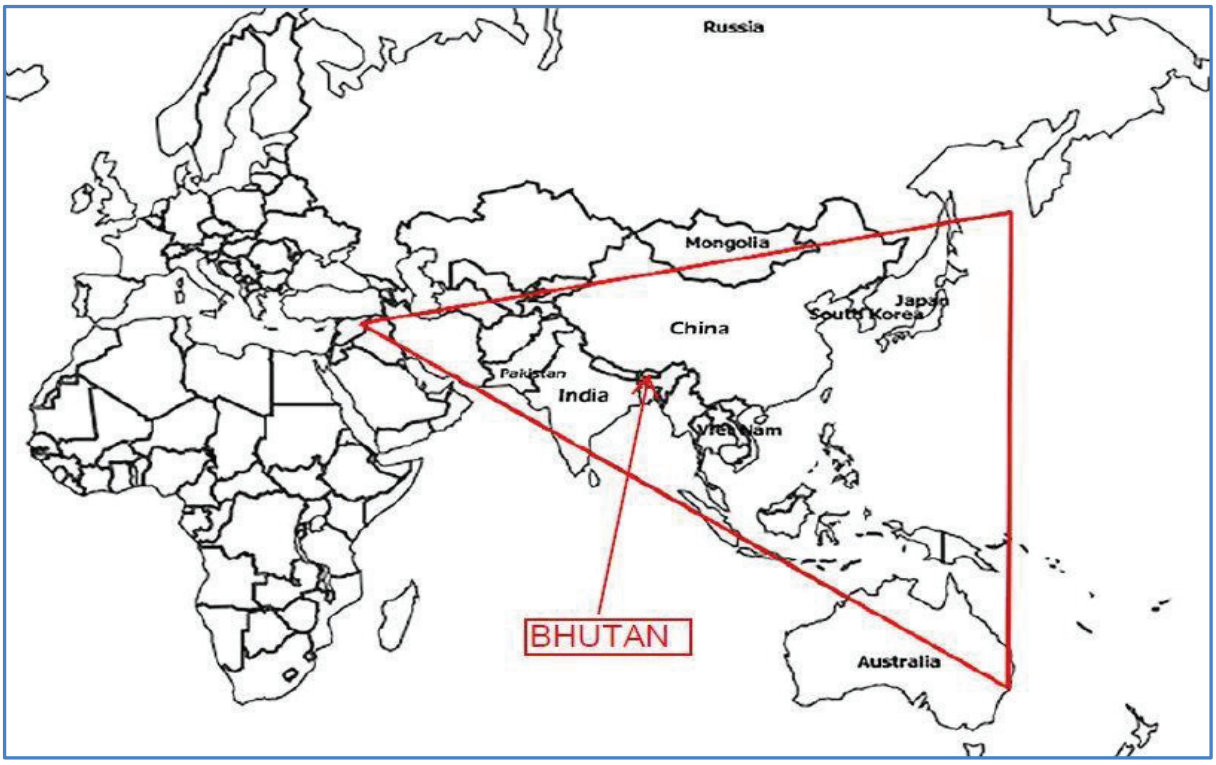


Figure 4.1. Map showing Bhutan as a part of the "tsutsugamushi triangle"

In the Asia-Pacific region, an estimated one million new cases occur annually and a billion people are at risk of the infection[270]. A recent systematic review reported that ST is a leading cause of treatable non-malarial febrile illness in Asia as indicated by prospective fever studies. In addition, seroepidemiological studies in Asia implicated that ST is common across Asia with a median seroprevalence of 22.2% (range 9.3%-27.9%)[184]. Q fever (QF) has a worldwide distribution[97] and have been reported almost anywhere they have been sought except New Zealand[101, 307].

At the time of commencing this project, data on rickettsial diseases in Bhutan was limited, with information restricted to ST and no data available for other rickettsial diseases or QF. There were no reports or any formal studies on rickettsial infections, or on QF in livestock. This indicated that rickettsial infections were highly neglected diseases in Bhutan with a large gap in understanding by the medical professionals and the public. Since Bhutan lies within the "Tsutsugamushi triangle" and other rickettsial infections have also been widely reported from this area, it was hypothesized that ST and other rickettsial diseases (as well as QF), may be endemic in Bhutan. Therefore, this project was undertaken as the first comprehensive study of rickettsial diseases and QF in Bhutan, intended to fill this gap and uncover the postulated hidden endemicity of rickettsial infections in the country.

4.2 Aims and Objectives

The overall aim of the project was to advance the understanding of the epidemiology of rickettsial infections in Bhutan with the following specific objectives:

a. Collate existing limited and scattered data on ST in Bhutan to understand the situation prior to the start of the current project
b. Investigate an outbreak of febrile illness with rash (? due to ST) in a remote primary school in Bhutan in 2014

c. Estimate the contribution of rickettsial diseases and QF as a cause of acute febrile illness in patients with fever of unknown origin attending 14 selected hospitals
d. Determine the rate of past exposure (by measuring seroprevalence) against rickettsial diseases and QF in the healthy Bhutanese population
e. Generate preliminary data on exposure of domestic animals to rickettsial bacteria

Chapter 5: Materials and methods

5.1 Study design

The majority of the activities in this project were carried out as a prospective, descriptive study designed to understand the epidemiology of rickettsial infections and Q fever (QF) in Bhutan. Two activities (collation of existing information on Scrub typhus (ST) and analysis of archived blood samples from an outbreak of febrile illness) were carried out retrospectively.

5.2 Study subjects and recruitment

The study subjects and their recruitment for each specific objective of the project have been described in the respective research publications included in this thesis under different chapters. The following provides a more detailed description of the study subjects and their recruitment process for each of the specific study objectives.

5.2.1 Collation of existing data on Scrub typhus

This part of the project involved a data search; compilation and analysis of laboratory records of samples tested between 2009 to 2014 in the public health laboratory, the National Referral Hospital and other hospitals; plus data from the Annual Health Bulletin (AHB) of the Ministry of Health and other documents obtained through an online search and personal communication with concerned officials in the public health departments.

5.2.2 Investigation of an outbreak of a febrile illness in a remote primary school

This was undertaken by reviewing the outbreak investigation report and communication with relevant officials involved in the investigation. Archived blood samples from the patients in the outbreak were retrieved, shipped to the ARRL and analyzed.

5.2.3 Assessing the case incidence amongst patients with acute undifferentiated fevers

As part of this objective, fourteen hospitals **(Fig 5.1),** mostly located in the south-central part of Bhutan were selected. Patients attending these hospitals between October 2014 to June 2015 and fulfilling the case definition were enrolled in the study.

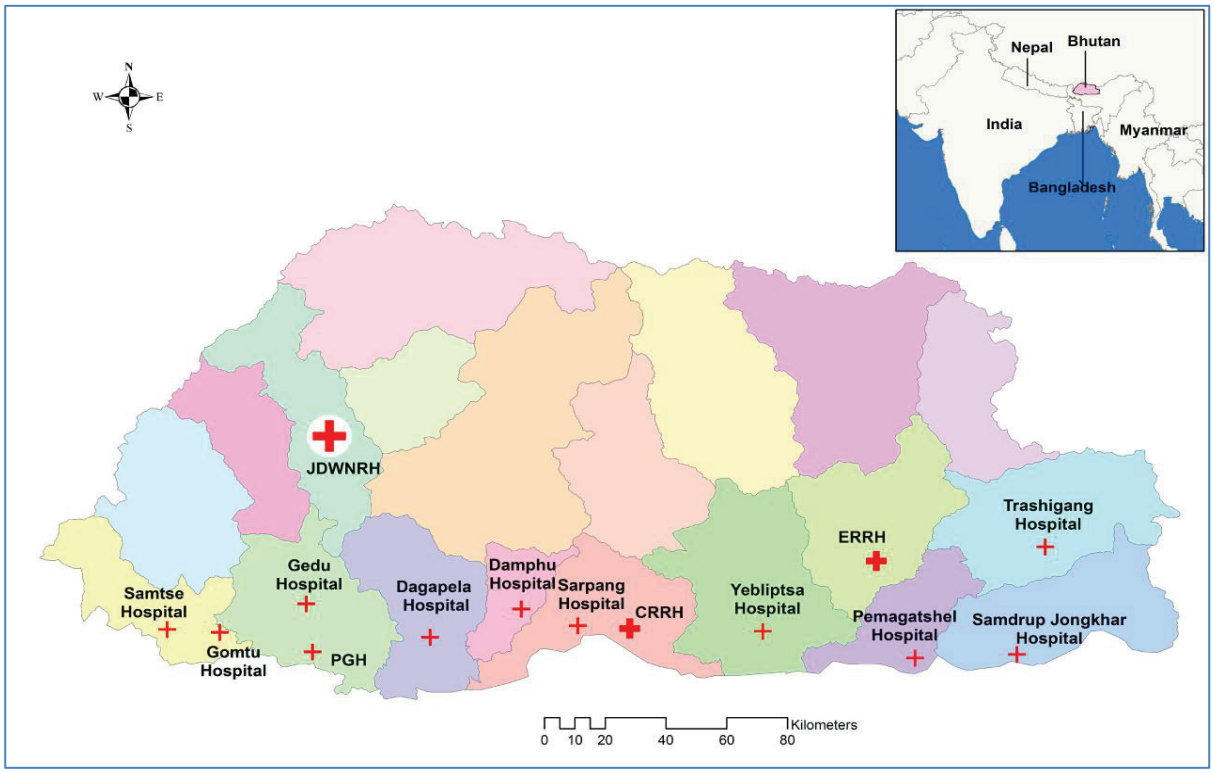


Figure 5.1: Map of Bhutan showing the 14 hospitals (study sites) (JDWNRH: Jigme Dorji Wangchuck National Referral Hospital; ERRH: Eastern Regional Referral Hospital; CRRH: Central Regional Referral Hospital; PGH: Phuentsholing General Hospital)

A case was defined as **"any patient attending one of the selected health centers with acute onset of fever for ≥ 4 days with one or more of headache, chills, myalgia/arthralgia, rash/eschar, lymphadenopathy and clinical exclusion of any obvious causes of fever such as tonsillitis, pharyngitis, otitis, urinary tract infection etc".**

In each hospital, physicians and other clinical and laboratory staff were briefed on the case definition and study recruitment process. When a treating physician came across a patient

fulfilling the study criteria, the patient was sent to the laboratory unit where study information was provided, informed consent given, clinico-demographic details obtained **(Appendix 1.)** and blood samples collected **(Appendix 2.).** Serum and whole blood in ethylene diamine tetra-acetic acid (EDTA) were stored at 4°C until shipment to the central laboratory, where the principal investigator (PI) was based. Forms and blood samples were shipped within a week of collection to the PI. At the central laboratory, serum was obtained from the clotted blood and buffy coat was extracted from the EDTA blood into 15% glycerol. Both the serum and buffy coat in glycerol were stored at -70°C until shipment to the ARRL.

5.2.4 Determination of seroprevalence amongst the healthy population

The subjects of this study were healthy people (≥ 13 years) living in the eight selected districts (8 urban and 8 rural sampling sites) as shown in **Fig 5.2**.

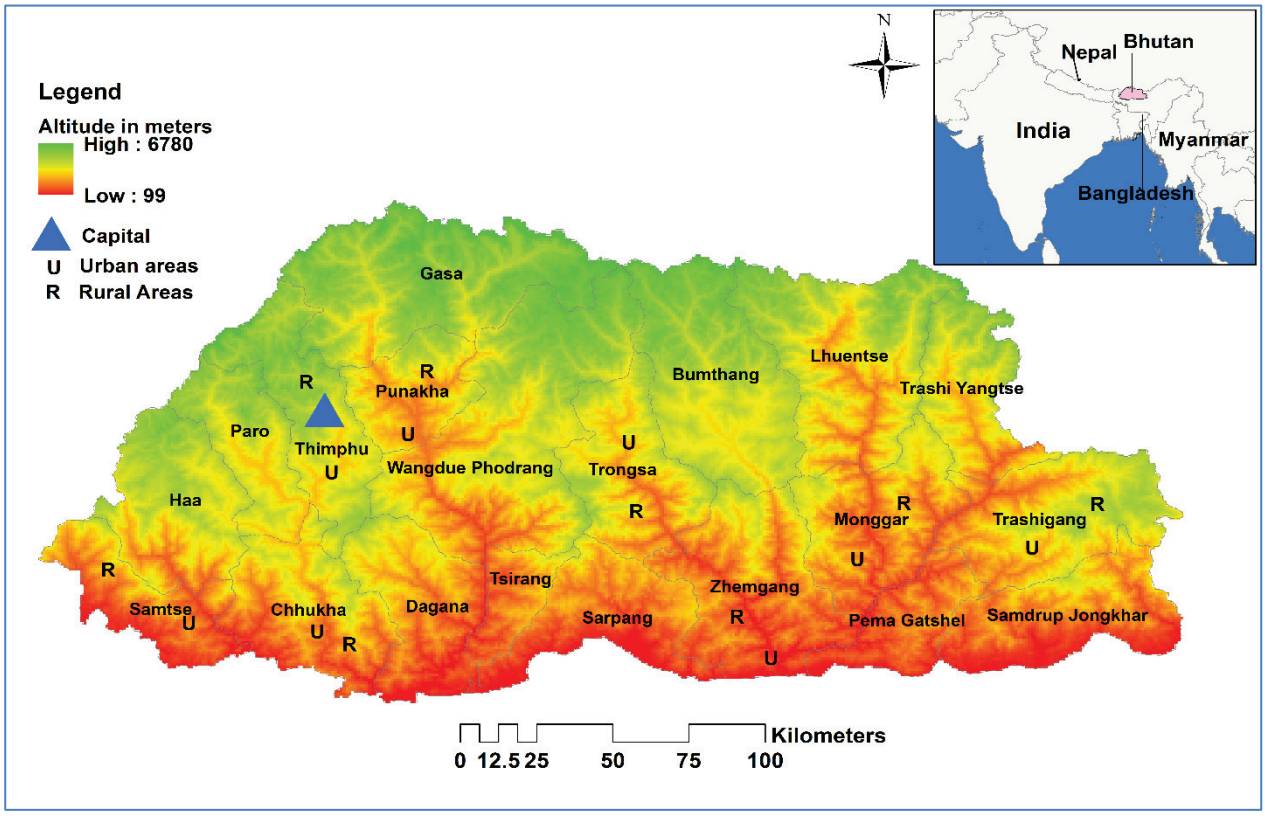


Figure 5.2. Urban (U) and rural (R) primary sampling units from the eight districts of Bhutan

Participants for this study were recruited as detailed in section **5.5** below. Participant selection and blood sample collection were performed according to guidelines detailed in **Appendix 3** and **Appendix 2.** Samples were collected by trained medical laboratory staff and the PI **(Fig 5.3 and 5.4).**

Figure 5.3. Sampling in rural Bhutan involved days of walking

Figure 5.4. Drawing blood sample from a participant at his home

5.3 Inclusion and exclusion criteria

5.3.1 For acute undifferentiated febrile cases in the selected hospitals

- Included: All consenting patients meeting the case definition in **5.2.3**.
- Excluded: Any patient who had been admitted to the hospital for a prolonged time with known chronic illness

5.3.2 For seroprevalence in the healthy population

- Included: All consenting, individuals who were selected from the particular household through the lottery method.
- Excluded: Chronic ill patients at home, children < 13 years

5.4 Animal studies

For animals studies, serum samples were opportunistically collected from domestic animals present in the same study sites as the human seroprevalence study in the eight districts (as in section 5.2.4). Animal species included cattle, dogs, goat, sheep, horses, yaks and cats. Samples were collected by livestock field staff as per sampling instructions in **Appendix 4.** Livestock officers and field staff were briefed on the objectives of the study and procedures invloved **Fig 5.5.**

Figure 5.5. Briefing to the livestock officers and field staff

5.5 Sample size and sampling strategy

For the study on acute febrile patients, no sample size was determined. Nevertheless, each of the 14 hospitals was expected to enrol at least fifty patients over the study period as estimated from previous acute fever registries. In addition, the sample size was not calculated for the animal studies since it involved opportunistic sampling of animals present in the locality of the human seroprevalence study.

However, the sample size was calculated for the seroprevalence study amongst the healthy people using a clustered, multi-staged sampling technique to be nationally representative, as follows:

5.5.1 Sample Size Calculation

Sample size to estimate the number of households to be surveyed with 95% confidence was calculated using the following formula and assumption.

Step 1

Sample size $n = \frac{Z^2 1-\alpha\ P(1-P)}{d^2}$

Where:

Z= level of confidence measure which represents the number of standard errors away from the mean. This describes the uncertainty in the sample mean or prevalence as an estimate of the population mean (normal deviate if alpha equals 0.05, Z = 1.96, for 95% confidence level)

P= The estimated prevalence of the disease within the target population. Values closest to 50% are the most conservative. The recommended value of 0.5 is included if there are no previous data on population, otherwise, the value closest to 0.5 is accepted from previous data.

d = Margin of error. The expected half-width of the confidence interval, with 0.05 being taken for this study.

Therefore, $\mathbf{n} = \frac{1.96 \times 1.96\ [0.5 \times (1-0.5)]}{0.05 \times 0.05}$

n= 384

Step 2

- Considering a design effect of 2.0 to address the issue of cluster sampling

  n= 384 x 2.0 =768

Step 3

- Adjusting for expected non-response to get the final sample size and expecting a 90% response rate, the above sample size was divided by the expected response rate.

  n = 768/0.9 = 853.4 ≈ **860**

  For each of the eight districts = 860/8 = 107.5 ≈ **108 (per district) = 864 (total)**

5.5.2 Sampling frame and sampling procedure

Administratively, Bhutan is divided into 20 districts. Each district is subdivided into either Geogs for rural settings or towns/cities for urban settings. There are altogether 205 Geogs and 33 towns. Each Geog is further sub-divided into Chiwogs (or villages) and on average 5-7 chiwogs make a Geog. There are 1044 chiwogs. In urban areas, towns/cities are divided into Enumeration Areas (EAs). There are 311 EAs for the 33 towns of Bhutan. Previous national surveys and other studies have used Chiwogs in rural areas and EAs in urban areas as the primary sampling units (PSUs) as practised by the Bhutan National Statistics Bureau (NSB).

A multi-stage sampling method was employed to select the districts with an urban and a rural primary sampling unit (PSU) selected from each district and the required number of households and eligible participants from each household in three stages as described below.

5.5.3 Type and number of PSUs selected (Stage 1)

For nationally representative data, the country was divided into four clusters as per the practice of the NSB: Eastern (5 districts), western (5 districts), southern (6 districts) and central (4 districts). Two districts were selected from each cluster for the study through a probability proportionate to size (PPS) method. After selection of the eight districts, an urban and a rural sampling unit was selected from each district using the same PPS method. Chiwogs in rural areas and an EA in urban areas were taken as the PSUs for each study district.

Based on the urban and rural population proportion of 30:70 in Bhutan, 30% of the participants were recruited from the urban settings, while the other 70% of the participants were taken from the rural settings. Thus, of the total sample size (864), 608 (70%) participants were taken from the rural frame and 256 (30%) were taken from the urban frame. Individually, each of the eight districts recruited a total of 108 participants (32 from an urban PSU and 76 from a rural PSU).

5.5.4 Selection of households (Stage 2)

After the PSUs in the eight districts were selected, the required households (32 in urban and 76 in the rural frame) were selected from each of the rural and urban PSUs. All households in the particular PSU were line-listed and the required number selected through a lottery method. When the selected PSU had less than the required number of households, a nearby PSU was merged and the required number of households selected accordingly. The list of households with a unique identification number (ID) developed during the previous national surveys (National Health Survey 2012 and Non-communicable disease STEPwise approach to surveillance (NCD-STEPS), 2014) or a recent listing of households available from the Basic Health Units was used as the sampling frame for this selection.

5.5.5 Selection of eligible participants at the household level (Stage 3)

After selection of the household, all eligible members (aged ≥13 years) present in the house during the visit were listed and one participant was selected through a lottery system.

SUMMARY OF SAMPLING STRATEGY

20 Districts (administrative districts)

Divided into 4 regions

Eastern (5 districts) **Central (4 districts)** **Western (5 districts)** **Southern (6 districts)**

2 districts selected from each region by PPS method

8 selected Districts

One urban and one rural sampling area selected from each district using the same PP method

(Total 8x2 = 16 sampling sites)

Total human serum samples to be collected as calculated = **860**
Samples to be collected from each district = 860/8 = 107.5 ≈ **108**
Considering the urban-rural population distribution of **30:70**

Eight selected district (108 participants each)

Urban frame (30%) **Rural frame (70%)**

32 samples **76 samples**

5.6 Demographic and clinical data collected

Basic demographic, clinical and environmental exposure information were collected from acute febrile patients **(Appendix 1)** and healthy subjects (**Appendix 3).** Information place of residence was collected from domestic animals **(Appendix 4).**

5.7 Collection, storage, and shipment of biological samples

For the specific studies, the following samples were collected:

1. A single serum sample for serology and EDTA blood for real-time polymerase chain reaction (qPCR) from acute febrile cases presenting to the 14 health centres (**Appendix 1 and 2**)
2. A single serum sample from healthy subjects for serology **(Appendix 2 and 3)**
3. Serum samples from domestic animals **(Appendix 2 and 4)**

The above samples were shipped in thermocol ice-packs (2-4^{o}C) from the study sites to the PI at the central laboratory. At the central laboratory, human and animal serum samples were directly stored at -70^{o}C. Buffy coat was extracted from the EDTA blood samples into 15% glycerol vials and these were also stored at -70^{o}C. At the end of the sampling period, all were samples were shipped to the Australian Rickettsial Reference Laboratory (ARRL), a nationally accredited laboratory for subsequent rickettsial testing. A material transfer agreement (MTA) **(Appendix 5)** was signed between the Bhutan national referral hospital and the ARRL (Australia) for the shipment of biological samples. Samples were packed with IATA packing instruction 650 and shipped to the AARL at room temperature according to recommended practice of the ARRL (**Fig. 5.6**)

Figure 5.6. Samples finally reach the Australian Rickettsial Reference Laboratory (ARRL)

5.8. Laboratory Methods

In this project, rickettsial antibodies were detected using immunofluorescence assay (IFA) and a qPCR was used to detect rickettsial DNA. In Australia, three IFA assays including those from PanBio, bio-Merieux and the ARRL are available. The three assays have not been formally compared[205]. Our study used the ARRL assay. In addition, qPCR positive samples were subjected to conventional PCR and gel electrophoresis for DNA sequencing. The Standard Operating Procedures (SOPs) of the ARRL were followed for all procedures.

5.8.1 Serology by Immunofluorescence assay (IFA)

The indirect immunofluorescence assay (IFA) titration, a technique that gives a quantitative value of the antibody concentration in the serum, is the currently accepted gold standard for rickettsial diagnosis. In this study, testing for antibodies was carried out by an indirect micro-IFA described previously[221]. Antibodies to the spotted fever group (SFG) rickettsia were tested

with *Rickettsia australis*, *R. honei*, *R. conorii*, *R. africae*, *R. rickettsii* and *R. felis* antigens; antibodies to the typhus group (TG) rickettsia with *R. prowazekii* and *R. typhi* antigens; antibodies to the scrub typhus group (STG) with *Orientia tsutsugamushi* (Gilliam, Karp and Kato strains) and *O. chuto* antigens, and antibodies to QF using *Coxiella burnetii* phase I and phase II antigens of the Nine Mile strain. All antigens were prepared in-house in the ARRL by culturing the respective organisms in L929 cell line and Roswell Park Memorial Institute medium (RPMI) (Invitrogen) with 5% fetal bovine serum (FBS). The same antigens were used for both the human and animal studies.

IFA slides were coated with the respective antigens (*Rickettsia, Orientia* and *Coxiella*) in duplicates and dried to be used immediately or stored frozen for future use. Serum samples (human or animal) were pipetted onto the fixed antigens at the required dilutions and incubated for a period of 40 minutes at 37°C. Unbound serum was removed by washing with 10% phosphate buffered saline (PBS) and dried. Following this, dried slides were treated with secondary conjugated antibodies; fluorescein-labelled anti-human antibodies (anti-IgM, anti-IgG, and anti-IgA as required) for human studies and anti-animal (such as anti-bovine, anti-dog, anti-goat, anti-horse and anti-cat) for animal studies from KPL Inc, United States of America (USA) (https://www.kpl.com/) to detect the respective antibody-antigen complexes. Unbound conjugated antibodies were washed off with 10% PBS and dried slides were observed under an ultraviolet (UV) light microscopy with Fluorescein isothiocyanate (FITC) filters at x400 magnification. Positive samples were identified by the presence of bright green fluorescence while negative samples lacked fluorescence **(Fig 5.7).** Samples were screened at low dilutions and titrated to an endpoint (titer) when positive. The detailed step by step procedures of preparing the specific antigens, controls and the IFA technique used in this project are given as appendices; Appendix 11: IFA procedure for *Rickettsia* and *Orientia* antibody detection, Appendix 12: IFA procedure for *Coxiella* antibody detection.

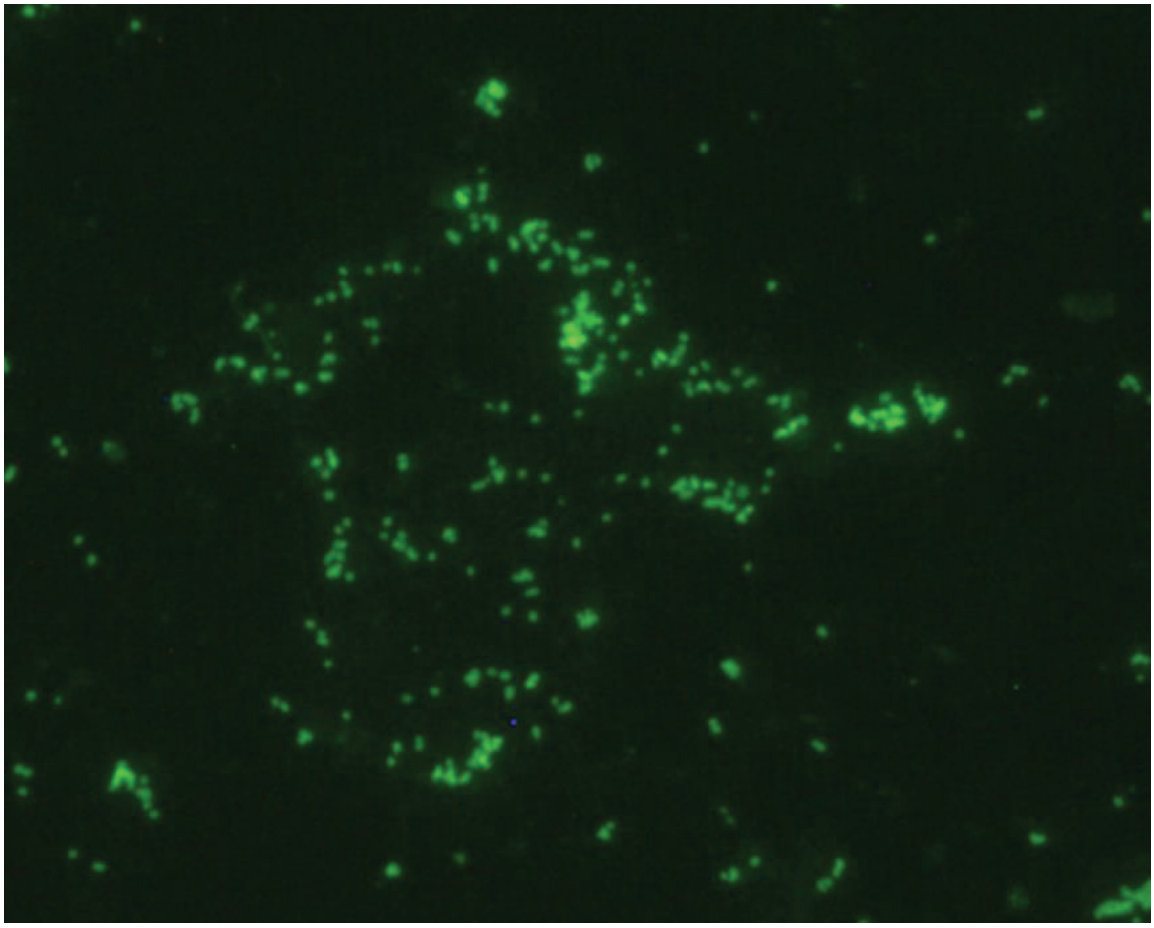


Figure 5.7 A positive sample demonstrated by the bright green fluorescence under UV light

Human antibody detection procedures and titre interpretations (diagnostic cut-offs) were solely based on the ARRL protocols but interpretations for animal serology were based on previous related studies in Australia for QF[330]; Brazil[331] and Sri Lanka[265] for *Rickettsia* and *Orientia.* Known positive and negative control wells were included and tested with every slide for both human and animal samples. For some animal species where no definite positive and negative controls were available (such as yaks), certain assumptions were made by using known positive and negative control human sera to ensure the quality of the antigens, conjugates and the test procedure, even though a non-human serum was being titrated. Samples and conjugates were

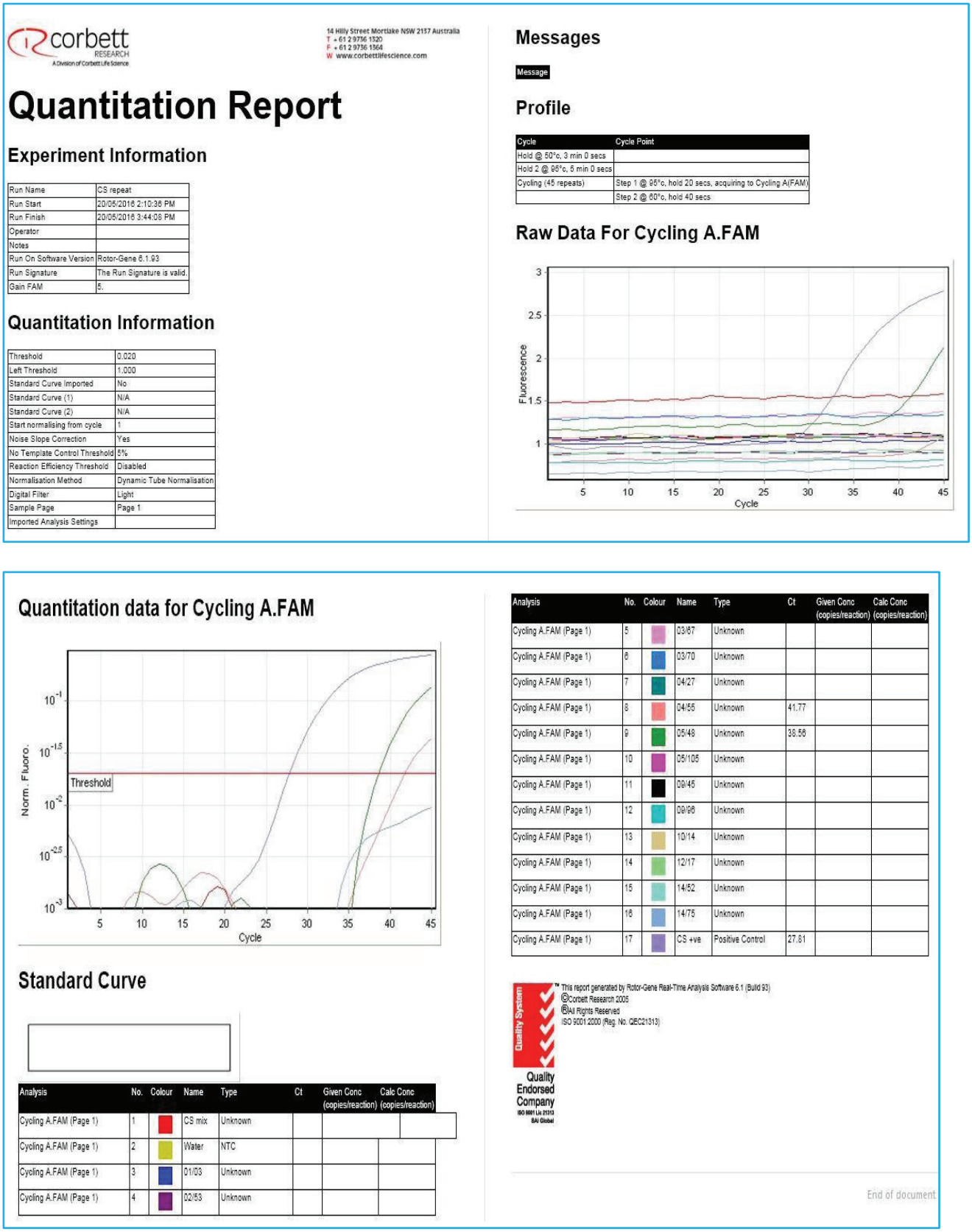
diluted in 2% casein PBS to limit nonspecific binding. All the samples and controls were tested in duplicate on every slide.

5.8.2 DNA extraction and detection by real-time PCR (qPCR)

Samples in this project consisted of buffy coat extracted from human blood in EDTA. DNA was extracted using HiYield™ DNA Mini Kit, YGB100**,** Real Genomics (Taiwan) as described in the manufacturer's instructions for specific samples with slight modifications as adapted in the ARRL protocol. The qPCR assays utilised in the ARRL have methodologies derived from different sources[332-334] but have been fully validated prior to being introduced into diagnostic testing in the laboratory. The detailed procedures of DNA extraction and qPCR are described in **Appendix 13.**

SFG and TG being genetically similar, were tested targeting the citrate synthase gene (CS-F 5'-TCG CAA ATG TTC ACG GTA CTT T-3', CS-R 5'- TCG TGC ATT TCT TTC CAT TGT G-3', CS-Probe 5'-FAM TGC AAT AGC AAG AAC CGT AGG CTG GAT G BHQ1-3')[333]. The 16S rDNA gene was targeted for *Orientia*[335](16S rDNA-F 5'- CTT ATT TGC CAG CGG GTA ATG C-3', 16S rDNA-R 5'-GGG CCA TGA TGA CTT GAC CTC-3', 16S rDNA-Probe 5'- FAM CCC ACC TTC CTC CGG CTT AGC ACC BHQ1-3') and the com 1 gene for *Coxiella* [334] (com1-F 5'-AAA ACC TCC GCG TTG TCT TCA-3', com1-R 5'GCT AAT GAT ACT TTG GCA GCG TAT TG-3', com1-probe 5'-FAM AGA ACT GCC CAT TTT TGG CGG CCA BHQ1-3') both designed using Primer Express (Applied Biosystems).

Any sample with cycling threshold (Ct) value of <35 was deemed positive, between 35-40 equivocal (repeated to determine their status) and >40 considered negative against the specific rickettsial agents tested **(Fig 5.8)**.



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Quantitation Report

Experiment Information

Run Name	CS repeat
Run Start	20/05/2016 2:10:36 PM
Run Finish	20/05/2016 3:44:08 PM
Operator	
Notes	
Run On Software Version	Rotor-Gene 6.1.93
Run Signature	The Run Signature is valid.
Gain FAM	5.

Quantitation Information

Threshold	0.020
Left Threshold	1.000
Standard Curve Imported	No
Standard Curve (1)	N/A
Standard Curve (2)	N/A
Start normalising from cycle	1
Noise Slope Correction	Yes
No Template Control Threshold	5%
Reaction Efficiency Threshold	Disabled
Normalisation Method	Dynamic Tube Normalisation
Digital Filter	Light
Sample Page	Page 1
Imported Analysis Settings	

Messages

Message

Profile

Cycle	Cycle Point
Hold @ 50°c, 3 min 0 secs	
Hold 2 @ 95°c, 5 min 0 secs	
Cycling (45 repeats)	Step 1 @ 95°c, hold 20 secs, acquiring to Cycling A(FAM)
	Step 2 @ 60°c, hold 40 secs

Raw Data For Cycling A.FAM

Quantitation data for Cycling A.FAM

Analysis	No.	Colour	Name	Type	Ct	Given Conc (copies/reaction)	Calc Conc (copies/reaction)
Cycling A.FAM (Page 1)	5		03/67	Unknown			
Cycling A.FAM (Page 1)	6		03/70	Unknown			
Cycling A.FAM (Page 1)	7		04/27	Unknown			
Cycling A.FAM (Page 1)	8		04/55	Unknown	41.77		
Cycling A.FAM (Page 1)	9		05/48	Unknown	38.56		
Cycling A.FAM (Page 1)	10		05/105	Unknown			
Cycling A.FAM (Page 1)	11		09/46	Unknown			
Cycling A.FAM (Page 1)	12		09/96	Unknown			
Cycling A.FAM (Page 1)	13		10/14	Unknown			
Cycling A.FAM (Page 1)	14		12/17	Unknown			
Cycling A.FAM (Page 1)	15		14/52	Unknown			
Cycling A.FAM (Page 1)	16		14/75	Unknown			
Cycling A.FAM (Page 1)	17		CS +ve	Positive Control	27.81		

Standard Curve

Analysis	No.	Colour	Name	Type	Ct	Given Conc (copies/reaction)	Calc Conc (copies/reaction)
Cycling A.FAM (Page 1)	1		CS mix	Unknown			
Cycling A.FAM (Page 1)	2		Water	NTC			
Cycling A.FAM (Page 1)	3		01/03	Unknown			
Cycling A.FAM (Page 1)	4		02/53	Unknown			

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Figure 5.8 An example of a analysis report of the real-time PCR in Corbett Research Rotor-Gene 6.1.93

5.8.3 Conventional PCR

All qPCR positive samples were subjected to conventional PCR intended for sequencing of the rickettsial bacteria. For this, the following primers were used (Table 5.1).

