Molecular epidemiology of spotted fever group rickettsiae in Zambia and development of multiplex LAMP for simultaneous detection of the rickettsiae and malaria parasites

（ザンビアにおける紅斑熱群リケッチアの分子疫学とマルチプレックス LAMP を利用したマラリア原虫との同時検出法の開発）

Lavel Chinyama Moonga

Division of Collaboration and Education,

Graduate School of Infectious Diseases,

Hokkaido University, Japan.

Table of Contents

Table of Contents **1**

List of publications related to the dissertation **4**

List of abbreviations **5**

1. General introduction **6**

1.1 *Rickettsia* genus classification and epidemiological distribution 6

1.2 Clinical presentation of rickettsiosis in human 11

1.3 Diagnosis and treatment of rickettsiosis 11

2. Chapter 1 ..... **13**

Molecular detection of *Rickettsia felis* in dogs, rodents and cat fleas from Zambia **13**

2.1 Background 13

2.2 Materials and methods 16

2.2.1 Animal and arthropod sample collection 16

2.2.2 PCR amplification and amplicon sequencing 18

2.2.3 Alignment and phylogenetic analysis 18

2.2.4 Deep sequencing of biotin synthase gene (*bioB*) positive samples using next generation sequencer 20

2.2.5 Criteria for species identification 21

2.2.6 Statistics 21

2.3 Results 22

2.4 Discussion 27

2.5 Conclusion 30

3. Chapter 2 ..... **31**

Molecular detection and characterization of *Rickettsia asembonensis* from human blood in Zambia **31**

3.1 Background 31

3.2 Materials and methods 33

3.2.1 Human blood samples 33

3.2.2 DNA extraction 35

3.2.3 PCR screening and multiple gene sequencing 35

3.2.4 *Rickettsia* quantity estimation from blood 37

3.4 Results 38

3.5 Discussion 43

3.6 Conclusion 45

4. Chapter 3 46

Development of a multiplex loop-mediated isothermal amplification (LAMP) method for simultaneous detection of spotted fever group rickettsiae and malaria parasites by dipstick DNA chromatography 46

4.1 Background 46

4.2 Materials and methods 49

4.2.1 Multiplex LAMP 49

4.2.2 Visualization of multiplex amplified products by dipstick DNA chromatography (C-PAS) 51

4.2.3 The determination of the sensitivity of multiplex LAMP assay using synthetic gene 53

4.2.4 The determination of the sensitivity of multiplex LAMP assay using genomic DNA from in vitro cultured *R. monacensis* or *P. falciparum* 53

4.2.5. Detection of *R. monacensis* and *P. falciparum* spiked with human blood from DNA card using heating block and dipstick chromatography 54

4.3 Results 55

4.3.1 Multiplex LAMP using single synthetic templates of *Rickettsia* or *Plasmodium* genes in plasmids 55

4.3.2 Optimization of multiplex LAMP using mixed synthetic templates of *Rickettsia* and *Plasmodium* gene, and discrimination by C-PAS 57

4.3.3 Validation of multiplex LAMP using genomic DNA from in vitro cultured *R. monacensis* or *P. falciparum* organisms 60

4.3.4 Comparative sensitivity of the multiplex LAMP system against real-time qPCR system 62

4.3.5. Simplified LAMP reaction using human blood spiked samples and field-deployable amplification methods 64

4.4 Discussion 66

4.5 Conclusion 68

5. General Summary 69

6. Acknowledgements 70

7. References 72

8. Summary in Japanese 88

List of publications related to the dissertation

The contents of this dissertation are based on research conducted during the period from April 2017 to March 2021, and part of the results have been published in the following publications.

The contents of Chapter 1 have been published in *Parasites and Vectors*.

Moonga LC, Hayashida K, Nakao R, Lisulo M, Kaneko C, Nakamura I, Eshita Y, Mweene AS, Namangala B, Sugimoto C, Yamagishi J. 2019. Molecular detection of *Rickettsia felis* in dogs, rodents, and cat fleas in Zambia. *Parasit Vectors* **12**:168.

The contents of Chapter 3 have been published in *Diagnostics*.

Moonga LC, Hayashida K, Kawai N, Nakao R, Sugimoto C, Namangala B, Yamagishi J. 2020. Development of a multiplex loop-mediated isothermal amplification (LAMP) method for simultaneous detection of spotted fever group rickettsiae and malaria parasites by dipstick DNA chromatography. *Diagnostics* **10**:897.

List of abbreviations

LAMP	Loop-mediated isothermal amplification
C-PAS	Chromatographic Printed Array Strip
SFG	Spotted fever group
RFLOs	*Rickettsia felis*-like organisms
TG	Typhus group
TRG	Transitional group
AG	Ancestral group
cox1	cytochrome *c* oxidase subunit 1 gene
cytb	cytochrome *b* gene
gltA	citrate synthase gene
ompA	outer membrane protein *A* gene
ompB	outer membrane protein *B* gene
bioB	biotin synthase gene
RfelB	*Rickettsia felis* specific qPCR based on *ompB*
BLAST	Basic Local Alignment Search Tool
NCBI	National Center for Biotechnology Information
MEGA7	Molecular Evolutionary Genetics Analysis version 7
htrA	17-kDa outer membrane antigen gene
MAFFT	Multiple Alignment using Fast Fourier Transform
dsDNA HS	double stranded DNA High Sensitivity
SNP	Single Nucleotide Polymorphism
UFI	Undifferentiated Febrile Illness
NAAT	Nucleic Acid Amplification-based test
LF	Loop forward
LB	Loop backward
FIP	Forward Inner Primer
BIP	Backward Inner Primer
F3	Outer forward primer
B3	Outer backward primer
CFI	Colori-Fluorometric Indicator
dNTP	deoxyribonucleotide triphosphate

1. General introduction

1.1 *Rickettsia* genus classification and epidemiological distribution

Rickettsiae organisms belong to the genus *Rickettsia*, and some of them are transmitted to humans via hemophagous arthropod vectors. The *Rickettsia* genus has traditionally been antigenically and phylogenetically classified into the spotted fever group (SFG), typhus group (TG), and ancestral group (AG) (Shpynov *et al*., 2015; Shpynov *et al*., 2018). However, sequence-based phylogenomics analysis recognizes the classification of the transitional group (TRG) as a fourth group closely related to SFG (Fournier *et al*., 2003; Murray *et al*., 2016) (Figure 1). The TRG classification generated controversy based on genetic and genomic criteria, and is not widely accepted (Sachman-Ruiz and Quiroz-Castañeda, 2018). Therefore, TRG will be considered as a subgroup of SFG and SFG without TRG as the "classical" SFG for descriptive purposes in this thesis. The "classical" SFG consists of *Rickettsia rickettsii*, *Rickettsia africae*, *Rickettsia conorii*, *Rickettsia japonica*, *Rickettsia heilongjiangensis*, *Rickettsia sibirica*, *Rickettsia slovaca*, *Rickettsia raoultii*, *Rickettsia aeschlimannii*, *Rickettsia monanensis*, *Rickettsia parkeri*, *Rickettsia massiliae*, *Rickettsia honei*, and *Rickettsia helvetica*. The primary host and vector for "classical" SFG are ticks. The TRG is comprised of *Rickettsia akari*, *Rickettsia felis,* and other *Rickettsia felis*-like organisms (RFLOs). *Rickettsia akari* is transmitted by mites while *R. felis* and RFLOs have a diverse vector and host range with fleas being the most common. *Rickettsia felis*-like organisms are composed of *Rickettsia asembonensis* and *Rickettsia senegalensis* with other closely related unspecified *Rickettsia* species. The typhus group consists of *Rickettsia typhi* and *Rickettsia prowazekii,* which are transmitted to humans via flea and human body louse, respectively. Humans play a primary role in the natural transmission cycle of *R. prowazekii* where it can persist in latent state (Bechah *et al*., 2008) while humans act as secondary host in the other *Rickettsia* spp. (Raoult, 1997). Currently, there are more than 20 *Rickettsia* species known to cause disease in humans (Table 1). The other *Rickettsia* species are described with unknown pathogenicity mainly because they have only been isolated from arthropods, and not been isolated or detected in humans. However, this list of human infective *Rickettsia* species may be updated in future as more studies are conducted, as was the case for *R. africae* (La Scola and Raoult, 1997). *Rickettsia africae* was first isolated from ticks and was considered non-pathogenic. *Rickettsia africae* was later recognized as human infective agent following isolation from human patient's blood (Kelly *et al*., 1992, 1996). The ancestral group comprises *Rickettsia bellii* and *Rickettsia canadensis* which are maintained in ticks and are not known to cause human rickettsiosis. Despite the unknown pathogenicity of some *Rickettsia* species,

their potential to infect humans cannot be overlooked. The probable human infection could occur through *Rickettsia* inoculation by an infected bloodsucking arthropod.

Rickettsia genus is spread worldwide except Antarctica. The majority of *Rickettsia* species are only found in their natural vector and/or mammalian host. Therefore, climatic conditions that limit vectors and natural host distribution make many *Rickettsia* species being region-locked (Abdad *et al.*, 2018). On the other hand, some *Rickettsia* species such as flea-borne *R. felis* and *R. typhi* transmitted by cosmopolitan vectors have a worldwide distribution (Abdad *et al*., 2011). Other vectors that have worldwide distributions are mites and lice, which transmit *R. akari* and *R. prowazekii*, respectively. *Rickettsia felis* has also been found in various arthropods including non-hematophagous booklice and mosquitoes, posing the potential to change the understanding of its ecology and transmission cycle (Behar *et al*., 2010; Socolovschi *et al*., 2012). Most SFG and AG rickettsiae are maintained by ixodid ticks hence their distribution is limited by the distribution of their host ticks (Table 1).

Table 1. Common *Rickettsia* species causing rickettsiosis in humans and their geographical distribution

Group	Species	Disease name	Main host	Distribution	Reference
Spotted fever group (SFG)	*Rickettsia rickettsii*	Rocky Mountain spotted fever	Tick	America	(Jongejan and Uilenberg, 2004; Piesman and Eisen, 2008)
	Rickettsia africae	African tick-bite fever	Tick	Sub-Saharan Africa, West Indies	(Piesman and Eisen, 2008; Lorusso *et al*., 2016)
	Rickettsia conorii	Mediterranean spotted fever	Tick	Europe, Asia, Africa	(Piesman and Eisen, 2008; Lorusso *et al*., 2016)
	Rickettsia japonica	Japanese spotted fever	Tick	Asia	(Mahara, 2006)
	Rickettsia heilongjiangensis	Far-Eastern tick-borne rickettsiosis	Tick	Russia, Asia	(Wood and Artsob, 2012; Abdad *et al.*, 2018)
	Rickettsia sibrica	Siberian tick typhus	Tick	Russia, Asia, Europe	(Fournier *et al*., 2005; De Sousa *et al*., 2008; Zhao *et al*., 2019)
	Rickettsia slovaca	Tick-borne lymphadenitis	Tick	Europe, Asia	(Ibarra *et al*., 2006; Abdad *et al*., 2018)
	Rickettsia raoultii	Tickborne lymphadenopathy	Tick	Europe, Asia	(Zhao *et al*., 2019)
	Rickettsia aeschlimanii	Unnamed rickettsiosis	Tick	Africa	(Pretorius and Birtles, 2002)
	Rickettsia monacensis	Unnamed rickettsiosis	Tick	Europe, Africa	(Jado *et al.,* 2007; Sfar *et al*., 2008)
	Rickettsia parkeri	*Rickettsia parkeri* rickettsiosis	Tick	America	(Bartlett, 2004)

Group	Species	Disease name	Main host	Distribution	Reference
SFG	*Rickettsia masilliae*	Unnamed rickettsiosis	Tick	Africa, America, Europe	(Merhej *et al*., 2014; Vitale *et al*., 2006)
	Rickettsia honei	Flinders Island spotted fever	Tick	Australia, Thailand	(Jiang *et al*., 2005; Stenos *et al*., 2003)
	Rickettsia helvetica	Unnamed rickettsiosis	Tick	Europe, Asia, Africa	(Sfar *et al.*, 2008; Abdad *et al.*, 2018)
Transitional group (TRG)	*Rickettsia felis*	Flea-borne spotted fever	Cat flea	Worldwide	(Angelakis *et al*., 2016)
	Rickettsia asembonensis	Unknown pathogenicity	Flea	Worldwide	(Maina *et al*., 2019)
	Rickettsia senegalensis	Unknown pathogenicity	Flea	America, sub-Saharan Africa	(Roucher *et al.*, 2012; Maina *et al.*, 2016a; Betancourt-Ruiz *et al.*, 2019)
	Rickettsia akari	Rickettsialpox	Mite	Worldwide	(Boyd 1997; Wood and Artsob, 2012)
	Rickettsia australis	Queensland tick typhus	Tick	Australia	(Sexton *et al*., 1991)
Typhus group (TG)	*Rickettsia typhi*	Murine typhus	Flea	Worldwide	(Walker and Fishbein, 1991)
	Rickettsia prowazekii	Epidemic typhus	Human	Africa, Asia, America	(Walker and Fishbein, 1991)
Ancestral group (AG)	*Rickettsia canadensis*	Unknown pathogenicity	Tick	North America	(Wood and Artsob, 2012)
	Rickettsia bellii	Unknown pathogenicity	Tick	North and South America	(Philip *et al*., 1983)

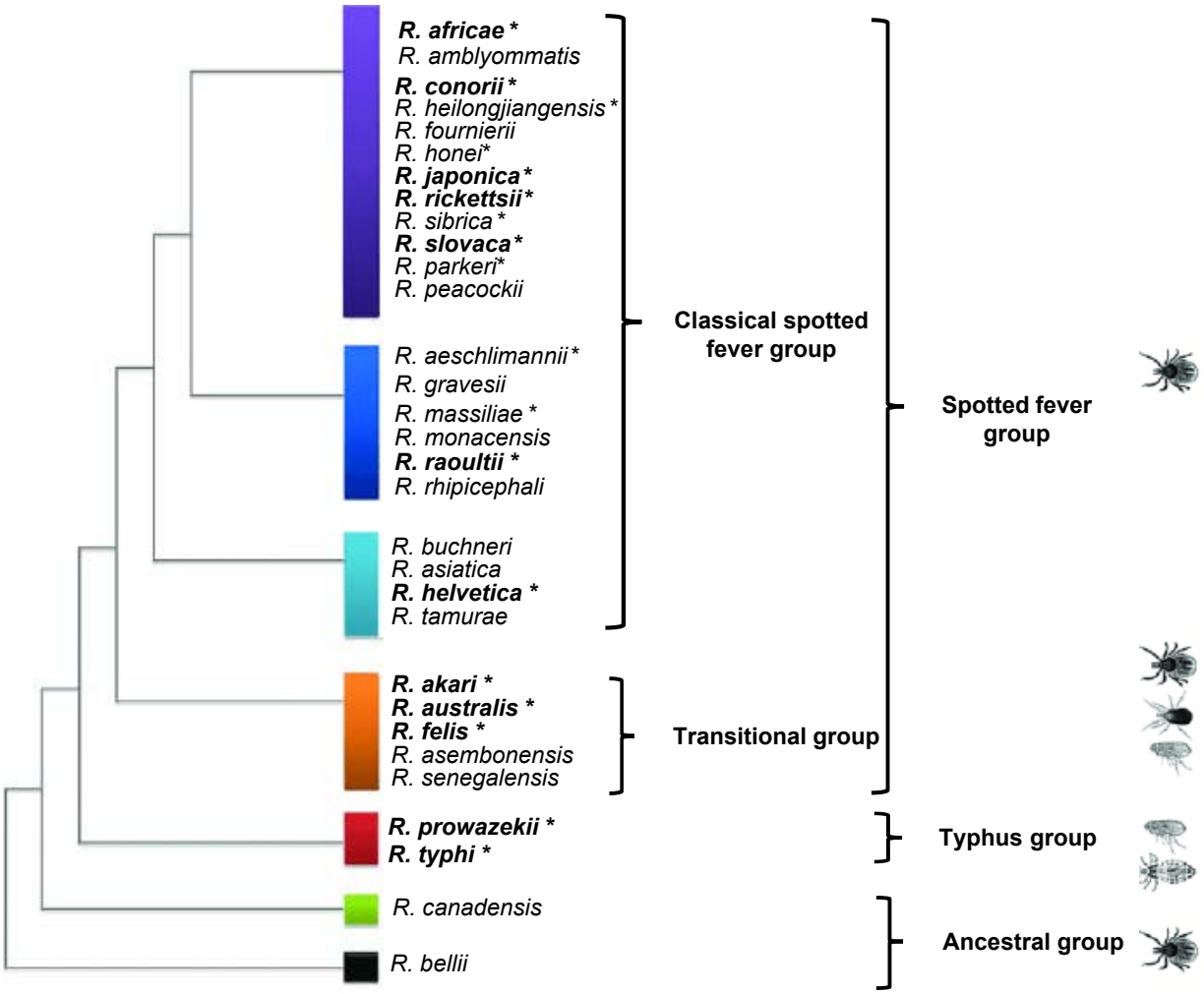

Figure 1. Phylogenetic tree of *Rickettsia* species based on concatenated *gltA* and *sca4* (Sekeyová *et al*., 2019). *Rickettsia* species are distributed into 4 groups: "Classical" SFG, TRG, TG, and AG. "Classical" SFG is mostly associated with ticks; TG with human body lice (*R. prowazekii*) and rat fleas (*R. typhi*); TRG with ticks, cat fleas, or mites; and AG with ticks. RFLO represents *Rickettsia felis*-like organisms which are *R. asembonensis* and *R. senegalensis*. Asterisks mark pathogenic species, and bold letters indicate species causing central nervous system infections.

1.2 Clinical presentation of rickettsiosis in human

The common clinical manifestation of rickettsiosis is fever. Other clinical symptoms associated with rickettsial infections include headache, malaise, muscle pain, and cutaneous eruption. Rickettsioses such as Rocky Mountain spotted fever and Mediterranean spotted fever have long been described exclusively based on clinical evidence of a characteristic rash. Rickettsiae invade endothelial cells and proliferate in the vascular endothelium resulting in vasculitis. Following vasculitis, skin rash develops, therefore the disease was named spotted fever. However, rash has been rarely reported in rickettsial infections (Colomba *et al*., 2006; Helmick *et al*., 1984). Rather, most clinical presentations of rickettsioses are non-specific and indistinguishable from other causes of febrile illnesses including those of more virulent disease such as Rocky Mountain spotted fever (Sexton and Corey, 2018). Flea-borne rickettsiae, specifically flea-borne spotted fever and Murine typhus, have many similarities including antigenic cross-reactivity and sharing the similar flea vector, hence not easily distinguishable (Schriefer *et al*., 1994; Williams *et al*., 1992). Few cases have presented with an 'eschar', which is a single and crusted cutaneous lesion surrounded by inflammation, thought to represent the site of inoculation via an arthropod (Jensenius *et al*., 2003). The term 'yaaf' was used to describe the vesicles of *R. felis* infection in Senegal (Mediannikov *et al*., 2013a). The neurologic signs including meningoencephalitis and subacute meningitis have also been reported in rickettsial infection cases (Mawuntu *et al*., 2020; Salva *et al*., 2014; Zavala-Velázquez *et al*., 2000).

1.3 Diagnosis and treatment of rickettsiosis

The gold standard for rickettsiosis diagnosis is serology. Immunofluorescence assay (IFA) is the reference serology method for the diagnosis of rickettsiosis. Seroepidemiological studies are also commonly conducted by using IFA (Jang *et al*., 2005; Robinson *et al*., 2019). The genus *Rickettsia* is classified into two major disease-causing antigenic groups namely the SFG and TG. However, there is considerable cross-reactivity between spotted fever group and typhus group antibodies in human sera (Paris and Dumler, 2016; La Scola and Raoult, 1997). Additionally, some cases of rickettsiosis have been reported to lack seroconversion, hence rendering serological methods ineffective. Regardless of the downsides, serological methods remain the diagnostic tool of choice due to their quick processing and minimal sample preparation time. Other serological tests that have been used include the Weil-Felix test, ELISA, and western immunoblot. The Weil-Felix test is the oldest but still serves as a useful and cheapest available tool for the laboratory diagnosis of rickettsial diseases despite its poor sensitivity and low specificity (Isaac and Varghese, 2004). ELISA and western immunoblot are

more applicable for seroepidemiological studies and more specific as western immunoblot can distinguish species by cross-adsorption (La Scola and Raoult, 1997).

Polymerase chain reaction-based methods are the essential methods for the detection of spotted fever group rickettsia particularly, during the early phase of infection before the development of detectable antibodies (Robinson *et al*., 2019; Santibáñez *et al*., 2013). However, PCR-based methods are used primarily for epidemiological purposes. PCR as a clinical diagnostic tool is not yet readily used as Point of Care Testing (POCT) by clinicians because few diagnostic laboratories have access to the equipment, laboratory experts, and the resources for PCR assays. Whole blood is the main sample type used for PCR detection of *Rickettsia* infection, because *Rickettsiae* are leaked into blood after endothelial vasculature damage. However, detection of *Rickettsia* in blood by PCR is limited by low quantities of rickettsial organisms in non-fatal cases which ranged from 8.40×10^1 to 3.95×10^5 copies/ml of blood, whilst in patients with fatal infections copy numbers ranged from 1.41×10^3 to 2.05×10^6 copies/ml of blood (Kato *et al*., 2016). Other sample types used for diagnosis of human rickettsiosis include tissue biopsies such as skin, eschar scrapings, and swabs. Eschar scrapings and swabs tend to have higher concentration of *Rickettsia* hence improving the sensitivity of detection by PCR.