Target gene and PCR type	Primers	Sequences
1. Citrate synthase (glt A)		
Nested PCR – 2 PCRs	Forward - RpCS.780	GACCATGAGCAGAATGCTTCT
	Reverse 1 - RpCS.1258n	ATTGCAAAAAGTACAGTGAACA
	Reverse 2 - RpCS.877p	GGGGGCCTGCTCACGGCGG
2. 17 kDa gene		
Nested PCR – 2 PCRs	Forward 1 - Rr17k.1p	TTTACAAAATTCTAAAAACCAT
	Forward 2 - Rr17k.90p	GCTCTTGCAACTTCTATGTT
	Reverse - Rr17k.539n	TCAATTCACAACTTGCCATT
3. Sca4 gene		
Single PCR	Forward - D1f	ATGAGTAAAGACGGTAACCT
	Reverse – D928r	AAGCTATTGCGTCATCTCCG
4. ompB gene		
Single PCR	Forward – 120-M59	CCGCAGGGTTGGTAACTGC
	Reverse – 120-807	CCTTTTAGATTACCGCCTAA
5. 56 kDa gene (*Orientia*)		
Single PCR	Forward	TACATTAGCTGCAGGTATGACA
	Reverse	CCAGCATAATTCTTTAACCAAG

PCR master mix and PCR conditions used were as follows:

Master mix preparation	PCR conditions
10X buffer – 2.5 µl	95°C – 5 min
$MgCl_2$ - 1 µl	95°C – 30 sec
dNTPs – 0.5 µl	55°C – 30 sec
Forward primer – 1.5 µl	72°C – 90 sec
Reverse primer – 1.5 µl	72°C – 7 min
Taq polymerase – 0.2 µl	4°C - ∞
Water – enough to make 25 µl reaction	
DNA – 4 µl	

5.8.4 Gel electrophoresis and DNA sequencing

Conventional PCR products were run in gel electrophoresis**.** A 1.2% gel was prepared by measuring out 0.6 gram of agarose powder to which 50 ml of 1xTAE and about 2µl SYBR (gel stain) was added. The mixture was heated in microwave oven unitl the agarose fully dissolved.

This was cooled for about five minutes (be able to touch with bare hands) and then poured into the gel prepration well (casting rays) with gel comb. After adequate setting, the comb was removed and the gel taken into the electrophoresis chamber. A 100 bp marker, samples with positive and negative controls were mixed with gel loading dye and loaded into the gel well. Electrophoresis was run for about 30-35 minutes at a current of 100 amp and 100 volts. This gel run was vidualised under the the transilluminator system**(Fig 5.9).** After successful detection in electrophoresis, PCR products were sent to Macrogen Inc, a South Korean biotechnology company (https://dna.macrogen.com/eng/) for DNA sequencing. Macrogen Inc. employs a single sequencing method to get DNA base pairs which can be used to analyse and built phylogeny trees to identify the bacterial species.

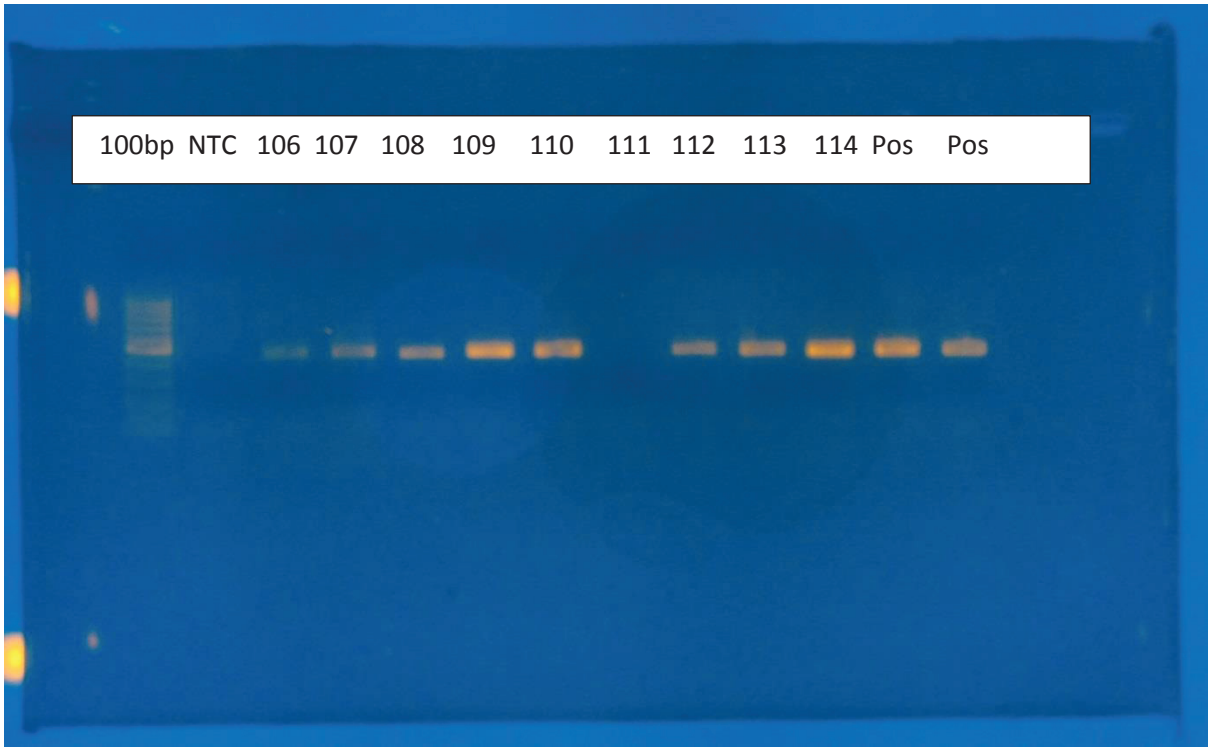

Figure 5.9 A gel run following conventional PCR (100bp: 100 base pair ladder; NTC: No template control; Samples: 106-114; Pos: Positive controls)

5.9 Ethics approval, consent and confidentiality

Ethics approval was granted by the Research Ethics Board of Health (REBH), Ministry of Health, Bhutan, for the human study (Ref: REBH/Approval/2014/019; **Appendix 6)** and the Council of RNR Research of Bhutan (CoRRB), Ministry of Agriculture and Forest, Bhutan, for the livestock study (Ref: CORRB/TCO/D-2/732; **Appendix 7)**. Ethics approval was also granted by the Human Research Ethics Committee (HREC), University of Newcastle, Australia (Ref: H-2016-0085; **Appendix 8)**.

The study information sheet and informed consent forms were printed both in English **(Appendix 9)** and in Dzongkha (the national language of Bhutan) **(Appendix 10).** Written informed consent was obtained from all participants. For illiterate subjects, the information and consent form were read to them by the person taking the consent in the presence of a witness. For children and minors (≤ 16 yrs.), informed consent was obtained from a parent or guardian. Personal details and sample identification were anonymized/coded to ensure patient confidentiality.

Chapter 6: Scrub typhus in Bhutan: a synthesis of data from 2009 to 2014

Information on scrub typhus (ST) is very limited in Bhutan. The few available reports were scattered and data from the hospitals have not been analysed. Importance of ST was highlighted only after a probable outbreak in a rural locality in 2009. After that the national Public Health Laboratory (PHL) initiated a small surveillance activity in Gedu, the locality where the 2009 outbreak occurred. Testing for ST was started in the PHL in 2010 and ST was included in the national notifiable diseases. However, notifications were weak, and the reports included overtly clinical cases due to the lack of laboratory test kits in the hospitals. There is slow but steady momentum in the awareness of the importance of ST amongst the healthcare workers. Laboratory supplies have improved and a good number of hospitals, especially in the southern part of the country with warm and humid climate now have the test kits in their annual supply lists.

This paper attempted to collate and synthesize all available data before the comprehensive study in this PhD project was undertaken. This chapter is presented as a research paper published in the WHO South East Asia Journal of Public Health. Available from: **http://www.who-seajph.org/temp/WHOSouth-EastAsiaJPublicHealth52117-1847155_050751.pdf**

Review

Scrub typhus in Bhutan: a synthesis of data from 2009 to 2014

Tshokey Tshokey, Tashi Choden, Ragunath Sharma

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ABSTRACT

Scrub typhus is an acute, febrile illness, caused by the bacterium *Orientia tsutsugamushi*, that affects millions annually in the endemic Asia-Pacific region. In untreated cases, the case-fatality rates range from 6% to 35%. In Bhutan, there was a probable outbreak in Gedu in 2009, which resulted in heightened awareness of the disease. Nevertheless, information on scrub typhus in Bhutan is limited and scattered and the epidemiology has yet to be established. To report the current picture of scrub typhus in Bhutan, this review gathered data from scholarly databases, surveillance reports, the *Annual health bulletin*, research publications and laboratory test reports from hospitals. The weight of evidence indicates an increasing burden of scrub typhus since the Gedu incident, coupled with increased awareness and testing. Another outbreak in a rural primary school in 2014 resulted in two deaths. More hospitals now have testing facilities and laboratory-confirmed cases have been increasing since 2009, with seasonal trends. This review highlights the need for in-depth surveillance and reporting, increased awareness among health-care workers, and initiation of prevention and control programmes in the country.

Key words: Bhutan, Gedu, *Orientia tsutsugamushi*, scrub typhus

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BACKGROUND

Scrub typhus is an acute febrile illness caused by the bacterium *Orientia tsutsugamushi*. The infection results from bites of chigger mites that are primarily associated with rodents of the genus *Rattus*. With an incubation period of 10–14 days, scrub typhus presents with acute onset of fever, headache, malaise, swollen lymph nodes and maculopapular rashes by the end of the first week of fever.[1] Without treatment, the case-fatality rate remains at 6–35% but can be as high as 60% in some outbreaks.[1] It has been described as a seriously neglected life-threatening tropical disease.[2] It is also a travel-associated disease,[3] and has great importance among military personnel.[4] Scrub typhus is known to be endemic to the Asia-Pacific region, covering a triangular area known as the "*tsutsugamushi* triangle", extending from Afghanistan and Pakistan in the west to China and Korea in the east and the islands of the south-western Pacific and northern Australia in the south. In this region, an estimated one million cases occur annually, especially among those engaged in logging, clearing of land and working in rice fields.[3] The disease is focally distributed throughout the region, from the low coastal lands to a height of over 3200 m in the Himalayas.[1] Scrub typhus is being increasingly reported from new areas within the endemic Asia-Pacific region and also beyond the described triangle, with recent descriptions in a patient from Dubai in the Middle East[5] and in children in Kenya, Africa.[6]

In the Indian city of Darjeeling, a study in 2005 showed that the incidence of scrub typhus increased from 2/100 000 population in July to 20/100 000 population in September and decreased to zero in December.[7] One of the first studies on scrub typhus in Bangladesh, a prospective sero-epidemiologic survey across six major teaching hospitals in Bangladesh using an immunoglobulin M (IgM) enzyme-linked immunosorbent assay, found that 24% (287/1209) had evidence of recent exposure to *Orientia tsutsugamushi*. Seropositivity differed between regions but there was no clustering of cases and no difference between urban and rural residents.[8] Cases of scrub typhus have been increasingly reported in mainland China, from 1248 in 2006 to 8886 in 2012.[9] There have been several studies on outbreak or clusters of scrub typhus cases from different states of India.[10–15] In a survey of the eastern Himalayas (Indian districts bordering the Bhutan foothills) for endemicity of scrub typhus among small mammals, chiggers were the most prevalent ectoparasites, with clusters of single hosts harbouring up to 600 mite larvae.[16] Of the 573 mammals sampled, 357 (62%) were infested with trombiculid larvae, and rodents and insectivores were both equally good hosts for larval mites, with 68% infestation in both cases.[16] Despite increasing reports of significant burden, the true incidence of human infections in south central Asia still remains unknown.[1]

Bhutan is a small Himalayan country between India and China. It has a population of about 750 000, with a population density

of 9 to 64 people/km^2 in different districts.[17] The country has over 70% forest cover and the economy is mostly agricultural, with 70% of the population living in rural areas. Bhutan can be conveniently divided into three regions: the north bordering China, dominated by the Great Himalayas with terrains and high mountains above 3700 m; the lesser Himalayas above 1500 m; and the Duars plain, bordering India in the south. With four distinct seasons, the mean daily temperature can vary from 5 °C in winters to 25 °C in summer and the average annual rainfall varies from less than 500 mm in the northern Himalayas and 500–1000 mm in the inner central valleys to 2000–5000 mm in the southern foothills.[1] Bhutan lies within the zone that is endemic for scrub typhus, and the environment of wide scrub vegetation, high humidity, extensive agricultural activities and abundant rodent population, especially in the middle and southern part of the country, makes it conducive for transmission of the organism, although the disease was not documented until recently. Earlier infections may have been missed, owing to a lack of diagnostic facilities and poor awareness among health-care workers. However, many non-malarial and non-typhoid febrile cases that responded rapidly to doxycycline or chloramphenicol in daily medical practice could have been scrub typhus. The disease was only highlighted with a probable outbreak of some febrile illness in 2009. The infection is now increasingly reported from many parts of the country.

Scrub typhus has been included in the national list of notifiable diseases since 2008 and reporting was initiated in 2010 but the notification system is weak and many health-care workers are unaware of the need to notify, resulting in minimal reports. The surveillance manual defines scrub typhus as "characterized by acute onset of fever after several days, headache, profuse sweating, myalgia, eschar, lymphadenopathy and rash" and defines a suspected case as "a case that is compatible with [the] clinical description".[18] Since 2010, reports on scrub typhus (as rickettsial diseases) have been included in the annual mortality and morbidity reports submitted by the district health offices to the Ministry of Health. These are compiled by the Health Management Information System, in the *Annual health bulletin*.[19–22] Almost all of these cases are clinically diagnosed cases, since most hospitals did not have laboratory tests until 2013. At present, the limited data available, in the form of departmental reports and publications, are scattered. This review aimed to compile all the available epidemiological data, to obtain a current picture of the disease in Bhutan.

METHODOLOGY

All available information on scrub typhus in Bhutan was gathered using web-based searches, surveillance and outbreak reports, the *Annual health bulletins* of the Ministry of Health, and laboratory test data from the Public Health Laboratory, the Jigme Dorji Wangchuck National Referral Hospital and other district hospitals. The terms "Bhutan" and "scrub typhus" or "*Orientia tsutsugamushi*" were used to search for any mention of the disease in Bhutan in scholarly databases. The sources of data obtained and used for this synthesis of information are presented in Table 1.

All laboratory-confirmed cases were tested by a single rapid antibody test kit that detects *O. tsutsugamushi* IgM, IgG and IgA antibodies (SD Bioline Tsutsugamushi assay, Standard Diagnostics Inc, Republic of Korea). The manufacturers of the test kit claim a sensitivity of 99%, a specificity of 96% and a serological agreement of 97.5% with indirect immunofluorescent assay, the accepted gold-standard method.

The current laboratory surveillance system was limited to selected district hospital laboratories in the southern part of Bhutan that collect serum samples from suspected patients and ship them to the Public Health Laboratory in Thimphu. However, any health centre can report any suspected or confirmed cases through the weekly, monthly or annual morbidity and mortality report to the Public Health Laboratory and/or Ministry of Health.

Table 1. Data sources obtained and used for the review

Topic of research/report	Author/year	Type of report/study population
Vector-borne Disease Control Programme tour report to Chukha Dzongkhag (27–31 July 2009)	Lhazeen, 2009	Personal communication, site visit and investigation report
Annual health bulletins (2011–2014)[19–22]	Health Management Information system, Ministry of Heath, Bhutan	Annual reports from health centres to the Ministry of Health
Outbreak investigation report on scrub typhus in Singye Namgyel Primary School, Wangduephodrang[23]	Phuentsho, 2015	Outbreak investigation report
Clinical characteristics of scrub typhus in Gedu and Mongar (Bhutan)[24]	Dorji, Wangchuk, Lhazeen, 2009	Surveillance report submitted to the Ministry of Health
Study on clinico-laboratory profile of children with scrub typhus	Bhandari, 2011	Personal communication, clinical study and oral presentation at the Second Annual Clinical Conference, JDWNRH, Thimphu, 2011

JDWNRH: Jigme Dorji Wangchuk National Referral Hospital.

Since there was no involvement of patients and only existing data were used, ethical clearance was not required by local guidelines. No personally identifying information was collected. Data analysis was descriptive of the number of cases reported, number of samples tested and annual and seasonal trends of scrub typhus.

RESULTS

Two probable outbreak investigation reports, one surveillance report, one hospital-based clinical research study, four Ministry of Health annual reports and one newspaper report of an outbreak were found.[19–24] Some laboratory reports were also obtained by referring to past records and personal communication with the laboratory staff concerned. No regional or international publications relating to scrub typhus in Bhutan were found.

Bhutan experienced two probable outbreaks of scrub typhus. The first occurred during July 2009 in Gedu, a locality in the south-western part of the country, but similar cases were also noted during the same month in the previous year (personal communication). Cases presented with fever, headache, joint pains and maculopapular rash. Initially, patients were managed as for dengue fever or dengue shock syndrome and some of them tested positive for IgM dengue antibody. During the incident, several people, mostly farmers from the locality, were seen in the outpatient unit, 18 were admitted and three lives were lost. Two of the deceased had signs and symptoms of dengue shock syndrome and also tested positive for dengue IgM. One of the deceased had enteric-fever-like symptoms and gastrointestinal perforation. Overall, the incident had a case-fatality rate of more than 10%. During the investigation, five of the seven febrile patients in the ward had typical eschar. No laboratory test for scrub typhus was carried out. With signs and symptoms of febrile illness, the presence of eschar in many cases, and rapid response to doxycycline in those treated, this was described as a probable outbreak of scrub typhus, the first recognition of the disease in the country. The second outbreak was recent and occurred at a remote boarding primary school in August to October 2014, at Athang, Wangduephodrang district in central Bhutan.[23] In this boarding school, there was abundant scrub vegetation and students lived in crowded rodent-infested hostels. The outbreak affected about 36 children, with seven hospital admissions and two deaths. The cases commonly presented with typical scrub-typhus-like symptoms and most had eschar but the two deceased had signs and symptoms of meningitis/encephalitis. Many cases had severe thrombocytopaenia and all of the 12 blood samples collected by the investigating team tested positive for scrub typhus with a rapid-test kit.

Following the report of high numbers of febrile cases in 2008 in Gedu, the Public Health Laboratory collected blood samples for scrub typhus from Gedu hospital in March 2009.[24] A few samples were also, coincidentally, received from Mongar hospital. Of the 33 serum samples sent to the reference laboratory outside Bhutan, 23 (69.7%) had acute scrub typhus infection as tested by indirect immunofluorescent assay.[24] Cases were seen in all age groups, including children as young as 1 year. One patient tested positive for three infections (dengue, scrub typhus and murine typhus). This was the first definite laboratory-confirmed case of scrub typhus in Bhutan using the gold-standard laboratory technique. At the same time, a hospital-based retrospective study looked at the clinico-laboratory profile of children with suspected scrub typhus in the Eastern Regional Referral Hospital, Mongar, between August and October 2009 (personal communication). Participants were severely ill children admitted to the ward with fever and thrombocytopaenia. Of the 18 children admitted, 12 had a scrub typhus test result and 10 (83%) of these were positive (personal communication). Three of the children with scrub typhus also tested positive for dengue fever. None were positive for malaria, typhoid or leptospirosis.

The Public Health Laboratory initiated testing for scrub typhus on samples from the district hospitals from 2009. However, without a properly established system, and fuelled by lack of awareness among health-care workers, very few samples were received initially. However, over the years, the number of referred samples and the number of positive results increased, as depicted in Table 2. The test positivity ranged between 22% and 60% among the cases sampled. It was noted that the number of samples increased from two per month in December/January to about 30–75 in June to October, on average, over the 4 years (2009–2012). By November, both the number of samples and the number of positive test results gradually decreased. This showed a seasonal trend of the disease in the hot monsoon and subsequent months, as shown in Fig. 1.

The Jigme Dorji Wangchuck National Referral Hospital took over the testing of scrub typhus from the Public Health Laboratory by end of 2012. The record showed an increase in the number of samples tested for scrub typhus from 269 in 2013 to 336 in 2014. Fig. 2 shows a summary of the total number of cases, depicting an increasing case-load, positivity and seasonal trend similar to the Public Health Laboratory results. Few district hospitals received the test kit by end of 2013 or early 2014. The five hospitals that submitted their test results for this study performed 560 tests in 2014. The Eastern Regional Referral Hospital tested 244 samples but could not provide the number of positive results. Thus, considering the four hospitals, the total number of samples tested was 316, with 38 (12%) positive results.

Table 2. Scrub typhus tests in the Public Health Laboratory with samples received from the district hospitals (2009–2012)

Year	Number tested	Number (%) positive
2009	5	3 (60)
2010	70	23 (33)
2011	153	55 (36)
2012	92	20 (22)
Total	320	101 (32)

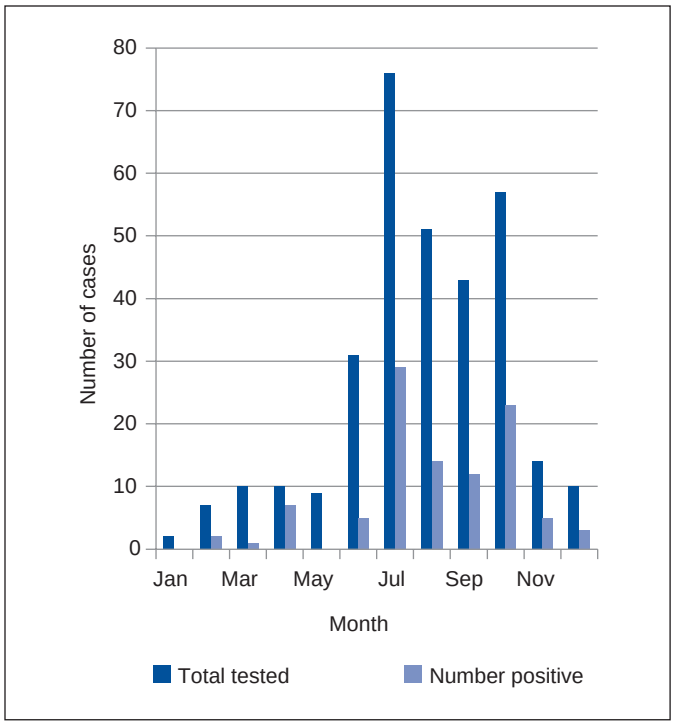

Fig. 1. The seasonal trend of scrub typhus tested in the Public Health Laboratory (2009–2012)

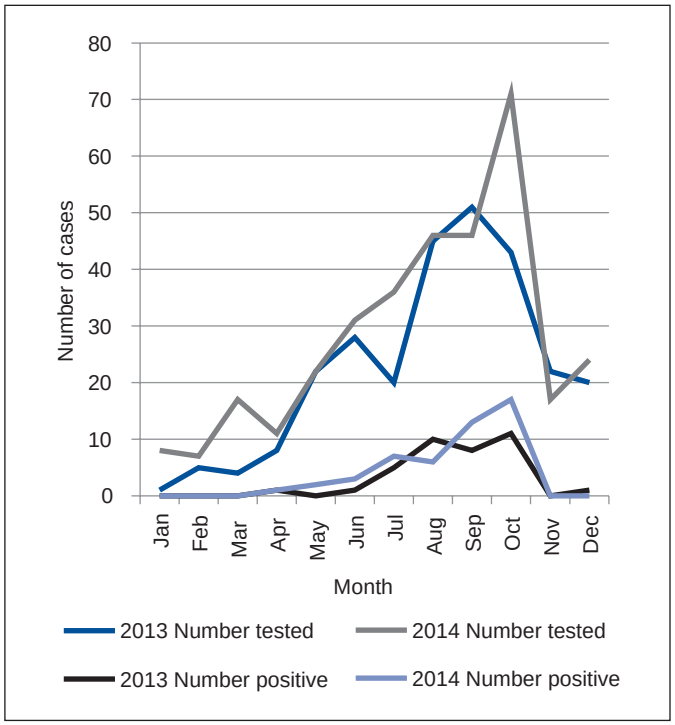

Fig. 2. Monthly number of samples and seasonal trend in the Jigme Dorji Wangchuck National Referral Hospital, Thimphu (2013 and 2014)

A review of the reports in the *Annual health bulletin* from 2011 to 2014 showed an increase in the number of cases reported, from 91 in 2010 to 351 in 2013 (see Table 3). The reports did not mention any demographic, seasonal or geographic details, or limits to the total number of cases each year. About 59% of the patients required hospitalization and 12% were children aged under 5 years. Despite the increase in reported cases, mortality was rare and sporadic, with only 2 (0.3%) deaths over the time period.

DISCUSSION

This review is the first of its kind to put the limited and scattered data on scrub typhus together. It has highlighted substantial facts about scrub typhus in Bhutan. Bhutan has environmental, climatic and occupational conditions that are favourable for transmission of *Orientia tsutsugamushi*, especially in the central and southern part of the country. Although awareness of the disease among health-care workers is increasing, the current surveillance activities, notification system and prevention and control programmes are inadequate.

Table 3. Rickettsial diseases reported in the *Annual health bulletin*, Ministry of Health, Bhutan (2011–2014)[19–22]

Year	Total number of cases reported	Inpatient	Outpatient	Deaths	Cases in children aged under 5 years
2010	91	70	21	0	14
2011	118	75	43	1	17
2012	218	146	72	0	21
2013	351	169	182	1	45
Total	778	460 (59.1%)	318 (40.9%)	2 (0.3%)	97 (12.5%)

Despite inclusion in the national manual of notifiable diseases in 2008[18] and initiation of reporting since 2010, the surveillance and reporting of scrub typhus has been weak, leading to minimal reports. It often happened that a manual or guideline was developed but this was not conveyed to field staff for proper implementation. In addition, without adequate laboratory support, febrile illnesses like malaria, enteric fever, leptospirosis, dengue and viral fevers, which remain prevalent, often challenge the diagnosis of rickettsial diseases. In the Bhutanese setting, it is still a clinical dogma to test every unexplained febrile case for malaria and enteric fever. During the past decade, the number of cases of malaria has dropped dramatically but the emergence of dengue fever since 2004 has complicated the matter.

The probable outbreak of scrub typhus in Gedu included some cases of dengue shock syndrome, supported by positive dengue IgM antibody. Apart from the presence of eschar in some cases, supported by a rapid response to doxycycline, there were no laboratory tests confirming scrub typhus in this cluster of febrile cases. Thus, this cluster could have been either dengue or scrub typhus or both. Similar cases in the future need to be taken more seriously and properly investigated, even at the cost of sending biological samples out of the country to establish the causal agent.

Documented cases of scrub typhus have presented with classical symptoms of fever, headache, skin rash and joint pain, with or without eschar in most cases. However, the two deceased during the outbreak in 2014 had meningitis-like signs and symptoms. Scrub typhus has been known to occur with symptoms of meningitis/encephalitis,[25–27] pneumonia and acute respiratory distress syndrome,[25,28,29] and multi-organ dysfunction syndrome.[25] All these support the fact that, in endemic areas, scrub typhus should be considered in the differential diagnosis of patients presenting with meningeal or respiratory symptoms or multi-organ dysfunction syndrome. One of the deceased in the Gedu incident had intestinal perforation and it is worth noting that scrub typhus can precipitate intestinal perforation.[30]

All laboratory tests reported here were solely based on one rapid-test kit and this has been the only test available, even to the present. Although they are rapid and easy to use, the rapid-test kits are not the ideal tool. With increasing reports of cases with sporadic outbreak potential, it would be valuable to have different diagnostic methods at different levels of health facilities, with, for example, rapid-test kits in the lowest-level health centres and more specific tests at the referral hospitals and the Public Health Laboratory. At present, only hospitals in the southern part of the country are supplied with the test kits, on the assumption that scrub typhus is prevalent only in a warm humid climate. This assumption needs to be proven, and until then test kits should be supplied to all hospitals in the country, to help understand the focus of the infection in different areas throughout the country.

There is an urgent need to invest in research on scrub typhus and other rickettsial diseases, to establish their true epidemiology and understand the social, environmental, occupational and behavioural determinants, in order to help develop prevention and control strategies. Health-care workers need to be educated and made aware of the disease and its differential diagnosis with other febrile illnesses. Laboratory test kits should be made available, to enable early diagnosis and prompt treatment of this serious but treatable disease. The general public needs to be educated on recognition of the disease, so they seek medical care early and take measures for prevention and control.

The study has some limitations. Firstly, the overall information available was very limited. No detailed demographic and clinical data could be extracted from the laboratory registers, thereby making inferences difficult. Test kits were not widely available, a situation that prevails even now, and the number of tests would have been an underestimate, owing to erratic supply of test kits. Many district laboratories could not give the actual number of tests performed, owing to poor record keeping. Unfortunately, all the laboratory data reported here relied on only one commercial rapid-test kit and it should be understood that such test kits are not the gold standard.

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Chaper 7: Scrub Typhus Outbreak in a Remote Primary School, Bhutan, 2014

In this chapter we report an outbreak of scrub typhus (ST) in a remote primary school that occurred in the year 2014. This was probably the second officially reported and investigated outbreak of ST in Bhutan. In this outbreak two students have lost their lives most likely due to central nervous system complications resulting from delayed medical care. The blood samples collected from the patients were shipped to the Australian Rickettsial Reference Laboratory (ARRL) and analysed for the presence of DNA and antibodies against Orientia.

This chapter is presented as a research letter published in the CDC Emerging Infectious Diseases (EID) journal available from **https://wwwnc.cdc.gov/eid/article/23/8/16-2021_article**

Scrub Typhus Outbreak in a Remote Primary School, Bhutan, 2014

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Scrub typhus in Bhutan was first reported in 2009. We investigated an outbreak of scrub typhus in a remote primary school during August–October 2014. Delay in recognition and treatment resulted in 2 deaths from meningoencephalitis. Scrub typhus warrants urgent public health interventions in Bhutan.

Scrub typhus, caused by the intracellular parasite *Orientia tsutsugamushi*, is a miteborne infection that largely occurs in the "tsutsugamushi triangle" (online Technical Appendix Figure 1, https://wwwnc.cdc.gov/EID/article/23/8/16-2021-Techapp1.pdf), where Watt et al. estimated ≈1 million cases occurred annually in 2003 (*1*). Infected persons commonly have fever, headache, conjunctival congestion, myalgia, lymphadenitis, rashes, and eschars with and without complications (*2*). Among untreated persons, the case-fatality rate is 6%–35% (*3*). In scrub typhus–endemic areas, central nervous system involvement occurs in ≈25% of patients (*4*). Consequently, scrub typhus should be considered in the differential diagnosis of aseptic meningitis.

During January–October 2016, Nepal reported scrub typhus in 37 districts, resulting in 8 deaths (*5*). Himachal Pradesh, India, reported 700 case-patients, 20 of whom died (*6*). In Bhutan, scrub typhus gained clinical attention after an outbreak in 2009 (*7*); earlier cases might have been missed because of low awareness.

During August–October 2014, a scrub typhus outbreak occurred in Singye Namgyal Primary School (SNPS), a remote community boarding school in the Wangduephodrang district of Bhutan (online Technical Appendix Figure 2). On August 17, three girls from SNPS reported 5–6 days of fever, headache, cough, and body aches and were treated symptomatically by the visiting health assistant from Kamichu Basic Health Unit (KBHU). Two of the girls recovered; the third was admitted to the KBHU on August 20 and transferred to Bajo Hospital (BH) the next day. By August 26, she experienced neck stiffness, irritability, and disorientation. Viral encephalitis was suspected, and she was referred to the Jigme Dorji Wangchuck National Referral Hospital (JDWNRH) in Thimphu on August 27. On admission, a serum sample tested positive for *O. tsutsugamushi* by rapid diagnostic test (RDT); she died the next day.

Another girl and her brother from SNPS were admitted to the Punakha district hospital on September 1 with similar symptoms. The boy was sent home with medications and recovered; his sister had meningeal symptoms and severe thrombocytopenia and was transferred to the JDWNRH on September 2, where she died on September 28. Specimens from both patients were *O. tsutsugamushi*–positive by RDT.

On September 22, a 10-year-old girl from SNPS was referred to JDWNRH with similar symptoms. Her serum specimen was *O. tsutsugamushi* positive, but she recovered with treatment.

After linking the 2 deaths and other cases, an investigation team visited the school during October 2–4. Case-patients were defined as any person from SNPS with fever, headache, and body ache with or without hemorrhagic manifestations currently or in the previous 2 weeks. Forty-one cases related to the outbreak were listed (online Technical Appendix Figure 3); blood samples were drawn from 21 students, 12 of whom were acutely ill, and 10 local residents. Results for all 31 were negative for malaria and dengue; the Widal test of serum samples for enteric fever from 1 student and 2 local residents showed high antibody titers against *Salmonella enterica* serotype Typhi. Serum samples from the 12 acutely ill students were also tested for *O. tsutsugamushi* by RDT (SD Bioline Tsutsugamushi Test; Standard Diagnostics, Yongin, South Korea) in the Bhutan Public Health Laboratory; all were positive. The 12 samples were taken to the JDWNRH for routine blood tests; results showed anemia in 5 patients, thrombocytopenia in 4, neutropenia in 3, lymphocytosis in 2, and neutrophilia in 2 (online Technical Appendix Table). The samples were also sent to the Australian Rickettsial Reference Laboratory (http://www.rickettsialab.org.au/), where they were tested for antibodies against *Orientia* by an indirect microimmunofluorescence assay (mIFA) (*8*). Of the 12 samples, 9 were positive (≥1:512 for IgM or ≥1:256 for IgG) (Table), including samples from all 6 students who had eschars. All samples from the 12 students were negative for *Orientia* DNA by using quantitative PCR.

Table. Antibody titers by indirect microimmunofluorescence assay of 9 students with diagnosis of scrub typhus, Bhutan, 2014*

		Orientia tsutsugamushi							
		Gilliam		Karp		Kato		*O. chuto*	
Patient ID	Age, y/sex	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM
2	6/M	256	512	256	512	256	128	<128	128
3	9/F	8,192	8,192	8,192	8,192	8,192	8,192	4,096	4,096
4	6/M	512	128	512	256	512	128	128	128
5	10/F	1,024	128	1,024	128	1,024	128	512	128
6	13/M	1,024	256	512	128	256	128	256	128
7	15/M	1,024	128	512	128	512	128	<128	<128
9	7/F	2,048	4,096	2,048	4,096	2,048	2,048	<128	<128
11	10/F	1,024	512	1,024	512	1,024	512	256	256
12	14/F	128	1,024	256	512	128	512	256	256

*ID, identification.

Of the acutely ill patients who had positive mIFA results, 67% had pathognomonic eschars, confirming the clinical diagnostic value in this sign of systemic infection. Thrombocytopenia as a sign of scrub typhus could be useful but is a less specific diagnostic indicator (*9*). There was only a 75% agreement between the rapid test kit and the precise mIFA, but RDTs were shown to be more useful in early detection (*10*).

The deaths of 2 children in this outbreak could have been prevented if the public had greater awareness of the signs and symptoms of scrub typhus. Lapses of 7–10 days from symptom onset to initial medical consultation and >1 month until the outbreak was investigated demonstrate the importance of training school health coordinators to identify and report incidences of abnormal medical findings to public health agencies, especially in remote, hard-to-reach areas. Parents delayed seeking medical advice, and in some cases, school staff had to persuade them to take their children for medical evaluation. Rapid medical care during illnesses should be encouraged through better community education.

Despite inadequate identification and reporting, there is increasing evidence of endemic scrub typhus in Bhutan. Outbreaks may be common but unrecognized, and past outbreaks may have been missed. Scrub typhus warrants a dedicated public health program or incorporation into the existing vectorborne disease control program in this country.