An alternative to the standard PCR assays, suitable for more field-based diagnosis, is the loop-mediated isothermal amplification assay (LAMP). LAMP is a simple and rapid molecular detection method employing an isothermal nucleic acid amplification technique (Notomi *et al*., 2000). LAMP has potential as POCT as it does not require sophisticated laboratory and operates with ease to use equipment. The LAMP methods for the detection of SFG rickettsia are increasingly being developed (Hanaoka *et al*., 2017; Noden *et al*., 2018; Pan *et al*., 2012). Since both PCR and LAMP methods are targeting conserved gene(s) among SFG, species differentiation by gel electrophoresis is not feasible. Therefore, further sequencing analysis is required to differentiate at species level. Such a species identification is important for the provision of epidemiological data but less valuable for treatment intervention.

Doxycycline is the first-line drug for the treatment of rickettsioses. Clinical signs such as fever usually subside within 24-72 hours after drug administration. Tetracyclines are also effective against rickettsiosis. Other drugs that have been used and showed efficacy include fluoroquinolones, chloramphenicol, and sulphonamides (Rolain *et al*., 1998).

2. Chapter 1

Molecular detection of Rickettsia felis in dogs, rodents and cat fleas from Zambia

2.1 Background

Rickettsiae are obligate intracellular Gram-negative bacteria that belong to the order Rickettsiales in the class Alphaproteobacteria. The genus *Rickettsia* is divided into four groups; among them is the TRG. The TRG consists of *R. akari*, *R. australis*, and *R. felis*. While cat fleas (*Ctenocephalides felis*) transmit *R. feli*s, *R. akari* and *R. australis* are transmitted by mites and ticks, respectively. *Rickettsia felis* is the causative agent of zoonotic "cat flea typhus" also known as "flea-borne spotted fever" in humans. The bacterium was first described in 1990 in the cytoplasm of midgut endothelial cells of cat flea (*C. felis*). The bacterium was provisionally named as "ELB agent" for the EL Laboratory (Soquel, CA) where it was first identified (Adams *et al*., 1990). It was later characterized and renamed as *R. felis* following approval by the International Society for Systematic and Evolutionary Biology. *Rickettsia felis* was subsequently realized as the causative agent for flea-borne spotted fever in humans (Bouyer *et al*., 2001; Raoult *et al*., 2001b; Schriefer *et al*., 1994). Since then *R. felis* has been reported worldwide (Parola, 2011).

Flea-borne spotter fever is one of the emerging rickettsioses to which little attention has been accorded. *Rickettsia felis* is classified in SFG rickettsiosis based on the clinical disease manifestation. The first human case of *R. felis* infection was reported in 1994 in Texas, USA (Schriefer *et al*., 1994), since then it remained neglected until it emerged as a cause of febrile illness in sub-Saharan Africa (Parola, 2011). It has since been reported in eastern and western sub-Saharan Africa (Berrelha *et al*., 2009; Maina *et al*., 2012; Mediannikov *et al*., 2013a) and North Africa (Hamzaoui *et al*., 2020). *Rickettsia felis* infections in humans have also been reported in the USA, Brazil, and Mexico with varying clinical signs and severity. Furthermore, cases of *R. felis* infection have been reported in Taiwan, Thailand, and South Korea, as well as in France, Germany, and Spain. (Angelakis *et al*., 2016; Reif and Macaluso, 2009). This worldwide distribution of *R. felis* infections in humans and a wide host and vector/potential vector range made the pathogen to be considered as a global threat to human health (Pérez-Osorio *et al*., 2008). Once a susceptible human is infected, SFG rickettsia mainly invades endothelial cells and causes vasculitis, acute flu-like symptoms, fever, chills,

headache, skin rash, and photophobia (Renvoisé *et al*., 2009) as the clinical manifestations of flea-borne spotted fever. Fever is the most common clinical symptom of *R. felis* infection worldwide. The disease can be self-limiting to severe if untreated (Mawuntu *et al*., 2020; Zavala-Castro *et al*., 2009).

Cat fleas have been considered as the hosts and biological vectors of *R. felis*. *Rickettsia felis* in cat fleas has been suggested to be both transovarially and transtadially transmitted (Wedincamp and Foil, 2002). However, recent nucleotide detection reports suggest the role of other arthropods including various flea species, ticks, mites, and booklice in the epidemiological cycle of *R. felis* (Rakotonanahary *et al*., 2017; Abarca *et al*., 2013; Tsui *et al*., 2007; Thepparit *et al*., 2011). In addition to cat fleas, *R. felis* has been reported in the following flea species: *Ctenocephalides canis*, *Ctenocephalides orientis*, *Anomipsyllus nudata*, *Archaeopsylla erinacei*, *Ctenophthalmus* sp., *Echidnophaga gallinacean*, *Xenopsylla cheopis*, *Xenopsylla brasilliensis*, *Tunga penetrans*, *Ceratophyllus gallinae*, *Spilospsyllus cuniculi,* and *Pulex irritans* (Hawley *et al*., 2007). *Rickettsia felis* has also been detected in *Aedes albopictus* mosquitoes in Gabon (Parola, 2011; Socolovschi *et al*., 2012) and was experimentally proven to be transmitted by *Anopheles gambiae* in a mouse model (Dieme *et al*., 2015). This assertion was further supported by the elucidation of frequent co-infection of febrile patients with *Plasmodium* spp. and *R. felis* in malaria-endemic areas of Senegal, suggesting co-transmission by mosquitos (Mediannikov *et al*., 2013b).

Domestic dogs and cats are considered to be the mammalian reservoir hosts for *R. felis* (Gracia *et al*., 2015; Hii *et al*., 2011), although the role of mammals as reservoir is still under debate since the pathogen are detected only by the molecular method and never been isolated from any mammalian blood. Other proposed mammalian reservoirs include domestic/peridomestic rodents, opossums, and raccoons which are suggested to maintain the pathogen in peridomestic areas (Panti-May *et al*., 2015; Sashika *et al*., 2010). What is unclear is the interaction between the domestic/peridomestic cycles for pathogen transmission and maintenance in nature. Cat fleas could be playing an essential role in both cycles and risking human exposure due to their indiscriminate feeding behavior (Boostrom *et al*., 2002). Therefore, understanding the epidemiology of *R. felis* is vital for implementation of effective control measures as host animals share habitat with humans.

Although *R. felis* is frequently detected in western and eastern sub-Saharan Africa, little is known about its epidemiology in Zambia and the public health risk it poses. On the other hand, the presence of other spotted fever group rickettsia have been reported from Zambia; 16.7% seroprevalence for antibodies against *R. conorii* in humans was reported (Okabayashi *et al*., 1999) although cross-reactivity of closely related *Rickettsia* species cannot

be excluded (Znazen *et al*., 2006). Later, molecular evidence of spotted fever group rickettsia closely related to *R. africae*, the causative agent for African tick bite fever was reported in free-ranging non-human primates in Mfuwe district near Luangwa national park, Zambia (Nakayima *et al*., 2014). With endemic malaria in Zambia, little attention has been given to other pathogens that cause febrile illness (Masaninga *et al*., 2013), hence possibility of misdiagnosis could be expected. A study in Kenya found strong evidence of the association of *R. felis* with a fever of unknown origin (Richards *et al*., 2010). To date, insufficient studies have been conducted to elucidate the prevalence of *R. felis* in animals, humans, and arthropods in Zambia. Understanding the epidemiology of *R. felis* in mammalian hosts and arthropod vectors/potential vectors is of prime importance. The epidemiological data is vital for predicting the potential risk to human transmission. This study aimed to detect and characterize spotted fever group rickettsia from animal hosts and possible arthropod vectors in Zambia by molecular techniques. The findings would provide the baseline for understanding the circulation of *Rickettsia* spp. in Zambia and risk of human exposure to the infection.

2.2 Materials and methods

2.2.1 Animal and arthropod sample collection

Blood samples from visibly healthy domestic dogs (*n* = 150) were collected from Lusaka district in Lusaka Province, and Mazabuka and Monze districts in Southern Province in 2015 (Figure 2). Genomic DNA was extracted from blood using the DNAzol kit (Molecular Research Center, OH, USA) according to the manufacturer's protocol. Genomic DNA samples (*n* = 106) from rodents were collected in a previous study (Nakamura *et al.*, 2013). The rodent species were identified by partial sequence of the cytochrome *c* oxidase subunit 1 gene (*cox1*) or cytochrome *b* gene (*cytb*). The analyzed species included *Mastomys* sp. (*n* = 77), *Steatomys* sp. (*n* = 10), *Saccostomus* sp. (*n* = 3), *Rattus rattus* (*n* = 3) and unidentified species in subfamily Gerbillinae (*n* = 13). The rodent samples were collected from Lusaka district in Lusaka Province and Namwala district in Southern Provinces of Zambia.

Samples of fleas (*n* = 53) infesting dogs in Mazabuka district, Southern Province were collected in 2016. The fleas were identified using morphological taxonomical keys (Marcos and Costa, 2012). Each single flea was separately homogenized and DNA was extracted. The flea species was then determined by sequencing the cytochrome *c* oxidase subunit 1 gene *cox1* using published primers (Lawrence *et al*., 2014) (Table 2). The identification of the flea species was done by sequence homology using the BLAST program in NCBI website (https//www.blast.ncbi.nlm.nih.gov/Blast.cgi). The species classification threshold was set above 97% homology (Hebert *et al.*, 2003). The sampled fleas belonged to the *Ctenocephalides felis strongylus* and *Echidnophaga gallinacean* species (Table 3). Mosquitoes of different species were collected by using the CDC light trap and Biogents (BG)-Sentinel traps in Lusaka, Mpulungu and Isoka districts of Zambia during the 2016-2017 period. They were then identified to species level using morphological taxonomic key. The mosquitoes were then pooled (*n* ≤ 10) based on identical sex and species, homogenized and subjected to DNA extraction using Takara SimplePrep reagent for DNA (Takara, Shiga, Japan).