Acknowledgments

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Scrub Typhus Outbreak in a Remote Primary School, Bhutan, 2014

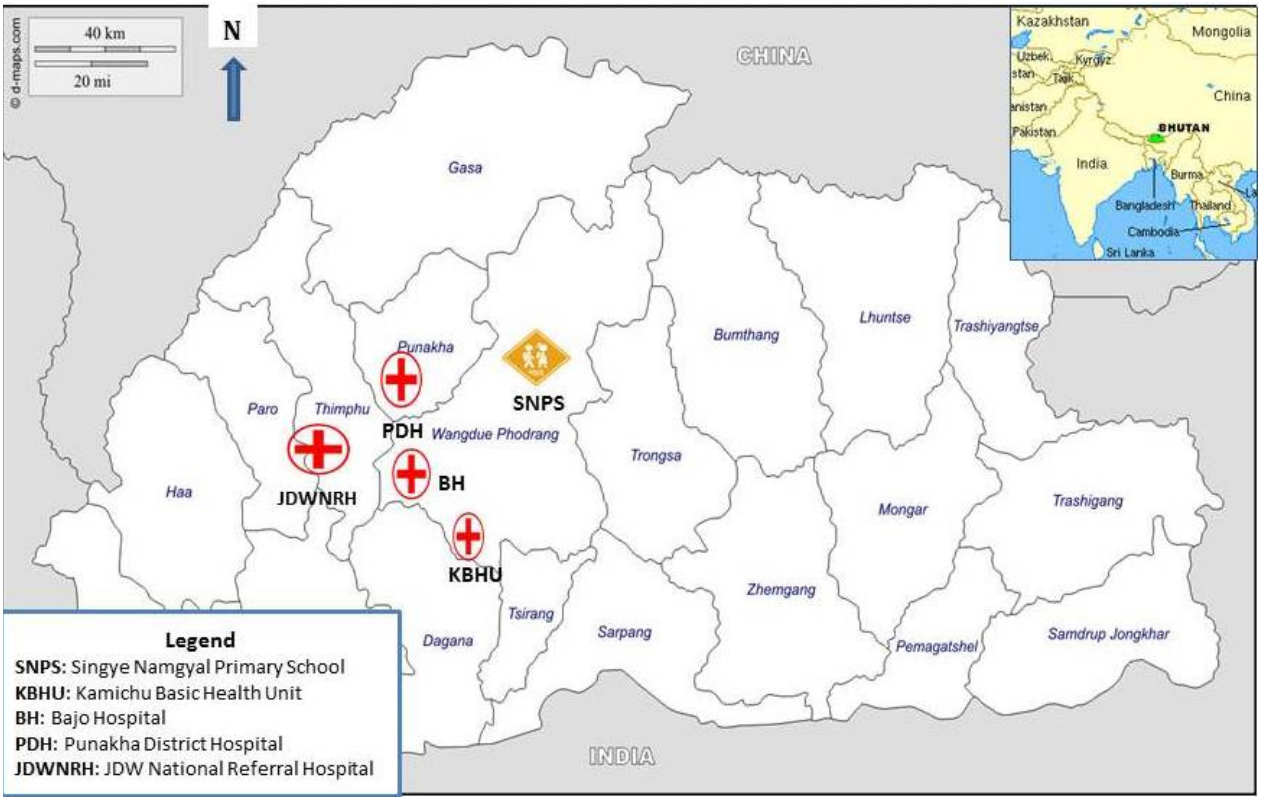
Technical Appendix

Technical Appendix Table. Complete blood count of 12 acutely ill children infected with scrub typhus, Bhutan, 2014*

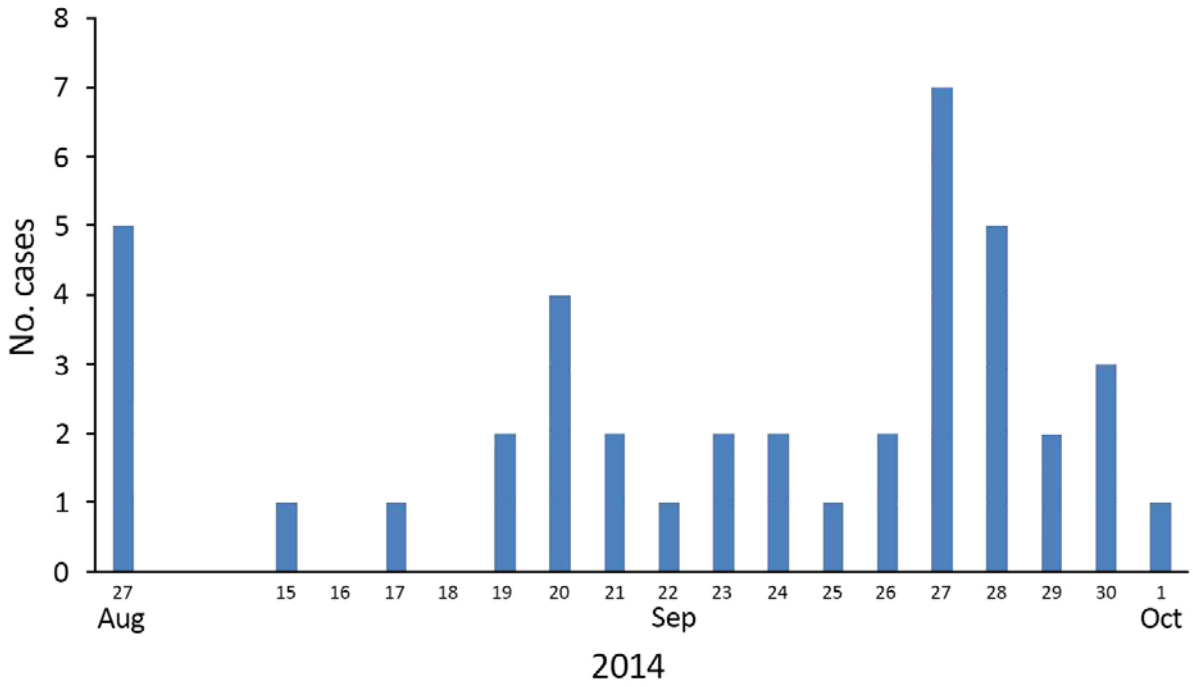
			Full blood count					
Pt. ID	Age (y)	Sex	**Total leucocyte count (10^3/μL) (NR=4–12)**	**Lymphocyte (%) (NR=20–50)**	**Neutrophil (%) (NR=40–65)**	**Platelet (10^3/μL) (NR=150–450)**	**Haemoglobin (g/dL) (NR=11–16)**	**Haematocrit (%) (NR=33–45)**
1	7	F	9.5	46	40	298	10.2	25
2†	6	M	6.5	47	39	272	10.0	30
3†	9	F	9.4	30	56	329	11.2	35
4†	6	M	8.1	34	45	169	12.0	36
5†	10	F	12.8	28	56	251	11.3	35
6†	13	M	4.5	21	70	121	13.2	41
7†	15	M	6.0	22	68	137	12.7	39
8	14	M	7.1	43	43	187	12.5	41
9†	7	F	7.9	31	58	92	12.2	35
10	8	M	4.6	57	31	193	9.3	29
11†	10	F	5.0	58	28	76	8.4	27
12†	14	F	11.4	32	59	209	9.9	30

*NR, normal range, Pt. ID, patient identification.
†Positive for scrub typhus by microimmunoflorescence.

Technical Appendix Figure 1. The tsutsugamushi triangle (in red). More than 1,000,000 cases of scrub typhus, which is a mite-borne infection caused by the bacterium, *Orientia tsutsugamushi*, were reported in this area during 2003.



Technical Appendix Figure 2. Map of Bhutan showing the school and health centers relevant to outbreak of scrub typhus.



Technical Appendix Figure 3. Clinical cases of scrub typhus identified among students of Singye Namgyal Primary School in the Wangduephodrang district of Bhutan during August 27–October 1, 2014. Symptom onset among the first 3 case-patients began 5–6 days before being reported on August 17; the first incidence of scrub typhus in this cohort was diagnosed on August 27.

Chapter 8: Rickettsial Infections and Q Fever Amongst Febrile Patients in Bhutan

This chapter reports on the case incidences of acute rickettsial infections (spotted fever group and typhus group rickettsia), scrub typhus (ST) and Q fever (QF) amongst patients who presented to fourteen Bhutanese hospitals with acute undifferentiated fever from October 2014 to June 2015. Demographic and clinical information and blood samples were collected from all consenting patients at each hospital, blood samples were shipped to the Australian Rickettsial Reference Laboratory (ARRL) and analysed for DNA and antibodies against the rickettsial diseases and QF.

This chapter is presented as a publication in the Tropical Medicine and Infectious Diseases (TMID) journal and available at **http://www.mdpi.com/2414-6366/3/1/12**

Tropical Medicine and Infectious Disease

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Article

Rickettsial Infections and Q Fever Amongst Febrile Patients in Bhutan

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Abstract: There is limited evidence of rickettsial diseases in Bhutan. We explored the contribution of rickettsioses as a cause of undifferentiated febrile illness in patients presenting to 14 Bhutanese hospitals from October 2014 to June 2015. Obvious causes of fever were excluded clinically. Clinico-demographic information and acute blood samples were collected. Samples were tested by immunofluorescence assay (IFA) and qPCR against scrub typhus group (STG), spotted fever group (SFG) and typhus group (TG) rickettsiae, and Q fever (QF). Of the 1044 patients, 539 (51.6%) were female and the mean age was 31.5 years. At least 159 (15.2%) of the patients had evidence of a concurrent rickettsial infection. Of these, 70 (6.7%), 46 (4.4%), 4 (0.4%), and 29 (2.8%) were diagnosed as acute infections with STG, SFG, TG, and QF respectively. Ten (1.0%) patients were seropositive for both SFG and TG. Seven of the 70 STG patients were positive by qPCR. Eschar ($p < 0.001$), myalgia ($p = 0.003$), and lymphadenopathy ($p = 0.049$) were significantly associated with STG, but no specific symptoms were associated with the other infections. Disease incidences were not different between age groups, genders, occupations, and districts, except for students with significantly lower odds of infection with STG (OR = 0.43; 95% CI = 0.20, 0.93; $p = 0.031$). Rickettsioses were responsible for at least 15% of undifferentiated febrile illnesses in Bhutan, scrub typhus being the commonest. Health authorities should ensure that health services are equipped to manage these infections.

Keywords: Bhutan; Q fever; rickettsial infections; scrub typhus; undifferentiated fever

1. Introduction

Rickettsia, *Orientia*, and *Coxiella* cause undifferentiated febrile illnesses of varying severity in humans. Diseases caused by *Rickettsia* and *Orientia* species are together referred to as rickettsioses, and Q fever (QF) caused by *Coxiella burnetii* is frequently included under rickettsioses [1]. Rickettsial diseases are both emerging [2] and re-emerging [3]. The genus *Rickettsia*, with more than 22 species, comprises several groups, two of which, the spotted fever group (SFG) and typhus group (TG), are the most important causes of human disease [4]. *Orientia*, with two known species, *O. tsutsugamushi* and *O. chuto* [5], form the scrub typhus group (STG). SFG and TG have a worldwide distribution and STG is mainly found in the Asia-Pacific region [1]. Human infection with QF occurs worldwide [6] except in New Zealand [7].

The detection and diagnosis of rickettsial diseases remains inadequate and is not widely available. Serological techniques can provide difficulties in interpretation and may cross-react with other antigens. However, serology is the most utilized technique and immunofluorescence assay (IFA) is regarded as the current gold standard. For diagnosis, the presence of IgM antibody indicates an acute/primary infection and IgG appears about two weeks after the infection. IgM may be present for months and IgG may remain detectable for years [8,9], thus confusing the diagnosis of an acute infection. The examination of both acute and convalescent sera is usually required for confirming an acute diagnosis [9]. Molecular techniques have been used for increasing the sensitivity/specificity of diagnosis, in identifying new rickettsial species, and also in epidemiological studies [8]. Rickettsial diseases are believed to be a significant cause of morbidity in south-east Asia [10]. Scrub typhus has been reported widely from countries neighboring Bhutan, with recent increases in cases and outbreaks [3,11–13]. There is limited recent data on Q fever in humans in the region compared to other rickettsial diseases. In Bhutan, scrub typhus is better known than other rickettsial infections. There were two recorded outbreaks of scrub typhus; one in 2009 [14] prompting preliminary scrub typhus surveillance [15], and another in 2014 [16,17]. A review noted that scrub typhus is being increasingly reported in Bhutan, with 91 cases in 2010 and 351 cases in 2013 [16], increasing to 605 cases in 2015 [18]. Although awareness is improving, there are no clinical guidelines supporting the diagnosis and treatment of rickettsial diseases in Bhutan. The first published seroprevalence study for Bhutan reported an overall seroprevalence of 49% against rickettsioses; the commonest being STG (22.6%), followed by SFG (15.7%), Q fever (6.9%), and TG (3.5%) [19]. There is still a huge gap in the understanding of causes of febrile illnesses in Bhutan. This current study was undertaken to investigate the potential contribution of rickettsial diseases amongst patients presenting with undifferentiated fever attending hospitals in Bhutan.

2. Materials and Methods

2.1. Setting and Study Sites

Bhutan is comprised of 20 administrative districts with an estimated population of 770,000 in 2016 [20]. Due to the landscape and harsh terrain, district population density varies between 9 and 64 people/km^2 [21]. The country has over 70% forest cover and about 70% of the population lives in rural areas. With four distinct seasons, the mean daily temperature varies between 5 °C in the winter to 25 °C in the summer. The annual average rainfall varies from less than 500 mm in the northern Himalayas, to 500–1000 mm in the inner central valleys, and 2000–5000 mm in the southern foothills [22]. Each district has one or more hospitals and medical treatment is provided free of charge by the government. Fourteen of the 28 hospitals in the country (Figure 1) were selected for this study. The hospitals were intentionally selected based on their size (larger hospitals), location (ensuring the more populous south-central region was appropriately represented), and previous records of scrub typhus occurrence. The 14 hospitals were located in 11 of the 20 districts, but with a combined estimated population of 573,000, they provide health services to about 75% of Bhutan's population [20].

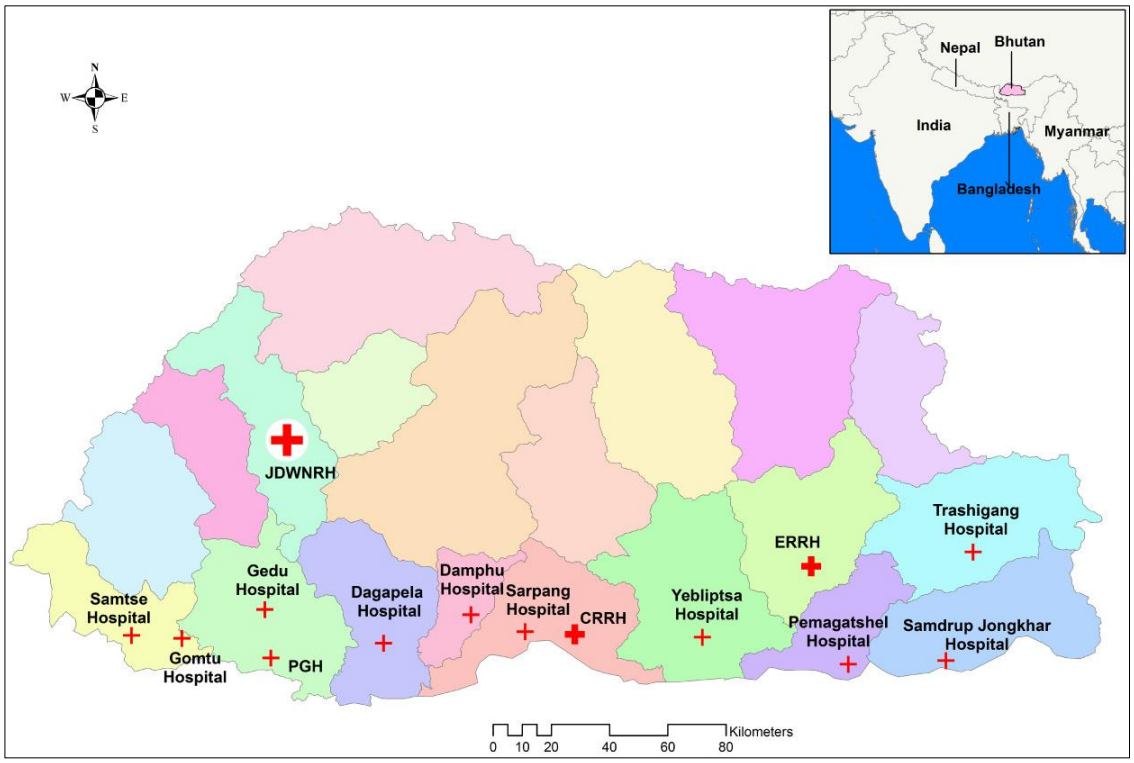

Figure 1. Map of Bhutan showing the 14 hospitals (study sites).

2.2. Study Design and Participants

A prospective descriptive study was carried out from October 2014 to June 2015. Patients (inpatients and outpatients) attending the 14 hospitals with 'undifferentiated fever of ≥4 days duration accompanied by one or more symptoms of headache, chills, myalgia, arthralgia, lymphadenopathy, or skin rash/eschar' were included in the study after excluding other obvious systemic or local causes of fever (such as respiratory tract infections, urinary tract infections, abscesses and cellulitis, otitis media, etc.) through clinical examination. Clinico-demographic information was recorded and a single acute EDTA blood sample and a serum sample was collected from each patient. No convalescent sera and treatment details were collected and patients were not followed up for treatment outcomes. Blood samples were stored at −70 °C until their shipment to the Australian Rickettsial Reference Laboratory (ARRL), an accredited reference center for rickettsial testing [23].

2.3. Laboratory Testing

At the ARRL, all samples were tested for antibodies to STG, SFG, TG, and QF by IFA [24], and for rickettsial (STG, SFG, and TG) and *Coxiella* DNA by qPCR. Antibodies against STG were tested using *O. tsutsugamushi* (Gilliam, Karp, and Kato strains) and *O. chuto* antigens; against SFG using *R. australis*, *R. honei*, *R. conorii*, *R. africae*, *R. rickettsii,* and *R. felis* antigens; against TG using *R. prowazekii* and *R. typhi* antigens; and against QF using *C. burnetii* phase I and II antigens. All antigens were prepared in-house in the ARRL by culturing the respective organisms in L929 cell line and RPMI media (Invitrogen) with 5% fetal bovine serum (FBS). Samples were screened at low dilutions and titrated to end titre if positive. Positive and negative control antigens were included and tested with every slide.

DNA was extracted from the buffy coat sample using a HiYield^{TM} DNA Mini Kit, YGB100, Real Genomics (Taipei, Taiwan), and tested using qPCR procedures established as the ARRL protocol [25,26]. Any samples with Ct values of <35 were deemed positive, between 35 and 40 equivocal (repeated to determine their status), and >40 negative, against the specific rickettsial agents. SFG and TG being genetically similar, were tested targeting the citrate synthase (CS) gene (CS-F 5′-TCG CAA ATG TTC

ACG GTA CTT T-3′, CS-R 5′-TCG TGC ATT TCT TTC CAT TGT G-3′, CS-Probe 5′-FAM TGC AAT AGC AAG AAC CGT AGG CTG GAT G BHQ1-3′) [26]. The *16S rDNA* gene was targeted for *Orientia* [27] (16S rDNA-F 5′-CTT ATT TGC CAG CGG GTA ATG C-3′, 16S rDNA-R 5′-GGG CCA TGA TGA CTT GAC CTC-3′, 16S rDNA-Probe 5′-FAM CCC ACC TTC CTC CGG CTT AGC ACC BHQ1-3′) and the com 1 gene for *Coxiella* [27] (com1-F 5′-AAA ACC TCC GCG TTG TCT TCA-3′, com1-R 5′-GCT AAT GAT ACT TTG GCA GCG TAT TG-3′, com1-probe 5′-FAM AGA ACT GCC CAT TTT TGG CGG CCA BHQ1-3′), both designed using Primer Express (Applied Biosystems, Foster City, CA, USA). Attempts at sequencing the DNA from the qPCR positive samples were not successful.

2.4. Defining Current Infections

In primary rickettsial infections, antibody responses usually appear by the second week of illness. While acute infections are best diagnosed by evidence of rising antibody titres (ideally by a four-fold rise) between acute and convalescent samples, it was not possible in this study with no convalescent sera available. When ascertaining acute infections using single acute serum samples, interpretation is potentially confounded by interfering background antibodies from previous exposures, especially in endemic areas [28], and high background seroprevalence has been proven to exist in Bhutan [19]. For diagnosing a current infection in this study, a high titre of IgM antibody with adequate time ($\geq$14 days of fever) for antibody development was considered appropriate. IgM antibody titres of $\geq$1:1024 were considered positive for *Rickettsia* (SFG and TG) and *Orientia* (STG), while IgM titres of $\geq$1:100 against *C. burnetii* phase I or II antigens or both were considered positive for Q fever. These titres were chosen at least four-fold above the standard positive titres of these assays to ensure confidence of the causative infection. When there was sero-reactivity against two or more antigen groups, the higher titre was considered the likely current infection. When the qPCR was positive, infection was considered current regardless of duration of illness and antibody titre since the presence of DNA denotes current infection.

2.5. Statistical Analysis and Determination of Associations

Data were analyzed using STATA software version 14. Clinical and demographic variables of interest were reported descriptively in frequencies and percentages. Associations between infections and patients' age groups, occupations, districts, and environmental exposures were explored using the Chi-squared test or Fisher's test as appropriate, considering a p value of $\leq$0.05 significant. Univariate logistic regression analysis was used to determine odds ratios (OR), and p values of <0.05 were considered significant for association of the variables against the infections.

2.6. Ethics, Consent and Confidentiality

This study was approved by the Research Ethics Board of Health (REBH), Bhutan (Ref: REBH/Approval/2014/019), and the Human Research Ethics Committee (HREC), University of Newcastle, Australia (Ref: H-2016-0085). All patients or parents/guardians agreed to participate in the study and provided informed consent before participation. All information and samples were anonymized.

3. Results

3.1. Demography

From a total of 1044 patients, 539 (51.6%) were females. With a mean age of 31.5 years (95% CI, 30.4, 32.5) and median age of 30 years, the youngest patient was one year of age and the oldest 88 years old. There was an almost equal number of farmers, office workers, and students (Table 1).

Table 1. Patient distribution by age group and occupation (n = 1044).

Occupation	Age Groups in Years					
	<13	**13–24**	**25–36**	**37–48**	**>48**	**Overall (%)**
Farmer	0	20	55	71	128	274 (26)
Office worker	0	22	134	82	31	269 (26)
Student	114	165	7	0	0	286 (27)
Housewife	0	17	62	33	37	149 (14)
Unemployed	0	5	5	4	2	16 (2)
Pre-school	50	0	0	0	0	50 (5)
Total (%)	164 (16)	229 (22)	263 (25)	190 (18)	198 (19)	1044 (100)

The 14 hospitals enrolled a median of 74.5 cases, with the highest enrolment of 122 patients from the Jigme Dorji Wangchuck National Referral Hospital (JDWNRH), Thimphu district, and the lowest of 46 from Damphu hospital, Tsirang district. Patients enrolled in the study had an overall mean of 6.8 days of fever (95% CI, 6.0, 6.5), with a maximum period of illness of 30 days.

3.2. Laboratory Findings

Of the 1044 patients, 70 (6.7%), 46 (4.4%), 4 (0.4%), and 29 (2.8%) were positive against STG, SFG, TG, and QF respectively. Ten (1.0%) patients had equal antibody titres against SFG and TG. Seven of the 70 patients positive for STG were tested positive by qPCR (4 positive only by qPCR and 3 positive by both qPCR and IFA). All other samples were tested negative by qPCR for SFG and TG *Rickettsia*, *Orientia,* and *Coxiella* DNA. This resulted in an overall case incidence of 15.2% in the 14 hospitals during the study period. The distribution of cases by age and occupation is presented in Table 2, and individual hospital cases are presented in Figure 2.

Table 2. Number of positive cases of each infection by age group and occupation.

Variable	**No. of Positives (%)**				
Age Group (yrs.)	STG (n = 70)	SFG (n = 46)	TG (n = 4)	SFG + TG (n = 10)	QF (n = 29)
<13	10 (14.2)	7 (15.2)	0 (0.0)	1 (10.0)	0 (0.0)
13–24	14 (20.0)	10 (21.7)	1 (25.0)	4 (40.0)	7 (24.1)
25–36	13 (18.6)	16 (34.8)	0 (0.0)	3 (30.0)	7 (24.1)
37–48	20 (28.6)	8 (17.4)	1 (25.0)	0 (0.0)	6 (20.7)
>48	13 (18.6)	5 (10.9)	2 (50.0)	2 (20.0)	9 (31.1)
Occupation					
Farmer	23 (32.9)	11 (23.9)	1 (25.0)	6 (60.0)	11 (37.9)
Office worker	21 (30.0)	9 (19.6)	2 (50.0)	1 (10.0)	8 (27.6)
Student	10 (14.3)	14 (30.4)	1 (25.0)	1 (10.0)	5 (17.2)
Housewife	10 (14.3)	8 (17.4)	0 (0.0)	1 (10.0)	3 (10.4)
Unemployed	1 (1.4)	3 (6.5)	0 (0.0)	0 (0.0)	2 (6.9)
Pre-school	5 (7.1)	1 (2.2)	0 (0.0)	1 (10.0)	0 (0.0)

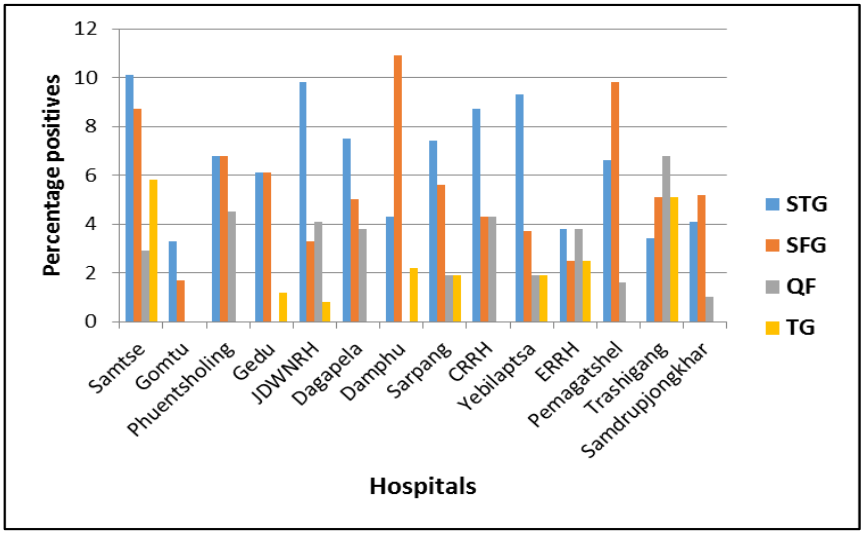

Figure 2. Overall seropositivity of the four infections in the 14 hospitals (STG: scrub typhus group; SFG: spotted fever group; QF: Q fever; TG: typhus group).

3.3. Clinical Presentations

The study recorded nineteen different presenting symptoms, including fever (100%, n = 1044), an essential inclusion criteria, headache (77%, n = 805), arthralgia (60%, n = 623), myalgia (29%, n = 298), rash (25%, n = 262), lymphadenitis (3%, n = 33), and eschar (3.8%, n = 40). Some patients also reported other symptoms like cough, anorexia, and backache. An eschar was seen in 44.3% (31/70) of STG patients, but not in acute cases of SFG, TG, and QF. The presence of an eschar ($p < 0.001$), myalgia ($p = 0.003$), and lymphadenopathy ($p = 0.049$) were significantly associated with STG (Table 3). No symptoms were statistically associated with the other infections.

Table 3. Association of signs and symptoms with STG, SFG, TG, and QF cases.

Signs and Symptoms		STG (n = 70)			SFG (n = 56)			TG (n = 14)			QF (n = 29)		
		Pos	Neg	p Value	Pos	Neg	p Value	Pos	Neg	p Value	Pos	Neg	p Value
Rash	Yes	23	240	0.126	11	252	0.325	4	259	0.769	8	255	0.763
	No	47	734		45	736		10	771		21	760	
Eschar	Yes	31	9	<0.001 *	0	40	0.125	0	40	0.452	0	40	0.276
	No	39	965		56	948		14	990		29	975	
Headache	Yes	55	750	0.763	48	757	0.115	13	792	0.158	22	783	0.871
	No	15	224		8	231		1	238		7	232	
Arthralgia	Yes	48	575	0.116	37	586	0.316	11	612	0.147	19	604	0.515
	No	22	399		19	402		3	418		10	411	
Myalgia	Yes	31	267	0.003 *	17	281	0.761	3	295	0.551	5	293	0.171
	No	39	706		39	706		11	734		24	721	
Lymphadenopathy	Yes	5	28	0.049 *	2	31	0.859	0	33	0.496	1	32	0.930
	No	65	944		54	955		14	995		28	981	
Others	Yes	8	99	0.736	10	97	0.054	1	106	0.700	1	106	0.221
	No	62	875		46	891		13	924		28	909	

Others: Cough, anorexia, backache, abdominal pain; * $p < 0.05$. (Note: cases with dual positivity for SFG and TG have been included in both SFG and TG.)

To compare signs and symptoms of rickettsioses to other non-rickettsial causes of undifferentiated fever, cases of all four infections (STG, SFG, TG, and QF) were pooled together (as rickettsioses) and their signs and symptoms compared with those negative patients (non-rickettsioses) as in Table 4. In this comparison, the presence of an eschar remained the only significant sign of a rickettsial infection ($p < 0.001$).

Table 4. Association of different signs and symptoms against rickettsioses and other causes of undifferentiated fever.

Signs and Symptoms		Diagnosis		*p* Value
		Rickettsioses	Non-Rickettsioses	
Rash	Yes	43	220	0.559
	No	116	665	
Eschar	Yes	31	9	<0.001 *
	No	128	876	
Headache	Yes	129	676	0.190
	No	30	209	
Arthralgia	Yes	106	517	0.051
	No	53	368	
Myalgia	Yes	53	245	0.149
	No	106	639	
Lymphadenopathy	Yes	8	25	0.145
	No	151	858	
Others	Yes	19	140	0.443
	No	140	797	

Others: Cough, anorexia, backache, abdominal pain; * $p < 0.05$.

3.4. Environmental Factors and Association with Rickettsial Infection

Information on exposure to environmental factors such as domestic animals, pets, bush/forest, and tick/flea/mite bites was collected. About 47% (493/1044) reported bush/forest contact, 45% (472/1044) animal contact, 13% (118/1044) tick bites, and 11% (107/1044) flea/louse bites, Of those with animal contact, almost 60% (282/472) did not specify the animal species and cattle 35% (167/472) was the commonest animal of contact. None of these factors was significantly associated with any of the four infections.

In logistic regression analysis for the association of age, gender, occupation, district of residence, and other environmental risk factors, none was significant against SFG, TG, and QF infections. However, with STG there was significantly lower odds of infection in students (OR = 0.43; 95% CI = 0.20, 0.93; $p = 0.031$) compared to other occupational groups, as shown in Table 5. All other factors were insignificant.

Table 5. Association of age, gender, occupation, and district of residence with acute STG cases.

Variables	OR	95% CI		*p* Value
Age group (yrs.)				
Children	Ref.			
13–24	1.00	0.43	2.32	0.995
25–36	0.80	0.34	1.87	0.608
37–48	1.81	0.82	3.99	0.140
Above 48	1.08	0.46	2.54	0.856
Gender				
Male	Ref.			
Female	0.88	0.54	1.42	0.596
Occupation				
Farmer	Ref.			
Office worker	1.08	0.58	2.00	0.802
Student	0.43	0.20	0.93	0.031 *
Housewife	0.85	0.39	1.86	0.683
Unemployed	0.79	0.20	6.26	0.821
Pre-school	1.31	0.47	3.66	0.604

Table 5. *Cont.*

District				
Samtse	Ref.			
Chukha	0.92	0.37	2.30	0.862
Thimphu	1.45	0.59	3.59	0.416
Dagana	1.08	0.37	3.16	0.887
Tsirang	0.61	0.13	2.91	0.532
Sarpang	1.19	0.49	2.93	0.699
Zhemgang	1.36	0.43	4.27	0.597
Mongar	0.52	0.14	1.98	0.337
Pemagatshel	0.94	0.28	3.17	0.915
Trashigang	0.47	0.10	2.24	0.341
Samdrupjongkhar	0.57	0.17	1.92	0.367

OR: odds ratio; 95% CI: 95% confidence interval; * $p < 0.05$.

The number of cases, particularly of STG and SFG, gradually decreased from October (30 STG and 13 SFG cases) to February (5 STG and 3 SFG cases), and then increased from March (5 STG and 4 SFG cases) to June (9 STG and 5 SFG cases), during and after the warm, rainy summer season. This occurrence correlated with the average monthly precipitation and temperature of Bhutan [29], indicating that STG and SFG occur mostly during the warm monsoon and following months (Figure 3 and Supplementary Table S1).

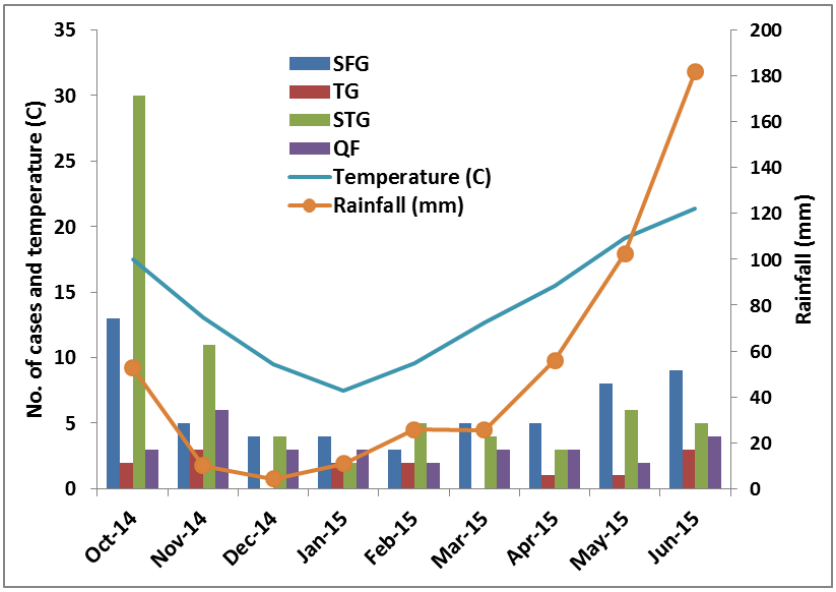

Figure 3. Cases by month in relation to average monthly temperature and precipitation.

4. Discussion

Rickettsioses appeared to be an important infectious disease in the 14 Bhutanese hospitals during the nine-month study period (October 2014–June 2015). In about 15% (at least one in every six) of the patients presenting with an undifferentiated febrile illness, there was evidence of a concurrent rickettsial infection, scrub typhus being the commonest. Although inferences may have been tempered by testing only single acute serum samples, the use of a high IgM titre (≥1:1024) and only including patients with an adequate duration for antibody response after the onset of illness (≥14 days) likely provided a good estimate of current rickettsial infections in this study. Although scrub typhus has been increasingly recognized over the past decade with two reported outbreaks [16,17], this is the first study on SFG, TG, and Q fever in Bhutan amongst acute febrile patients in the hospital setting.

The mean duration of illness before seeking medical attention was 6.8 days and there were patients who visited hospitals as late as 30 days after the onset of illness (range 4–30 days). Neglect of treatment could have serious outcomes [14,16,17] in an otherwise easily treatable rickettsial infection. Such practices have huge implications in a highly spiritual and religious society like Bhutan, where some sections of society are still known to seek medical attention only after exhausting home remedies. Presenting signs and symptoms in this study were similar to studies from India [30,31], Taiwan [32], and Thailand [33]; fever (100%) being the most common followed by headache and arthralgia. Our study, however, did not record other signs and symptoms, such as jaundice, ocular congestion, hepatomegaly, and splenomegaly, that were reported as significant in other studies [34,35].

In Bhutan, a largely rural country with high livestock ownership, participants indicating bush/forest contact of only 47% and animal contact of only 45% (472/1044) was unexpected, and possibly underreported. Finding no association between environmental factors and these vector-borne diseases probably indicates that people who work in offices or attend schools may nevertheless come into regular contact with domestic animals and vegetation, albeit to a lesser extent than that of farmers. Students revealing lower odds of infection with STG in this study should be interpreted with caution. The degree to which students may be exposed to the infections is completely dependent on the location of schools, and environmental and living conditions can vary widely. Outbreaks of scrub typhus [17] have occurred in schools in remote parts of Bhutan in the past.

STG was the most commonly identified rickettsiosis in this study, similar to reports from Sri Lanka [36] and south India [37]. An eschar was seen in 44.3% (31/70) of STG patients in this study, compared to 93% (243/261) in a study in Taiwan [38], 40% (59/146) in Thailand [33], and 10% (2/21) in an Indian study [39]. Although current findings are similar to others, it is crucial to understand that eschars occur at the site of chigger bites in often concealed body areas and can be easily missed without a detailed physical examination. A Thai study reported eschars to be mostly located in the perineal, inguinal, and buttock areas in males, and on the head and neck in females [33]. The significant association of an eschar with STG positivity ($p < 0.001$) in this study substantiates its usefulness in making a clinical diagnosis of scrub typhus, if present. Additionally, myalgia ($p = 0.003$) and lymphadenopathy ($p = 0.049$) could be clinically useful diagnostic symptoms of scrub typhus in the Bhutanese setting. However, only eschar ($p < 0.001$), but not myalgia ($p = 0.149$) and lymphadenopathy ($p = 0.145$), would useful in differentiating rickettsioses from other non-rickettsial causes of undifferentiated febrile illnesses.

SFG rickettsiosis was the second commonest infection in this study. This was in contrast to findings in central India [40] and Africa [41,42] where SFG cases were more common than STG amongst febrile patients. Because of fewer positive SFG cases, no significant correlations could be derived. In this study, *R. typhi* (causing endemic/murine typhus) and *R. prowazekii* (causing epidemic typhus) were individually tested but considered together as TG rickettsiae due to cross-reactivity in IFA. However, due to past evidence in Bhutan [15] and the non-epidemic nature of the current illnesses, the cases in this study were probably murine typhus. Epidemic typhus, once dreaded for causing epidemics, now seem to have become less prevalent than other rickettsial diseases as shown in this and other similar studies [40–43]. This could perhaps be due to a general improvement in living conditions and hygiene, resulting in reduced flea and louse infestations. However, certain populations, such as those in prisons, refugee camps, orphanages, and boarding schools, may still be at risk. Nevertheless, a study in Nepal's capital, Kathmandu, detected 17% seropositivity against murine typhus [44]. The incidence of QF at 2.8% of acute infections in this study was lower than expected but reflects a study in Nepal, a country with similar geographical conditions to Bhutan, where only one of 125 acute undifferentiated fever patients tested positive (0.8%) for QF [44]. This low incidence could be explained by the fact that, although livestock ownerships is high, domestic animals (the common sources of infection) are primarily free-range and found only in small herds mostly restricted to small land holdings, unlike large high-density commercial farms in developed countries. Studies on coinfections of rickettsial diseases are limited, but dual infection with STG and QF was seen in 3.6% (5/137) of patients in

Taiwan [32]. Although coinfections with these arthropod-borne diseases are possible, in our study, only 10 (1.0%) patients had equal antibody titres against SFG and TG rickettsiae. These were more likely due to cross-reacting antibodies rather than concurrent infections.

As reported in other studies [16], STG and SFG rickettsioses showed seasonal variation with greater activity in wet, warmer months, but the current study was limited to nine months (October–June) due to time and financial constraints. However, seasonal variations were shown in previous studies in temperate regions [1,4]. The seasonality of SFG rickettsioses may be explained by the activity of the tick vectors, particularly the adults, which are more active during the spring and early summer [4], and that of STG due to the growth of vegetation following the warm monsoon [45].

In situations where diagnostic facilities are limited, greater clinical awareness and a higher index of suspicion among healthcare workers could increase case detection and reduce morbidity and mortality, as rickettsial infections respond rapidly to appropriate antibiotic therapy. A previous Bhutanese study recounted that many non-malarial, non-typhoidal febrile cases which responded readily to doxycycline or chloramphenicol may have been rickettsial infections [16]. For Bhutan, the increasing number of cases may be due to uncovering a longstanding neglected endemic situation rather than a re-emergence of the infections. Public health authorities should ensure that health services are equipped to diagnose and treat these cases. Further studies are indicated to explore risk factors and biosocial characteristics of disease transmission to identify appropriate preventive and control measures. Additionally, studies should attempt speciation of the rickettsial agents and the emergence of antibiotic resistance as detected in other countries. Similar studies of longer duration across all seasons with patient follow-up to obtain convalescent samples and response to treatment should confirm the results of the current study.

This study has several important limitations. The case definition was overtly clinical and other possible causes of undifferentiated febrile illness such as malaria, typhoid fever, dengue fever, leptospirosis, and brucellosis were not excluded as test kits were not available. Additionally, only single acute blood samples were collected and tested, making it difficult for a definitive serological diagnosis of acute/current infection to be made due to background antibodies that are known to interfere with the diagnosis of the current infection. Because of the low positivity rate, especially of QF and TG rickettsioses, data should be interpreted with caution.

5. Conclusions

This study, the first of its kind in Bhutan, found that at least 15% (one in every six) of the undifferentiated febrile illnesses in the 14 Bhutanese hospitals were due to a rickettsiosis, scrub typhus being the commonest. Since the 14 hospitals in the study represented about 75% of Bhutan's population, the findings may likely be representative of the country, especially in the absence of any other similar studies at present. The findings of this study may warrant the Bhutan Ministry of Health to recognize the burden of rickettsioses and intervene to reduce their threat through the development of clinical guidelines and community education.

Supplementary Materials: The following are available online at http://www.mdpi.com/2414-6366/3/1/12/s1, Supplementary S1: Raw data, Supplementary S2: Table showing monthly cases of SFG, TG, STG and QF with corresponding average month temperature and rainfall of Bhutan.

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work; Tshokey Tshokey analyzed the data and wrote the first draft of the paper; Stephen R. Graves, John Stenos, David N. Durrheim, and Keith Eastwood reviewed and corrected the paper. All authors reviewed and approved the manuscript.

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Supplementary table 1: Monthly cases of SFG, TG, STG and QF with corresponding average month temperature and rainfall of Bhutan

Months	SFG	TG	STG	QF	Temperature (C)	Rainfall (mm)
Oct-14	13	2	30	3	18	53
Nov-14	5	3	11	6	13	10
Dec-14	4	0	4	3	10	4
Jan-15	4	2	2	3	8	11
Feb-15	3	2	5	2	10	26
Mar-15	5	0	4	3	13	26
Apr-15	5	1	3	3	16	56
May-15	8	1	6	2	19	103
Jun-15	9	3	5	4	21	182

SFG: Spotted fever group; TG: Typhus group; STG: Scrub typhus group; QF: Q fever; C: degree Celsius; mm: millimeter

Chapter 9: Seroprevalence of rickettsial infections and Q fever in Bhutan

This chapter reports the first seroprevalence study of rickettsial diseases and Q fever in Bhutan. Healthy Bhutanese people ≥13 years of age living in the eight of the twenty representative districts selected through a probability proportionate to size (PPS) method Bhutan were proportionately sampled. The sampling methods considered rural and urban populations separately through a clustered multi-stage sampling method. Demographic information and blood samples were collected from all selected and consenting subjects, serum samples were shipped to the Australian Rickettsial Reference Laboratory (ARRL) and tested for IgG antibodies against the rickettsial diseases and QF for evidence of past exposure.

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RESEARCH ARTICLE

Seroprevalence of rickettsial infections and Q fever in Bhutan

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Abstract

Background

With few studies conducted to date, very little is known about the epidemiology of rickettsioses in Bhutan. Due to two previous outbreaks and increasing clinical cases, scrub typhus is better recognized than other rickettsial infections and Q fever.

Methodology

A descriptive cross-sectional serosurvey was conducted from January to March 2015 in eight districts of Bhutan. Participants were 864 healthy individuals from an urban (30%) and a rural (70%) sampling unit in each of the eight districts. Serum samples were tested by microimmunofluorescence assay for rickettsial antibodies at the Australian Rickettsial Reference Laboratory.

Results

Of the 864 participants, 345 (39.9%) were males and the mean age of participants was 41.1 (range 13–98) years. An overall seroprevalence of 49% against rickettsioses was detected. Seroprevalence was highest against scrub typhus group (STG) (22.6%) followed by spotted fever group (SFG) rickettsia (15.7%), Q fever (QF) (6.9%) and typhus group (TG) rickettsia (3.5%). Evidence of exposure to multiple agents was also noted; the commonest being dual exposure to STG and SFG at 5%. A person's likelihood of exposure to STG and SFG rickettsia significantly increased with age and farmers were twice as likely to have evidence of STG exposure as other occupations. Trongsa district appeared to be a hotspot for STG exposure while Punakha district had the lowest STG exposure risk. Zhemgang had the lowest exposure risk to SFG rickettsia compared to other districts. People living at altitudes above 2000 meters were relatively protected from STG infections but this was not observed for SFG, TG or QF exposure.

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Conclusion

This seroprevalence study highlights the endemicity of STG and SFG rickettsia in Bhutan. The high seroprevalence warrants appropriate public health interventions, such as diagnostic improvements and clinical treatment guidelines. Future studies should focus on vector profiles, geospatial, bio-social and environmental risk assessment and preventive and control strategies.

Author summary

Rickettsial infections including scrub typhus and Q fever are not widely recognised in Bhutan although the country is situated in an endemic Asian region. With two recorded outbreaks, scrub typhus is known to occur but other rickettsial infections and Q fever have not been recorded. In this first seroprevalence study of rickettsial infections, an overall seroprevalence of 49% was detected against rickettsioses amongst 864 participants. Seroprevalence was highest against scrub typhus group (STG) (22.6%) followed by spotted fever group (SFG) rickettsia (15.7%), Q fever (QF) (6.9%) and typhus group (TG) rickettsia (3.5%). Evidence of exposure to multiple agents were also noted; the commonest being dual exposure to STG and SFG at 5%. A person's likelihood of exposure to STG and SFG significantly increased with age and farmers were twice as likely to have evidence of STG exposure as other occupations. Trongsa district in central Bhutan appeared to be a hotspot for STG exposure. People living at altitudes above 2000 meters were relatively protected from STG infections but this was not observed for SFG, TG and QF exposure. The findings from this study may direct future research on rickettsioses in Bhutan. These neglected tropical diseases warrant specific public health interventions in Bhutan.

Introduction

Rickettsial infections including scrub typhus and Q fever are usually referred to as rickettsiosis [1]. Rickettsioses are zoonotic infections transmitted to humans through bites of infected ticks, fleas, lice and mites or through aerosols generated during exposure to infected placentas and birth fluids of mammals in the case of QF [2]. The family Rickettsiaceae includes two genera, *Rickettsia* and *Orientia*, which include many human pathogens some of which cause lethal infections with up to 30% mortality without treatment [3, 4]. The genus *Rickettsia* has more than 20 species making up several groups among which the spotted fever group (SFG) and typhus group (TG) are established human pathogens [4, 5]. The SFG rickettsia includes the etiologic agents of Rocky Mountain spotted fever (*R. rickettsii*) and Mediterranean spotted fever (*R. conorii*) and many others. The TG rickettsia include agents of epidemic (*R. prowazekii*) and endemic (*R. typhi*) typhus [4]. *Orientia* has two species; *O. tsutsugamushi* and *O. chuto* [6], together forming the scrub typhus group (STG). *Coxiella burnetii* is the causal agent of Q fever (QF). Of all the methods to detect rickettsial infections, antibody detection by serology is the most commonly used, microimmunofluorescence assay (IFA) being the currently accepted gold standard [7]. After an infection, IgM can be detectable for months and IgG for years [7, 8].

SFG and TG rickettsia occur worldwide and are a significant cause of morbidity in southeast Asia [9]. STG was originally thought to be confined to the Asia-Pacific region but now

has been reported from the Middle East [6], Africa [10, 11] and South America [12]. Q fever has a worldwide distribution [13] except New Zealand [14] although fears of its introduction have been raised [15]. Rickettsioses are both emerging and re-emerging infections [16, 17]. Despite being endemic in Asia and causing significant burden to public health, true prevalence studies of these infections are limited. In India, rickettsial diseases including scrub typhus have been documented in several states from all parts of the country [1]. A seroepidemiology study in northeast India, in areas bordering Bhutan reported a sero-prevalence of 30.8%, 13.8% and 4.2% against STG, SFG and TG respectively [18]. In Darjeeling, another Indian district near Bhutan, a 2005 study reported an overall incidence of STG at 34 cases/100,000 population/pa, varying from 2 cases/100,000 population in July to 20/100,000 population in September and decreasing to zero in December [19]. Q fever has been under-reported from India and recent data are lacking [20]. A Chinse study reports an overall Q fever prevalence of 10% and highlights it as an under-reported and underdiagnosed illness [21].

Although situated in the endemic Asia Pacific region, Bhutan has reported scrub typhus cases only since 2009 [22] and SFG, TG and QF have not been reported to date. Rickettsial diseases (excluding Q fever) have been included in the national notifiable diseases since 2010 with increasing reports, mostly scrub typhus, from 118 cases in 2011 to 605 cases in 2015. Despite the increasing notifications and improving awareness, there are currently no clinical guidelines on management of rickettsial infections in Bhutan, and awareness needs improving. There are no reports of Q fever in Bhutan owing to the lack of diagnostic facility both in the human and animal sector at present. Therefore, a serological investigation was undertaken to determine the seroprevalence of rickettsial infections including QF in Bhutan.

Methods

Setting

Bhutan is composed of 20 districts and 205 subdistricts with an estimated population of 770,000 in 2016 [23]. The Bhutan national census in 2005 reported on 1044 rural villages/chiwogs and 311 urban towns as primary sampling units (PSUs). Population density in different districts vary between 9–64 people/km^2 [23]. For this study, the 20 districts were stratified into four regions; eastern (5 districts), central (4 districts), western (5 districts) and southern (6 districts) as defined by the Bhutan National Statistical Bureau (NSB) [23] for their national surveys. From each region, two districts were selected with a probability proportionate to size (PPS) method, selecting eight of twenty districts for the study (Fig 1). A rural and an urban PSU were selected from each district by the same PPS method. To assess the influence of altitude on exposure, altitude of places were arbitrarily grouped into low (<1000 meters), medium (1000–2000 meters) and high altitude (>2000 meters).

Study design, participants and sample size

This descriptive cross-sectional sero-survey was carried out from January to March 2015, during the dry winter and early spring season. The sample size was calculated using a multi-stage cluster sampling method. Persons <13 years were excluded due to the possible risk of complications during blood sampling in remote areas. The sample size needed to estimate the number of households to be surveyed with a 95% confidence interval and other assumptions (50% prevalence rate, 0.05 margin of error, a design effect of 2 and an expected rate of participation of 90%) was calculated to be 864. Based on Bhutan's urban-rural population proportion of 30:70 [23], 30% of the participants were taken from urban and the remaining from rural settings; therefore, of the 864 households selected, 256 were from urban and 608 from rural

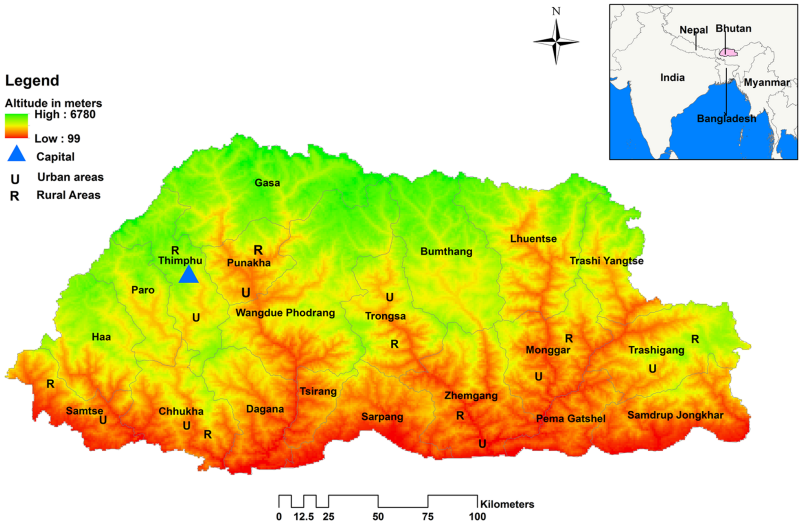

Fig 1. Map of Bhutan showing the eight selected districts with the location of urban (U) and rural (R) primary sampling units. (Map generated using the ESRI's ArcMap 10.3.1 for desktop software. An electronic map of district boundaries of Bhutan in shapefile format was obtained from Global Administrative Areas database (http://www.gadm.org/country). Figure created solely for this manuscript and has not been used for any other publications or documents).

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settings. Each of the eight selected districts contributed 108 households (76 rural and 32 urban households). The households were taken from the household list with unique identification numbers developed during the previous national surveys (National Health Survey 2012 and NCD STEPS Survey 2014). When a selected PSU had a lesser number of households than required, a nearby PSU was added. After selection of the household, all eligible members (≥13 years) present in the house were listed and one member was selected for the study through a lottery system. After selection, written consent was obtained; demographic details and environmental exposure history were taken by trained laboratory personnel through a face-to-face interview and blood samples were collected. Serum was extracted and stored at -70˚C until shipment to Australia.

Serological testing

Serum samples were shipped at room temperature to the Australian Rickettsial Reference Laboratory (ARRL) [24], a nationally accredited laboratory for rickettsial testing, where serological testing was carried out by indirect microimmunofluorescence assay (IFA) [25]. Antibodies against SFG rickettsia were individually tested using *R. australis*, *R. honei*, *R. conorii*, *R. africae*, *R. rickettsii* and *R. felis* antigens; TG rickettsia using *R. prowazekii* and *R. typhi* antigens; STG using *O. tsutsugamushi* (Gilliam, Karp and Kato strains) and *O. chuto* antigens, and QF using *C. burnetii* phase I and phase II antigens. Samples were initially screened at low dilutions and titrated to end-point (titre) when positive. With slight modification from the usual ARRL interpretation criteria [24, 25], antibody titres of ≥1:256 for IgG and/or ≥1:1024 for IgM against any of the SFG, TG and STG antigens were considered positive for the rickettsial group agents. Similarly an antibody titre of ≥1:50 for IgG or IgA and ≥1:100 against IgM against *C. burnetii* phase I or II or both were considered positive for Q fever. Positive and negative control wells were included in each slide during testing.

Statistical analysis

Data were entered into an Excel spreadsheet and analysed using STATA software version 14. Chi-squared or Fischer's exact test was used to explore the association between seropositivity and study variables considering p values of ≤ 0.05 significant. Univariate logistic regression was used to determine crude odds ratio (COR) and p values. All variables with p values 0.2 or less in the univariate analysis were taken for multivariate logistic regression to determine adjusted OR (AOR) and corresponding p values of <0.05 considered significant.

Ethics and consent

The study was approved by the Bhutan Research Ethics Board of Health (REBH) (Ref: REBH/Approval/2014/019) and the Human Research Ethics Committee (HREC), University of Newcastle, Australia (Ref: H-2016-0085). All individuals or parent/guardian provided written consent before participation.

Results

Demography

A total of 864 participants were enrolled from the eight districts and all selected candidates consented to the study. There were 345 (39.9%) males and the mean age of participants was 41.1 (range 13–98) years. Most participants belonged to the age group of 26–40 years. Farmers 414 (47.9%) were the highest group by occupation (Table 1).

Serology

In seropositive participants, most had IgG antibody titres of 1:256 or 1:512 against STG, SFG and TG rickettsia and titres of 1:100 or 1:200 against *Coxiella* phase II IgG, IgA or IgM. A very

Table 1. Participants distribution by gender, occupation and location in different age groups (N = 864).

Variable	Age group (years)				
	13–25	26–40	41–55	>55	Overall (%)
Total (%)	163 (19)	311 (36)	210 (24)	180 (21)	864 (100)
Gender					
Male	59	112	97	77	345 (40)
Female	104	199	113	103	519 (60)
Occupation					
Farmers	63	113	114	124	414 (48)
Herders	1	13	16	11	41 (5)
Employees	17	87	32	8	144 (17)
Students	45	0	0	0	45 (5)
Housewives	21	90	37	34	182 (21)
Unemployed	16	8	11	3	38 (4)
Sampling unit					
Urban	39	121	52	44	256 (30)
Rural	124	190	158	136	608 (70)
Altitude					
Low	28	54	36	22	140 (16)
Medium	106	213	139	126	584 (68)
High	29	44	35	32	140 (16)

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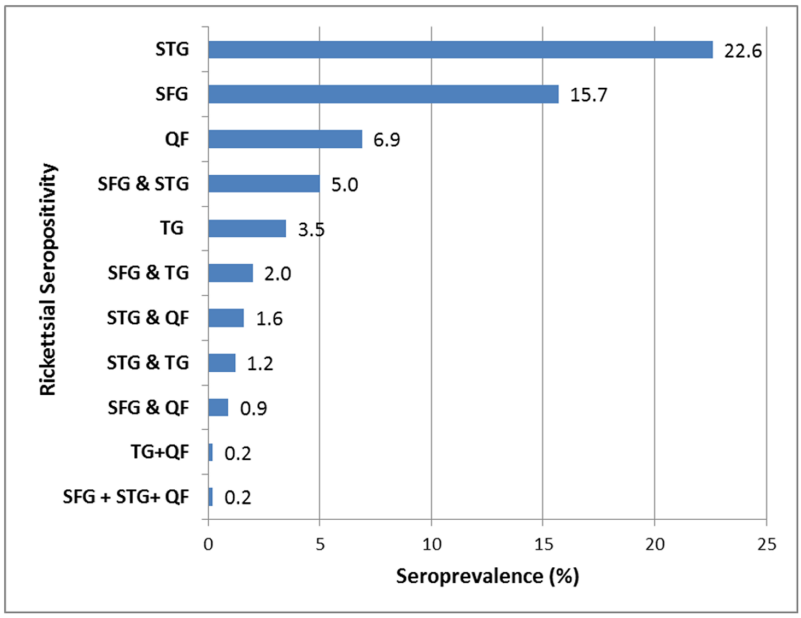

Fig 2. Overall seroprevalence of rickettsial infections in Bhutan. (STG, Scrub Typhus Group; SFG, Spotted Fever Group; TG, Typhus Group; QF, Q Fever).

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small number of high antibody titres of up to 1: 2048 in STG (≈3%) and SFG (≈ 0.1%) were seen in participants. Overall, the most prevalent rickettsial infection was STG (22.6%) and the least prevalent was TG rickettsia (3.5%). Evidence of past exposure to multiple agents was also seen; the commonest being dual exposure to SFG and STG (5%) (Fig 2).

Seropositivity rates were not significantly different between males and female for all four infectious agents. The prevalence of each infection, especially STG and SFG, appeared to increase with age and farmers exhibited the highest seropositivity rates.

Sociodemographic and environmental determinants of exposure risk

Thirty percent (256) of the participants were from urban areas. Among all participants, 550 (63.7%) reported having animal contact and almost half (426) had pets at home. In addition, 620 (71.8%) reported contact with vegetation and forest during their daily activities, 205 (24.3) recollected suffering from febrile illness in the past, 337 (40.8%) had past tick bites, 153 (18.0%) had an eschar in the past and 202 (23.6%) had past flea bites.

Many of the demographic and environmental variables showed significant baseline correlation with seropositivity against each infection in Chi-squared or Fisher's exact test (Table 2) and a few were statistically significant in the logistic regression analysis.

The comparative seropositivity in the urban and rural sampling units of the eight districts and the overall national prevalence of all four infections are shown in Fig 3 and the estimated proportion of each infection at different sampling units (urban and rural) of the eight districts presented in Fig 4. Significant epidemiological factors and seropositivity are shown for STG (Table 3) and SFG (Table 4). No factors showed association with QF or TG rickettsia seropositivity, likely due to the small number of seropositives.

The prevalence of STG seropositivity increased with age. The odds of exposure to STG infection was significantly higher in farmers compared to other occupations. Punakha district had the lowest risk of exposure to STG infections while people living in Trongsa district were

Table 2. Baseline correlation of seropositivity with different variables.

Variables	STG		SFG		TG		QF	
	Positive	*p* value	Positive	*p* value	Positive	*p* value	Positive	*p* value
Gender								
Male (n = 345)	76 (22.0)	0.757	54 (15.6)	0.954	9 (2.6)	0.258	25 (7.2)	0.776
Female (n = 519)	119 (22.9)		82 (15.8)		21 (4.0)		35 (6.7)	
Age groups (yrs)								
13–25 (n = 163)	23 (14.1)	0.001*	13 (8.0)	0.003*	5 (3.1)	0.107	12 (7.4)	0.619
26–40 (n = 311)	61 (19.6)		45 (14.5)		6 (1.9)		17 (5.5)	
41–55 (n = 210)	56 (26.7)		39 (18.6)		8 (3.8)		16 (7.6)	
> 55 (n = 180)	55 (30.6)		39 (21.7)		11 (6.1)		15 (8.3)	
Occupation								
Farmers (n = 414)	122 (29.5)	<0.001*	79 (19.1)	0.031*	16 (3.9)	0.14	29 (7.0)	0.103
Herders (n = 41)	8 (19.5)		5 (12.2)		3 (7.3)		6 (14.6)	
Office workers (n = 144)	14 (9.7)		15 (10.4)		2 (1.4)		5 (3.5)	
Students (n = 45)	3 (6.7)		2 (4.4)		0 (0.0)		3 (6.7)	
Housewives (n = 182)	39 (21.4)		31 (17.0)		6 (3.3)		12 (6.6)	
Unemployed (n = 38)	9 (23.7)		4 (10.5)		3 (7.9)		5 (13.2)	
Districts								
Chukha (n = 108)	32 (29.6)	<0.001*	29 (26.9)	0.006*	6 (5.6)	0.009*	9 (8.3)	0.034*
Mongar (n = 108)	13 (12.0)		13 (12.0)		2 (1.9)		13 (12.0)	
Punakha (n = 108)	10 (9.3)		18 (16.7)		10 (9.3)		3 (2.8)	
Samtse (n = 108)	25 (23.1)		22 (20.4)		2 (1.9)		2 (1.9)	
Thimphu (n = 108)	5 (4.6)		8 (7.4)		3 (2.8)		12 (11.1)	
Trashigang (n = 108)	19 (17.6)		18 (16.7)		5 (4.6)		6 (5.6)	
Trongsa (n = 108)	46 (42.6)		14 (13.0)		2 (1.9)		8 (7.4)	
Zhemgang (n = 108)	45 (41.7)		14 (13.0)		0 (0.00)		7 (6.5)	
Sampling units								
Rural (n = 608)	159 (26.2)	<0.001*	106 (17.4)	0.035*	26 (4.3)	0.065	41 (6.7)	0.72
Urban (n = 256)	36 (14.1)		30 (11.7)		4 (1.6)		19 (7.4)	

*p<0.05

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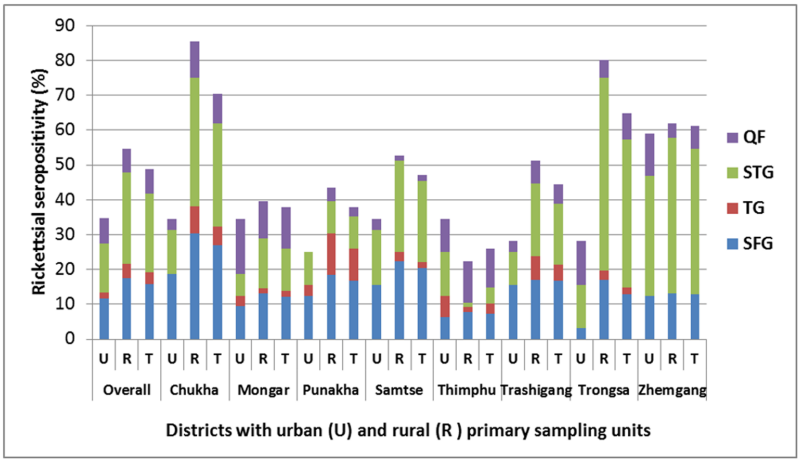

Fig 3. Prevalence of rickettsial seropositivity in different districts of Bhutan. (U, Urban; R, Rural; T, Total).

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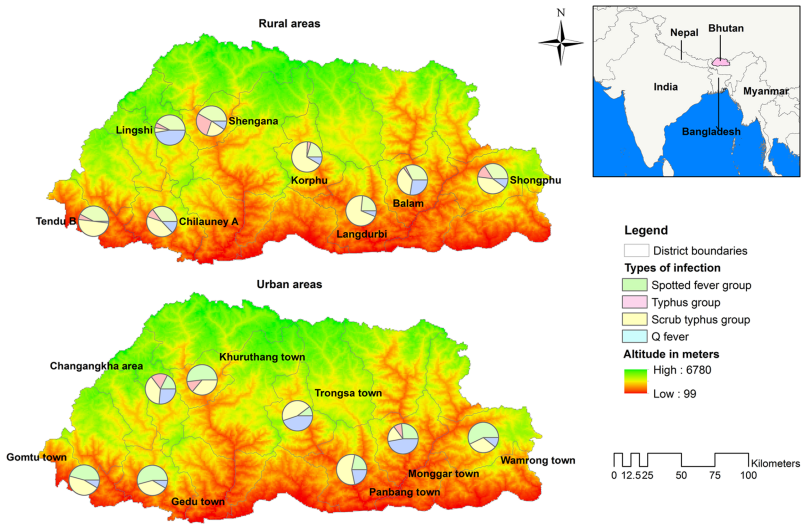

Fig 4. Prevalence estimates of the four infections in the urban and rural sampling units of the eight districts in Bhutan. (Map generated using the ESRI's ArcMap 10.3.1 for desktop software. An electronic map of district boundaries of Bhutan in shapefile format was obtained from Global Administrative Areas database (http://www.gadm.org/country). Figure created solely for this manuscript and has not been used for any other publications or documents).

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three times more likely to be infected as those in other districts. Contact with domestic animals more than doubled the odds of exposure to STG. People residing at high altitude had 89% lower odds of being exposed to STG compared to those residing at lower altitude (AOR 0.11, p 0.002, 95% CI 0.03, 0.44), (Table 3).

SFG rickettsial seropositivity prevalence also increased with age. A person over 55 years of age was three times more likely to have been exposed to SFG than the younger age groups. Compared to other districts, Zhemgang district had a significantly lower odds of exposure to SFG rickettsia (AOR 0.34, p value = 0.010, 95% CI 0.15, 0.77). Altitude did not affect the prevalence of SFG (Table 4).

Discussions

This study revealed an overall seroprevalence of 48.7% against rickettsioses in Bhutan with the highest prevalence to scrub typhus (22.6%) followed by SFG rickettsia (15.7%), Q fever (6.9%) and TG rickettsia (3.5%). Evidence of past exposure to two or more rickettsial agents was seen in 11.1% of the participants depicting probable dual or multiple infections in an endemic setting or possibly cross-reacting antibodies. This is the first seroprevalence study on rickettsioses in Bhutan and may be used as baseline data for subsequent studies in this country although it is recognised that prevalence estimates may vary when measured at different times of the year. The limitations of the findings from the exclusion of children (<13 years old) should be borne in mind. This was required to avoid risks of complications during blood sampling especially in remote areas where medical assistance is hard to obtain. Unintended but unavoidable exclusion of potential participants could have also occurred due to a member of the household being away from home during sampling. Information on past fevers, tick bites and eschar might have had drawbacks due to participants failing to comprehend technical terms. In addition, inter-district, urban versus rural as well as high versus low altitudes comparisons was not precise due to the highly variable landscape within and between districts. Unavailability of adequate local data on climatic conditions, environmental and geospatial information at primary

Table 3. Epidemiological factors associated with seropositivity to Scrub typhus group (STG) rickettsioses in Bhutan.

Variables	COR	95% CI		*p* value	AOR	95% CI		*p* value
Age group (years)								
13–25	Ref.							
26–40	1.49	0.88	2.50	0.138	1.91	1.01	3.63	0.048*
41–55	2.21	1.29	3.79	0.004*	2.37	1.24	4.54	0.009*
> 55	2.68	1.56	4.61	<0.001*	3.14	1.59	6.20	0.001*
Occupation								
Office workers	Ref.							
Herders	0.58	0.26	1.29	0.183	1.94	0.62	6.09	0.254
Farmers	0.26	0.14	0.47	<0.001*	2.43	1.13	5.20	0.023*
Students	0.17	0.05	0.56	0.004*	1.26	0.29	5.47	0.755
Housewives	0.65	0.43	0.99	0.043	1.97	0.94	4.13	0.074
Unemployed	0.74	0.34	1.62	0.453	2.26	0.76	6.75	0.144
Districts								
Chukha	Ref.							
Mongar	0.33	0.16	0.66	0.002	0.42	0.17	1.02	0.056
Punakha	0.24	0.11	0.52	<0.001*	0.21	0.08	0.54	0.001*
Samtse	0.72	0.39	1.31	0.281	0.82	0.40	1.68	0.584
Thimphu	0.12	0.04	0.31	<0.001*	0.96	0.17	5.33	0.962
Trashigang	0.51	0.27	0.97	0.039	0.75	0.32	1.72	0.493
Trongsa	1.76	1.00	3.09	0.048*	3.44	1.53	7.74	0.003*
Zhemgang	1.70	0.97	2.98	0.066	1.63	0.81	3.28	0.174
Altitude								
Low	Ref.							
Medium	0.70	0.47	1.05	0.085	0.84	0.45	1.57	0.577
High	0.15	0.07	0.32	<0.001*	0.11	0.03	0.44	0.002*
Animal contact								
No	Ref.							
Yes	1.66	1.17	2.36	0.005	1.68	1.09	2.58	0.018

COR, crude odds ratio; AOR, adjusted odds ration;
*p<0.05

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sampling unit (urban and rural) level made it impossible to explain inter-district differences of exposure to the infections.

The findings in this study were similar to a seroprevalence study in north-east India, that reported the highest seroprevalence against STG (30.8%) followed by SFG (13.8%) and TG (4.2%) [18]. The similarity is noteworthy due to the proximity of these areas to Bhutan. Similar occurrences of these infections in neighbouring countries may benefit from coordinated cross-border prevention and control activities.

The odds of exposure sequentially increased with increasing age of participants in case of STG and SFG rickettsiae. This mirrors the situation in endemic areas where increasing number of people would be exposed to the infections as they advance through life leading to an accumulation of older seropositive people in the community. In south-east Asia, murine typhus was reported more in urban dwellers, while STG and SFG were more prevalent in rural dwellers [9]. However, in Bhutan, this study did not find any significant differences in any of the four infections between urban and rural residents probably reflecting similar environmental conditions between the two populations. This is supported by finding no significant

Table 4. Epidemiological factors associated with seropositivity to Spotted fever group (SFG) rickettsiosis in Bhutan.

Variables	COR	95% CI		*p* value	AOR	95% CI		*p* value
Age group (years)								
13–25	Ref.							
26–40	1.95	1.02	3.73	0.043*	1.68	0.81	3.48	0.159
41–55	2.63	1.35	5.12	0.004*	2.16	1.04	4.48	0.040*
> 55	3.19	1.64	6.23	0.001*	2.87	1.35	6.09	0.006*
Districts								
Chukha	Ref.							
Mongar	0.37	0.18	0.77	0.007*	0.46	0.19	1.14	0.094
Punakha	0.54	0.28	1.06	0.072	0.53	0.22	1.24	0.141
Samtse	0.70	0.37	1.31	0.263	0.7	0.34	1.47	0.348
Thimphu	0.22	0.09	0.50	<.001*	1.78	0.16	19.28	0.634
Trashigang	0.54	0.28	1.06	0.072	0.68	0.29	1.61	0.386
Trongsa	0.41	0.20	0.82	0.012*	0.62	0.25	1.54	0.301
Zhemgang	0.41	0.20	0.82	0.012*	0.34	0.15	0.77	0.010*
Altitude								
Low	Ref.							
Medium	0.66	0.42	1.03	0.067	0.82	0.43	1.59	0.564
High	0.23	0.11	0.51	<0.001*	0.11	0.01	1.07	0.058

COR, crude odds ratio; AOR, adjusted odds ration;
*p<0.05

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differences between occupational groups for all infections, with the exception of STG where farmers had higher seropositivity rates compared to other occupations. There were differences between a few districts for STG and SFG infections, highlighting hot spots for these two infections. Trongsa district in central Bhutan appeared to be a hotspot for STG infection and Punakha district exhibited significantly low odds of exposure. STG exposures were significantly low amongst participants in high altitude areas. This may be explained by cold weather at high altitude areas not favouring mite survival. Of all the districts, Zhemgang in south-central Bhutan had the lowest odds of exposure to SFG rickettsia. Unlike STG infections, altitude had no effect on SFG, TG and QF exposure. This could be due to different tick species at different altitudes transmitting different infections. Expanding primary and secondary clusters of STG infections were also reported in China [26]. Such clusters or hotspots would benefit from focused public health interventions especially where resources are limited as in the case of Bhutan. Targeting prevention and control activities in hotspot areas could be effective and cost saving.

Antibody titres of 1:256 or 1:512 were the commonest observed antibody levels amongst the participants. A small number of participants with higher antibody titres of 1:1024 or 1:2048 may have been due to recent infections (symptomatic or asymptomatic) or due to recurrent subacute exposures stimulating antibody production. Cross-reactions between antibodies within the rickettsial species, especially between SFG and TG rickettsia, are known to occur. Therefore, persons with mixed antibodies may not necessarily have had multiple infections but may be due to cross-reacting antibodies resulting from the one infection. This could also explain some of the observed multi-species exposures. Background antibody levels in endemic situations are known to interfere with serological diagnosis of acute infections especially with rapid point-of-care diagnostics [17]. This is worthy of note in the Bhutanese setting where only rapid point-of-care diagnostics are available currently. There is urgent need to improve diagnostic facilities in Bhutan to provide more specific assays such as the microimmunofluorescence assay and molecular

diagnostics, especially in the main centres. Point-of-care diagnostics could still be useful in the smaller districts and remote health centres for ease of use.

Rickettsioses have been associated with poor maternal and neonatal outcomes including stillbirth and low birth weights [27, 28] in endemic situations. The role of rickettsioses in the high maternal and neonatal mortality and morbidities in Bhutan deserves to be studied. Scrub typhus has also been known to involve the central nervous system manifesting as meningoencephalitis [29–31] and STG has been recently reported as a significant cause of encephalitis in northeast India [32]. This is important in the Bhutanese context in light of establishing the causes of acute encephalitis syndromes including meningococcal infection, Japanese encephalitis and other viral meningitis syndromes which are poorly understood at present. Documented deaths in Bhutan due to proven scrub typhus had resulted from meningoencephalitis and gastrointestinal perforation [22], which are known to be severe complications of scrub typhus. Understanding these occurrences in endemic areas could be helpful in averting preventable deaths from such complications.

Rickettsioses are important causes of illnesses in international travellers [33]. In a study on the spectrum of illness amongst ill returned travellers from six GeoSentinel sites, rickettsial infections were significant causes with 17 and 32 patients/1000 cases returning from south central and south-east Asia respectively [34]. Bhutan is an emerging destination for international travellers involving in activities like camping, trekking, cultural and rural home-stays. The high prevalence of rickettsioses could potentially expose travellers to these infections. Therefore travellers should be aware of the risk and become educated on preventive measures. In addition, educating travellers would keep them vigilant for any febrile illnesses during travel or upon returning to their home countries, enabling them to provide a detailed travel history and for their treating doctor to include rickettsial infections in their differential diagnoses.

There are limited prevalence studies on rickettsioses in south-east Asia [9]. Studies are even scantier in south and central Asia including Bhutan where most published studies were focused on clinical cases and acute febrile patients. Therefore, a prevalence study of these neglected but re-emerging infections in these endemic areas should be carried out with active regional collaborations and participations. This first seroprevalence study in Bhutan highlighted the endemicity of rickettsioses especially STG and SFG rickettsia. Findings on TG rickettsia and Q fever should be interpreted with caution due to the detection of fewer positive cases. This high rickettsial seroprevalence needs attention from the Bhutan Ministry of Health such as appropriate public health interventions, diagnostic improvement and clear clinical treatment guidelines. Future studies should focus on vector profiles, geospatial, bio-social and environmental risk assessment and preventive and control strategies formulation.