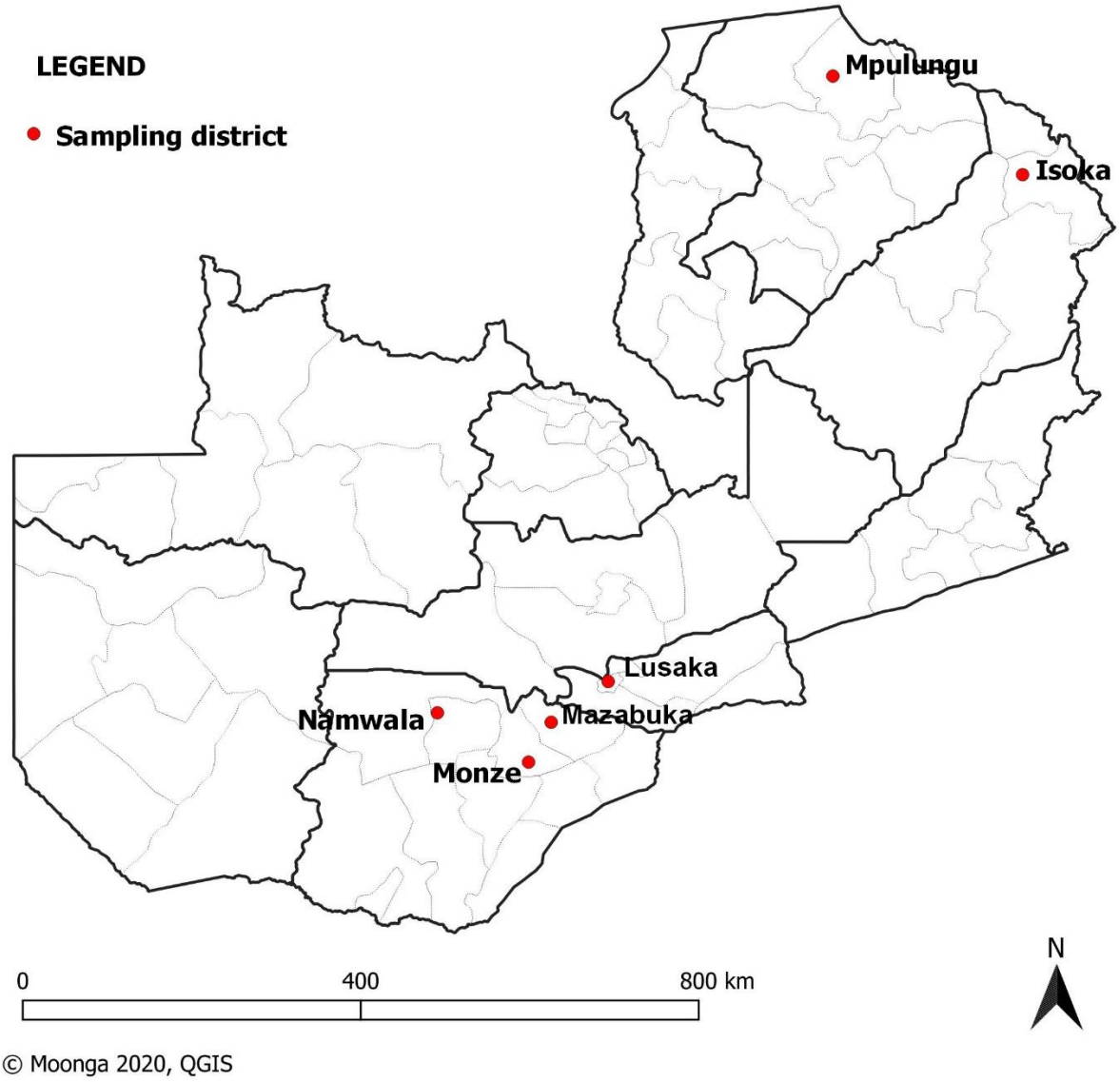

Figure 2. Map of sample collection sites. Dog, rodent, flea, and mosquito samples were collected from six districts. Red points on the map indicate the collection sites. QGIS version 3.10.7 was used for creating the map (QGIS Geographic Information System. Open Source Geospatial Foundation Project. http://qgis.osgeo.org).

2.2.2 PCR amplification and amplicon sequencing

Conventional PCR targeting the partial citrate synthase gene (*gltA*) for *Rickettsia* genus was performed using reported primers (Wa *et al*., 2013), Ampdirect plus buffer (Shimadzu, Tokyo, Japan), and BioTaq Polymerase (Bioline, London, UK). The PCR amplification was performed at 94°C DNA denaturation, 54°C annealing and 72°C extension temperatures for 35 cycles. The amplicons were electrophoresed on a 1.2% agarose gel stained with GelRed (Biotium, CA, USA) and visualized under UV light. The samples positive for *gltA* PCR were processed for PCR targeting the outer membrane protein *A* gene (*ompA*) and outer membrane protein *B gene* (*ompB*) using primers designed in this study. The primers targeting *ompA* and *ompB* were designed by using the Primer-blast tool in NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The *ompA* and *ompB* sequences for *R. felis* accession number CP000053 downloaded from GenBank were used as references for primer design. The PCRs were conducted as described above with modification of annealing temperature (48°C for the *ompA* and *ompB*) and amplicons were electrophoresed on 2% and 1.2% agarose gels, respectively. *Rickettsia monacensis* DNA extracted from cell culture (kindly provided by Dr. Ryo Nakao, Hokkaido University) was used as positive control and nuclease-free water as a negative control in all assays. All samples that were *gltA* PCR positive were further subjected to *R. felis* and *R. asembonensis* specific qPCR targeting biotin synthase gene (*bioB*) and *R. felis* specific unique segment of outer membrane protein gene (*RfelB*) using primers and probe described previously (Odhiambo *et al*., 2014; Socolovschi *et al*., 2010). All primers and probes used are shown in Table 2.

PCR products were treated with ExoSap-IT (ThermoFisher Scientific, MA, USA) according to the manufacturer's instructions. DNA concentration was quantified using a Nanodrop spectrophotometer. Amplicons were sequenced by Sanger sequencing using BigDye Terminator v3.1 cycle sequencing kit (ThermoFisher Scientific, USA) with the same primers as used for PCR amplification and analyzed on a 3500xl Genetic Analyzer (Applied Biosystems, CA, USA). The DNA sequences obtained were submitted to the DNA Data Bank of Japan (DDBJ) (http://www.ddbj.nig.ac.jp) under accession numbers LC431490 – LC431502.

2.2.3 Alignment and phylogenetic analysis

Obtained nucleotide sequences were assembled and aligned with relevant sequences from BLAST in NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using ClustalW aligner. Phylogenetic analysis of sequences obtained in the present study along with reference sequences was performed by Neighbor-Joining method supported by 500 bootstrap replicates using MEGA 7.0.2.1 software (Kumar *et al*., 2016).

Table 2. Primer sets used for PCR amplification and amplicon sequencing

Target gene	Target organism	Annealing temperature (°C)	Amplicon size (bp)	Oligonucleotide sequence (5'-3')	Reference
Citrate synthase gene (*gltA*)	Spotted fever group rickettsia	54	581	Forward: CGAACTTACCGCTATTAGAATG Reverse: CTTTAAGAGCGATAGCTTCAAG	(Wa *et al*., 2013)
Outer membrane *A* protein gene (*ompA*)	Spotted fever group rickettsia	48	258	Forward: TGCAGGGCTTAGATATTCGGC Reverse: AAGCTGTTGGTAAAGGAGCA	(Moonga *et al*., 2019, Chapter 1 in this thesis.)
Outer membrane protein *B* gene (*ompB*)	Spotted fever group rickettsia	48	776	Forward: GGACCTGAAGCTGGAGCAAT Reverse: CTGTCAGGCTGGCTGATGAA	(Moonga *et al.*, 2019, Chapter 1 in this thesis.)
Citrate synthase gene (*gltA*)	Spotted fever group rickettsia	50	290	Forward: AGGGATTGCCTCACTTTGGG Reverse: CTTTAAGAGCGATAGCTTCAAG	This study
Biotin synthase gene (*bioB*)	*R. felis* and *R. asembonensis*	60	120	Forward: ATGTTCGGGCTTCCGGTATG Reverse: CCGATTCAGCAGGTTCTTCAA Probe: FAM-GCTGCGGCGGTATTTTAGGAATGGG-TAMRA	(Socolovschi *et al*., 2010)
R. felis specific fragment of *ompB* gene (*RfelB*)	*R. felis*	60	97	Forward: TAATTTTAACGGAACAGACGGT Reverse: GCCTAAACTTCCTGTAACATTAAAG Probe: FAM-TGCTGCTGGTGGCGGTGCTA-BHQ	(Odhiambo *et al*., 2014)
Cytochrome *c* oxidase subunit I gene (*cox1*)	Flea	50	552	Forward: AGAATTAGGTCAACCAGGA Reverse: GAAGGGTCAAAGAATGATGT	(Lawrence *et al*., 2014)

2.2.4 Deep sequencing of biotin synthase gene (*bioB*) positive samples using next generation sequencer

2.2.4.1 Illumina paired-end library preparation

The biotin synthase gene (*bioB*) qPCR positive samples (*n* = 18), which were positive for either *R. felis* and/or *R. asembonensis,* were further analyzed by MiSeq deep sequencing of *gltA*. The sequencing library was prepared by a two-step PCR protocol according to the Illumina 16S sequencing library preparation protocol with minor modification. In the first PCR, a primer set targeting the spotted fever group-specific gene *gltA* was used. The *gltA* primer set was modified from those previously reported (Wa *et al*., 2013). The primers contain Illumina-tail sequences at the 5' end of each primer (Forward: ACACTCTTTCCCTACAC GACGCTCTTCCGATCTNNCTTTAAGAGCGATAGCTTCAAG and Reverse: GTGACTGGA GTTCAGACGTGTGCTCTTCCGATCTNNAGGGATTGCCTCACTTTGGG). The first PCR was conducted in 20 µL reactions containing 1 µL of 10 µM of each of the forward and reverse primers. Other reagents in the reaction mix included 10 µL of 2× Ampdirect Plus buffer (Shimadzu, Japan), 0.1 µL of 5 U/µL Bio Taq polymerase (Bioline, UK), 6.9 µL of distilled water and 1 µL of template DNA. The PCR cycling conditions were the same as described above for *gltA* PCR amplification. After the first PCR, the amplicons were electrophoresed in 1.2% agarose gel and purified by Wizard SV gel and PCR clean-up system (Promega, WI, USA) to remove free primers, primer dimers, and PCR reagents. The second PCR was performed in 10 µL reactions containing 1 µL of 10 µM Illumina dual-index primer mix (i5 and i7 primers), 1.2 µL of 25 mM $MgCl_2$, 0.4 µL of 10 mM of dNTP mix, 0.1 µL of 5 U/µL KAPA Taq polymerase (Kapa Biosystems, MA, USA), 4 µL of 5× buffer, and 1 µL of diluted amplicon template from first PCR. The PCR protocol included incubation at 95°C for 3 minutes, followed by 15 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, and a final extension at 72°C for 5 minutes. The dual index primers contain 8 bp unique nucleotides which allow the pooling of multiple libraries. The dual indexed libraries were then pooled in equal volumes. The pooled libraries were analyzed in 1.2% agarose gel without adding gel green intercalating dye and purified from the agarose gel by Wizard SV gel and PCR clean-up system (Promega, USA).

2.2.4.2 MiSeq sequencing

The purified sequencing libraries were quantified by using a Qubit fluorometer dsDNA HS assay kit (ThermoFisher Scientific, USA). The library concentration was then adjusted to the final concentration of 4 nM using distilled water. The pooled libraries were denatured with NaOH, diluted with hybridization buffer, and were sequenced on the MiSeq platform (Illumina,

CA, USA). The sequencing was conducted using a MiSeq sequencing reagent v3 (Illumina, USA) and a 10% PhiX DNA spike-in control for low diversity libraries.

2.2.4.3 Bioinformatics analysis

The data was quality filtered to remove reads of quality lower than 20 per read quality score and minimum length of 200 base pairs by Afterqc (Chen *et al*., 2017). Only forward reads were used for further analysis because of poor quality of reverse reads. The reads were then mapped to the customized *Rickettsia* database using Bowtie2 (Langmead and Salzberg, 2012). The 290 bp target region of the *gltA* is 94.8% (275/290 bp) homologous for *R. felis* and *R. asembonensis*. The *Rickettsia* database was created by combining *gltA* sequences of *R. felis* and *R. asembonensis* with accession numbers CP000053 and JP966774, respectively. The alignment criterion was set to sensitive local alignment option in Bowtie2 with 97% sequence similarity allowing 8 bp mismatches to call to species level. Uniquely mapping reads were then called for either *R. felis* or *R. asembonensis*.

2.2.5 Criteria for species identification

A sample was considered positive to species level based on sequence homology to reference sequence and species-specific qPCR analysis. Based on this criteria, a sample was considered *R. felis* positive when obtained *gltA* sequences by Sanger sequencing had homology of more than 99% to *R. felis* strain URRXCal2 reference sequence (GenBank CP000053) and *R. felis* unique *RfelB* was positive by qPCR. Additionally, deep sequence analysis of *gltA* amplicon was also performed. Detection of reads uniquely mapping to either *R. felis* and/or *R. asembonensis* was considered a confirmatory positive result for either of the species or coinfected with both. Other *Rickettsia* species were identified based on percentage similarity with top hit on BLAST analysis.

2.2.6 Statistics

Statistical analysis was performed by the Fischer's exact test to determine the statistical difference in *Rickettsia* sp. prevalence among dogs from densely populated urban areas of Lusaka district and sparsely populated rural areas of Monze and Mazabuka districts. Differences were considered significant at p-value ≤ 0.05.

2.3 Results

Rickettsia felis was detected in seven out of 150 samples from dog blood and 12 out of 106 rodent tissue samples (Table 3). The obtained sequences of *gltA* fragment from dog and rodent samples were 99-100% identical to *R. felis* strain URRXCal2 (CP000053) and further confirmed for *R. felis* by qPCR targeting *bioB* and *RfelB.* Among the blood samples from dogs in Lusaka, Mazabuka, and Monze, it was found that dogs from urban areas of Lusaka (12%, 6/50) had a significantly higher prevalence of *Rickettsia* sp. than dogs from semiurban areas of Mazabuka (2%, 1/50) and Monze (0%, 0/50).

Among the analyzed cat flea samples, seven out of 53 samples were positive for *R. felis* (Table 3). Interestingly, all positive samples were co-infected with *R. asembonensis*. The coinfection of *R. felis* and *R. asembonensis* was confirmed by *R. felis* specific qPCR targeting *RfelB,* and sequence homology for *gltA* gene, respectively. The presence of *R. asembonensis* was further confirmed by sequence homology based on *ompA* and *ompB*. The sequences of *gltA*, o*mpA,* and *ompB* fragments obtained from cat fleas were 100%, 99%, and 100% similar to *R. asembonensis* sequences of accession numbers JN315968, KY650698, and KY650699, respectively. Additionally, 15 cat flea samples showed positive only for *R*. *asembonensis*.

To assess the co-infection nature of *R. felis* and *R. asembonensis*, the *bioB* qPCR positive (*n* = 18) cat flea samples were further analyzed for *gltA* deep sequencing using MiSeq. The *bioB* qPCR is specific for both *R. felis* and *R. asembonensis* hence only *bioB* positive samples (*n* = 18) were analyzed for possible co-infection. The *gltA* sequence reads uniquely aligned to either *R. felis* or *R. asembonensis* were considered belonging to that respective species. Based on deep sequencing analysis, seven out of 18 cat flea samples were co-infected with *R. felis* and *R. asembonensis.* The other ten samples were detected with only *R. asembonensis*. No reads were retrieved from sample labeled Mon13 (Figure 3) despite visible band on gel electrophoresis after index PCR. The error could have resulted from indexing error. The two *R. felis* unique *RfelB* qPCR positive samples are confirmed to be coinfected with *R. felis* and *R. asembonensis* as shown in samples labeled Mon05 and Mon18 (Figure 3).

The *gltA* gene sequences from dog blood and rodent samples phylogenetically clustered with *R. felis* species regardless of the sampling location (Figure 4). In contrast, those of cat flea samples were clearly separated into two distinct clades containing *R. felis* and *R. asembonensis*. The *gltA* gene sequences obtained from rodent samples were more diversified from the reference sequences previously identified in cat fleas.

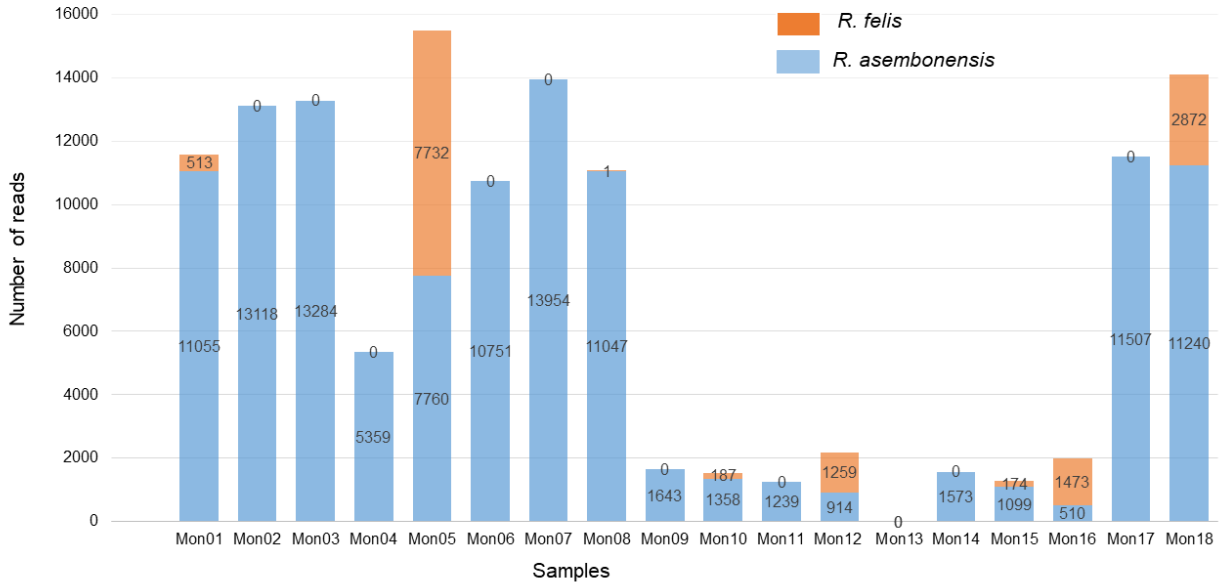

Figure 3. The number of uniquely mapped reads of *gltA* PCR amplicon from *Rickettsia bioB* positive cat fleas (*n* = 18). The number of uniquely mapped reads to each respective species of *R. felis* and *R. asembonensis* are shown in orange and blue color, respectively.

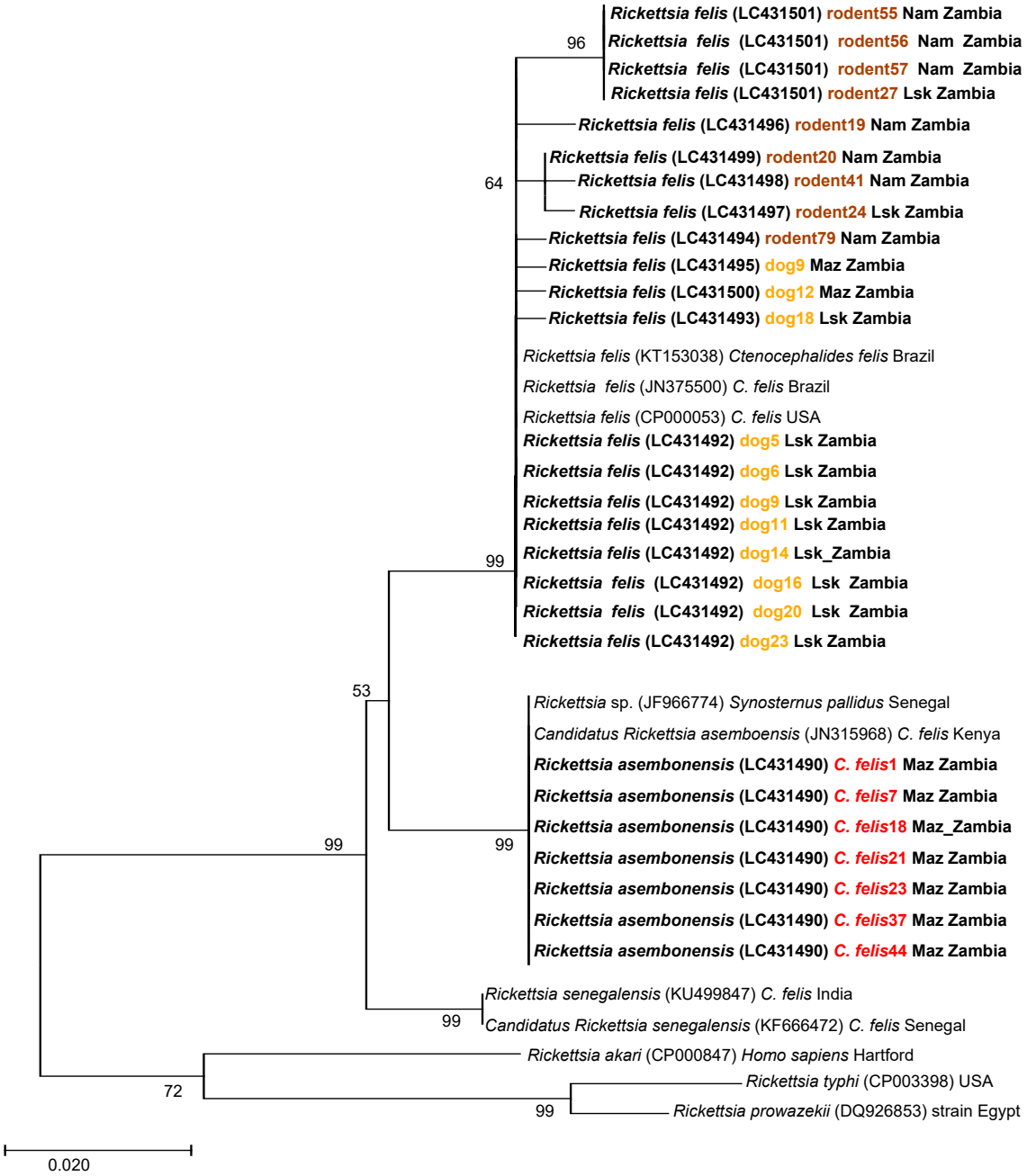

Figure 4. Phylogenetic tree based on *gltA* sequences detected in cat fleas, dogs and rodents in Zambia. The tree was based on 581 bp fragment of *gltA* gene. Bold labels represent sequences detected in this study in the *R. felis* and *R. asembonensis* clades. The detected species name, accession number, host (in color) and place of sample collection are indicated in the label in that order. Typhus group represents the outgroup.