Supporting information

S1 Metadata.
(XLSX)

S1 STROBE Checklist.
(DOC)

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Chapter 10: Serological Evidence of *Rickettsia, Orientia,* and *Coxiella* in Domestic Animals from Bhutan: Preliminary Findings

This chapter presents the first (preliminary) report on serological evidence of past exposure of domestic animals from Bhutan to *Rickettsia, Orientia* and *Coxiella*. Serum samples were opportunistically collected from domestic animals residing in urban and rural areas of the eight selected districts of Bhutan where a human seroprevalence study on *Rickettsia, Orientia* and *Coxiella* was carried out (presented in chapter 9). Serum samples were shipped to the Australian Rickettsial Reference Laboratory (ARRL) and tested for IgG antibodies against *Rickettsia, Orientia* and *Coxiella*.

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Serological Evidence of *Rickettsia*, *Orientia*, and *Coxiella* in Domestic Animals from Bhutan: Preliminary Findings

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Abstract

There is no information on rickettsial diseases in domestic animals in Bhutan. This study provides preliminary serological data on exposure of domestic animals to *Rickettsia*, *Orientia*, and *Coxiella.* Animal sera were collected opportunistically from Bhutan and tested in the Australian Rickettsial Reference Laboratory for IgG antibodies against spotted fever group (SFG) and typhus group (TG) *Rickettsia,* scrub typhus group (STG), and Q fever (QF). Of the 294 animals tested, 136 (46%) showed serological evidence of past exposure to one or more rickettsiae: 106 (36%), 62 (21%), 45 (15%), and 11 (4%) being positive against SFG *Rickettsia*, *Orientia*, TG *Rickettsia,* and *Coxiella*, respectively. Dogs appeared to exhibit the highest seropositivity against SFG (55%) and TG *Rickettsia* (45%), horses against STG (91%), while goats were mostly positive for *Coxiella* (9%). Dogs also appeared to have high risk of being exposed to SFG *Rickettsia* (odd ratios [OR] 5.71, 95% confidence interval [CI] 3.02–10.80, $p < 0.001$), TG *Rickettsia* (OR 48.74, 95% CI 11.29–210.32, $p < 0.001$), and STG (OR 6.80, 95% CI 3.32–13.95, $p < 0.001$), but not against QF (OR 1.95, 95% CI 0.42–8.95, $p = 0.390$). Differences in seropositivity rates between animal species may have been significant for SFG, TG, and STG, but not for QF. The differences in the seropositivity rates of the four infections between districts appeared to be significant for TG and STG, but not for SFG and QF. The seropositivity rates of domestic animals to the four rickettsial infections were consistent with similar studies on the human population in the same areas and appear to demonstrate a high prevalence of exposure to rickettsiae in Bhutan. These preliminary findings constitute baseline data for Bhutan. The findings of this study call for an increased human-livestock sector collaboration in rickettsial diseases research aimed at developing diagnostic and therapeutic guidelines and formulating preventive and control measures through a One Health approach.

Keywords: Bhutan, domestic animals, Q fever, rickettsial diseases, scrub typhus

Introduction

Rickettsia, *Orientia*, and *Coxiella* are important arthropod-borne zoonotic human pathogens. The genus *Rickettsia* has more than 22 species, making up several groups, among which are the spotted fever group (SFG) and typhus group (TG) (Sahni et al. 2013, Valbuena 2013). The SFG rickettsiae comprise *multiple species* causing mainly tick-borne infections such as Rocky Mountain spotted fever, rickettsialpox, and other spotted fevers. The TG rickettsiae consist of only two species; *Rickettsia prowazekii* (louse-borne) and *Rickettsia typhi* (flea-borne) causing epidemic and murine/endemic typhus, respectively. The genus *Orientia* consists of two species; *Orientia tsutsugamushi* and *Orientia chuto* (Izzard et al. 2010). These two species form the scrub typhus group (STG) and cause scrub typhus in humans. *Orientia* naturally inhabit several different species of *Leptotrombidium* mites, found both in the environment and on mammals, commonly rodents (Coleman et al. 2003). The genus *Coxiella,* with *Coxiella burnetii* the only species pathogenic for vertebrates, causes Q fever (QF) in humans, a worldwide zoonosis. *Coxiella* is known to cause coxiellosis in animal species ranging from

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domestic and wild mammals, birds, reptiles, and arthropods such as ticks (Honarmand 2012). Animal infections due to *C. burnetii* are often asymptomatic (Honarmand 2012), but infertility and abortions have been reported in numerous mammalian species (Marenzoni et al. 2013). Outbreaks of coxiellosis in domestic animals results in economic loss from death, abortions, reduced fertility, decreased milk production, and culling of infected animals as well as other activities associated with controlling the outbreak (Van Asseldonk et al. 2013).

Bhutan is a small Himalayan kingdom situated between India in the south and China in the north. It has an estimated human population of 770,000 in 2016 (National Statistics Bureau 2016) living in 20 districts. In a serosurvey among 864 healthy individuals, sampled equally from eight selected districts in Bhutan, an overall seroprevalence of 49% against rickettsioses was detected, represented by 22.6%, 15.7%, 3.5%, and 6.9% against STG, SFG, TG, and QF, respectively (Tshokey et al. 2017). As of 2016, Bhutan's livestock population largely comprised cattle (303,374) followed by yaks (49,617), goats (39,513), cats (33,866), pet dogs (28,630), horses (18,890), pigs (15,324), and sheep (11,277) (Livestock Statistics 2016). There are few buffaloes (532) and the poultry population (1,038,553) has increased recently due to the mushrooming of poultry farms all over the country. There are currently no data on rickettsioses in domestic animals in Bhutan. This preliminary study was undertaken to determine the serological evidence of rickettsial infections in domestic animals in Bhutan.

Materials and Methods

Study design, location, and animal sampling

This study was an opportunistic sampling of animals and not representative of the whole country or all animal species. It was conducted in the areas where a human seroprevalence study (Tshokey et al. 2017) was carried out as part of the same project between January and April 2015. For the human study, eight districts from Bhutan's 20 districts were selected through a probability proportionate to size method. From each district, a rural and an urban area were selected by the same method, resulting in 16 sampling sites in total (8 urban and 8 rural). Blood samples were collected from domestic animals in those same areas by trained livestock staff. Serum was stored at −70°C, until shipment to the Australian Rickettsial Reference Laboratory (ARRL) for serological testing. For this animal study, a sample size was not predetermined since it was a preliminary study designed only to demonstrate evidence of exposure of domestic animals to rickettsial pathogens in the areas where a high human rickettsial seroprevalence had been detected.

Serological testing

Serum samples were shipped at room temperature to the ARRL, a nationally accredited diagnostic laboratory, for rickettsial serological testing. Testing for IgG antibodies was carried out by indirect microimmunofluorescence assay (IFA) (Graves et al. 1991) using in-house prepared rickettsial antigens; SFG *Rickettsia* consisted of *Rickettsia australis*, *Rickettsia honei*, *Rickettsia conorii*, *Rickettsia africae*, *Rickettsia rickettsii*, and *Rickettsia felis* antigens; TG *Rickettsia* consisted of *R. prowazekii* and *R. typhi* antigens; STG consisted of *O. tsutsugamushi* (Gilliam, Karp, and Kato strains) and *O. chuto* antigens, and QF using *C. burnetii* phase I and phase II antigens of the Nine Mile strain. Known positive and negative control animal sera for the specific animal species being tested were included on each slide during testing. For some animal species where no definite positive and negative controls were available (such as yaks), certain assumptions were made by using known positive and negative control human sera to ensure the quality of the antigens, conjugates, and the test procedure, even though a nonhuman serum was being titrated.

IFA slides were coated with antigens from SFG and TG *Rickettsia*, *Orientia*, and *Coxiella* (phase I and II). Fluorescein-labeled IgG antibodies against the animal species tested (anti-bovine, anti-dog, anti-goat, anti-horse, anti-cat) from KPL were used to detect the IgG antibody-antigen complexes following established protocols in the ARRL and a previous related study (Muleme et al. 2016). Samples were initially screened at low dilutions (1:32 for *Rickettsia* and *Orientia* and 1:80 for *Coxiella*) and titrated to end-point titer when positive. Serum samples were pipetted onto the fixed antigens on the slide wells in duplicates and incubated for 40 min at 37°C. Unbound antibodies were removed by washing in 10% phosphate-buffered saline (PBS). Following this, dried slides were treated with secondary conjugated antibodies and incubated under similar conditions. Unbound conjugated antibodies were washed off with 10% PBS and dried slides were observed under an ultraviolet light microscopy with FITC filters at 400× magnification. Positive samples were identified by the presence of bright green fluorescence, while negative samples lacked any fluorescence. Based on similar past studies, antibody titers of ≥1:64 (Nanayakkara et al. 2013, Cunha et al. 2014) against any of the SFG, TG, and STG antigens were considered positive for the rickettsial group agents, and antibody titers of ≥1:160 (Muleme et al. 2016) against *C. burnetii* phase I or II or both were considered positive for QF.

Statistical analysis

Data were entered into an Excel spreadsheet and analyzed with STATA software version 14 (StataCorp). Descriptive statistics were carried out to calculate the frequencies and percentages of each of the variables such as animal species sampled from each district and seropositivity rates. Chi-squared or Fischer's exact test was used to explore the association between seropositivity and different study variables considering p values of <0.05 statistically significant. Univariate logistic regression analysis was attempted with seropositivity status of each rickettsial disease (seropositive vs. seronegative) as an outcome and the animal species and study sites as an explanatory variable. The odds ratios (OR) and the corresponding 95% confidence intervals (CIs) and p values were calculated. Variables with a p value of <0.05 were considered to be significantly associated with the seropositivity of the rickettsial disease.

Ethics approval

This study was approved by the Council for RNR Research of Bhutan (CoRRB), Ministry of Agriculture and Forests, Royal Government of Bhutan, through approval no. CORRB/TCO/D-2/732.

Results

A total of 294 domestic animals from 7 species were opportunistically sampled from an urban and rural study site of the 8 districts (Table 1). More than half, 164 (56%) of the animals were sampled from rural areas and the remainder from urban areas.

Of the 294 sampled animals, 136 (46%) showed serological evidence of past exposure to one or more rickettsial agents. Individually, 106 (36%), 62 (21%), 45 (15%), and 11 (4%) were positive for IgG antibodies against SFG *Rickettsia*, *Orientia*, TG *Rickettsia*, and *Coxiella*, respectively. Among those seropositive against more than one agent, the commonest was dual seropositivity against SFG and TG (15%), followed by SFG and STG (13%). The highest antibody titers observed against antigen groups for SFG, TG, and STG was 1:512 and for *Coxiella* was 1:320 (Table 2). The distribution of the 136 seropositive cases within the approximate location of the sampling sites is shown in Fig. 1. These seropositivity rates in domestic animals when compared to seropositivity in humans (Tshokey et al. 2017) from the same study sites (Table 3) showed that rural Samtse and rural Chukha districts in the south-western Bhutan had higher SFG prevalence and rural Trongsa and rural Zhemgang in central Bhutan had higher STG exposure in both human and animal populations. In addition, although generally of low prevalence in both human and animal populations, QF seemed to be more prevalent in rural Mongar, rural Punakha, and rural Chukha districts (Table 3).

Seropositivity rates against the four different rickettsial infections among the sampled animal population of different species are presented in Table 4. Only positive sera are presented in this table. Dogs exhibited the highest seropositivity against SFG *Rickettsia* (55%) and TG *Rickettsia* (45%); horses against STG (91%), and goats against QF (9%). Differences in seropositivity rates between animal species may have been significant for SFG, TG, and STG, but not for QF. The differences in the seropositivity rates of the four infections between districts may have been significant for TG and STG, but not for SFG and QF (Table 4). These comparative seropositivity data between different animal species and different study sites should be interpreted with caution due to the overall small sample size and variable number of each animal species sampled from each study sites.

Given the unequal number of animal species sampled from each study site, logistic regression analysis would have been questionable and thus data are not presented in detail. However, for a closer comparison, a few comparisons with high odds ratios were probably significant and worth mentioning. Compared to other animals, dogs appeared to have a high risk of being exposed to SFG (OR 5.71, 95% CI 3.02–10.80, $p < 0.001$), TG (OR 48.74, 95% CI 11.29–210.32, $p < 0.001$), and STG (OR 6.80, 95% CI 3.32–13.95, $p < 0.001$), but not against QF (OR 1.95, 95% CI 0.42–8.95, $p = 0.390$). In addition, the differences in seropositivity against STG between animals from urban and rural areas appeared to be significant with rural animals at double the risk of being exposed compared with urban animals (OR 2.07, 95% CI 1.14–3.78, $p = 0.017$). This difference was not seen for SFG, TG, or QF.

Discussions

This is the first serological study of rickettsial diseases and QF in domestic animals in Bhutan. A high seropositivity of

Table 1. Animal Species Sampled from the Urban and Rural Study Sites in the Eight Study Districts of Bhutan

Study sites	*Cattle*	*Dog*	*Goat*	*Sheep*	*Horse*	*Yak*	*Cat*	*Total (%)*
Mongar	20	8	0	0	0	0	1	29 (9.9)
Urban	10	8	0	0	0	0	1	19 (6.5)
Rural	10	0	0	0	0	0	0	10 (3.4)
Punakha	7	9	5	0	0	0	3	24 (8.2)
Urban	0	0	0	0	0	0	0	0 (0.0)
Rural	7	9	5	0	0	0	3	24 (8.2)
Samtse	21	15	20	0	0	0	0	56 (19.0)
Urban	11	8	10	0	0	0	0	29 (9.9)
Rural	10	7	10	0	0	0	0	27 (9.1)
Trashigang	12	12	0	0	1	0	0	25 (8.5)
Urban	0	11	0	0	0	0	0	11 (3.7)
Rural	12	1	0	0	1	0	0	14 (4.8)
Chukha	20	0	20	20	0	0	0	60 (20.4)
Urban	11	0	10	10	0	0	0	31 (10.5)
Rural	9	0	10	10	0	0	0	29 (9.9)
Trongsa	10	10	0	0	0	0	0	20 (6.8)
Urban	10	0	0	0	0	0	0	10 (3.4)
Rural	0	10	0	0	0	0	0	10 (3.4)
Zhemgang	20	10	0	0	10	0	0	40 (13.6)
Urban	10	0	0	0	0	0	0	10 (3.4)
Rural	10	10	0	0	10	0	0	30 (10.2)
Thimphu	1 0	20	0	0	0	10	0	40 (13.6)
Urban	10	10	0	0	0	0	0	20 (6.8)
Rural	0	10	0	0	0	10	0	20 (6.8)
Total (%)	120 (40.8)	84 (28.6)	45 (15.3)	20 (6.8)	11 (3.7)	10 (3.4)	4 (1.4)	294 (100)

Table 2. Antibody Titers in All Animals Against the Four Rickettsial Antigen Groups

Rickettsial antigen groups	IgG antibody titer[a]					
	<1:32 (Screening negative)	1:32	1:64	1:128	1:256	1:512
SFG						
Rickettsia australis	223 (75.8)	45 (15.3)	9 (3.1)	10 (3.4)	7 (2.4)	0 (0.0)
Rickettsia honei	196 (66.8)	21 (7.1)	21 (7.1)	23 (7.8)	18 (6.1)	15 (5.1)
Rickettsia conorii	196 (66.7)	21 (7.1)	34 (11.6)	23 (7.8)	14 (4.8)	6 (2.0)
Rickettsia africae	185 (62.9)	11 (3.7)	17 (5.8)	44 (15.0)	28 (9.5)	9 (3.1)
Rickettsia rickettsii	185 (62.9)	2 (0.7)	10 (3.4)	30 (10.2)	36 (12.2)	31 (10.6)
Rickettsia felis	228 (77.6)	21 (7.1)	39 (13.3)	4 (1.4)	1 (0.3)	1 (0.3)
TG						
Rickettsia prowazekii	223 (75.8)	25 (8.5)	27 (9.2)	15 (5.1)	2 (0.7)	2 (0.7)
Rickettsia typhi	226 (76.9)	26 (8.8)	27 (9.2)	12 (4.1)	1 (0.3)	2 (0.7)
STG						
Orientia tsutsugamushi (Gilliam strain)	204 (69.4)	29 (9.9)	13 (4.4)	15 (5.1)	20 (6.8)	13 (4.4)
O. tsutsugamushi (Karp strain)	219 (74.5)	18 (6.1)	22 (7.5)	13 (4.4)	14 (4.8)	8 (2.7)
O. tsutsugamushi (Kato strain)	240 (81.6)	2 (0.7)	20 (6.8)	20 (6.8)	11 (3.8)	1 (0.3)
Orientia chuto	272 (92.5)	10 (3.4)	7 (2.4)	4 (1.4)	1 (0.3)	0 (0.0)

Coxiella burnetii	IgG antibody titer			
	<1:80	1:80	1:160	1:320
C. burnetii phase II antigen	273 (92.9)	10 (3.4)	10 (3.4)	1 (0.3)

[a]Sera were screened at a dilution of 1:32 (SFG, TG, and STG) and 1:80 (*C. burnetii*) and titrated to end-titer if positive. A titer of ≥1:64 (for SFG, TG, and STG) and ≥1:160 (for *C. burnetii*) was taken as positive.
SFG, spotted fever group; STG, scrub typhus group; TG, typhus group.

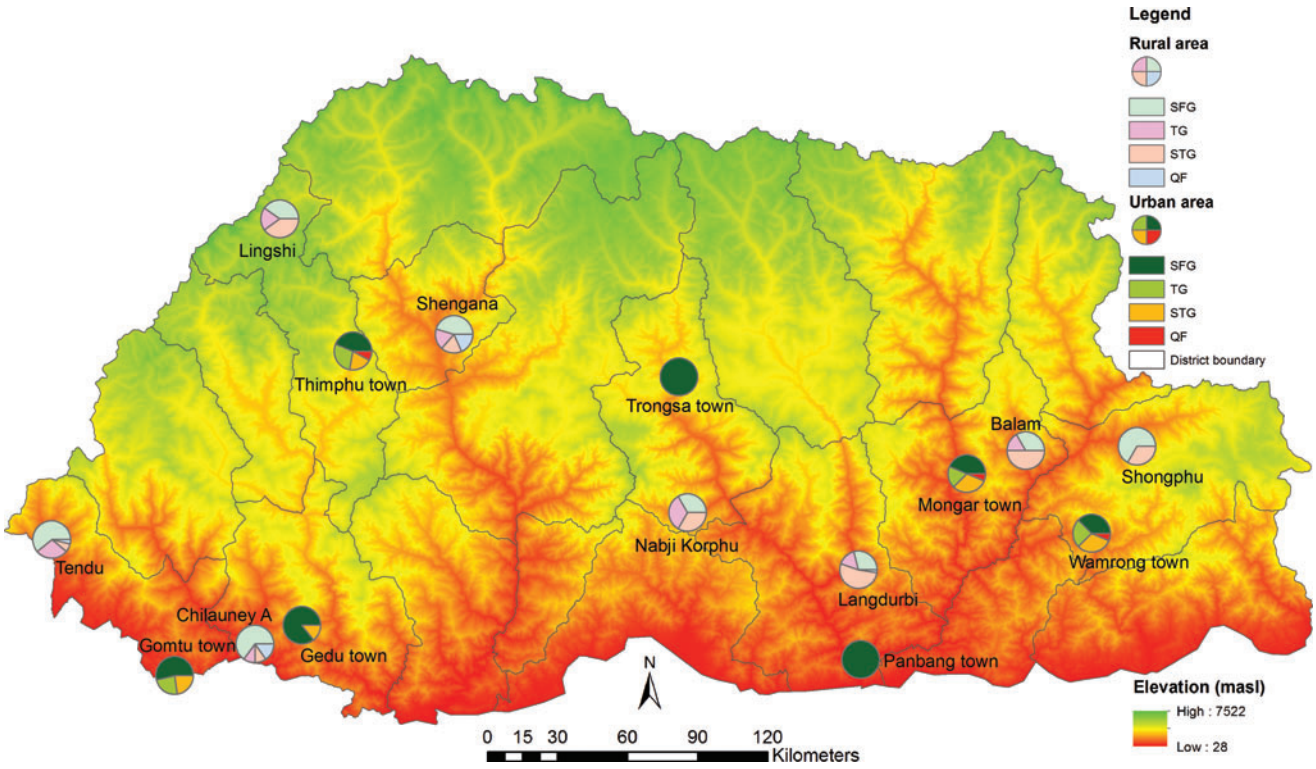

FIG. 1. Map of Bhutan showing pie charts demonstrating proportion of animals seropositive against SFG, STG, TG, and QF in each urban and rural sampling area. Seronegative animals are not shown on the map. Pie chart location provides an approximate location of sampling sites. QF, Q fever; SFG, spotted fever group; STG, scrub typhus group; TG, typhus group. Color images available online at www.liebertpub.com/vbz

Table 3. Comparison of Seropositivity Rates Against Four Rickettsiae Among Human and Animal Population in the Study Sites/Districts

	SFG positive (%)		*STG positive (%)*		*TG positive (%)*		*QF positive (%)*	
Districts	*Animal*	*Human*	*Animal*	*Human*	*Animal*	*Human*	*Animal*	*Human*
Mongar	9 (31.0)	13 (12.0)	8 (27.6)	13 (12.0)	4 (13.8)	2 (1.9)	1 (3.4)	13 (12.0)
Urban	7 (36.8)	3 (9.4)	5 (26.3)	2 (6.3)	3 (15.8)	1 (3.1)	1 (5.3)	5 (15.6)
Rural	2 (20.0)	10 (13.2)	3 (30.0)	11 (14.5)	1 (10.0)	1 (1.3)	0 (0.0)	8 (10.5)
Punakha	5 (20.8)	18 (16.7)	2 (8.3)	10 (9.3)	2 (8.3)	10 (9.3)	2 (8.3)	3 (2.8)
Urban	0 (0.0)	4 (12.5)	0 (0.0)	3 (9.4)	0 (0.0)	1 (3.1)	0 (0.0)	0 (0.0)
Rural	5 (20.8)	14 (18.4)	2 (8.3)	7 (9.2)	2 (8.3)	9 (11.8)	2 (8.3)	3 (4.0)
Samtse	24 (42.9)	22 (20.4)	5 (8.9)	25 (23.2)	11 (19.6)	2 (1.9)	1 (1.8)	2 (1.9)
Urban	7 (24.1)	5 (15.6)	3 (10.3)	5 (15.6)	3 (10.3)	0 (0.0)	0 (0.0)	1 (3.1)
Rural	17 (63.0)	17 (22.4)	2 (7.4)	20 (26.3)	8 (29.6)	2 (2.6)	1 (3.7)	1 (1.3)
Trashigang	10 (40.0)	18 (16.7)	7 (28.0)	19 (17.6)	4 (16.0)	5 (4.6)	1 (4.0)	6 (5.6)
Urban	6 (54.5)	5 (15.6)	5 (45.5)	3 (9.4)	4 (36.4)	0 (0.0)	1 (9.1)	1 (3.1)
Rural	4 (28.6)	13 (17.1)	2 (14.3)	16 (21.1)	0 (0.0)	5 (6.6)	0 (0.0)	5 (6.6)
Chukha	19 (31.7)	29 (26.9)	3 (5.0)	32 (29.6)	2 (3.3)	0 (0.0)	3 (5.0)	9 (8.3)
Urban	6 (19.4)	6 (18.8)	1 (3.2)	4 (12.5)	0 (0.0)	6 (7.9)	0 (0.0)	1 (3.1)
Rural	13 (44.8)	23 (30.3)	2 (6.9)	28 (36.8)	2 (6.9)	6 (5.6)	3 (10.3)	8 (10.5)
Trongsa	9 (45.0)	14 (13.0)	6 (30.0)	46 (42.6)	6 (30.0)	2 (1.9)	0 (0.0)	8 (7.4)
Urban	3 (30.0)	1 (3.1)	0 (0.0)	4 (12.5)	0 (0.0)	0 (0.0)	0 (0.0)	4 (12.5)
Rural	6 (60.0)	13 (17.1)	6 (60.0)	42 (55.3)	6 (60.0)	2 (2.6)	0 (0.0)	4 (5.3)
Zhemgang	13 (32.5)	14 (13.0)	20 (50.0)	45 (41.7)	6 (15.0)	0 (0.0)	1 (2.5)	7 (6.5)
Urban	2 (20.0)	4 (12.5)	0 (0.0)	11 (34.4)	0 (0.0)	0 (0.0)	0 (0.0)	4 (12.1)
Rural	11 (36.7)	10 (13.2)	20 (66.7)	34 (44.7)	6 (20.0)	0 (0.0)	1 (33.3)	3 (4.0)
Thimphu	17 (42.5)	8 (7.4)	11 (27.5)	5 (4.6)	10 (25.0)	3 (2.8)	2 (5.0)	12 (11.1)
Urban	11 (55.0)	2 (6.3)	5 (25.0)	4 (12.5)	7 (35.0)	2 (6.3)	2 (10.0)	3 (9.4)
Rural	6 (30.0)	6 (7.9)	6 (30.0)	1 (1.3)	3 (15.0)	1 (1.3)	0 (0.0)	9 (11.9)
Overall	106 (36.1)	136 (15.7)	62 (21.1)	195 (22.6)	45 (15.3)	30 (3.5)	11 (3.7)	60 (6.9)
Urban	42 (32.3)	30 (11.7)	19 (14.6)	36 (14.1)	17 (13.1)	4 (1.6)	4 (3.1)	19 (7.4)
Rural	64 (39.0)	106 (17.4)	43 (26.2)	159 (26.2)	28 (17.1)	26 (4.3)	7 (4.3)	41 (6.7)

Human data from Tshokey et al. (2017).
QF, Q fever.

domestic animals against rickettsial infections especially SFG (36%) and STG (21%) was observed. The findings of this study were consistent with the findings of a seroprevalence study of the same infections in the human population (Tshokey et al. 2017) from the same study sites. While the seropositivity of SFG was higher in animals, STG was higher in the human population. This could be due to differences in vectors and their exposure risks; tick infestation of domestic animals being high in Bhutan favoring tick-borne SFG in animals, while human exposure to rodents (host for mites) would possibly explain human mite-borne STG. This difference also possibly suggests that the extent to which human disease occurs may not always be proportionate to exposure in animals although animals and their arthropod vectors act as the reservoirs for these zoonoses. The observed dual seropositivity against SFG and TG of 15% was likely due to cross-reacting antibodies between the two rather than true exposure to both groups. However, dual seropositivity of about 13% against SFG and STG reveals the true proportion of exposure to both these agents in domestic animals since SFG and STG antigens are distinct and serological cross-reactions are unlikely. The key message from this and the related human study is that STG and SFG are widely prevalent in both the human and animal population in Bhutan, with some likely hot-spots. Findings on TG *Rickettsia* and QF were less convincing in both the human and animal populations due to low seropositivity rates.

There is overall limited data on animal seroprevalence to *Rickettsia* and *Orientia*, especially in Asia, compared to *Coxiella* which have been studied widely. Nevertheless, studies on *Coxiella* have concentrated on goats, sheep, cattle, and horses but no other animal species. The seropositivity rate of SFG (36%) among domestic animals in this study was higher than that reported in Mongolia ($\approx$20%). In the Mongolian study, seroprevalence rate was 30%, 13%, 21%, 35%, and 2% for cattle, goats, sheep, horses, and camels, respectively (von Fricken et al. 2018). Although data would be specific to the study sites rather than nationally representative, the prevalence of SFG in Bhutanese dogs (55%) was similar to Sri Lankan dogs ($\approx$42%), whereas seropositivity against STG appeared to be higher in Bhutanese dogs (45%) than Sri Lankan dogs (24%) in the areas studied (Nanayakkara et al. 2013). These findings suggest that dogs may act as an important reservoir of rickettsial infections in Bhutan.

In the case of *Coxiella*, seropositivity rate was low in the sampled domestic animals in this study (4%) as well as in the human population (7%) in the same areas (Tshokey et al. 2017). The seropositivity of QF ($\approx$4%) in this study appeared lower than that reported in studies from India at 14%

Table 4. Differences in Seropositivity Rates Against the Four Rickettsiae by Animal Species and Study Sites

	SFG (n = *106*)		*STG* (n = *62*)		*TG* (n = *45*)		*QF* (n = *11*)	
Variables	*Positive*	p	*Positive*	p	*Positive*	p	*Positive*	p
Animal species								
Cattle (*n* = 120)	21	<0.001*	13	<0.001*	2	<0.001*	3	0.444
Dog (*n* = 84)	46		38		38		4	
Goat (*n* = 45)	22		0		5		4	
Sheep (*n* = 20)	10		1		0		0	
Horse (*n* = 11)	5		10		0		0	
Yak (*n* = 10)	2		0		0		0	
Cat (*n* = 4)	0		0		0		0	
Study sites (8 districts)								
Mongar (*n* = 29)	9	0.522	8	<0.001*	4	0.041*	1	0.846
Urban (19)	7		5		3		1	
Rural (10)	2		3		1		0	
Punakha (*n* = 24)	5		2		2		2	
Urban (0)	0		0		0		0	
Rural (24)	5		2		2		2	
Samtse (*n* = 56)	24		5		11		1	
Urban (29)	7		3		3		0	
Rural (27)	17		2		8		1	
Trashigang (*n* = 25)	10		7		4		1	
Urban (11)	6		5		4		1	
Rural (14)	4		2		0		0	
Chukha (*n* = 60)	19		3		2		3	
Urban (31)	6		1		0		0	
Rural (29)	13		2		2		3	
Trongsa (*n* = 20)	9		6		6		0	
Urban (10)	3		0		0		0	
Rural (10)	6		6		6		0	
Zhemgang (*n* = 40)	13		20		6		1	
Urban (10)	2		0		0		0	
Rural (30)	11		20		6		1	
Thimphu (*n* = 40)	17		11		10		2	
Urban (20)	11		5		7		2	
Rural (20)	6		6		3		0	

*p value <0.05 indicates significant differences in seropositivity rates between different animal species or the eight study sites (districts).

in animals although these had reproductive disorders (Vaidya et al. 2010); at 14% in Tibetan sheep in China (Yin et al. 2015b) and 14% in free-range yaks in China (Yin et al. 2015a). Unlike sheep and yaks in China, none of the few sheep and yaks in the current study showed evidence of exposure to *Coxiella*. A meta-analysis on *C. burnetii* in horses world-wide reported a seroprevalence of 16%, although none of the 122 cases of equine abortion, stillbirth, or neonatal foal death was positive for *Coxiella* DNA (Marenzoni et al. 2013). The few Bhutanese horses in this study did not have evidence of exposure to *Coxiella*. With this apparently low prevalence of QF, for Bhutan, a developing country with numerous other priorities, investing in QF vaccines for domestic animals and veterinarians may not merit immediate consideration. This preliminary data, however, need to be confirmed with larger nationally representative studies. Nevertheless, other activities aimed at adequate case identification and management are likely to be important with clear strategies for prevention and control through close human-animal sector collaboration.

Although some apparently significant findings have been seen in this first study in animals, percentage seropositivity results and their significance should be interpreted with caution due to wide variations in the number of animals sampled from each area and within each species. For instance, yaks being found only in the highlands, the 10 yaks tested were from one specific area in Thimphu district only and 10 of the 11 horses tested were from the one sampling area in Zhemgang district. However, these preliminary findings are the baseline data for future research into the epidemiology of rickettsial diseases in Bhutanese domestic animals.

In Bhutan, although some human-livestock sector collaborations were initiated recently, there are no ongoing collaborative activities specific to research and management of rickettsioses between the two sectors. Currently, diagnostics is limited to the use of rapid immunochromatographic test kits for scrub typhus alone in the human sector with none available in the livestock sector for rickettsial diseases. Clinical management, prevention, and control guidelines are nonexistent in both sectors. This study has brought together key personnel from both the sectors and the data generated should prompt taking these typical ''One Health'' collaborative activities further.

This study has significant limitations, the main one being the opportunistic sampling strategy. Assumptions were made

during testing due to unavailability of known positive and negative controls for some animal species (*e.g.*, yaks). The number of animals sampled from each district or sampling areas and within each animal species was not uniform between sampling areas (no fixed sample size), thereby making comparisons contentious. For some animal species, there were only a few animals sampled and consequently few or no seropositive cases obtained.

Conclusion

In conclusion, this study presents preliminary data on rickettsial diseases in Bhutanese domestic animals and these results constitute baseline data for Bhutan. A high proportion of different animal species were seropositive against SFG and STG, but with fewer animals seropositive against TG and QF. These findings were consistent with the seroprevalence of the same infections in human population in the same areas of Bhutan. Findings from this study and related studies in the human population appear to demonstrate high prevalence of exposure to rickettsiae in both human and animal populations in Bhutan. They may be causally related but further studies would be needed to confirm it. These results call for increased collaboration between the human and animal sectors in rickettsial diseases research aimed at developing diagnostic and treatment guidelines and formulating prevention and control measures through a One Health approach.

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Chapter 11: Discussions

11.1 Healthcare situations in Bhutan

Bhutan is a small Himalayan kingdom situated between China in the north and India in the south, with an estimated population of 770,000[336] living in the 20 administrative districts **(Fig 11.1)**. The country has a total area of about 38,390 square kilometres. Currently, about 70% of this area is under forest cover[337] and Bhutan's constitution mandates that at least 60% of the total area remain forested at all times to come[338]. Environmentally, about 70% of the Bhutanese population is rural and agriculture is the main sources of income. About 90% of the rural population own livestock as primary or supplementary sources of living. Bhutan's recent general literacy rate of 71%[336] is comparable with neighbouring countries but stands low compared to developed countries.

Figure 11.1. Map of Bhutan with 20 administrative districts in relation to China in the north and India in the south (from https://d-maps.com)

In Bhutan, primary healthcare is provided free of cost by the state as mandated by the kingdom's constitution[338]. Healthcare is delivered through health facilities categorized from the highest to the lowest level as the National Referral Hospital (NRH), Regional Referral Hospitals (RRH), General Hospitals (GH), District Hospitals (DH), Basic Health Unit I (BHU-I) and Basic Health Unit II (BHU-II). All these categories have one or more Outreach Clinics (ORCs) where community health staffs from the hospitals visit monthly to provide services related to maternal and child health, routine immunizations and attend to minor ailments. As of 2018, there are 29 hospitals (including NRH, RRH, GH and DH), 211 BHUs (I and II) and over 550 ORCs[339]. The category of the health facility also determines the level of care and type of medicines available in that facility as directed by the national essential medicines list[340]. There are no private medical clinics/hospitals and the few private pharmacy retailers mostly deal with medicines which are not available in the essential medicines list or different formulations of those medicines available from government hospitals (such as syrups for children).

With only 345 medical doctors (all categories) and 1264 nurses (all categories) in the country as of 2017[339], there is a continuing shortage of healthcare professionals in Bhutan. Although the situations have improved in the last decade, it was common for a lone doctor in a district hospital to manage all types of clinical cases both in the outpatient and inpatient units, round the clock. There are a small number of clinical specialists even at the referral hospital level and almost all DHs are managed by general medical graduates. All DH laboratories are basic and tests for infectious diseases are limited to a few immunochromatographic/rapid test kits (for dengue fever, malaria, enteric fever, scrub typhus (ST), human immune deficiency virus (HIV), hepatitis B, hepatitis C and syphilis) and microscopy (for malaria and tuberculosis). Only laboratories in the NRH and RRH are equipped with some higher tests than those available in DHs including enzyme immunoassays (EIAs) and microbiological culture facilities. There are

a few clinical practice guidelines on infectious diseases including tuberculosis, malaria and HIV developed by the respective public health programs of these infectious diseases. There is a national antibiotic guideline and a guideline on scrub typhus has been drafted in 2017. There are no surveillance or clinical practice guidelines specific to the rickettsial disease and QF in both the human and animal sectors. Currently, there is poor awareness amongst health professionals on rickettsial infections and QF.

11.2 Challenges in the diagnosis and management of infectious diseases in Bhutan

Bhutan has a conducive environment for infectious diseases due to many factors. Sanitation and living conditions are still poor in many parts of the country. Food and water-borne diseases are still widespread. Due to the high forest cover, many agricultural activities and livestock ownership, there is close interaction between humans, animals (including wildlife) and the natural vegetation which poses significant risks of exposure to vector-borne diseases including rickettsial diseases **(Fig 11.2)**. In addition, health literacy is low and ignorance on health and diseases hampers prevention and control practices at an individual and community level. Seeking prompt medical attention after falling ill remains challenging. In a highly religious, spiritual and traditional society like Bhutan, the practice of seeking medical intervention only after the complete exhaustion of home and traditional remedies are still prevalent. High terrains and long distance from health facilities add to these problems. Delayed consultation with health professionals leads to serious complications from the infections, such as the four deaths that occurred in the two outbreaks of ST[173, 341] resulting into complications of the central nervous system (meningoencephalitis).