One hundred ninety of pooled samples from different mosquito species; *Culex* spp., *Aedes* spp., and *Anopheles* spp., were all negative by *gltA* PCR (Table 3). Among the analyzed mosquito samples, 154 out of 190 samples representing 81.1% proportion, were collected from Lusaka district, the same place where *R. felis* was detected in dogs and rodents. *Culex* spp. were the common species sampled from Lusaka district. There were 134 out of 154 *Culex* spp. samples representing 87% while the others were *Anopheles* and *Aedes* spp. In contrast to Lusaka district, 29 out of 33 (87.8%) of the samples from Mpulungu district were of *Aedes* spp. suggesting that *Culex* spp. are more predominant in urban Lusaka township, although sampling bias could not be ruled out.

Table 3. The prevalence of spotted fever group rickettsia detected in Zambia and their hosts

Sampling district	**Sampled animal or arthropod species**	**Number of Samples**	**Number of positives for *R. asembonensis* (%)**	**Number of positives for *R. felis* (%)**	**Number of positives for both *R. felis* and *R. asembonensis* (%)**
Mazabuka	*Canis familiaris*	50	0	1 (2.0)	0
	Ctenocephalides felis strongylus	48	22 (45.8)	7 (14.5.2)	7 (14.5)*
	Echidnophaga gallinacean	5	0	0	0
Lusaka	*Canis familiaris*	50	0	6 (12.0)	0
	Mastomys spp.	33	0	2 (6.1)	0
	Culex spp.	134	0	0	0
	Anopheles spp.	9	0	0	0
	Aedes spp.	11	0	0	0
Namwala	*Mastomys* spp.	44	0	10 (22.7)	0
	Gerbil	13	0	0	0
	Saccostomus spp.	3	0	0	0
	Rattus rattus	3	0	0	0
Monze	*Canis familiaris*	50	0	0	0
Mpulungu	*Culex* spp.	4	0	0	0
	Aedes spp.	11	0	0	0
Isoka	*Culex* spp.	3	0	0	0
Total by host species	***Ctenocephalides felis strongylus***	**48**	**22 (45.8)**	**7 (14.5)**	**7 (14.5)**
	Canis familiaris	**150**	**0**	**7 (4.6)**	**0**
	***Mastomys* spp.**	**77**	**0**	**12 (15.5)**	**0**

* Two positives by *R. felis* specific qPCR, and all positive for coinfection by deep-sequencing.

2.4 Discussion

The studies of neglected zoonotic pathogens in animals and blood-sucking arthropods are vital in the control and eradication of zoonotic diseases. This study revealed the presence of *R. felis*, a known human pathogen, in domestic dogs from Zambia. The detected genotype is identical to the reported one deposited in GenBank which was isolated from cat fleas and other hematophagous arthropods worldwide. This highlights the significance of domestic dogs as potential mammalian reservoirs of *R. felis* as experimentally suggested (Ng-Nguyen *et al*., 2020). It is therefore important for dog owners to be aware of *Rickettsia* infection risk as concurrent *R. felis* infections in a positive dog and its infected owners have already been reported (Oteo *et al*., 2006).

Rickettsia felis was more prevalent in urban Lusaka district as compared to semiurban Mazabuka and Monze districts. The higher prevalence of *R. felis* in urban areas could probably be attributed to high density of outdoor and stray dogs that roam the streets without adequate ectoparasite control (Islam and Chizyuka, 1983). The high dog population density and closeness of households in urban areas with stray dogs could favor the survival of ectoparasites resulting in high prevalence of arthropod-borne pathogens (Galay *et al*., 2018). However, due to lack of dog population data in Zambia, direct inference of dog population density to high *Rickettsia* infection rate could only be speculated.

It is known that cat flea is a vector for flea-borne spotted fever causative agent. In this study, *R. felis* was also detected in cat fleas in the same sampling place where dogs were positive for *R. felis*. This molecular-based evidence implies that *R. felis* is circulating with domestic dogs serving as reservoirs and cat fleas as vectors in Zambia. This is of public health significance as the bacteria is potentially maintained among domestic dogs and cat fleas as earlier reported (Hii *et al*., 2011). This results in a potential risk of human infections through cat flea bites as they feed indiscriminately, hence effective vector-borne infection control measures are necessary to prevent zoonotic outbreaks.

A high prevalence of *R. asembonensis* (45.8%) was also observed with relatively low prevalence of *R. felis* (14.5%) in cat fleas. This result is in accordance with previous studies that found up to 100% prevalence of *R. asembonensis* in cat fleas with low *R. felis* prevalence (Bai *et al*., 2017; Jiang *et al*., 2013; Oteo *et al*., 2014; Rust, 2017; Rzotkiewicz *et al*., 2015; Sansyzbayev *et al*., 2016; Silva *et al*., 2017). The *R. asembonensis gltA* sequences detected in cat fleas showed close relatedness to *R. asembonensis* detected in cat fleas collected from human habitats in Kenya (Jiang *et al*., 2013). The phylogenetic clustering of *R. asembonensis* together with those from Kenya and Senegal suggests similar genotype of *R. asembonensis*

in cat fleas from sub-Saharan Africa (Figure 3). Interestingly, two of the cat flea samples positive for *R. felis* by *RfelB* qPCR were also positive for *R. asembonensis* as confirmed by *gltA* sequencing analysis. These findings suggest evidence of coinfection of *R. felis* and *R. asembonensis* in cat fleas. In conventional Sanger sequencing method, no *gltA* sequences of *R. felis* were obtained from these *RfelB* qPCR positive cat fleas, probably because the dominant species in the co-infected flea samples were *R. asembonensis* and no *R. felis gltA* were sequenced. In fact, *R. felis gltA* sequences were obtained from the same samples by *gltA* deep sequencing using MiSeq. The detection of seven samples positive for *R. felis gltA* sequences by deep sequencing suggests that deep sequencing by next-generation sequencer is more powerful than Sanger sequencing in detecting coinfection. *Rickettsia asembonensis* is a potential endosymbiont of fleas (Perotti *et al*., 2006; Rzotkiewicz *et al*., 2015), therefore, a higher population in cat fleas is expected. The cat fleas that harbor *R. asembonensis* have indiscriminate bloodmeal sources including humans. The risk of human infection is possible during cat flea bloodmeal from humans because *R. asembonensis* was detected in cat fleas in human dwellings (Jiang *et al*., 2013; Maina *et al*., 2016). Despite the risk of infection, the pathogenicity of *R. asembonensis* to humans remains unknown, therefore, further studies on their pathogenicity are warranted.

This study also presents molecular evidence of *R. felis* in *Mastomys* species of rodents in Zambia suggesting them as potential reservoirs. Rodents have been implicated as reservoir hosts for diverse zoonotic pathogens ranging from viruses, protozoa, fungi, helminths to bacteria (Han *et al*., 2015) including *Rickettsia* spp. (Panti-May *et al*., 2015). Limited studies have been conducted to understand the role of rodents in epidemiology and ecology of *R. felis* (Gajda *et al*., 2017; Minichová *et al*., 2017; Panti-May *et al*., 2015; Rzotkiewicz *et al*., 2015), despite many studies having detected *R. felis* DNA in fleas infesting rodents (Bai *et al*., 2017; Eremeeva *et al*., 2008; Reif and Macaluso, 2009; Rzotkiewicz *et al*., 2015). This result adds *Mastomys* sp. to the rodent species proposed to be potentially maintaining *R. felis* in nature. Rodents are suggested to be vital for the maintenance of *R. felis* resulting in sporadic cases of rickettsioses in humans as they share habitat with humans and dogs in the presence of fleas vectors (Bonwitt *et al*., 2017). Therefore, the interplay among mammalian hosts, fleas, and humans needs to be clarified to understand the zoonosis transmission cycle and possible control measures.

Many other blood-sucking arthropods with the potential to transmit *Rickettsia* are endemic in Zambia including mosquitoes. It is necessary to survey neglected vector-borne zoonoses such as spotted fever rickettsioses which pose a potential emerging public health threat in the human population. Based on this precedence, *Culex, Aedes,* and *Anopheles* spp.

were screened for *Rickettsia* spp. but were all negative. This suggests that mosquitos may not be playing an essential role in the epidemiology of *R. felis* in Zambia. The role of these mosquito species in *R. felis* epidemiology is still unclear despite recent advances in transmission potential studies, hence further studies are needed (Dieme *et al*., 2015; Mediannikov *et al*., 2013b; Socolovschi *et al*., 2012). Additionally, the primary food source for both male and female mosquitoes is nectar and plant juices. Female mosquitoes feed on blood meal for reproductive nutrition. Humans are not primary hosts for *R. felis*, hence transmission to mosquito vectors could be rare. The primary mammalian reservoir hosts for *R. felis* are dogs, cats and rodents on which mosquitoes are not primarily known to acquire a blood meal (Zhang *et al*., 2019; Zhu *et al*., 2017). However, sampling bias could not be ruled out as the majority of the sampled mosquitoes were *Culex* species while *R. felis* has been commonly detected in *Anopheles* and *Aedes* spp. (Barua *et al*., 2020; Socolovschi *et al*., 2012).

2.5 Conclusion

In this chapter, we provide evidence of the occurrence of *R. felis* in domestic dogs, rodents and cat fleas for the first time in Zambia. In particular, the genotype identified from the dogs was identical to a reported genotype detected in cat fleas. Therefore, the maintenance and transmission of *R. felis* in dogs and cat fleas in Zambia are surmised. Besides, the potential of rodents as reservoirs is not negligible even though observed genotypes in *Mastomys* sp. were slightly diversified from the reported genotype in cat fleas. Interestingly, cat fleas were coinfected with *R. asembonensis* of unknown pathogenicity with a potential to infect humans. These findings provide evidence of the potential circulation of *R. felis* in Zambia and the risk of human infection from cat fleas during bloodsucking. Further studies of potential flea-borne rickettsioses in humans are needed to understand the epidemiology and possible control strategies.