Figure 11.2. A typical rural village in Bhutan by the riverside

Bhutan is a developing country with a lot of priorities. Due to limited financial and human resources, diagnostic laboratories in the hospitals are equipped only with the basic test parameters. As of now, there are no dedicated research bodies or designated funding for research in any organizations. As a result, the epidemiology of many diseases remains unknown in the country. Because of the unique geographical and demographic features, the epidemiology of diseases from the neighbouring countries may not always be similar to Bhutan. This leads to widespread blind therapeutic practices and poor control and preventive measures of many easily treatable and preventable infectious diseases. In addition, without adequate laboratory support, febrile illnesses like malaria, enteric fever, leptospirosis, dengue and viral fevers, which remain prevalent, often challenge the diagnosis of rickettsial diseases. In the Bhutanese setting, it is still a clinical dogma to test every unexplained febrile case for malaria and enteric fever (especially in the endemic areas). As per the World Health

Organization (WHO), about 70% of the 1.8 billion people in the ten countries of the WHO South-East Asia Region (WHO/SEAR), are at some risk for malaria with 26% at high risk. However, the WHO recently reported significant progress in malaria control programs with seven countries reporting reduced malaria incidences. Three countries including Bhutan are in the pre-elimination and elimination stages[342]. In Bhutan, this reduced incidence of malaria has resulted from an effective malaria control program in the country. Many hospitals in Bhutan still use the obsolete Widal test for typhoid fever due to its cheap and easy availability. Throughout the country, many febrile cases were treated (and re-treated) as typhoid fever based on the Widal test results which detects high antibody titres even in patients treated several times for the same illness. A fever study directed at patients with clinically suspected enteric fever found only 24% (109/457) testing positive for antibodies against *Salmonella typhi* and revealed a huge gap in the understanding of causes of fever in the Bhutanese patients[343]. It is a general understanding that sanitation and water supply have improved in the country and this would have led to a decline in food and water-borne infections like *S. typhi.* An outbreak of a febrile illness in a locality (later established as scrub typhus[341]) and the introduction of a rapid test kit for scrub typhus in some health centres complicated the 'testing typhoid fever dogma' and raised some awareness amongst medical professionals. Many cases that would have been treated as typhoid fever in the absence of a scrub typhus test kit, now tested positive for scrub typhus. In many instances both the Widal test and scrub typhus test were positive. Situations in a few hospitals were further complicated by the supply of a rapid leptospirosis test kit through some surveillance project. Unfortunately, the emergence of dengue fever and chikungunya with several outbreaks since 2004 has complicated the matter further.

As malaria and typhoid fever, the assumed main causes of undifferentiated fever in the country decline, it is timely that other causes of fever are sought after, and appropriate medical and public health interventions undertaken. Therefore, this study on rickettsial infections and QF

was apt, timely and crucial to fill the emerging gap in the understanding of the etiologic agents of undifferentiated fevers in Bhutan. This project is the first comprehensive study on the epidemiology of *Rickettsiae, Orientia* and *Coxiella* in Bhutan. The comprehensiveness comes from including all important rickettsial pathogens (including *Coxiella*), enumerating both case incidence (acutely ill patients) and prevalence (healthy people) in the community, and including both human and animal subjects. Additionally, the limited and scattered data on ST was synthesized and an outbreak of ST investigated. All these components of the project have successfully generated the first baseline data for Bhutan on rickettsial diseases, ST and QF and established their endemicity in the country. The findings of this project should guide the development of therapeutic guidelines and initiate prevention and control measures. It should also prompt further research activities both in the human and livestock sectors.

11.3 Rickettsial diseases

Rickettsiae are emerging pathogens with continuing discovery of new species which currently stands at a total of 30 species, 20 known to be pathogenic[344]. Preceding to the current study, the only report of *Rickettsia* in Bhutan was from a small surveillance conducted by the National Public Health Laboratory (PHL) wherein 33 blood samples from febrile patients from Bhutan were sent to the Wellcome Trust-Mahosot Hospital-Oxford University-Mahosot Hospital Tropical Medicine Research Collaboration, Vientiane, Lao PDR for testing. Twelve of the 33 (36.4%) patients had high IgG and IgM antibody titers against *R. typhi* (murine typhus)[256]. However, almost all these 12 seropositive patients also had high antibody titers against *O. tsutsugamushi*. Since the patients were from the area where an outbreak of ST has taken place in the previous months, it was thought that the presence of antibodies against *R. typhi* was due to recent/past exposures and the current episodes of illness were from ST. Regardless of the

final diagnosis, those laboratory results have proven that murine typhus was prevalent in Bhutan.

The current study (work included in this thesis), looked at the two pathogenic groups of *Rickettsia*, the Spotted Fever Group (SFG) and the Typhus Group (TG), but did not consider individual species. To differentiate individual species with serological methods is unreliable due to cross-reactions between the different species within the groups and between the two groups. At least 15% (159/1044) of patients with acute undifferentiated fevers attending 14 hospitals in Bhutan were due to a rickettsiosis of which 46 (4.4%) were due to SFG, 4 (0.4%) due to TG and 10 (1%) due to both[172]. It is most likely that those patients positive for both SFG and TG were due to cross-reactions rather than dual infections. About 49% of 864-healthy Bhutanese had evidence of past exposure to a rickettsiosis of which 15.7% were exposed to SFG and 3.5% to TG[258]. In animals, a seropositivity rate of 46% (106/ 294) was detected; 106 (36%) and 45 (15%) being positive against SFG and TG respectively[345]. The TG *Rickettsia* detected in this study were most probably *R. typhi* rather than *R. prowazekii*. This assumption is based on the previously documented prevalence of *R. typhi* and the nature of the illnesses which could have been more severe and greater in number with likely outbreaks. In countries neighbouring Bhutan, a high *R. typhi* seropositivity rate of 66.6% (805/1209) in Bangladesh[263] and 17% (21/125) in Nepal[264] were reported among febrile patients in hospitals. Among healthy people in northeast India, bordering Bhutan, a seroprevalence of 13.8% and 4.2% against SFG and TG respectively have been reported[259]. These data together imply that the region is highly endemic in rickettsial diseases and a collaborative effort is warranted to focus on national and cross-border prevention and control activities.

In the past, vector-borne zoonoses like rickettsioses were not thought important in travel medicine. However, reports of travel-associated rickettsial infections, mainly murine typhus caused by *R. typhi*, Mediterranean spotted fever caused by *R. conorii*, African tick bite fever

caused by *R. africae*, and ST caused by *O. tsutsugamushi* are on the rise[346]. Bhutan is an emerging destination for travellers from all over the world. Tourist activities like trekking, mountain biking, hiking and community-home-stays are high-risk activities for vector-borne infections, especially in a country with high vegetation coverage like Bhutan. Understanding the epidemiology of these infections in the country would be helpful to provide travel advisories to the potential travellers to Bhutan. This knowledge would also enable travellers to be aware of such infections and be able to alert their physicians in case of any illnesses after they return to their home country. Currently, Bhutan lacks such travel advisories and other public health services to foreign travellers and initiating such a system would benefit the increasing international and local travellers.

R. felis is an emerging rickettsial pathogen with a worldwide distribution in mammals, humans, and ectoparasites. The clinical manifestations of *R. felis* infections resemble those of murine typhus and dengue, which makes them difficult to diagnose without an appropriate laboratory test. For this reason, infections due to this emergent pathogen are likely underestimated and misdiagnosed. Further research should be conducted to determine the actual incidence of *R. felis* infection in humans, the spectrum of clinical signs and symptoms, and the severity of this infection and also to assess the impact on public health[147]. A travel related infection from *R. felis* was reported in a 57-year-old Italian woman who became unwell 14 days after returning from a two-weeks travel to Nepal[150]. The high prevalence (46%) of *R. felis* infection in Bangladesh suggests that this infection is endemic in the country and might be associated with contact between humans of low socioeconomic status and a large number of stray cats and dogs[148]. In contrast, cases of *R. felis* infection in humans reported to date in China, Taiwan, Thailand, and Laos have been very few. These data clearly indicate that R. felis is prevalent in the south and east Asian region. Infections due to *R. felis* could also be prevalent in a

developing country like Bhutan, especially in places where there are poor living conditions and high contact with cats and dogs.

Diagnostic challenges of rickettsial diseases impact their recognition, identification and differentiation of rickettsial species causing different clinical symptoms[347]. Serological methods are the most commonly used method, but it is difficult or impossible to identify the infecting rickettsial species because of cross-reactivity among antigens of pathogens within the same genus. For instance, *R. felis* was earlier misdiagnosed as *R. typhi* probably because it responds serologically as though it were a TG Rickettsia showing strong cross-reaction[261]. As a result, most studies have not characterized the *Rickettsia* species involved in their studies. Despite this drawback, serology remains the diagnostic tool of choice due to its quick turnaround time, relatively easy procedure and comparatively low cost compared to molecular diagnostics.

Evidence on laboratory-acquired infections (LAIs) from *O. tsutsugamushi* and *R. typhi* have been enumerated. From 1931 to 2000, a record of 25 cases of LAIs with 8 (32%) deaths from *Orientia* and 35 cases due to *R. typhi* (with no deaths) was published. The deaths from ST occurred in the pre-antibiotic era. Findings indicated that the highest risk activities were working with infectious laboratory animals involving significant aerosol exposures, accidental self-inoculation or bite related infections. A risk-based biosafety approach for in vitro and *in vivo* culture of *Orientia* and *R. typhi* would require only high-risk activities be performed in high containment BSL3 laboratories. However, the authors felt that relatively low-risk activities including inoculation of cell cultures or the early stages of *in vitro* growth using low volumes/low concentrations of infectious materials can be performed safely in BSL2 laboratories within a biological safety cabinet[348]. Additionally, *Coxiella* is a category B bioterrorism agent[100, 101] as per the CDC classification and is considered highly infectious. The

risks associated with LAIs from these organisms make it unfeasible for suitable laboratory work to be carried out in low resource countries with poorly equipped laboratories.

11.4 Scrub typhus

Compared to other rickettsial diseases and QF, ST was better known among health professionals in Bhutan. This could have resulted from an outbreak of ST (in 2009) in a rural locality which affected several people with 18 hospital admissions and three deaths[341]. Two of the deaths had symptoms like dengue shock syndrome and the third had an intestinal perforation. During this outbreak, cases were first managed as dengue fever and dengue shock syndrome and a few even tested positive for dengue IgM with a rapid test kit. No test for ST was conducted due to unavailability of test kits. However, due to the presence of ST-like symptoms with typical eschars in many cases that responded well to doxycycline, it was concluded to be an outbreak of ST. Another outbreak of ST in 2014 in a remote primary school affected over 40 people and two students lost their lives. Both deaths have resulted from central nervous system (CNS) complications presenting as meningoencephalitis and delayed medical attention. Meningoencephalitis is a common complication of ST with several published cases and studies[180, 288, 349]. Therefore, it is crucial to include ST in the differential diagnosis of meningitis/encephalitis syndromes, especially in the endemic areas. The third death in the first outbreak due to intestinal perforation should also be explained by ST related pathology since ST has been found to precipitate intestinal perforation[350].

Eschar **(Fig 11.3)** was seen in about 70% of the cases in the first outbreak and 67% of the cases in the second outbreak. Presence of an eschar is a clinically useful observation and considered pathognomonic for ST[351]. Presence of an eschar was also significantly associated with ST (p<0.001) in the Bhutanese patients[172]. A patient can have single or multiple eschars at different sites of the body at presentation[352] and it is important to conduct a detailed physical

examination since eschars may occur at concealed body areas and can be easily missed. A Thai study reported eschars to be mostly located in the perineal, inguinal, and buttock areas in males, and on the head and neck in females[282]. A significant difference in the distribution of eschars was also observed between males and females in India with a preponderance of the chest and abdomen (42.3%) among females and the axilla, groin and genitalia (55.8%) in males[351]. Eschars are the site of the proliferation of *O. tsutsugamushi* and large amounts of the bacteria are present in them. Therefore, eschars are not only valuable for clinical diagnosis but are also a good specimen for *Orientia* DNA detection by PCR for early diagnosis as well as in differentiating other eschar-like crusted lesion[353]. However, an eschars or eschar-like lesions can also be seen in other rickettsial infections such as Thai Tick typhus or Flinders Island spotted fever (caused by *R. honei*)[136], Mediterranean Spotted Fever (caused by *R. conorii*)[129] and North Asian tick typhus/Siberian tick typhus (caused by *R. sibirica*)[145].

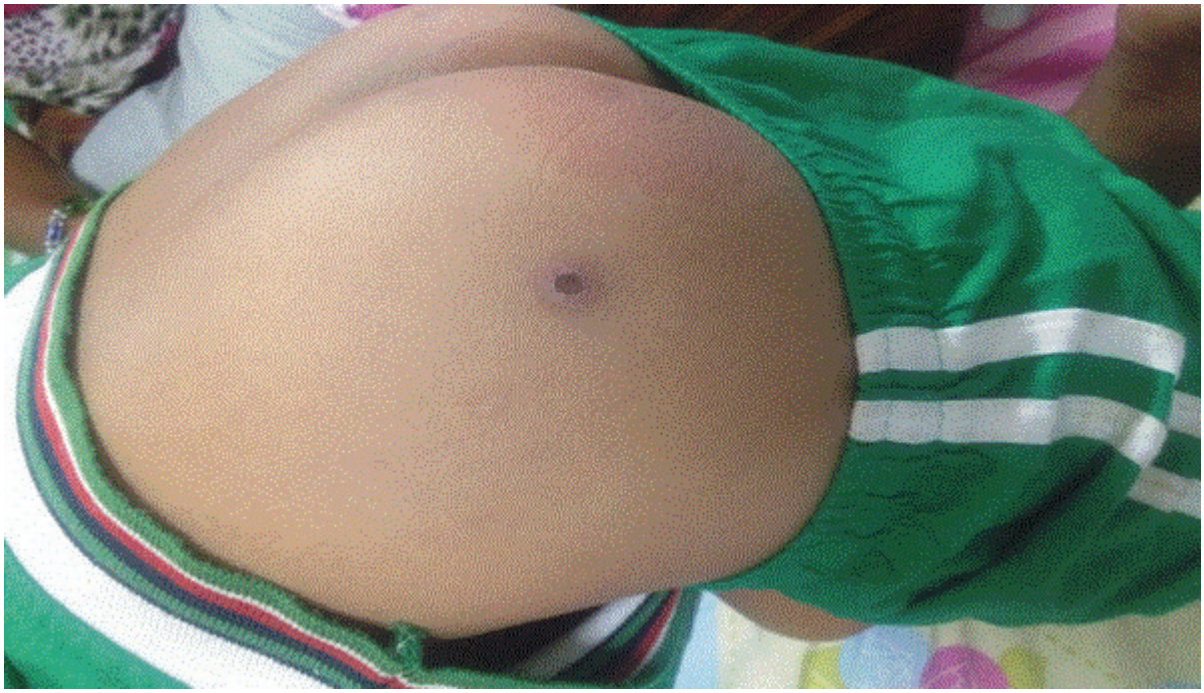

Figure 11.3. An eschar on the buttock of a male student during the outbreak of scrub typhus.in a remote primary school in Bhutan, 2014

True burden assessment of ST and other rickettsial infections including QF are lacking. The highly cited estimation that about one million cases occur annually with one billion people at risk of ST in the Asia Pacific region in 2003 [270] is probably obsolete and needs a serious review; firstly the claim was based on expert opinions during that time rather than true burden assessment, secondly ST has emerged and re-emerged in recent times and an exact national, regional or international burden assessment has never been conducted and may be impossible at best. Inaccessibility to healthcare, non-specific presentation of the infection and lack of diagnostic tests could result in many cases being undiagnosed and unreported. Therefore, the estimate of experts from 2003, maybe a huge underestimate of the actual disease occurrence. Despite the high burden of ST infection in Asia, only a few countries (South Korea, Japan, China and Thailand) have an established national surveillance system in place[184]. This has resulted in a huge gap in generating true estimates in individual countries and the region. Even countries with surveillance a system in place have no uniform diagnostic and reporting criteria and comparing reports between countries may be faulty. ST was included in the Bhutan national notifiable diseases list since 2008[272] but notification did not pick up. This resulted probably from the failure to disseminate the disease notification manual to the responsible medical professionals. This is typical of a developing country situation where a team develops some guideline or manual, but it remains in the office of the different departments within the Ministry of Health (MoH), citing financial constraints to circulate and brief/train field staffs on the documents. Such trends add a lot to the failure of public health activities, but this could be improved with the organized management of resources (human and financial), responsibility and accountability fixation in the respective organizations.

The recent emergence of ST in previously unknown areas and re-emergence in previously known areas have many explanations. The improved diagnostics methods, increased investigations and greater awareness amongst medical professionals[354] explain a large part of

the developments while climate change, global warming and changing land-use patterns with increased deforestations played a role in increasing the risk of contact with scrub vegetations and rodent populations[280, 355, 356]. This is true even for the Bhutanese setting. Awareness and diagnostics have increased after the first outbreak of ST in 2009. The MoH with the support of the World Health Organization Bhutan country office has organized few meetings and initiated the development of a clinical guideline on ST diagnosis and management which would go a long way in improving diagnosis and management of the infection. There is also increasing urbanization and deforestation resulting in increasing encroachment into vegetation areas. However, since the constitution of Bhutan mandates a forest cover of at least 60% at all times to come, deforestation is not seen as an emerging threat to changing disease modality in the country.

Availability and affordability of the diagnostic assays in a clinical or research laboratory are one of the most challenging factors, especially in a developing country. Although there are increasing penetration of more reliable diagnostic methods in many countries, many of the studies from the Indian subcontinent were based on the old Weil-Felix method or a point of care rapid test kits which have poor sensitivity and specificity. This probably makes the published results poorly comparable and questionable reliability. However, in the absence of the 'tests of choice', they are invaluable to establish the presence of the infections and inform the scientific community and the public on the diseases in a hospital, region or the country. Current diagnosis of ST in Bhutan is solely based on a rapid test kit that detects *O. tsutsugamushi* IgM, IgG and IgA antibodies (*SD Bioline Tsutsugamushi assay, Standard Diagnostics Inc, Republic of Korea*). The manufacturers of the kit claim a sensitivity of 99%, a specificity of 96% and a serological agreement of 97.5% with the indirect immunofluorescent assay, the accepted gold-standard method. However, in the samples from the outbreak of ST in 2014, there was only 75% agreement between this rapid test kit and the IFA assay of the ARRL.

Nevertheless, such rapid test kits are indispensable in a setting like Bhutan with limited resources for their low cost, ease of use and rapid results. In a remote area setting, higher sensitivity of a test kit resulting in overdiagnosis could be more useful and practical to save lives and avert complications rather than to underdiagnose and keep potential cases untreated. Yet, the choice of a test kit should be made after successful comparative studies of the different products in the local Bhutanese settings, unlike the current trend of selecting them based on the quoted price by the suppliers.

In the clinical setting, diagnosis of a rickettsial infection or ST with a single acute serum sample can be problematic and faulty especially in an endemic area. During a long-term (over 46 months) follow up of antibody levels in patients with ST, IgM levels slowly declined but remained elevated above the diagnostic cut-off for up to 12 months post-infection. In contrast, IgG levels continued to rise with a peak at 10 months, followed by a gradual decline over several months, remaining above the cut-off threshold for more than three years in a majority of the patients[72]. These finding adds emphasis to the unreliability of ST diagnosis with elevated IgM in a single serum sample as it remains elevated for up to 12 months after the infection and reaffirms the usefulness of serum IgG levels as an indicator of past infection. Similarly, when two groups of military personnel (first group consisting of actively deployed but healthy and second group with acute febrile illness) in northern Sri Lanka were tested and compared for rickettsial infections; 84% (48/57) of the healthy military personnel had serological evidence of exposure to rickettsioses and 67.3% (33/49) of the second group were serologically positive for acute rickettsioses[260]. This finding indicated that the pre-existing high seroprevalence of SFG rickettsioses in the endemic setting is likely to interfere with the serological diagnosis of acute SFG rickettsioses in this population. Therefore, it is essential to acquire two (an acute and a convalescent) samples to truly diagnose a current acute episode of the infection. However, this is practically challenging in a setting like Bhutan, where people, especially from

remote places travel for hours to reach a health centre for medical consultation and rush back at the earliest possible to attend to their daily chores and farms, especially in outpatients.

There are several unfolding issues on ST. Emerging drug-resistance in *Orientia* poses a great challenge to the management of otherwise an easily treatable infection. The first report of chloramphenicol and doxycycline-resistant strains of *O. tsutsugamushi* in 1996 in Chiangrai, in northern Thailand[238] and a suspicion of possible drug-resistant infection 2003 in south India[290] is worrying. If such resistant strains emerge or spread, it would be devastating for endemic countries (including Bhutan) with the added burden of testing drug resistance on top of their existing poor diagnostic facilities. Mortality estimates are higher in untreated cases[184] and a drug-resistant bacterium will have the same mortality rates even with timely diagnosis and treatment. Conventional drug sensitivity testing methods do not work for intercellular organisms such as *Rickettsia, Orientia* and *Coxiella* and complex and expensive methods are not practical in low-income countries. There is an increasing concern on the adverse effects of typhus (ST and murine typhus) on pregnancy although there is no data on whether typhus occurs more or less commonly in pregnancy. Data collected in the last 18 years included only 97 pregnancies, 82 with known outcomes, including two maternal deaths. Miscarriage was reported in 17.3% (14/81) and poor neonatal outcomes in 41.8% (28/67). There was no significant improvement in neonatal outcomes (p=0.610) with the use of azithromycin[241]. There is underdiagnosis and under-recognition on the effects of these infections in pregnancy and evidence supporting the use of azithromycin, the most commonly used treatment, is weak. The gap in understanding calls for urgent collaborative, prospective clinical trials in pregnant women to reduce the burden of adverse maternal and newborn outcomes and to determine the safety and efficacy of different antimicrobial treatment regimen. There is a scientific concern on whether people living with HIV would be more prone to the intracellular rickettsial infections since the cell-mediated immunity is affected during HIV infection. This was found

unlikely as evidenced by reports of similar seroprevalence rates to healthy population were demonstrated in HIV patients[357]. This is an important concept for the HIV endemic countries and for Bhutan since the number of HIV cases are gradually increasing in the country.

11.5 Q fever

Prior to the current project, there were no reports of any sorts on Q fever (QF) from human as well as the livestock sector in Bhutan. Published data on QF is also scanty from countries neighbouring Bhutan including India, Bangladesh and Nepal with slightly more work done in China to the north of Bhutan. This is a clear demonstration of how neglected the infection is in these countries and the region. *Coxiella burnetii*, the causal agent of QF infects various hosts, including humans, ruminants (cattle, sheep, goats), dogs, cats, birds, rodents, reptiles and ticks. The organism is excreted in urine, milk, faeces, and birth products. These products, especially the latter, contain large numbers of bacteria that become aerosolized[97] and human infections occur mainly through these aerosols. In this context, Asian countries like Bhutan with huge livestock ownership have a high risk of acquiring the infection from the farm and pet animals. However, good data is lacking in these developing countries compared to the developed countries. Reports on sexual transmission of *C. burnetii* among humans were initially described in infected Polish sheep shearers in Spain who transmitted the infections to their wives upon returning to their home country[358]. In one case, the infection occurred fifteen days after coitus, when his wife developed serologically proven acute QF. *C. burnetii* DNA was detected by PCR performed on husband's semen samples obtained at 4 and 15 months after the onset of acute QF[359]. This finding may suggest that clinical guidelines should include advice on sexual restraint or use of protection during coitus at least until DNA is negative in subsequent PCRs. In Bhutan (work included in this thesis), only about 3% of the 1044 patients with acute undifferentiated fevers attending 14 hospitals were due to QF (case incidence)[172] but about 7%

of the healthy Bhutanese showed serological evidence of past exposure to *C. burnetii* (seroprevalence)[258]. This probably reflects the build-up of people with exposure to the bacterium as they age. Preliminary reports suggest that 4% of the 294 Bhutanese domestic animals have been exposed to *C. burnetii* (seropositivity). Of the seven animal species tested, goats (9%) showed the highest seropositivity against QF compared to other animals[345]. In one of the first studies in Bangladesh, a serological evidence of *C. burnetii* infection in cattle and goats were found to be 0.7% (8/1149); 0.7% (4/620) in cattle and 0.8% (4/529) in goats[328]. The presence of QF in domestic animals in Bangladesh was further supported by detecting it in three species of domestic ruminants with a seroprevalence of 9.5%, 3.3% and 3.6% in sheep, goats and cattle.

Q fever prevalence was reported to be higher in animals (15% in cattle and 12% in goats) compared to the human population (10%) in China[327]. Outbreaks of Q fever have been reported mostly from European countries[202] and study data are mostly available from developed countries. However, QF outbreaks may have occurred in the past and continue to occur in developing countries like Bhutan which may be missed due to low index of suspicion of healthcare and livestock workers and lack of laboratory facilities to investigate abnormal or suspicious occurrences in the community. Outbreaks of Q fever in domestic animals result in an economic loss from the death, abortions and culling of the suspected animals as well as other activities associated with controlling the outbreak[360].

Emerging and unresolved issues on QF include the mechanism and management of Q fever fatigue syndrome (QFS) and another on the definition and management of chronic QF. The latter is a result of conflict between the French team[199] and the Dutch team[198] on the definition and clinical diagnostic criteria on the currently used concept of chronic Q fever. Even though there is disagreement between the two teams, the main concept is the need for a review of the current definition of chronic QF to have a more practical and easier management guideline.

Therefore, the international body of experts should come together to work on an evidence-based criterion for diagnosis and treatment of this complex disease.

11.6 Conclusion

Rickettsioses (including ST and QF) are emerging and re-emerging infections with increased cases and outbreaks, especially in the Asia-Pacific countries. These infections have not received the warranted recognition in terms of research funding and diagnostic development, remaining severely neglected in Bhutan and its neighbouring countries (India, Nepal and Bangladesh). With this clear delineation of the burden of these infections, the Ministry of Health, Bhutan, will want to take appropriate actions to ensure health services in the country are equipped to manage these neglected tropical diseases appropriately. The actions would include: increased awareness among health professionals through continuing education and training; clinical guideline development; ensuring diagnostics are available in all hospitals; and strengthening surveillance and case notification. Countries should collaborate to share best evidence and practice models, with regional and cross-border collaborations facilitated by the World Health Organization. Multi-sectoral involvement adopting a "One health" approach would be most beneficial. In Bhutan, the MoH could find that incorporating the activities related to rickettsioses into the existing Vector-borne Diseases Control Program would be synergistic and potentially cost-beneficial.

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LIST OF APPENDICES

Appendix 1: Demography/clinical information for acute febrile cases

Case no: _____ Date: _____

SECTION 1: SUBJECT INFORMATION

i. AGE:

ii. **GENDER (tick):** 1. Male ( ) 2. Female ( )

iii. **CURRENT RESIDENCE**:

iv. **ORIGINAL HOME ADRESS:**

v. **OCCUPATION (tick):**

1. Farmer ( ) 2. Herder ( ) 3. Government/Private employee ( ) 4. Student ( )

5. Housewife ( ) 6. Unemployed ( )

vi. ESTIMATED DURATION OF ILLNESS (in days): ……

SECTION 2: OCCUPATIONAL/LEISURE HABITS	**Yes (1)**	**No (2)**	**Don't know (3)**
i. In your work or leisure activities, do you come in contact with animals? If yes, specify the animal/animals. …….			
ii. In your work or leisure activities do you come in contact with forests/bushes frequently?			
iii. Do any of your family members currently suffer from similar illness?			
iv. Have you been bitten by a tick/mite before the illness?			
v. Does your house or any family member have fleas/louse infestation?			
vi. Do you have pets at home? If yes, specify …….			

SECTION 3: SIGNS AND SYMPTOMS (Please tick all present)

1. Fever: ( ) 2. Skin rash ( ) 3. Eschar (skin sore) 4. Headache ( )
5. Joint pains ( ) 6. Muscle ache ( ) 7. Enlarged lymph nodes ( ) 8. Others …….

SECTION 4: SPECIMAN COLLECTED

1. Blood: ( ) 2. Swab from eschar ( ) 3. Skin biopsy from eschar : ( ) 4. Ecto-parasite (tick/mites): ( )

Appendix 2: Standard Operating Procedure for biological sample collection

(Use with other relevant appendices)

A. Blood sample collection from febrile patients attending the selected hospitals

- Make sure the subject meets the case definition
- Explain about the study and get the informed consent signed/thumb impression
- Take the demographic and clinical data (appendix 1)
- Explain the blood collection procedure
- Label the blood collection tubes
- Put the tourniquet in place and clean the collection site in the cubital fossa with 70% alcohol and let it dry
- Draw at least 10 ml of blood and dispense 5 ml into EDTA (pink/purple cap) and 5 ml in plain tubes (red cap)
- Separate serum from the plain vial
- Seal the sample tubes properly and store both at 4^{o}C (lower compartment/door of household refrigerator) until dispatch to Principal Investigator (PI)
- Keep the informed consent form and appendix 1 safely.
- Dispatch both in 4^{o}C (ice packs in thermocol boxes) with the forms

B. **Serum sample from healthy subjects in the community**

- Make sure the subject is selected by the sampling criteria
- Explain about the study and get the informed consent signed/thumb impression
- Take the demographic and clinical data (appendix 3)
- Explain the blood collection procedure
- Label the blood tube appropriately
- Put the tourniquet in place and clean the collection site in the cubital fossa with
- 70% alcohol and let dry
- Draw at least 6 ml of blood and dispense into plain tubes (red cap)
- Seal the sample tubes properly and store at 4^{o}C (lower compartment/door of
- household refrigerator if available and in cold box if refrigerator not available)
- Keep the informed consent form and appendix 3 safely.
- Transport and dispatch in 4^{o}C (ice packs in thermocol boxes) with the forms to PI

C. Blood and ectoparasites from animals

- Take the details of animal types and location (appendix 4)
- Label the sample containers for blood and tube with 100% ethanol for parasites
- Collect blood and/or parasite from the animal and put into the respective container
- Seal the parasite container properly and store at room temperature
- Extract serum from the blood and store at 2-4^{o}C (thermocol boxes)
- Dispatch the samples in thermocol boxes to the PI.

Appendix 3: Demographic/clinical information from healthy subjects

No: _____(*Use same number on serum container as well)* **Date: _____**

SECTION 1: SUBJECT INFORMATION

vii. AGE:

viii. **GENDER (tick):** 1. Male ( ) 2. Female ( )

ix. **CURRENT RESIDENCE**:

x. **ORIGINAL HOME ADRESS:**

xi. **OCCUPATION (tick):**

1. Farmer ( ) 2. Herder ( ) 3. Government/Private employee ( ) 4. Military ( ) 5. Student ( )

6. Housewife ( ) 7. Unemployed ( )

SECTION 2: OCCUPATIONAL/LEISURE HABITS	**Yes (1)**	**No (2)**	**Don't know (3)**
vii. In your work or leisure activities, do you come in contact with animals? If yes, specify the animal/animals. …….			
viii. In your work or leisure activities do you come in contact with forests/bushes frequently?			
ix. Did you suffer from any febrile illness in the recent past?			
x. Have you been bitten by a tick/mite in the past?			
xi. Did you anytime in the past have **eschar** on body following known/unknown insect bites?			
xii. Does your house or any family member have fleas/louse infestation?			
xiii. Do you have pets at home? If yes, specify ..…….			

Appendix 4: Information to be collected for domestic animals

ANIMAL NUMBER: …… ***(Write same number on the serum and parasite sample containers).*** **DATE: ……..**

SECTION 1: ANIMAL INFORMATION

xii. **ANIMAL LOCATION:**

1.Village/town name:

2. District:

xiii.ANIMAL SPECIES (Please tick √):

1. Dog ( ) 2. Cat ( ) 3. Cow/Ox ( ) 4. Horse ( ) 5. Sheep ( )
6. Goat ( ) 7. Buffalo ( ) 8. Yak ( ) 9. Others (specify) ……..

SECTION 2: SAMPLES COLLECTED

A. **Serum collected: Yes/No *(please circle)***

B. PARASITE COLLECTED (Tick √ all parasites collected & note how many of each)

2. Tick: ( ) ……how many? ……..
3. Mites: ( ) ……how many? …….
4. Fleas: ( ) …….how many? ……
5. Unknown/unidentified: ( ) ……. How many?

(Note: All parasites from the same animal to be collected in the same container and labeled properly)

SECTION 3: ANY ADDITIONAL COMMENTS

Appendix 5: Material transport agreement for sample shipment to Australia

MTA Reference Number: MTA/JDWNRH-ARRL/Rickettsia/2014-2018

SCHEDULE OF PARTICULARS

Subject to the terms and conditions of this Agreement, the JDWNRH hereby agrees to provide, and ARRL hereby agrees to accept, the Materials and Information specified below for such Purposes of Use and subject to such Restrictions on Use as specified below.
In this Agreement, the following expressions shall have the following meanings:

1. "Providing Institute":
Jigme Dorji Wangchuk National Referral Hospital (JDWNRH), Menkhang Lam, Thimphu, Bhutan.
2. "ARRL":
The Australian Rickettsial Reference Laboratory, DHRI, Barwon Health, Geelong Hospital Bellerine Street, PO BOX 281, Geelong VIC 3220, Australia.
3. "Materials":
Samples of human and animal blood, ticks and mites, lice and louse, skin biopsy and skin swabs collected during a PhD study project between the Institute and ARRL will be transported.
4. "Purposes of Use":
The Materials are provided to ARRL for testing of rickettsial diseases as part of a PhD project and for storage till such a time as required for the purpose of the project.
5. "Restrictions on Use":
The Materials shall not be used for any purpose other than the Purposes of Use.
7. "Term of Agreement":
This Agreement shall remain in full force and effect as from the date of its signature by both parties, for duration of 4 years.
8. "Material transport Charges":
Charges for transport are covered by the research cost allotted for the project.
9. "General Conditions":
The General Conditions attached hereto form an integral part of this Agreement.

This Agreement was duly signed on behalf of the parties as follows:

Signed for and on behalf of ARRL:	Signed for and on behalf of Providing Institute:
	_____
	Providing Institute's Responsible Scientist
Name: Prof. Stephen Graves	Name: Dr. Tshokey, Head of laboratory, JDWNRH
Title: Director, ARRL & PhD Supervisor	Title: PhD student
Date:	Date:

GENERAL CONDITIONS

1. Use

1.1 Other than for and within the Purposes of Use, the Materials and Information shall not be transferred, offered for sale or otherwise used, without the prior written agreement of the Institute.

1.2 ARRL shall allow only parties who have a need to know for the Purposes of Use and who are bound by similar obligations of confidentiality and Restrictions on Use as contained in this Agreement to have access to Materials and Information.

1.3 ARRL shall require any party handling and/or using the Materials and Information to comply with all relevant laws, rules and regulations applicable to the use of infectious substances and other biological materials.

2. Confidentiality

2.1 The Information may incorporate confidential information of the Institute. Accordingly, if and to the extent any such Information is clearly marked by the Institute as "confidential", ARRL shall during the Term of this Agreement and for a period of 5 years following its termination, treat such Information confidential and only disclose it under like obligations of confidentiality and Restrictions on Use as those contained herein. ARRL shall be deemed to have fulfilled its obligations, if it exercises at least the same degree of care in maintaining confidentiality as it would in protecting its own confidential information.

3. Rights

3.1 Except for the rights explicitly granted to ARRL hereunder (including, but not limited to, physical ownership of Materials transferred to ARRL hereunder, and the right to use such Materials and the Information for the Purposes of Use), nothing contained in this Agreement shall be construed as conveying any rights under any patents or other intellectual property which either party may have or may hereafter obtain.

4. Publications

4.1 Subject to the Institute's proprietary rights, the results obtained through use of the Materials within the Purposes of Use may be published by the PhD candidate and other individuals involved in the research jointly.

5. Warranties and Liabilities

5.1 The Institute makes no warranty of the fitness of the Materials for any particular purpose or any other warranty, either express or implied. However, to the best of the Institute's knowledge, the use of the Materials and/or Information within the Purposes of Use shall not infringe on the proprietary rights of any third party.