3. Chapter 2

Molecular detection and characterization of *Rickettsia asembonensis* from human blood in Zambia

3.1 Background

In chapter one, two flea-borne rickettsiae namely *R. felis* and *R. asembonensis* were detected in cat fleas collected in Zambia. Therefore, the second chapter focused on the epidemiology of flea-borne rickettsiae in humans. Flea-borne rickettsiae are circulating in various flea species and are transmitted to humans, causing disease. The well-known flea-borne rickettsiae are *Rickettsia typhi* belonging to the typhus group causing murine typhus, and *R. felis*, causing flea-borne spotted fever classified in the transitional group based on genetic characterization (Blanton and Walker, 2017; Fournier *et al*., 2003). Murine typhus is distributed along warm coastal regions throughout the world, where the primary mammalian reservoir (*Rattus* spp.) and flea vector (*Xenopsylla cheopis*) thrive while *R. felis* is distributed worldwide as it is maintained and transmitted by cat fleas with worldwide distribution. Besides, there are recently identified *Rickettsia* species classified as *Rickettsia felis*-like organisms (RFLOs), which are closely related to *R. felis*. *Rickettsia asembonensis* and *R. senegalensis* are the commonly identified RFLOs closely related to *R. felis* (Billeter *et al*., 2016; Maina *et al*., 2016). *Rickettsia felis* is known to cause mild to severe rickettsiosis in humans (Mawuntu *et al*., 2020; Rauch *et al*., 2018; Zavala-Castro *et al*., 2009), but the pathogenicity of RFLOs remains unknown despite the widespread detection in hematophagous arthropods.

Rickettsia asembonensis was first described in cat fleas infesting dogs from Asembo, Kenya, (Jiang *et al*., 2013), and those collected from human dwellings in Senegal (Roucher *et al*., 2012). *Rickettsia asembonensis* was also detected in cat fleas infesting dogs in Zambia (Moonga *et al.*, 2019, Chapter 1 in this thesis). Other sub-Saharan African countries that reported *R. asembonensis* in fleas include Uganda, Rwanda, and South Africa (Kolo *et al*., 2016; Nziza *et al*., 2019; Palomar *et al*., 2017). Before these reports of *R. asembonensis* characterization, *Rickettsia* spp. closely related to *R. asembonensis* were already detected by molecular techniques from fleas in various countries, mainly in the Asian region (Foongladda *et al*., 2011; Parola *et al*., 2003; Tay *et al*., 2014). Furthermore, reports of the presence of *R. asembonensis* in fleas emerged from other countries worldwide such as the USA, Peru, Brazil, and Ecuador (Dall'Agnol *et al*., 2017; Loyola *et al*., 2018; Oteo *et al*., 2014; Reeves *et al*.,

2005). *Rickettsia asembonensis* was also reported in Germany and Hungary (Gilles *et al*., 2009; Hornok *et al*., 2010). Despite cat fleas being the frequent host of *R. asembonensis*, other flea species have been reported to harbor it. The other flea species detected with *R. asembonensis* are *C. canis*, *C. orientis*, *X. cheopis*, *X. ramesis*, *X. gerbilli*, *E. gallinacean*, *P. simulans,* and *Synosternus pallidus*. *Rickettsia asembonensis* has also been detected in *Archaeopsylla erinacei*, *Ceratopsyllus fasciatus*, *Orchopeas howardi*, *Nosopsyllus laeviceps,* and *Coptopsylla lamellifer* (Jiang *et al*., 2013; Maina *et al*., 2019). The majority of the flea species have a worldwide distribution and are ubiquitous bloodsuckers feeding on domestic animals such as dogs, cats, and rodents. More importantly, they parasitize humans, since most of these flea species are found in human dwellings (Roucher *et al*., 2012).

Rickettsia asembonensis has been reported with high prevalence in fleas up to 100% (Jiang *et al*., 2013; Rzotkiewicz *et al*., 2015) with a lower prevalence of *R. felis*, which is a known human pathogen and has been detected in dogs and rodents as potential mammalian hosts (Moonga *et al.*, 2019, Chapter 1 in this thesis). Mammalian hosts of *R. asembonensis* have not been identified yet. However, *Rickettsia* sp. closely related to *R. asembonensis* was detected in cat fleas collected from cynomolgus monkey (*Macaca fascicularis*) in Malaysia (Tay *et al*., 2014). Later, it was identified in the blood of cynomolgus monkeys from Malaysia (Tay *et al*., 2015), and from the blood of cats and their fleas in Thailand, linking them as potential mammalian reservoir hosts (Phoosangwalthong *et al*., 2018).

Human infection closely related to *R. asembonensis* was detected in the blood of a patient from Malaysia with fever, myalgia, arthralgia, mild headache, conjunctival suffusion, and the presence of petechiae on his limbs (Kho *et al*., 2016). *Rickettsia asembonensis* DNA was again reported in a malaria patient presenting with fever and chills in Malaysia (Tay *et al*., 2019). The bacterium was also isolated in cell cultures from the blood of four humans with non-specific acute febrile syndrome and confirmed by sequencing in Peru (Palacios-Salvatierra *et al*., 2018). These reports suggest the possible role of *R. asembonensis* in febrile illness. However, the significance of RFLOs and other *Candidatus* Rickettsiae as human pathogens remains uninvestigated. Nevertheless, these agents are distributed throughout the world, thus have a probability of causing global public health concern/nuisance. This negligence is partly due to their unknown pathogenicity. Hence, our study aimed at investigating the prevalence of SFG rickettsia in humans with clinical symptoms in Zambia.

3.2 Materials and methods

3.2.1 Human blood samples

Residual blood samples from patients submitted for routine investigation in the University Teaching Hospital, Lusaka, Zambia (UTH) were secondarily sampled. The study was retrospective; therefore, results did not impact patient management. All age groups with various disease conditions were sampled. Available relevant clinical information was extracted from clinical records, among them was sickle cell anemia, fever, malaria, septicemia, meningitis and others (Table 4). Additionally, dried blood spots on Whatman FTA cards collected during the Zambia National Malaria survey were purposively included in the study to assess *Rickettsia* infection in healthy individuals from rural areas of Zambia. A total of 1,153 samples (519, 234, 200, and 200) were collected from Lusaka district, Chongwe district, Eastern and Central provinces of Zambia representing the urban, semiurban and rural populations respectively. All samples were collected in the year 2019. This research was conducted under the authorization of The National Health Research Authority, Lusaka Zambia, and the University Zambia Biomedical Research Ethics Committee under the reference number: 007-10-18.

Table 4. Clinical information of the screened samples

Sampling place	Sample type	Clinical manifestation category	Number of samples	Total samples per location
Lusaka district	Blood with EDTA	Febrile illness	15	519
		Neurological signs (Meningitis)	9	
		Septicemia	20	
		Anemia	19	
		Sickle cell anemia	41	
		HIV	18	
		Respiratory infection	43	
		Others	52	
		No clinical information provided	302	
Chongwe district	Blood with EDTA	Malaria	18	234
		Meningitis	3	
		Anemia	17	
		Bleeding	5	
		Respiratory infection	7	
		Sickle cell anemia	4	
		Others	32	
		No clinical information provided	148	
Eastern province	Dried blood spot	Healthy individuals	200	200
Central province	Dried blood spot	Healthy individuals	200	200

3.2.2 DNA extraction

Genomic DNA was extracted from 200 µL of blood by DNAzol BD Reagent (Invitrogen, MA, USA) according to the manufacturer's protocol with 200 µL elution by nuclease-free water. DNA from dried blood spots was extracted by the modified boiling method (Choi *et al*., 2014). In brief, two punched out filter papers of 3 mm of the dried blood spots were soaked in 40 µL of nuclease-free water in the PCR tube. The sample was then incubated at 60°C for 30 minutes and further boiled at 99°C for 10 minutes. The extracted DNA was stored in -80°C freezer until use for PCR screening analysis.

3.2.3 PCR screening and multiple gene sequencing

All DNA samples were subjected to PCR screening targeting the *gltA* of *Rickettsia* genus using modified primers described previously (Wa *et al*., 2013). The *gltA* PCR positive samples were further subjected to multiple gene amplification, namely 17-kDa common antigen gene (*htrA*), *ompA* and *ompB*. The amplified products were sequenced using previously published primers shown in Table 5 to species level. Briefly, PCR products were treated with ExoSap-IT (ThermoFisher Scientific, USA) according to the manufacturer's instructions. DNA concentrations were measured using a Nanodrop spectrophotometer (Thermo Fisher Scientific, USA). A hundred nanograms of the amplicons were cycle-sequenced by BigDye Terminator v3.1 cycle sequencing kit (Thermo Fisher Scientific, USA) with the same primers as used for PCR amplification and analyzed on a 3300 Genetic Analyzer (Thermo Fisher Scientific, USA). Additionally, *gltA* PCR positive samples were tested for malaria by nested PCR, as previously described (Singh *et al*., 1999).

The determined nucleotide sequences were subjected to BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to search for homologous sequences and aligned with related sequences using MAFFT version 7. The multiple gene sequences were then phylogenetically analyzed by the Neighbor-Joining method using MEGA 7.0.2.1 software with bootstrap support of 500 replicates (Kumar *et al*., 2016).

Table 5. Molecular characterization of the detected *R. asembonensis* by multiple-gene sequencing

Target gene (Primer reference)	**Details**	**ID: UTH_185**	**ID: UTH_231**
Citrate synthase gene (*gltA*) (Wa *et a*l., 2013)	Amplicon size	581 bp	581 bp
	Percentage identity to reference	100 %	100 %
	Reference name (accession number)	*Rickettsia asembonensis* (JF966774)*	*Rickettsia asembonensis* (JF966774)*
17kDa common antigen gene (*htrA*) (Labruna *et al*., 2004)	Amplicon size	550 bp	550 bp
	Percentage identity to reference	100 %	100 %
	Reference name (accession number)	*Rickettsia asembonensis* (MN003387)**	*Rickettsia asembonensis* (MN003387)**
Outer membrane protein *A* gene (*ompA*) (Moonga *et al.*, 2019, Chapter 1 in this thesis)	Amplicon size	280 bp	280 bp
	Percentage identity to reference	100 %	100 %
	Reference name (accession number)	*Rickettsia asembonensis* (KY650698)***	*Rickettsia asembonensis* (KY650698)***
Outer membrane protein *B* gene (o*mpB*) (Moonga *et al.*, 2019, Chapter 1 in this thesis)	Amplicon size	770 bp	770 bp
	Percentage identity to reference	100 %	100 %
	Reference name (accession number)	*Rickettsia asembonensis* (KY650699)***	*Rickettsia asembonensis* (KY650699)***

**Rickettsia asembonensis* from *Synosternus pallidus*, Senegal. (Roucher *et al*., 2012)

***Rickettsia asembonensis* from *C. felis*, Mexico (Sánchez-Montes *et al*., 2020)

****Rickettsia asembonensis* from cat flea, Peru (Loyola *et al.*, 2018)

3.2.4 *Rickettsia* quantity estimation from blood

The *Rickettsia* burden in the blood of *Rickettsia* positive samples was estimated by measuring the rickettsial DNA amount using quantitative real-time PCR. A single copy *ompA* of SFG *Rickettsia* was amplified by conventional PCR using published primers (Moonga *et al*., 2019, Chapter 1 in this thesis). The amplicons were then purified by Wizard SV Gel and PCR Clean-Up System (Promega, USA). The amplicons were quantified by the dsDNA HS assay kit with Qubit fluorometer (ThermoFisher Scientific, USA), and the copy number of *ompA* was estimated. The quantified *ompA* standard PCR product was then serially diluted for the derivation of the standard curve. Then, quantitative real-time PCR targeting *ompA* was conducted on *gltA* PCR positive DNA samples, and the copy number of *Rickettsia* genome in the original blood sample was estimated.