Appendix 6: Ethics approval from the Research Ethics Board of Health, Bhutan

ROYAL GOVERNMENT OF BHUTAN
MINISTRY OF HEALTH
RESEARCH ETHICS BOARD OF HEALTH
THIMPHU : BHUTAN
P.O. BOX : 726

REBH/Approval/2014/019 **15th January, 2015**

REBH Approval Letter

PI: Dr. Tshokey **Institute:** Microbiology Unit, Department of Laboratory Medicine, JDWNRH	**Study Title**: A study on epidemiological and genotypic characterization of rickettsial diseases and Q fever in Bhutan
Co-PI: 1. Prof. Stephen R. Graves - University of Newcastle, 2. Prof. David N Durrheim - University of Newcastle, 3.Prof. John Stenos – Murdoch university, and 4. Dr. Keith Eastwood - Hunter New Engla Population Health NSW: Australia	
Mode of Review: ✓*Full Board Review (Meeting No. 20th and 21st)* ☐*Expedited Review*	
Decision: Approved with conditions	
List of document(s) approved:	
Protocol	Version No.02 Dated: December 20, 2014
Informed Consent Form	Version No.01 Dated: August 8, 2014
Tools (Questionnaire/forms)	Version No.02 Dated: December 20, 2014

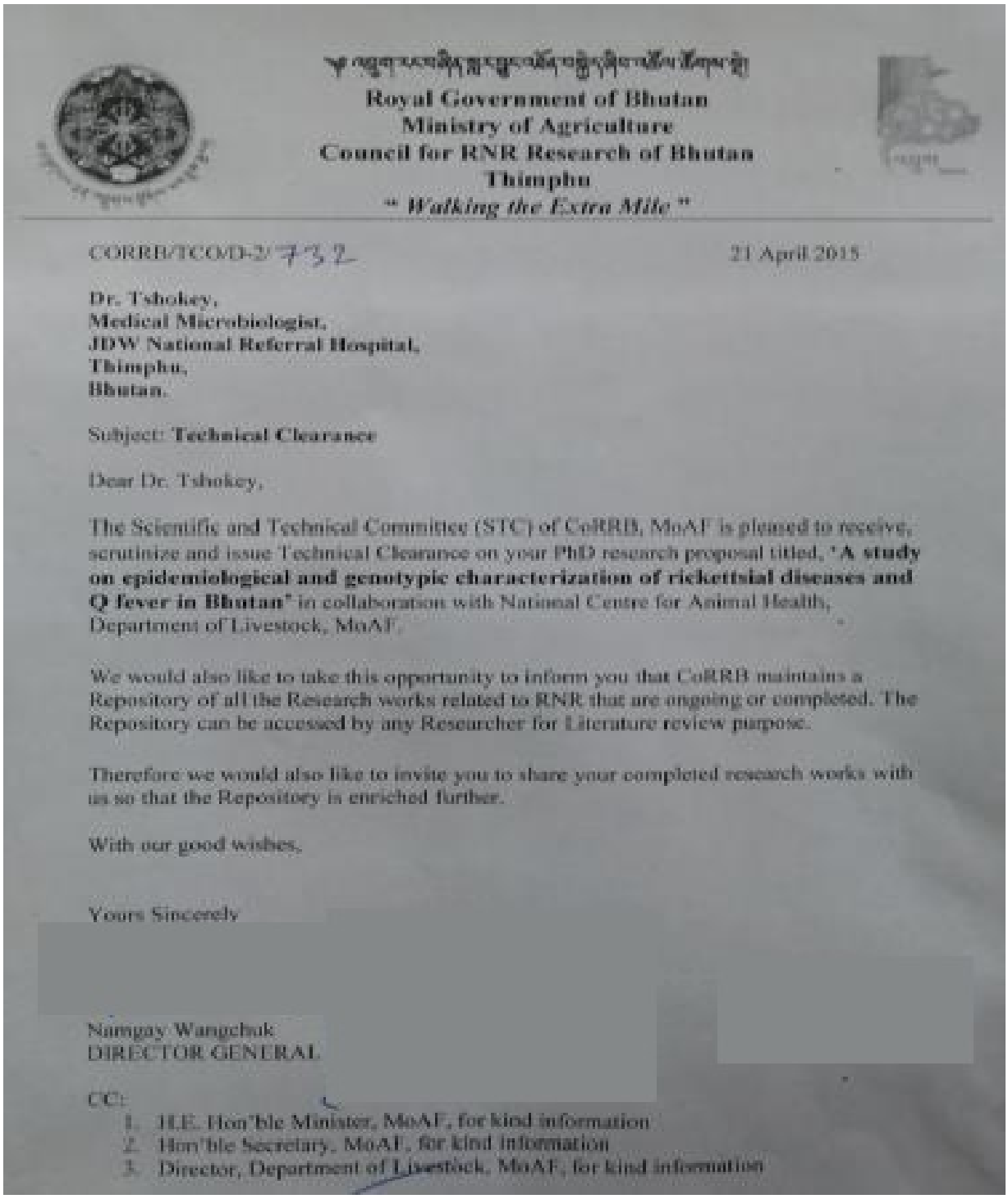
Conditions for Approval

1. *This approval is granted for the scientific and ethical soundness of the study. The PI shall be responsible to seek all other clearances/approvals required by law/policy including NSB Clearances, permission from the study sites, and administrative approval before conducting the study.*
2. *No biological material shall be used for other research purpose beyond which is specified in this protocol.*
3. *Any new research study with stored biological material from this study will need a new approval from the REBH before study begins.*
4. *Any changes to the proposal or to the attachments (informed consent and research tools such as forms) should be approved by REBH before implementation*
5. *Report serious adverse events to REBH within 10 working days after the incident and unexpected events should be included in the continuing review report or the final report.*
6. *Final report of the study both in soft and hard copy must be submitted to REBH at the end of the study before publishing.*
7. *This approval is valid till 14th January, 2016. The PI has to apply for the continuing review two months before this validity expires, if the study continues beyond the approved period.*

(Dr. Pakila Drukpa)
Chairperson-REBH
For further information please contact: *mongal56@health.gov.bt; REBH Member Secretary*

PABX: + 975-2-322602, 322351, 328091, 328092, 328093 (Extension 333)Fax: 324649

Appendix 7: Ethics approval from the Council for RNR Research of Bhutan



Royal Government of Bhutan
Ministry of Agriculture
Council for RNR Research of Bhutan
Thimphu
" Walking the Extra Mile "

CORRB/TCOMD-2/ 732 21 April 2015

Dr. Tshokey,
Medical Microbiologist,
JDW National Referral Hospital,
Thimphu,
Bhutan.

Subject: **Technical Clearance**

Dear Dr. Tshokey,

The Scientific and Technical Committee (STC) of CoRRB, MoAF is pleased to receive, scrutinize and issue Technical Clearance on your PhD research proposal titled, **'A study on epidemiological and genotypic characterization of rickettsial diseases and Q fever in Bhutan'** in collaboration with National Centre for Animal Health, Department of Livestock, MoAF.

We would also like to take this opportunity to inform you that CoRRB maintains a Repository of all the Research works related to RNR that are ongoing or completed. The Repository can be accessed by any Researcher for Literature review purpose.

Therefore we would also like to invite you to share your completed research works with us so that the Repository is enriched further.

With our good wishes,

Yours Sincerely

Namgay Wangchuk
DIRECTOR GENERAL

CC:
1. HE. Hon'ble Minister, MoAF, for kind information
2. Hon'ble Secretary, MoAF, for kind information
3. Director, Department of Livestock, MoAF, for kind information

Appendix 8: Ethics approval from the Human Research Ethics Committee, University of Newcastle, Australia

HUMAN RESEARCH ETHICS COMMITTEE

THE UNIVERSITY OF NEWCASTLE AUSTRALIA

Notification of Expedited Approval

To Chief Investigator or Project Supervisor:	**Conjoint Professor Stephen Graves**
Cc Co-investigators / Research Students:	**Dr . Tshokey** **Dr John Stenos** **Conjoint Professor David Durrheim** **Doctor Keith Eastwood**
Re Protocol:	**A study on the epidemiology of rickettsial diseases and Q fever in Bhutan**
Date:	**12-Aug-2016**
Reference No:	**H-2016-0085**
Date of Initial Approval:	**11-Aug-2016**

Thank you for your **Response to Conditional Approval** submission to the Human Research Ethics Committee (HREC) seeking approval in relation to the above protocol.

Your submission was considered under **Expedited** review by the Chair/Deputy Chair.

I am pleased to advise that the decision on your submission is **Approved** effective **11-Aug-2016**.

In approving this protocol, the Human Research Ethics Committee (HREC) is of the opinion that the project complies with the provisions contained in the National Statement on Ethical Conduct in Human Research, 2007, and the requirements within this University relating to human research.

Approval will remain valid subject to the submission, and satisfactory assessment, of annual progress reports. *If the approval of an External HREC has been "noted" the approval period is as determined by that HREC.*

The full Committee will be asked to ratify this decision at its next scheduled meeting. A formal *Certificate of Approval* will be available upon request. Your approval number is **H-2016-0085**.

If the research requires the use of an Information Statement, ensure this number is inserted at the relevant point in the Complaints paragraph prior to distribution to potential participants You may then proceed with the research.

The scope of this approval is limited to the analysis of samples previously collected and the reporting of the study outcomes.

Conditions of Approval

This approval has been granted subject to you complying with the requirements for *Monitoring of Progress*, *Reporting of Adverse Events*, and *Variations to the Approved Protocol* as detailed below.

PLEASE NOTE:
In the case where the HREC has "noted" the approval of an External HREC, progress reports and reports of adverse events are to be submitted to the External HREC only. In the case of Variations to the approved protocol, or a Renewal of approval, you will apply to the External HREC for approval in the first instance and then Register that approval with the University's HREC.

- ***Monitoring of Progress***

Other than above, the University is obliged to monitor the progress of research projects involving human participants to ensure that they are conducted according to the protocol as approved by the HREC. A progress report is required on an annual basis. Continuation of your HREC approval for this project is conditional upon receipt, and satisfactory assessment, of annual progress reports. You will be advised when a report is due.

- ***Reporting of Adverse Events***

1. It is the responsibility of the person **first named on this Approval Advice** to report adverse events.
2. Adverse events, however minor, must be recorded by the investigator as observed by the investigator or as volunteered by a participant in the research. Full details are to be documented, whether or not the investigator, or his/her deputies, consider the event to be related to the research substance or procedure.
3. Serious or unforeseen adverse events that occur during the research or within six (6) months of completion of the research, must be reported by the person first named on the Approval Advice to the (HREC) by way of the Adverse Event Report form (via RIMS at https://rims.newcastle.edu.au/login.asp) within 72 hours of the occurrence of the event or the investigator receiving advice of the event.
4. Serious adverse events are defined as:
    - Causing death, life threatening or serious disability.
    - Causing or prolonging hospitalisation.
    - Overdoses, cancers, congenital abnormalities, tissue damage, whether or not they are judged to be caused by the investigational agent or procedure.
    - Causing psycho-social and/or financial harm. This covers everything from perceived invasion of privacy, breach of confidentiality, or the diminution of social reputation, to the creation of psychological fears and trauma.
    - Any other event which might affect the continued ethical acceptability of the project.

5. Reports of adverse events must include:
    - Participant's study identification number;
    - date of birth;
    - date of entry into the study;
    - treatment arm (if applicable);
    - date of event;
    - details of event;
    - the investigator's opinion as to whether the event is related to the research procedures; and
    - action taken in response to the event.

6. Adverse events which do not fall within the definition of serious or unexpected, including those reported from other sites involved in the research, are to be reported in detail at the time of the annual progress report to the HREC.

- ***Variations to approved protocol***

If you wish to change, or deviate from, the approved protocol, you will need to submit an *Application for Variation to Approved Human Research* (via RIMS at https://rims.newcastle.edu.au/login.asp). Variations may include, but are not limited to, changes or additions to investigators, study design, study population, number of participants, methods of recruitment, or participant information/consent documentation. **Variations must be approved by the (HREC) before they are implemented** except when Registering an approval of a variation from an external HREC which has been designated the lead HREC, in which case you may proceed as soon as you receive an acknowledgement of your Registration.

Linkage of ethics approval to a new Grant

HREC approvals cannot be assigned to a new grant or award (ie those that were not identified on the application for ethics approval) without confirmation of the approval from the Human Research Ethics Officer on behalf of the HREC.

Best wishes for a successful project.

Professor Allyson Holbrook
Chair, Human Research Ethics Committee

For communications and enquiries:
Human Research Ethics Administration

Research Services
Research Integrity Unit
NIER, Block C
The University of Newcastle
Callaghan NSW 2308
T +61 2 492 17894
Human-Ethics@newcastle.edu.au

RIMS website - https://RIMS.newcastle.edu.au/login.asp

Linked University of Newcastle administered funding:

Funding body	Funding project title	First named investigator	Grant Ref

Appendix 9: Information sheet and informed consent form (English version)

EPIDEMIOLOGY OF RICKETTSIAL DISEASES IN BHUTAN
Information and Informed consent form

PART I: INFORMATION SHEET

Introduction:

Rickettsial diseases are a group of infections caused by bacteria belonging to the rickettsial family. There are many genus and species causing infection in the group. The diseases are transmitted by ticks, mites, fleas and lice of human and mammals. At present we know only of scrub typhus and murine typhus being reported in Bhutan. They are diseases causing fever, rash, joint and muscle pains like malaria. They may involve multiple organs as well. In 2008 there was an outbreak of these infections and some lives were lost before we could confirm the diagnosis. So far we do not know much detail of these diseases in Bhutan though cases are being reported from many hospitals.

Dr. Tshokey, a Microbiologist working in JDWNRH, in collaboration with the Australian Rickettsial Reference Laboratory (ARRL) in Victoria, Australia will conduct this study to understand the epidemiology of these diseases in Bhutan as part of a PhD program with the Faculty of Health and Medicine, University of Newcastle, Australia.

Process and participation in the study:

You are being invited as a subject for this study. If you agree to participate, a blood sample and some details will be taken. If you have any skin lesions, a swab may be taken. These samples will be taken to ARRL and tested for presence of present or past infections with this group of bacteria.

Please note that your participation in this study is completely voluntary and you can decide to participate or not. Even if you decide not to participant, you will receive the care and treatment like any other patient in this hospital.

Duration:

It will take a maximum of 5 minutes to complete the questionnaire and collect a blood sample.

Benefits and risks of participation:

Your participation in this study will not have any direct personal benefits but the information from this study will help the people and country in the future. There are no additional risks for participating in this project than seeking routine care from hospitals.

Reimbursements and financial benefits:

There will be no incentives for taking part in this project.

Confidentiality:

For this study only an untraceable study number will be used and the information will be kept confidential. Your name will not be associated with the results of this study.

Whom to contact:

If you have any questions, please ask now or later at the following;

Dr. Tshokey, Phone no: 17464788, email: doc_tshokey@yahoo.com

Please note that this study was reviewed & approved by REBH, an ethical committee to make sure that research participants are protected from harm & their biological samples are not misused.

PART II: INFORMED CONSENT FORM

I have read the foregoing information or it has been read and explained to me. I have had the opportunity to ask questions and all my doubts have been cleared to my satisfaction. I consent voluntarily to participate in this study.

Name: ……..
Signature: …….
Date: ……

If illiterate: Witness statement

I have witnessed the exact reading of the consent form to the potential participant and the individual has had the opportunity to ask questions. I confirm that the individual has given the voluntary consent.

Name of Witness: ……
Signature of witness: ……
Date: …….

Thumb print of participant

If children (≤16 yrs.): Parent/guardian consent

I have read or been explained the foregoing information. My child and I had the opportunity to ask questions and all our doubts have been cleared to our satisfaction. I consent voluntarily for my child to participant in this study.

Name of Parent/guardian: ……
Signature/thumb impression of parent/guardian: ……
Date: …….

Statement of Researcher/person taking consent

I have accurately read out the information sheet to the potential participant and I confirm that the participant was given enough opportunity to ask questions and all questions were clearly answered to the best of my knowledge. I confirm that the individual has not been forced to consent and the consent was given freely and voluntarily.

Name of Researcher/person taking the consent: ……..
Signature: …….
Date: ……

Appendix 10: Information sheet and informed consent form (Dzongkha version)

དོན་ཚན་༡་ཡིན་དོན་ཤོག་ཁྲམ།

ངོ་སྤྲོད་གསལ་བཤད།

Rickettsia གི་ནད་གཞི་འདི་ འགྲོ་བ་མི་དང་ ནོར་སེམས་ཅན་ལས་བརྒྱན་པའི་ ཀེན་ཙ་དང་ ཤིག་དང་ཀི་ཤིག་གིས་ཕར་ཚུར་སྤེལ་བའི་ནད་གཞི་ཅིག་ཨིན། ནད་གཞི་འདི་ཕྱམ་པ་མ་འདུག་ལེ་ཤུ་ཡོད། འབྲུག་རྒྱལ་ཁབ་ནང་དུ་སྤྱོ་ནད་གཞི་འདི་གི་སྐོར་ལས་ལེགས་ཤོམ་སྤེ་ཉ་མ་གོ་བར་ཡོད།ནད་གཞི་འདི་ཐོབ་པའི་རྟགས་མཚན་ཡང་ གཟུགས་དྲོད་འབར་རྒྱབ་ ཤུང་ཚོག་ངང་ལག་ཚོག་ན་ནེ་དང་ གཟུགས་ཁར་སྤུ་རོག་ ཡང་ན་ རྐ་འཐོན་ནེ་ཚུ་ཨིན། སྤྱི་ལོ་ ༢༠༠༤ ནད་གཞི་འདི་ལས་བརྟེན་མི་དག་པ་ཅིག་ལུ་ཤི་རྒྱུན་ཡང་བྱུང་ཡི། ད་སྤྱོ་ནད་གཞི་འདི་གི་སྐོར་ལས་ལེགས་ཤོམ་སྤེ་ཉ་མ་གོ་རྒྱང་ སྐན་ཁང་སོ་སོ་ལས་ ནད་གཞི་འདི་ཐོན་པའི་སྐན་ཞུ་ཐོབ་སྤེ་རང་ཡོད།

ནད་འཐུབ་ཀྱི་མཁས་མཚོག་ཅོག་ཏར་ མཚོ་སྐྱིང་ཀྱིས་ ཞས་ཀེ་ལི་ཡ་ལུ་ཡོད་པའི་ Rickettsia ནད་གཞི་བརྟག་དཔྱད་གཞི་རྟེན་གསུག་ལག་དང་གཅིག་ཁར་མཐུན་འཐེལ་ཀྱི་ཐོག་ལས་ ནད་གཞི་འདི་གི་སྐོར་ལས་ཞིབ་འཚོལ་འབད་ནི་ཨིན།

དཔེ་ཞིབ་ཐུ་སྐོའི་ལམ་ལུགས།

ཁྱེད་ཀྱིས་ལས་རིམ་འདི་ནང་འཐེལ་གཏོགས་འབད་གནང་པ་ཅིན་ཁྱེད་ཀྱིས་ང་བཅས་ལུ་ཁྱེད་རའི་ཁག་ཐེབ་དགོཔ་མ་ཚད་དེ་བཀོད་ཚུ་ཡང་ འབད་འོང།ཁྱེད་ཀྱི་ཁག་འདི་ནང་Rickettsia འབྲུ་ནད་ཚུ་ཞིབ་འཚོལ་འབད་འོང།

རང་གི་སྤྲོ་འདོད་དང་འཁྲེལ་ བཅའ་མར་གཏོགས་དགོས།

བརྟག་ཞིབ་ལས་རིམ་འདི་ནང་ བཅའ་མར་གཏོགས་ནི་འདི་ གཙོ་བོ་ར་ རང་སོའི་སྤྲོ་འདོད་དང་བསྟུན་དགོ། ལས་རིམ་འདི་ནང་ བཅའ་མར་གཏོགས་ནི་དང་ མི་གཏོགས་ནིའི་ཐང་ ཁྱེད་རའི་སྤྲོ་འདོད་དང་འཁྲེལ་དགོ། བཅའ་མར་མ་གཏོགས་ཏེ་འབད་རུང་ དེ་སྤྱོ་ཚུན་ཚོད་ཁྱེད་རའི་དོན་ལས་ གསོ་བའི་ལས་ཚན་འདི་ལས་ གསོ་བའི་མཐུན་རྐྱེན་དང་རྒྱབ་སྐྱོར་ཚུ་ ཐོབ་སྤེ་ཡོད་མེ་ཚུ་ འཕྲོ་མཐུད་དེ་ར་ཐོབ་ནི་མ་གཏོགས་ འདི་ལུ་ཐོ་ཐོག་དགོཔ་གི་ནི་ཡང་མེད།

དྲྭ་ཡུན།

བརྟག་ཞིབ་ཀྱི་དྲི་བ་དྲིས་ལན་ཚུ་ འབད་ནིའི་དོན་ལུ་ དྲྭ་ཡུན་སྐར་མ་ཁ་དེ་ཅིག་ལས་བརྐལ་ མི་འགོར།

ཉེན་ཁ་དང་ལེ་ཤས།

བརྟག་ཞིབ་ལས་རིམ་འདི་ལས་བརྟེན་པའི་ ཉེན་ཁའི་འབྱུང་ཉེན་གྱི་ཡང་མེད།བརྟག་ཞིབ་ལས་རིམ་འདི་ནང་བཅའ་མར་གཏོགས་མི་ལུ་ དོ་རྒྱུང་གི་ཁེ་ཕན་ཚུ་ གཅིག་ཡང་མེད་རྐྱངམ་ བརྟག་ཞིབ་ལས་རིམ་འདི་ལས་ཐོབ་མེ་ བང་དོན་ཚུ་ མ་འོངས་པ་ལུ་ Rickettsia ནད་གཞི་བརྟག་ཞིབ་ཐོག་ལས་བཀག་ཐབས་ལུ་ཕན་ནི་ཨིན།

ཟང་འཐུས་སྤྱོད་ལེན།

བརྟག་ཞིབ་ལས་རིམ་འདི་ནང་ བཅའ་མར་གཏོགས་པའི་སྐབས་ ཟང་འཐུས་སོགས་ག་ནི་ཡང་མེད་རྐྱངམ་ ཁྱེད་ཀྱིས་ གོ་སྐབས་བྱིན་མེ་ལུ་ བཀའ་དྲིན་དགའ་ཚོར་ཞུ་ནི་ཨིན།

གསང་ཐུ།

ཁྱོད་ཀྱིས་བྱིན་མི་གནས་ཚུལ་དང་གྲུབ་འབྲས་ཚུ་ཐུ་གསང་འབད་འབད་བཞག་ནི་ཨིན་པའི་ཁར་ གྲུབ་འབྲས་སྐན་ཞུ་ནང་ལུ་མེ་ངོམ་གཱ་ཨིན་ན་ངོ་སྤྲོད་འབད་འདི་བཀོད་ནང་ལས་བྱུང་པའི་གནས་ཚུལ་གསལ་བཀོད་ཚུ་གསང་ཨ་ངོ་སྤྲོད་འབད་འོང་ཁར་གཏན་ཚིག་ཏུ་ ཁྱེད་ཀྱིས་ཉམས་ཅིག་ལག་ཁེན་འཐབ་ནི་ཨིན།

ཁས་ལེན་མི་འབད་ནི་ ཡང་ན་ ཕྱིར་འཐོན་འབད་ཚོགས་པའི་ཐོབ་དབང་།

ཁྱེད་ར་ ཞིབ་འཚོལ་འདི་ནང་ བཅའ་མར་གཏོགས་ནིའི་ སྤྲོ་འདོད་མེད་པ་ཅིན་ ཁིན་ཅིག་མིན་ཅིག་ ཁས་ལེན་འབད་དགོཔ་མེད། དེ་སྟེ་ཁས་ཁས་ལེན་མ་འབད་མི་གིས་ ཁྱེད་རའི་དོན་ལུ་ གཞོ་བའི་ལས་ཁང་འདི་ལས་ ཐོབ་སྟོལ་ཡོད་མི་ སྐན་བཅོས་ཀྱི་མཐུན་རྐྱེན་ཚུ་ཡུལ་གཞོན་པ་ རྩ་ལས་མེད། ཁྱེད་ར་ ནམ་ར་ཨིན་རུང་ བརྟག་ཞིབ་ལས་རིམ་ནང་ལས་ གཞོད་སྐྱོན་གཱ་ནི་ཡང་མེདཔ་ཁཱ་ ཕྱིར་འཐོན་འབད་ཚོགས།

འཐེལ་བ་འཐབ་སྟ།

ཁྱེད་ར་ལུ་ དྲི་བ་བཀོད་དགོས་རེ་ཡོད་པ་ཅིན་ ད་སྤྲོ་ ཡང་ན་ ཤུལ་ལས་དང་ ཐ་ན་ བརྟག་ཞིབ་ལས་རིམ་ འགོ་བསྡུགས་ཚར་ཏེ་ཡོད་རུང་ དྲི་བ་ཚུ་བཀོད་ཚོག། དྲི་བ་ཚུ་ ཤུལ་ལས་བཀོད་ནིའི་སྤྲོ་འདོད་ཡོད་པ་ཅིན་བརྒྱུད་འཕྲིན་གི་ཐོག་ལས་ ཁོག་གི་མི་ངོ་མ་དང་གཅིག་ཁར་ འཐེལ་འཐབ་ཚོགཿ

ཞིབ་འཚོལ་ལས་རིམ་གྱི་གཙོས་འཆར་འདི་ ཞིབ་འཚོལ་ནང་བཅའ་མར་གཏོགས་མི་ཚུ་གིས་ རྩིན་སྐྱོན་ལས་སྲུང་སྐྱོབ་འབད་ཚུགསཔ་ངེས་བརྟན་འབད་ནི་འི་ ཁག་འགན་འབག་མི་ ཚོགས་ཆུང་ཅིག་ཨིན་མི་ **ཞར་ཕྱི་བི་ཨེཆ་** གིས་བསྒྲིར་ཞིབ་དང་ ཚ་འཇོག་གྲུབ་གྲུབ་ཨིན།

དོན་ཚན་༢་ཡིཨ་ ལས་ལེན་འཛིན་ཨོག།

ང་གིས་ གོང་གསལ་བརྗ་དོན་ཚུ་ ལྷག་བལྟ་ཡི་ ཡང་ན་ ང་ལུ་ ལྷག་སྐྱེ་གོ་བརྗ་འཛོད་བྱིན་ཡི། ང་ལུ་ དེ་གི་སྐོར་ལས་ དྲི་བ་ཚུ་བགོད་ནི་འི་གོ་སྐབས་ཡང་ བྱིན་ཡོད་པ་ཚང་ ང་གིས་ བགོད་མི་དྲི་བ་ཚུའི་ལན་གསལ་ཡང་ བསམ་པ་རྩོམས་ཏོག་ཏོ་སྟེ་ བྱིན་ཡི། ང་གིས་ ང་རའི་རང་མོས་ཁོ་ནའི་ ཐོག་ བརྟག་ཞིབ་ལས་རིམ་འདི་ནང་ གལ་གཏོགས་པ་གཅིག་སྟེ་ བཅའ་མར་གཏོགས་ནིའི་ ཁས་སྐང་ཕྱལཝ་ཨིན།ཁས་ལེན་གནད་རྟགས་བགོད་ཡོང་བའི་ཡིག་འདྲ་ཅིག་མི་ངོམ་རང་སོར་ལུ་སྟོང་ནི་ཨིན།

མིངཔ།.....མིང་རྟགསཔ།.....

ཁས་ལེན་ཕྱལ་བའི་ཚེས་གྲངསཔ།...../...../.....

ཁ་བྱངཔ།.....

འཛིའཁྲག་གི་ཤེས་ཡོན་མེད་མི་ཅིག་ཨིན་པ་ཅིན།

ང་གིས་ བརྟག་ཞིབ་ལས་རིམ་ནང་ བཅའ་མར་གཏོགས་མི་ལུ་ ཁས་ལེན་འཛིའཁྲག་གི་ནང་དོན་ཚུ་ ནོར་འཁྲུལ་མེད་པར་ ལྷག་སྐྱེ་བྱིན་སར་མཐོང་ཡོདཔ་མ་ཚད་ མི་ངོ་དེ་ལུ་ དྲི་བ་ཚུ་བགོད་ནི་འི་གོ་སྐབས་ཡང་ བྱིན་སར་མཐོང་ཡི། དེ་འབདཝ་ལས་ མི་ངོ་དེ་གིས་ ཁོ་རའི་སྟོལ་འདོད་ལྟར་ ཁས་སྐང་ཕྱལ་ཡོདཔ་ དེས་བདེན་ཞིན་པའི་ ཐེངས་འདིའི་ནང་ ཁས་ལེན་ཕྱལ་ཡོདཔ་ དང་ ཁས་ལེན་ཕྱལ་ཡོདཔ་ཅིའི་ཡང་ཞུ།

འཛོམས་དཔང་གི་མིང་:.....དང་ བཅའ་མར་གཏོགས་མིའི་ལག་མཚུ།

འཛོམས་དཔང་གི་མིང་རྟགས.....

ཚེས་གྲངས་.....

ཉེན་མ་/སྐྱེས་/སྐྱི་ལོ།

ན་ཚུང་ལོ་ ༡༨ མན་ཚང་ཞིན་པ་ཅིན་ཁམ་གྱིས་ ལས་ལེན/ངོས་ལེན་འབད་ཡོད་པའི་རྟགས།

ང་གིས་ བརྗ་དོན་ཨོག་ཁྲམ་འདི་ནང་བགོད་དི་ཡོད་མིའི་བརྗ་དོན་ཚུ་ ལྷག་སྐྱེ་ཀ་གི་ཡོདཔ་མ་ཚད་ ཡང་ན་ ལྷག་སྐྱེ་ཀ་གི་བསྒྲག་ཡོད་པའི་ཁར་ དྲི་བ་ཡང་བགོད་ནི་འི་གོ་སྐབས་ཐོབ་ལས་ དེ་གི་ཨ་ལོ་འདི་ བརྟག་ཞིབ་ལས་འདི་ནང་ བཅའ་མར་གཏོགས་ནིའི་ངོས་ལེན་འབདཝ་ཨིན།

ཁམ/ངོ་ཚབ་ཀྱི་མིང་.....

ཁམ/ངོ་ཚབ་ཀྱི་རྟགས་ ལག་མཐིལ.....

སྐྱེ་ཚེས.....

ཞིབ་འཚོལ་འབད་མི་/མོས་མཐུན་ལས་སྐང་ ལེན་མིའི་ངག་བརྗོད།

ང་གིས་ ཁོས་འབབ་ཡོད་མི་བཅའ་མར་གཏོགས་མི་ལུ་ བརྗ་དོན་ཨོག་ཁྲམ་འདི་ ནོར་འཁྲུལ་མེད་པར་ ལྷག་སྐྱེ་བྱིན་ཡོདཔ་མ་ཚད་ བཅའ་མར་གཏོགས་མི་འདི་ལུ་ བརྟག་ཞིབ་ལས་རིམ་གྱི་སྐོར་ དྲི་བ་ཚུ་བགོད་ཡང་བྱིན་ཀེ་ དེས་ལན་ཚུ་ཡང་ ང་ར་གིས་ གང་ཨེས་གང་སྟོན་ཐུགས་ ཨོག་ འཛོལ་བ་དང་འཁྲུལ་བ་མེད་པ་སྟེ་ ལན་གསལ་བྱིན་ཡི། དེ་འབདཝ་ལས་ མི་ངོ་དེ་ལུ་ ལྷག་སྐྱེ་འབད་འགོལ་ཐུགས་ ཁོ་རའི་ རང་མོས་དང་སྟོ་འདོད་ཨོག་ ལྷག་སྐྱེ་འབད་ཡོདཔ་ དེས་བདེན་ཞིན་པའི་ ངོ་སྐྱོར་འབད་ཚུགས།

ཞིབ་འཚོལ་པ་/མོས་མཐུན་ལས་སྐང་ལེན་མི་

མི་ངའི་མིངཔ།.....

ཞིབ་འཚོལ་པ་/མོས་མཐུན་ལས་སྐང་ལེན་མི་

མི་ངའི་མིང་རྟགསཔ།.....

ཚེས་གྲངསཔ།.....ཉེན་མ་/སྐྱེས་/སྐྱི་ལོ།

Appendix 11: IFA procedure for *Rickettsia* and *Orientia* antibody detection

Preparation and calibration of antigen

- The required rickettsial antigens (SFG, TG and STG antigens) were received directly from the ARRL's BCL3 laboratory in Newcastle, NSW.
- The inactivated antigens were serially diluted to establish the appropriate concentration for screening by IFA using the standardised positive control.
- Serial dilutions of 1:2 are performed and the optimum concentration selected in terms of peak fluorescence strength according to the concentration of visualised rickettsia and numbers of cells.

Preparation and calibration of positive controls

- Known positive human sera kept frozen in the ARRL were used as positive controls.
- Positive sera for SFG, TG and STG rickettsia were thawed and titrated 1:2 from 1:128 until an endpoint was reached.
- They were diluted in PBS so as to reach a fluorescence endpoint of 1:600.
- The three sera were then pooled, aliquoted in 5 µl volumes and stored frozen for future use.
- During use, they were thawed and diluted to 1:100 to effectively give a positive result for each respective antigen close to the endpoint of reactivity.

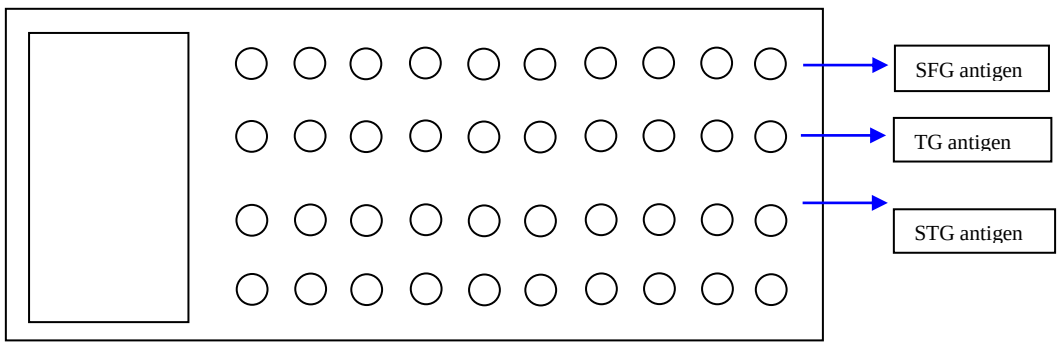
Preparation of screening slides

Material required:

- SFG, TG and STG mixed antigen
- 1 × sterile 40 well slide
- Sterile transfer pipettes
- Acetone

Method:

- The SFG, TG & STG antigens were thawed
- The first three rows of the slide were spotted with the respective antigens as follows:



- Slides were dried and fixed in acetone for 2 min
- After allowing acetone to evaporate, slides were used immediately or stored at –70°C for later use

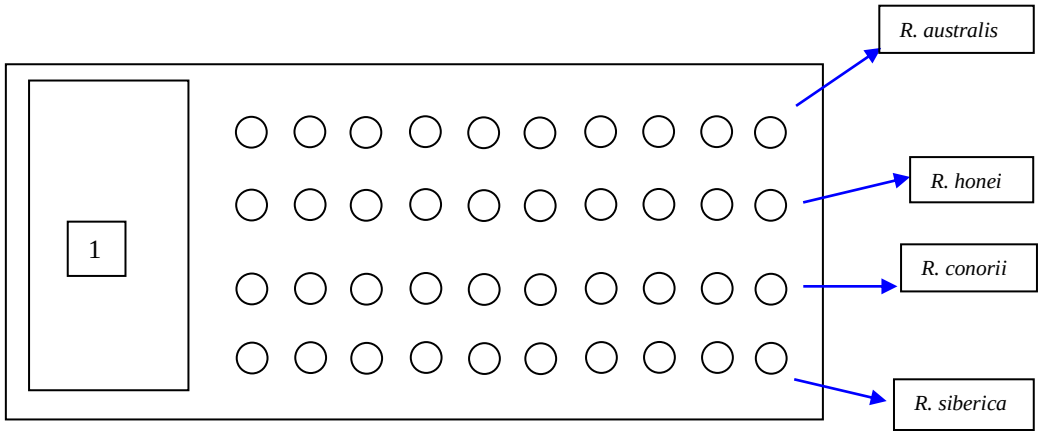
Preparation of Titration Slides

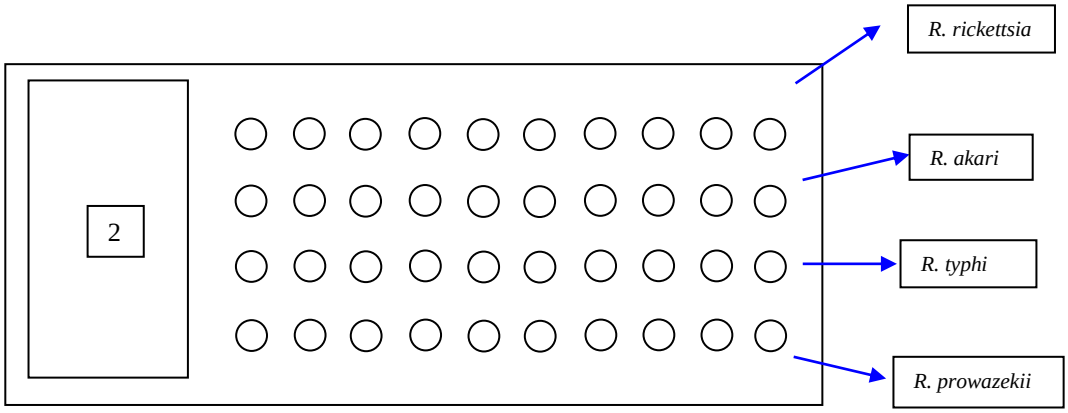
Materials required:

- *Rickettsial antigens:*
  - ✓ *R. australis*
  - ✓ *R. honei*
  - ✓ *R. conorii*
  - ✓ *R. siberica*
  - ✓ *R. rickettsiae*
  - ✓ *R. akari*
  - ✓ *R. typhi*
  - ✓ *R. prowazekii*
- *Orientia antigens:*
  - ✓ *O. tsutsugamushi* (Gilliam, Karp and Kato strains)
  - ✓ *O. chuto*
- 40 well slide
- Sterile transfer pipettes
- Acetone

Method:

- Rickettsial antigens were thawed
- Antigens were spotted as follows for Rickettsia:





- Slides were fixed in acetone for 10 mins
- After allowing acetone to evaporate slides can be used immediately or stored at –70^{o}C for later use

Preparation of PBS and 1/10 PBS

Materials required:

- Phosphate buffered Saline (Oxoid, Code BR14a)
- 100 ml of distilled deionised water for each tablet

Method:

- A tablet of PBS was added for every 100 ml of water in a beaker
- The beaker was placed on a magnetic stirrer and spun until tablets completely dissolved
- The PBS solution from this were transferred storage bottles
- PBS was diluted to 1/10 with the water and stored in 1/10 PBS container.

Preparation of 2% Casein Buffer

Materials required:

- 50 ml centrifuge tube
- 1g skim milk powder
- Sterile PBS
- 56°C water bath

Method:

- One gram of skim milk powder was weighed and placed into the 50 ml centrifuge tube
- Sterile PBS was added up to 50 ml mark
- This was placed in the 56°C water bath for 30 mins to dissolve the powder

- The solution was ready to use or stored at 4^{o}C for later use.

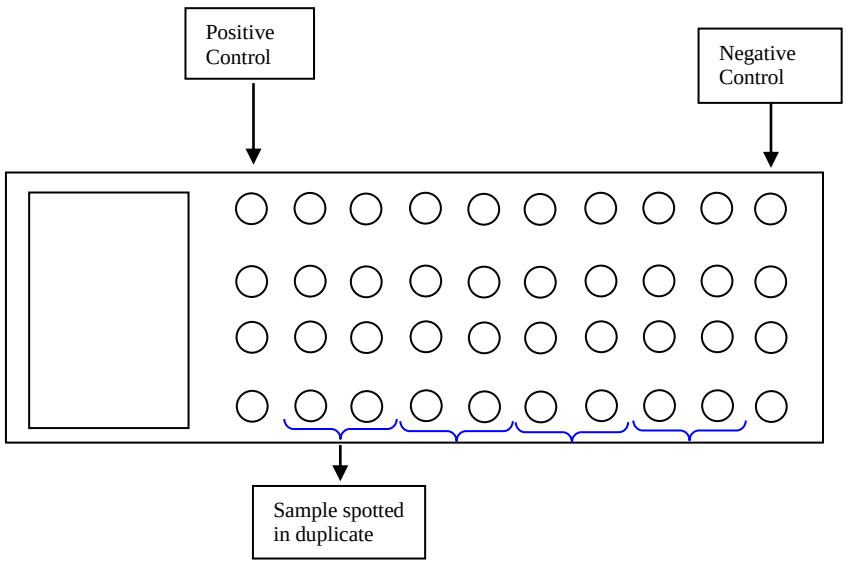
Rickettsial screening of samples by IFA

Materials required:

- Sample(s)
- IFA Screening record sheet
- Rickettsial screening slide (thawed)
- 1% Casein buffer
- ELISA Plate
- 2-20 µL Eppendorf pipettes
- 20-200 µL Eppendorf pipettes
- Sterile PBS
- Positive control (diluted 1 in 100 with 2 % casein)
- Negative control (diluted 1 in 100 with 2 % casein)
- Conjugate FITC anti-human IgA, IgM and IgG (diluted 1 in 100 with 2 % casein) (KPL Cat No. 02-10-07)
- Mounting fluid
- Cover slip
- UV Lamp Microscope

Method:

- Samples were labelled with specified numbers
- 2µl sample was diluted with 254µl 2% casein buffer per well on the ELISA plate for each sample; samples and controls spotted as follows on the rickettsial screening slide:



- Slides were incubated for 30 mins in humid condition
- After incubation slides were washed in 1/10 PBS on magnetic stirrer
- Excess PBS were shaken off and slides air dried
- Conjugates were added onto all wells of the screening slide
- Conjugated slides were incubated for 30 mins in humid condition and washed
- Dried slides were mounted with mounting fluid and cover slip
- Slides were viewed at X 400 with UV lamp microscope
- Positive results showed fluorescence and negative results did not fluorescent
- Positive samples were titrated against each antigen to get end-titre

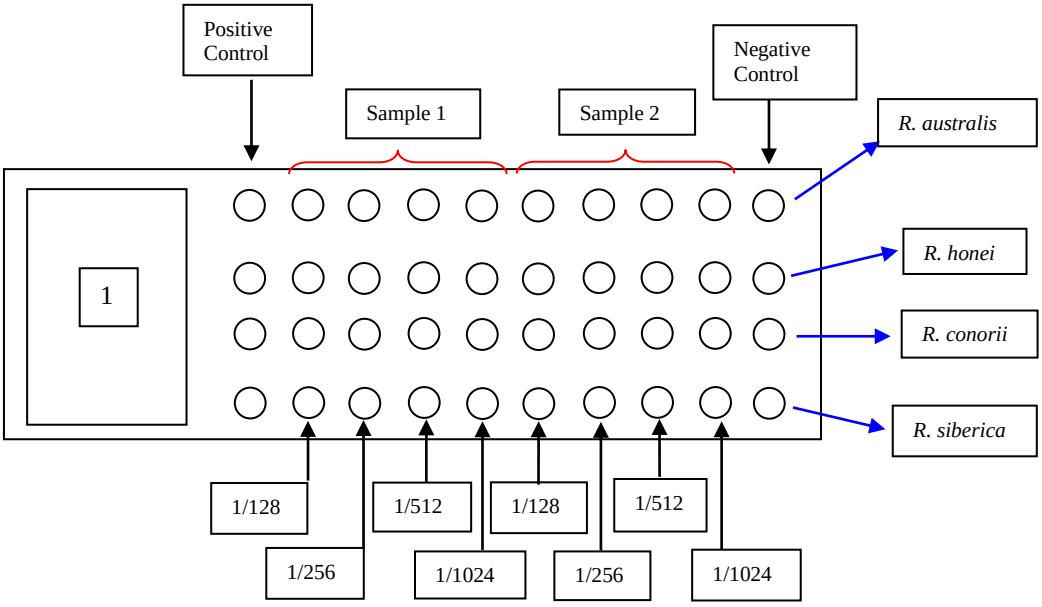
Rickettsial titration of screening positive samples

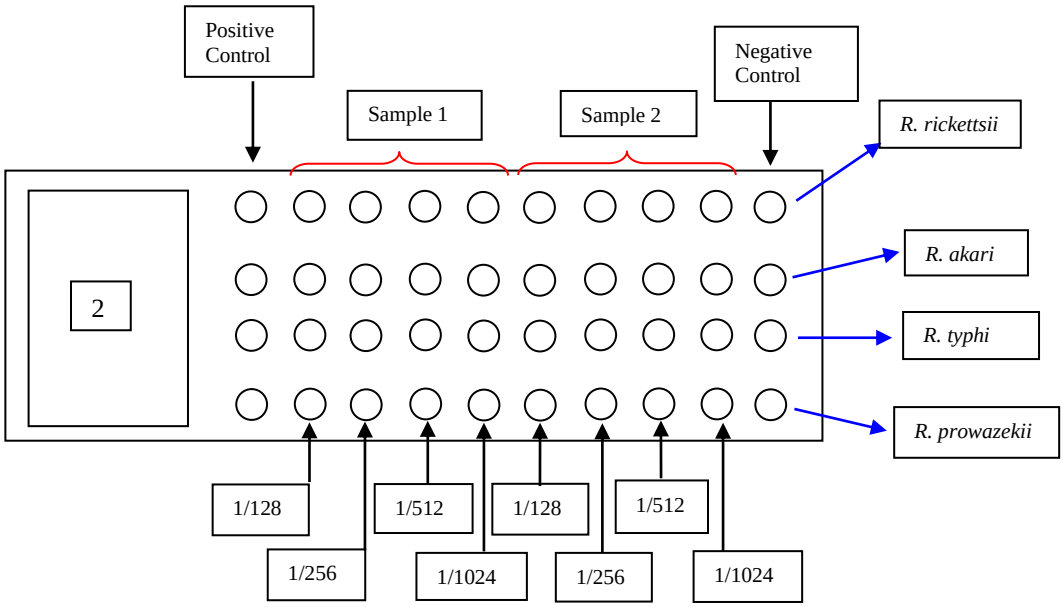
Materials required:

- As above plus IFA Titration record sheet and Rickettsial titration slide (thawed)

Method:

- Samples were labelled as required
- 2µl samples diluted with 254µl 2% casein buffer to give a dilution of 1:128 in the first well
- 120µl of diluent was added to each of three wells to perform serial dilutions from the 1:128 well by transferring 120µl from this dilution to the 2nd well (1:256), mixing and then transferring 120µl to the 3rd well (1:512), mixing and transferring 120 µl to the 4th well (1:1024) and more if required





- Slide were incubated for 30 mins in humid condition
- After incubation slides were washed in 1/10 PBS on magnetic stirrer and allowed to dry
- Conjugates were added onto all wells of the slide and incubated for 30 mins in humid condition
- Slides were washed in PBS by magnetic spin and dried
- Dried slides were mounted with mounting fluid and cover slip and viewed at X 400 with UV lamp microscope
- Positive results were indicated by fluorescence up to the specific dilution and negative results did not show fluoresce.

Appendix 12: IFA procedure for *Coxiella* antibody detection

Preparation and calibration of *Coxiella* antigen

- *Coxiella burnetii* Phase 1 (Virion-Serion, Cat. # 1227) and *Coxiella burnetii* Phase 2 (Virion-Serion, Cat. # 1123) antigens and Sirion RF absorbent (Cat # Z200) were obtained from DKSH Australia Pty Ltd (www.dksh.com.au/research)
- These were diluted, aliquoted and stored at -20^{o}C until use
- The appropriate antigen strains were taken out of –20^{o}C freezer and thawed
- 1 ml of sterile dH_2O was added into the vial to resuspend the pellet of antigen.
- The mixture was transferred to a 1.5 ml Eppendorf tube and spun for 1 minute in a microfuge at maximum speed.
- The supernatant was removed, and 1 ml of sterile PBS used to resuspend the pellet. The tube was then spun again for one minute repeating this step again.
- Serial dilutions of this material were performed and the most appropriate concentration for screening selected. This was done by IF using the standardised positive control.
- Serial dilutions of 1:2 were performed and the optimum concentration selected in terms of peak fluorescence strength in relation to concentration of visualised *Coxiella* antigen and numbers of cells. The ARRL had optimized the working solution concentrations for phase 1 and phase 2 Q fever screening as 1:4 and 1:8 for titration.

Preparation and calibration of positive controls

- Positive human sera kept frozen were used for this
- Positive sera for phase 1 & phase 2 *C. burnetii* were thawed and titrated 1:2 from 1:25 until an endpoint was reached.
- They were then diluted in PBS so as to reach a fluorescence endpoint of 1:400.
- The two sera were then pooled, aliquoted in 5 µl volumes and used immediately or stored frozen for later use.
- Once thawed they were diluted 1:100 to effectively give a positive result for each respective antigen close to the endpoint of reactivity.

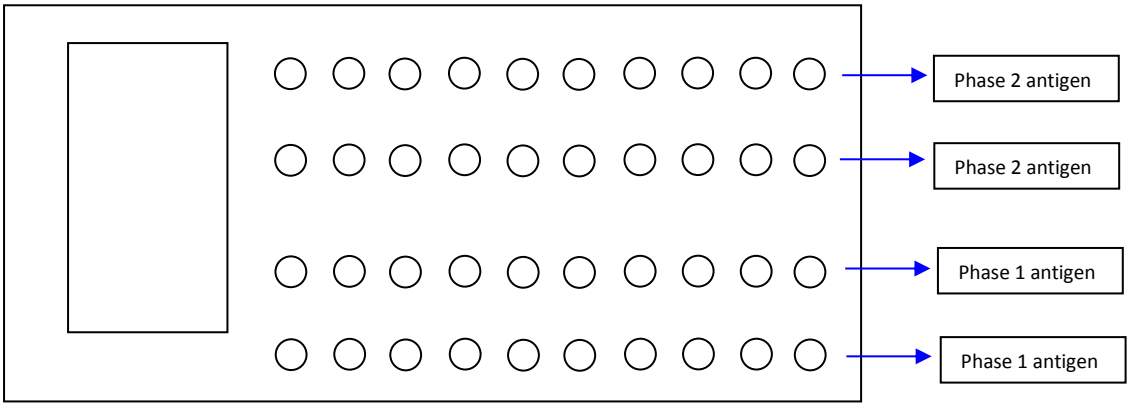
Preparation of QF screening slide

Material required:

- Phase 1 and 2 *C. burnetii* antigen
- sterile 40 well slide
- Sterile transfer pipettes
- Acetone

Method:

- Phase 1 & 2 antigen were thawed as required
- Antigens were spotted on to the entire rows as follows:



- Slides were fixed in acetone
- After drying, slides were used immediately or stored at –70^{o}C for later use

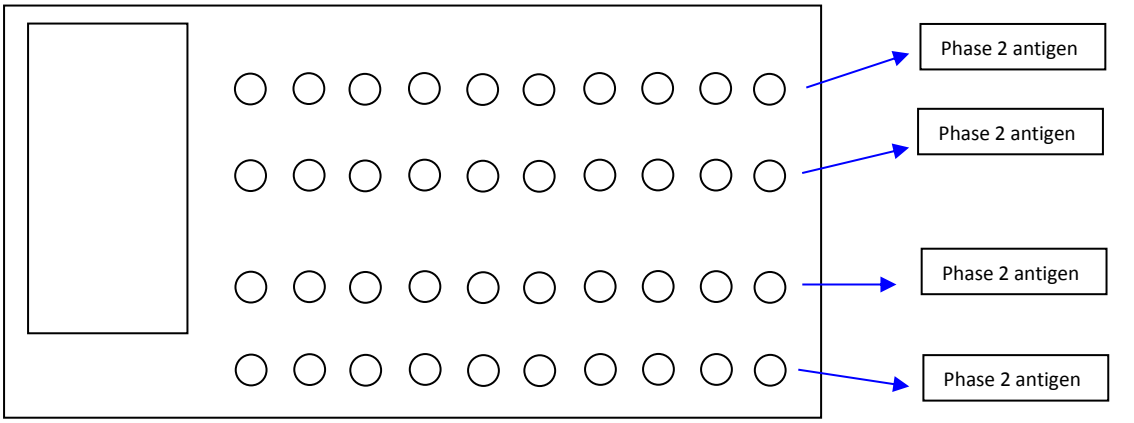
Preparation of Titration Slides

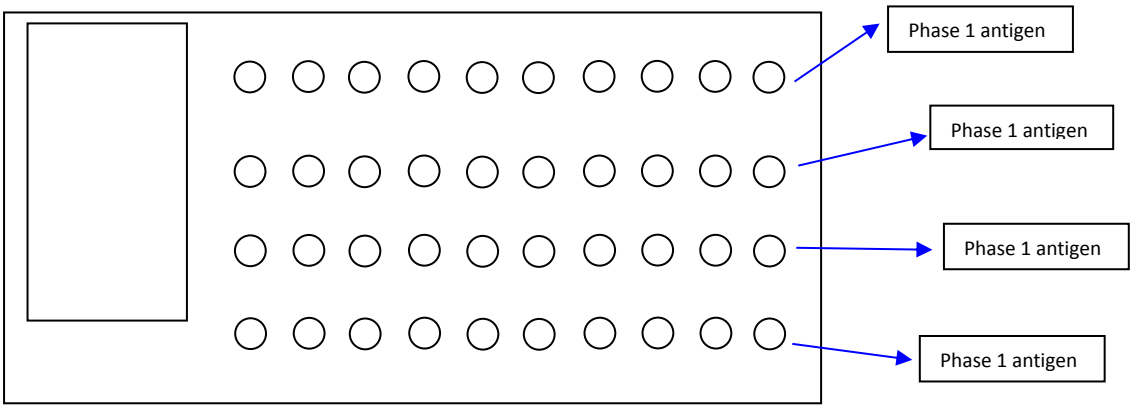
Material required:

- Phase 1 and 2 *C. burnetii* antigens
- Sterile transfer pipettes
- Acetone

Method:

- Phase 1 and 2 antigens were thawed as required
- Spotted entire rows with the antigens as follows:





- Slides were fixed in acetone and dried
- Slides were used immediately or stored at –70°C for later use

PBS, 1/10 PBS and 2% Casein were prepared as detailed in Appendix 11

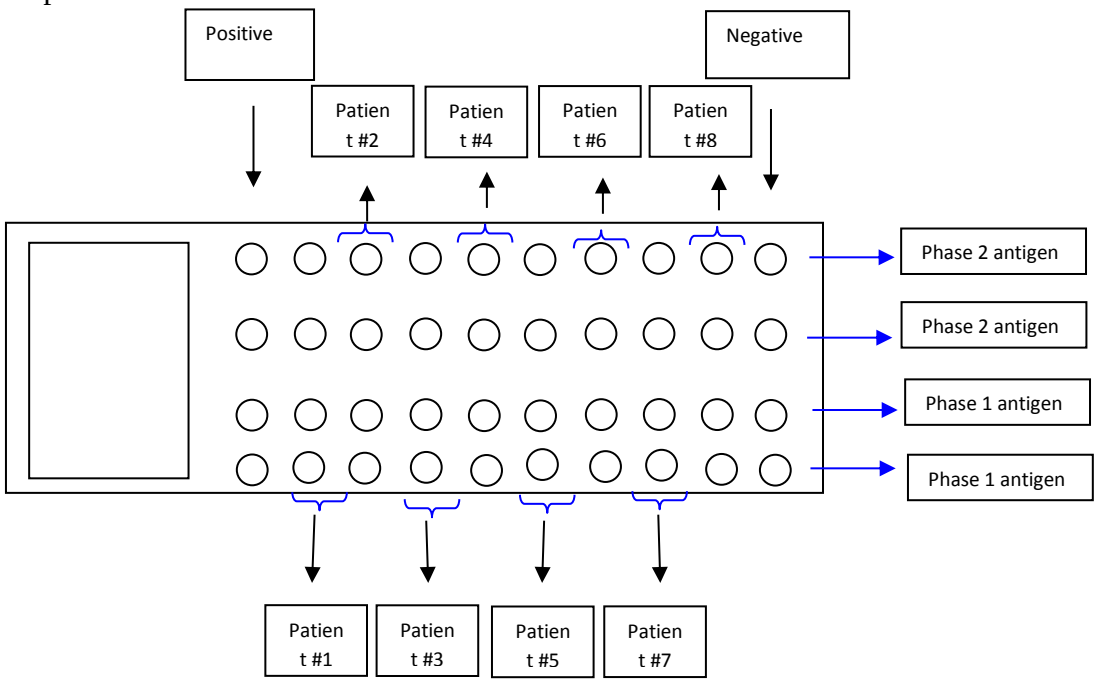
Q Fever screening by IFA

Materials required:

- Sample (s)
- Q Fever IFA screening record sheet
- Q Fever screening slide (thawed)
- 2% Casein buffer
- ELISA Plate
- 2-20 µL and 20-200 µL Eppendorf pipettes
- Sterile PBS
- Positive and negative control (diluted 1 in 100 with 2 % casein)
- Conjugate FITC anti-human (diluted 1 in 100 with 2 % casein) for:
- Total (IgA + IgG + IgM) antibody (H&L) (KPL Cat # 02-10-07)
- Mounting fluid and cover slip
- UV Lamp Microscope

Method:

- Samples were labelled with respective numbers
- Diluted 4µl sample with 96µl 2% casein buffer per well on the ELISA plate for each sample as follows:



- Slides were incubated for 30 mins in humid condition
- After incubation, slides were washed in 1/10 PBS on magnetic stirrer and dried
- Conjugates were added onto all wells of the screening slide
- Slides were incubated for 30 mins in humid condition and washed in PBS by magnetic spin
- Dried slides were mounted with mounting fluid and cover slip
- Slides were observed at X 400 with UV lamp microscope
- Positive results were indicated by fluorescence and negative results without fluorescence.
- Positive samples were titrated to get end titres

Q Fever titration by IFA

Materials required:

- Q Fever IF screening record sheet
- Q Fever titration slide (thawed)
- 2% Casein buffer
- ELISA Plate
- 2-20 µL and 20-200 µL Eppendorf pipettes
- Sterile PBS
- Positive and negative controls (diluted 1 in 100 with 2 % casein)
- Rheumatoid Factor Removal Reagent (RFRR) (Chemicon Cat #RFRR05)
- Conjugate FITC anti-human (diluted 1 in 50 with 2 % casein) for:
- IgA (KPL Cat # 02-10-01)
- IgM (KPL Cat # 02-10-03)
- Conjugate FITC anti-human (diluted 1 in 100 with 2 % casein) for:
- IgG (KPL Cat # 02-10-02)
- Total IgA + IgG + IgM (H&L) (KPL Cat # 02-10-07)
- Mounting fluid and cover slip
- UV Lamp Microscope

Method:

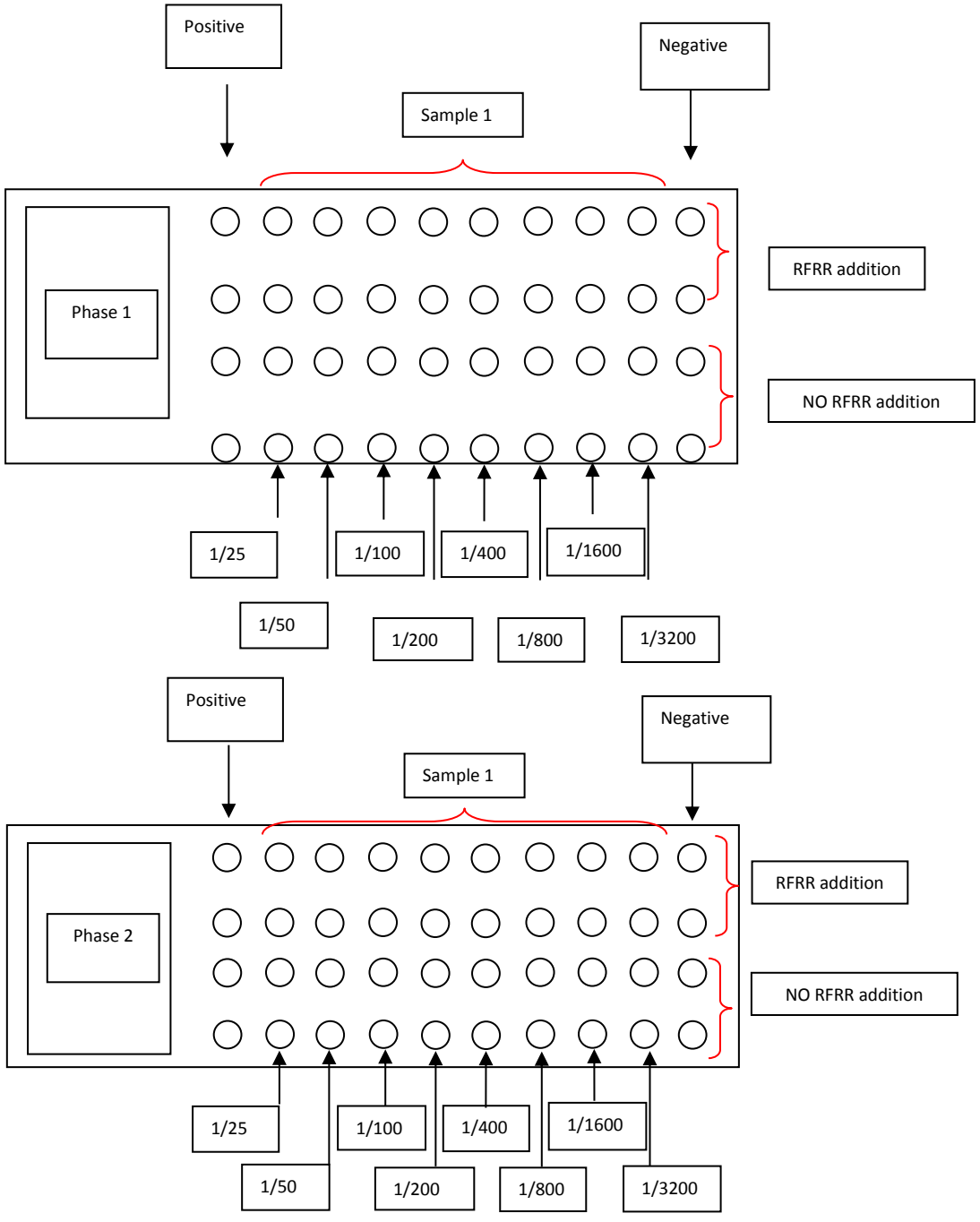
- Samples were labelled with sample numbers; in duplicate: one row of 8 wells for the addition of RFRR and one row for NO addition of RFRR

- For the row with RFRR addition, 4µl sample with 28µl of RFRR and 68µl 2% casein buffer were added to give a dilution of 1:25.

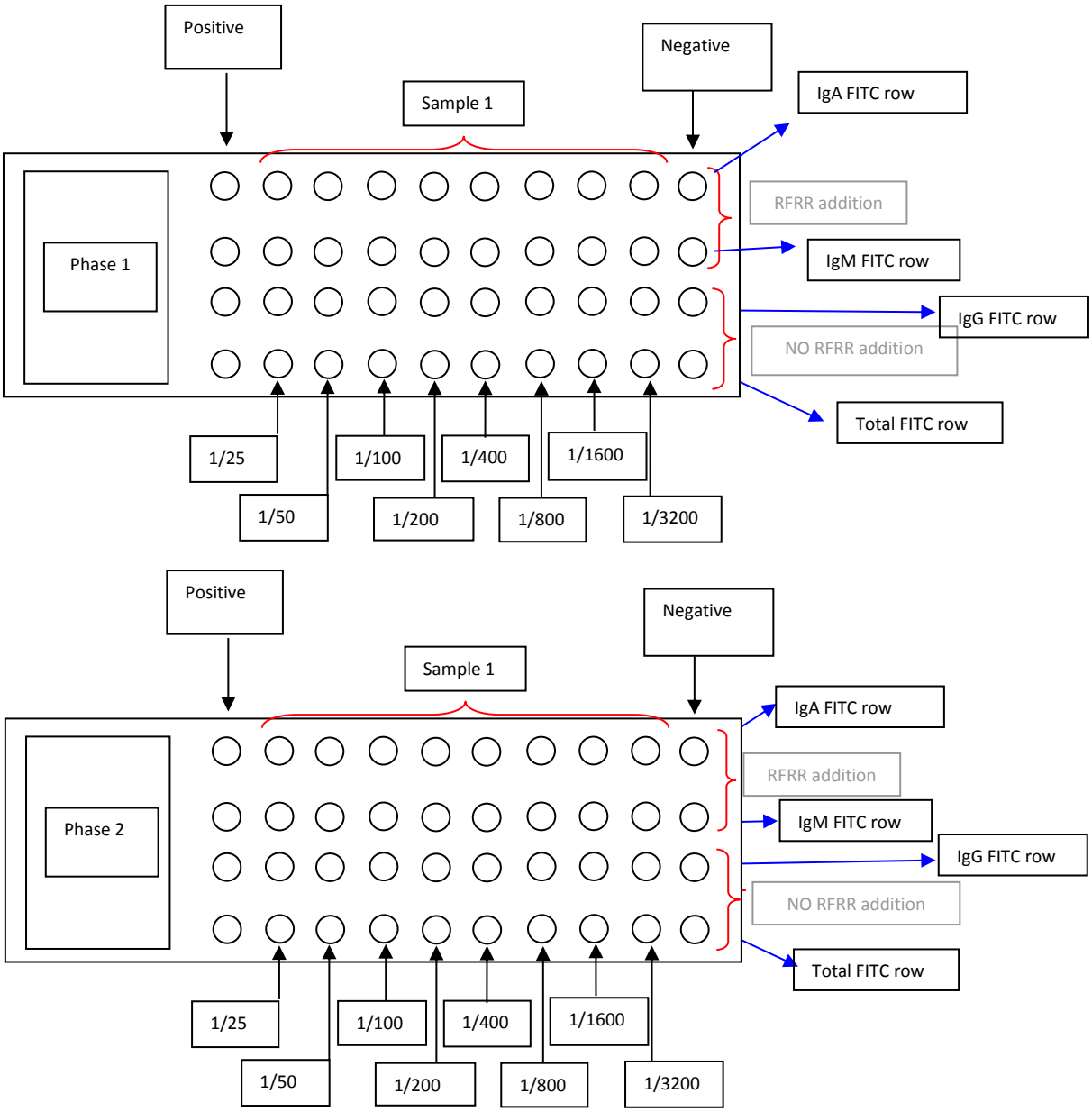
- To the next 7 wells 50µl of diluent was added to each. Serial dilution was performed from the 1:25 well by transferring 50µl from this dilution to the 2nd well (1:50),

mixing and then transferring 50µl to the 3rd well (1:100), mixing and transferring 50 µl to the 4th well (1:200) and so on until the last (8th well) dilution is 1:3600.

- For row with NO addition of RFRR, 4µl sample was diluted with 96µl 2% casein buffer to give a dilution of 1:25.
- 50µl of diluent was added to each of next 7 wells. Perform serial dilutions from the 1:25 well by transferring 50µl from this dilution to the 2nd well (1:50), mixing and then transferring 50µl to the 3rd well (1:100), mixing and transferring 50 µl to the 4th well (1:200) and so on until the last (8th well) dilution is 1:3600.



- Slides were incubated for 30 mins in humid condition and washed in PBS
- Conjugates were added onto all wells of the titration slides as follows:



- Slides were incubated for 30 mins in humid condition and washed in PBS
- Dried slides were mounted with mounting fluid and cover slip
- Slides were viewed under X 400 with UV lamp microscope
- Positive results were visualized with fluorescence and negative results lacked fluorescence

Appendix 13: DNA extraction and PCR protocol for *Rickettsia, Orientia* and *Coxiella*

Materials required

- Biological Safety Cabinet Class 2
- Laminar Flow Cabinet
- Transfer pipets
- Sterile PBS
- 100% ethanol
- Thermomixer (Eppendorf Thermomixer comfort)
- Pipets: 1-10µL, 2-20µL, 20-200µL and 100-1000µL and sterile filtered tips x 2 sets (one set designated to Biological Safety Cabinet for Sample prep and DNA extraction, the other designated to the Lamina Flow Cabinet for PCR)
- Real Genomics – Genomic DNA Extraction Kit (YGB100) :
    - RBC Lysis Buffer, GB Buffer, GT Buffer, Wash Buffer (Concentrated)
    - Elution Buffer, GD Column, 2ml Collection Tube
    - Eppendorf Microcentrifuge Tube
- 2X Platinum qPCR super mix-UDG (Master mix by Invitrogen)
- 50mM $MgCl_2$ (Invitrogen)
- Rotor Gene RG-3000 (Corbett Research)
- Primers and probe as described in following table: Primers from Invitrogen (https://www.thermofisher.com/au/en/home/brands/invitrogen.html) and Probes from Biosearch Technologies (https://www.biosearchtech.com/).

Primers and probes	**Sequences**	**Stock concentration**
Rickettsia		
CS Forward	TCG CAA ATG TTC ACG GTA CTT T	2µM
CS Reverse	TCG TGC ATT TCT TTC CAT TGT G	2µM
CS Probe	FAM TGC AATAGC AAG AAC CGTAGG CTG GATG BHQ1	2µM
Orientia		
16S rDNA Forward	CTT ATT TGC CAG CGG GTA ATG C	2µM
16S rDNA Reverse	GGG CCA TGA TGA CTT GAC CTC	2µM

16S rDNA Probe	FAM CCC ACC TTC CTC CGG CTT AGC ACC BHQ1	2μM
Coxiella		
Com 1 Forward	AAA ACC TCC GCG TTG TCT TCA	4μM
Com 1 Reverse	GCT AAT GAT ACT TTG GCA GCG TAT TG	4μM
Com 1 Probe	FAM AGA ACT GCC CAT TTT TGG CGG CCA BHQ1	4μM
htpAB Forward	GTG GCT TCG CGT ACA TCA GA	10μM
htpAB Probe	FAM AGC CAG TAC GGT CGC TGT TGT GGT BHQ1	10μM
htpAB Reverse	CAT GGG GTT CAT TCC AGC A	10μM

Methods

- All steps other than centrifugation and incubation steps should be performed inside the Biological Safety Cabinet.

Procedure for the extraction of DNA from buffy coat samples

- Ensure that Biological Safety Cabinet is clean and has been under UV light since last use (at least 10 minutes).
- Turn on thermomixer to 70°C
- Add ethanol (96-100%) to Wash Buffer (Wash 2) according to the Real Genomics Kit handbook.

RBC lysis

- Take 5 ml of RBC lysis buffer into a 10 ml conical centrifuge tube
- Add buffy coat sample into the tube containing RBC lysis buffer and shake vigorously
- Keep at room temperature for 15-30 minutes
- Centrifuge at 5000 x g for 5 minutes
- Decant the supernatant (by tilting the tube) and wash the pellet in about 5 ml of sterile PBS (repeat wash as required to get a clear pellet)
- In the last decant, leave about 400-500 µl of PBS and suspend the pellet well
- This can be used for DNA extraction, for culture or stored for future use

Cell Lysis

- Add 200µl GB Buffer to the sample tube and mix by vortexing
- Use a negative water control for each extraction batch
- Use a positive organism control for each extraction batch
- Incubate the sample mixture in the Thermomixer at 70°C for 10 minutes while shaking at 1200 x g until the sample lysate is clear.
- During this incubation, pre-heat required Elution Buffer (50µl per sample) in the Thermomixer

DNA Binding

- Add 200µl of 100% Ethanol to the sample lysate and mix immediately by vortexing for 10 seconds
- Place a GD Column on a 2ml Collection tube (labelled with the appropriate sample number)
- Apply the total mixture (including any precipitate) from step 4 to GD Column.
- Close the cap and centrifuge at full speed (14,000 x g) for 5 minutes
- Discard the flow-through and place the GD Column back in the Collection tube

Wash

- Add 500µl of Wash Buffer (Wash 1) into the column
- Centrifuge at 7,500 x g for 30 seconds
- Discard the flow-through and place the GD Column back in the Collection tube
- Repeat the Wash step. Add 500µl of Wash Buffer (Wash 2) (ethanol added) to wash again
- Place the GD Column back into a new collection tube
- Centrifuge at full speed for 3 minutes to dry the column matrix

DNA Elution

- Transfer dried GD Column into a clean 1.5ml microcentrifuge tube (pre-labelled with appropriate details)
- Add 50µl of preheated elution buffer into the centre of the column
- Stand for 2 minutes until elution buffer is absorbed by the matrix
- Centrifuge at 7,500 x g for 30 seconds to elute purified DNA

- Use extracted DNA for PCR or store in freezer until needed for PCR.
- Spray and wipe down floor of hood with 70% Ethanol and leave under UV light for >10 minutes.
- Also spray and wipe surfaces of centrifuge with 70% Ethanol

DNA detection using the real time PCR

- Clean and decontaminate the Laminar flow cabinet and the Biological Safety Cabinet with UV light (at least 10 minutes from last use)
- Thaw all reagents and samples. Master mix is kept in 500µL aliquots and kept in the -20°C freezer. Primers are kept in working concentrations in -20°C freezer, and stocks are kept in the -70°C freezer to minimise freeze thaw cycles.
- Fill out PCR work sheet for samples, positive and negative controls and use to calculate amounts required of each reagent for master mix.
- The reagents were combined manually or mixed with the liquid handling robot (CAS 1200).

Make up master mix for number of samples (x), controls (1 positive per run and 1 negative for every 3 samples) and an additional 2, as per the following table:

For Rickettsial PCR (targeting the Citrate Synthase gene) and *Orientia* (targeting 16S rDNA)

	Working concentrations	**Final Concentration**	**Amount**
Master mix	2X	1X	12.5µL
$MgCl_2$	50mM	2mM	1µL
Forward	2µM	200nM	2.5µL
Reverse	2µM	200nM	2.5µL
Probe	2µM	200nM	2.5µL
DNA			4µL

For Coxiella PCR (targeting the htpAB and com1 gene)

	Stock	**Final Concentration**	**Amount**
Master mix	2X	1X	12.5µL
$MgCl_2$	50mM	4.5mM	0.75µL
Forward	10µM	1000nM	2.5µL
Reverse	10µM	800nM	2µL
Probe	5µM	250nM	1.25µL
Water			1µL
DNA			5µL

- Place **21µL** of Rickettsial PCR mix, or **20µ**L of Coxiella mix into tubes.
- Move sealed tubes containing the master mix to sample preparation area.
- Place **4µL** of sample for Rickettsial PCR or **5µL** for Coxiella PCR into tube and close the cap.
- Repeat for all samples in PCR run.
- Add 4µL of fresh **dH_2O** into 1st negative Rickettsial control, or 5µL into 1st negative Coxiella control, and close the cap.
- Add 4µL of **known negative** sample into 2nd negative Rickettsial control, or 5µL into 2nd negative Coxiella control, and close the cap.
- Carefully take PCR samples to PCR room, open the Rotor Gene RG-3000 and gently place samples as number on the work sheet into rotor. Be careful to **handle tubes by the tops** so no fingerprints will interfere with the fluorescence.
- Open the Rotor-Gene 6 programme on the computer and select New.
- If the last run is the same type then select 'New', if not open a run of the same type and proceed to the Quick Start Wizard.

- Select tube size / rotor size
- Sample set up, rotor map or table. The programme will then offer a table to fill in what is in each tube (In case of CAS1200 preparation, select 'import' followed by the appropriate filename to retrieve the PCR sample reaction data). Fill out using the Worksheet completed in step 38. Ensure that all samples are "turned on" and allowed to run.

- Ensure that the thermo programme is as follows:

Thermo Programme: for CS, Com 1 and 16s program

		Temperature	Time
	First hold	50°C	3 minutes
	Second hold (denaturation)	95°C	5 minutes
Cycles	Denaturation	95°C	20 seconds
	Annealing and Extension	60°C	40 seconds (acquire FAM)
X 65 cycles			

Thermo Programme: for htpAB

		Temperature	Time
	First hold	50°C	3 minutes
	Second hold (denaturation)	95°C	5 minutes
Cycles	Denaturation	95°C	20 seconds
	Annealing and Extension	60°C	40 seconds (acquire ROX)
X 65 cycles			

- When everything is set select "Start Run"
- The programme will then ask you to name the file for the results to go in, select you file and for the title press Ctrl + V.
- Spray and wipe down floor of cabinet with 70% Ethanol and leave under UV light for >10 minutes.
- When run is completed, fill out Worksheet with +/- and the Ct of any positives. The worksheet can then be used as a record of the run.
- Run is valid if the positive controls are positive, and all negative controls are negative.
- Any sample with Ct value of < 35 is taken as positive, 35-40 as equivocal and repeated, > 40 as negative