3.4 Results

Among the 1,153 samples screened, two samples were positive for *gltA* PCR. Regionally, two out of 519 samples from the urban region of Lusaka district were *gltA* PCR positive representing 0.37% prevalence. The semiurban and rural settlements, which are Chongwe district ($n = 234$), and Eastern ($n = 200$) and Central provinces ($n = 200$) samples were all negative. Malaria PCR conducted on *gltA* PCR positive samples were negative.

The two *gltA* PCR positive samples were from the urban region of Lusaka district. Both cases were females aged 42 and 45 years old (Table 6). Case UTH_185 had a medical record of anemia and weight loss, while case UTH_231 had no clinical information provided at the time of sampling. Rickettsial load in the patient blood was estimated to be 8.9×10^5 and 2.1×10^6 copies of genomic DNA per mL of blood for case UTH_185 and UTH_231, respectively.

The obtained sequences from the two patients were 100% identical to each other in all the four genes analyzed. Further sequence analysis of the *gltA* sequences showed 100% similarity to *R. asembonensis* identified in fleas from human dwelling, and domestic dogs in Senegal, Kenya, and Zambia deposited as accession numbers JF966774, JN315968, and LC431490 (Jiang *et al*., 2013; Moonga *et al*., 2019, Chapter 1 in this thesis; Roucher *et al*., 2012), respectively. Comparative analysis of the sequenced *gltA* gene to those isolated from Peru (accession number KY650697) and other American regions revealed 99.8% homology (Figure 6). These two *gltA* PCR positive samples were further examined by multiple gene sequence analyses of partial *ompA*, *ompB*, and *htrA*. The sequenced multiple partial genes showed 100% homology to previously reported *R. asembonensis* from Peru and Mexico of accession numbers KY650698, KY650699, and MN003387, respectively (Table 5). Phylogenetic analysis of the partial *gltA* showed that sub-Saharan Africa isolates from cat fleas clustered together with those of *R. asembonensis* detected in humans. The sub-Saharan Africa isolates consistently showed one nucleotide difference in the partial *gltA* sequence as compared to other regions (Figure 6). Further phylogenetic analysis of *htrA* sequences showed clustering with previously published sequences of *R. asembonensis* without any difference (Figure 7).

Table 6. Characterization of individuals detected with *Rickettsia asembonensis*

Case ID	UTH_185	UTH_231
Age	42	45
Sex	Female	Female
Residential area	Lusaka	Lusaka
Clinical manifestation	Anemia and weight loss.	No information
Estimated rickettsia genome copies/mL blood	8.9×10^5	2.1×10^6
Malaria PCR result	Negative	Negative

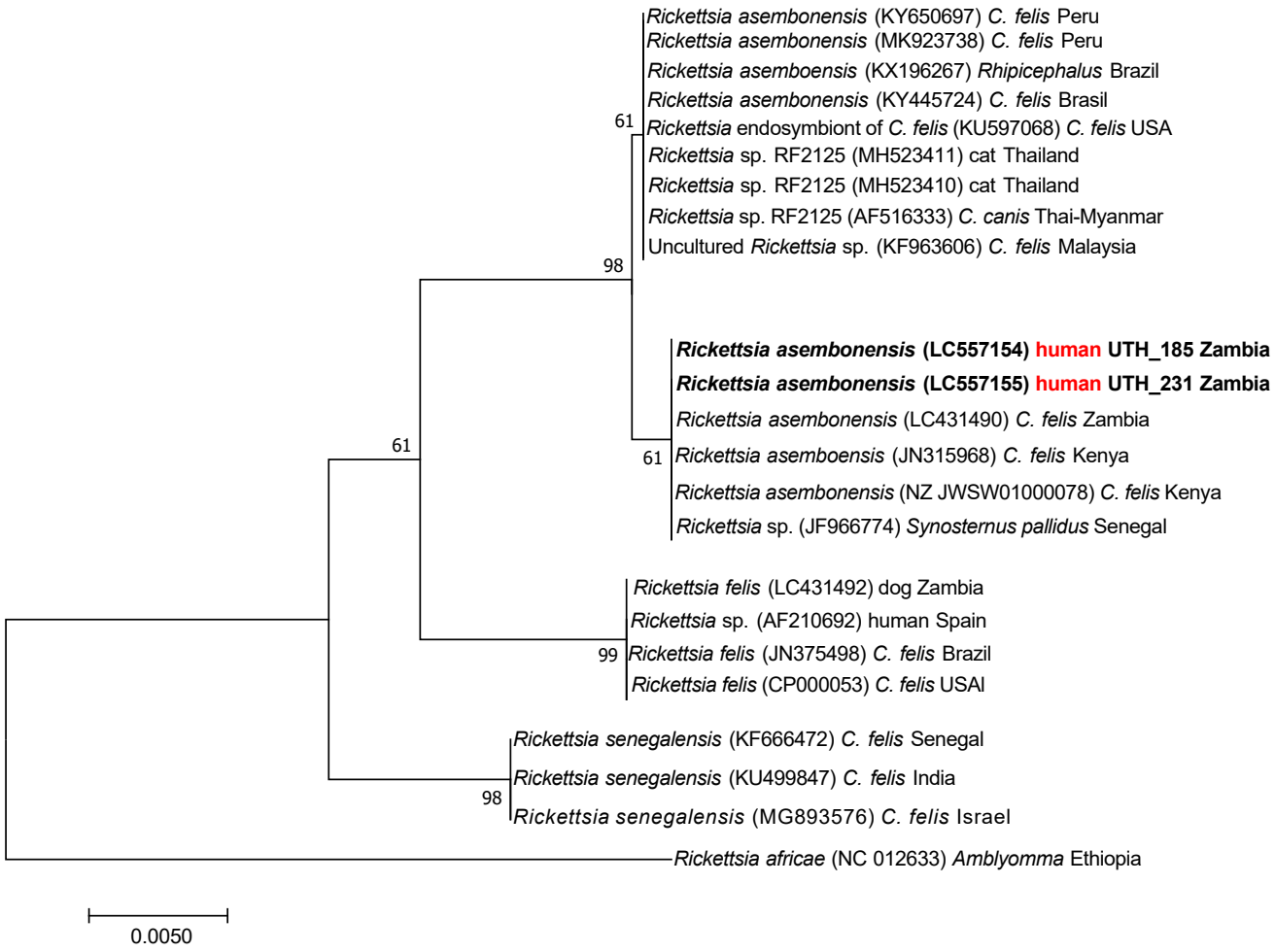

Figure 5. The phylogenetic tree showing the detected *Rickettsia* sequences based on the sequences of the *gltA*. The phylogenetic tree shows the clustering of *R. asembonensis* detected in Zambia together with those from sub-Saharan Africa while separated from those of other regions. The sequences obtained in this study are indicated in bold letters with the species name, accession number, and country of detection mentioned in the name in the same order. Scale bar indicates nucleotide substitutions per site.

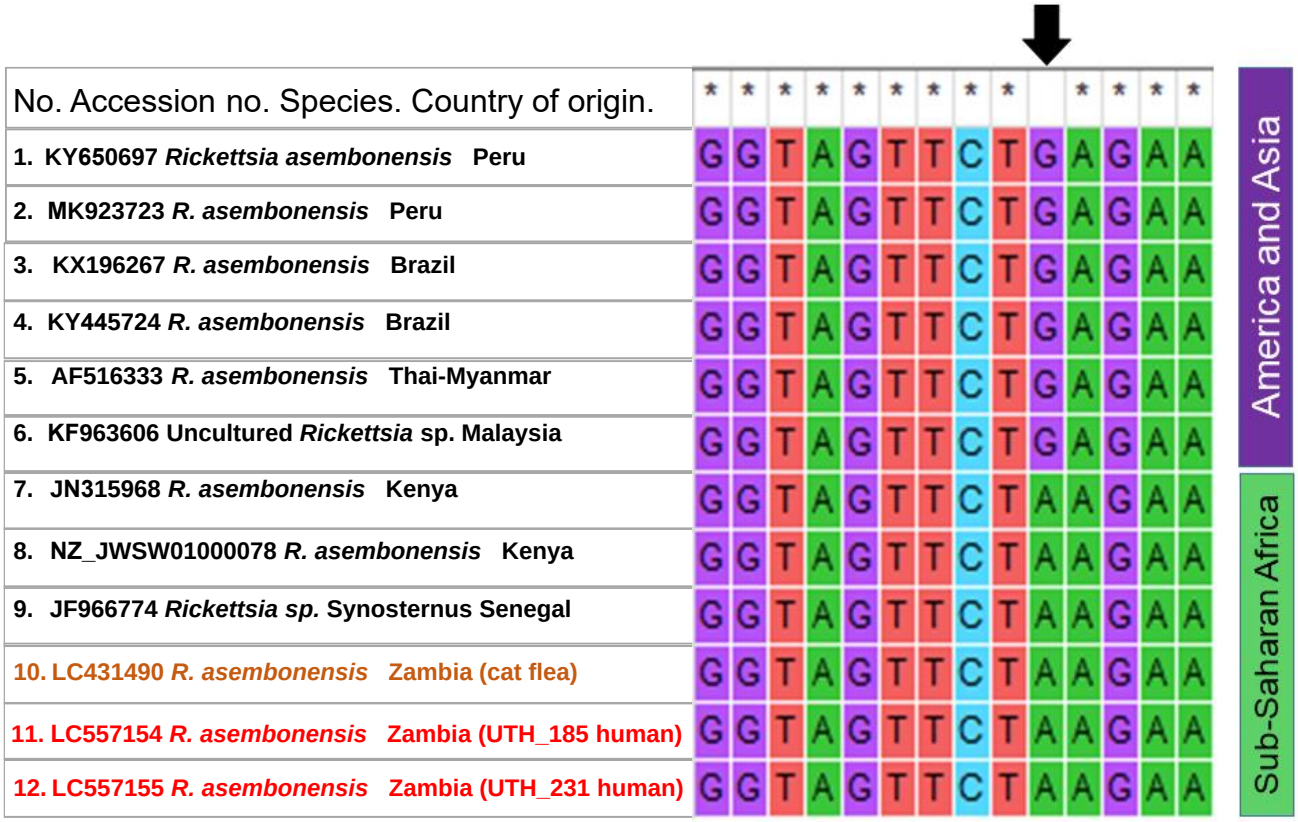

Figure 6. Multiple sequence alignment of partial *gltA* of *R. asembonensis* from different regions showing a single nucleotide polymorphism shown by the black arrow. Sequences in red color are those detected in humans from Zambia and showed clustering with those from sub-Saharan African origin. The sequence in yellow was detected in cat fleas collected from dogs in Zambia (chapter 1 in this thesis). There is only one variable site in the 581 base pair partial *gltA* sequences of *R. asembonensis*. Other variable sites not shown do not change amino acid transcripts.

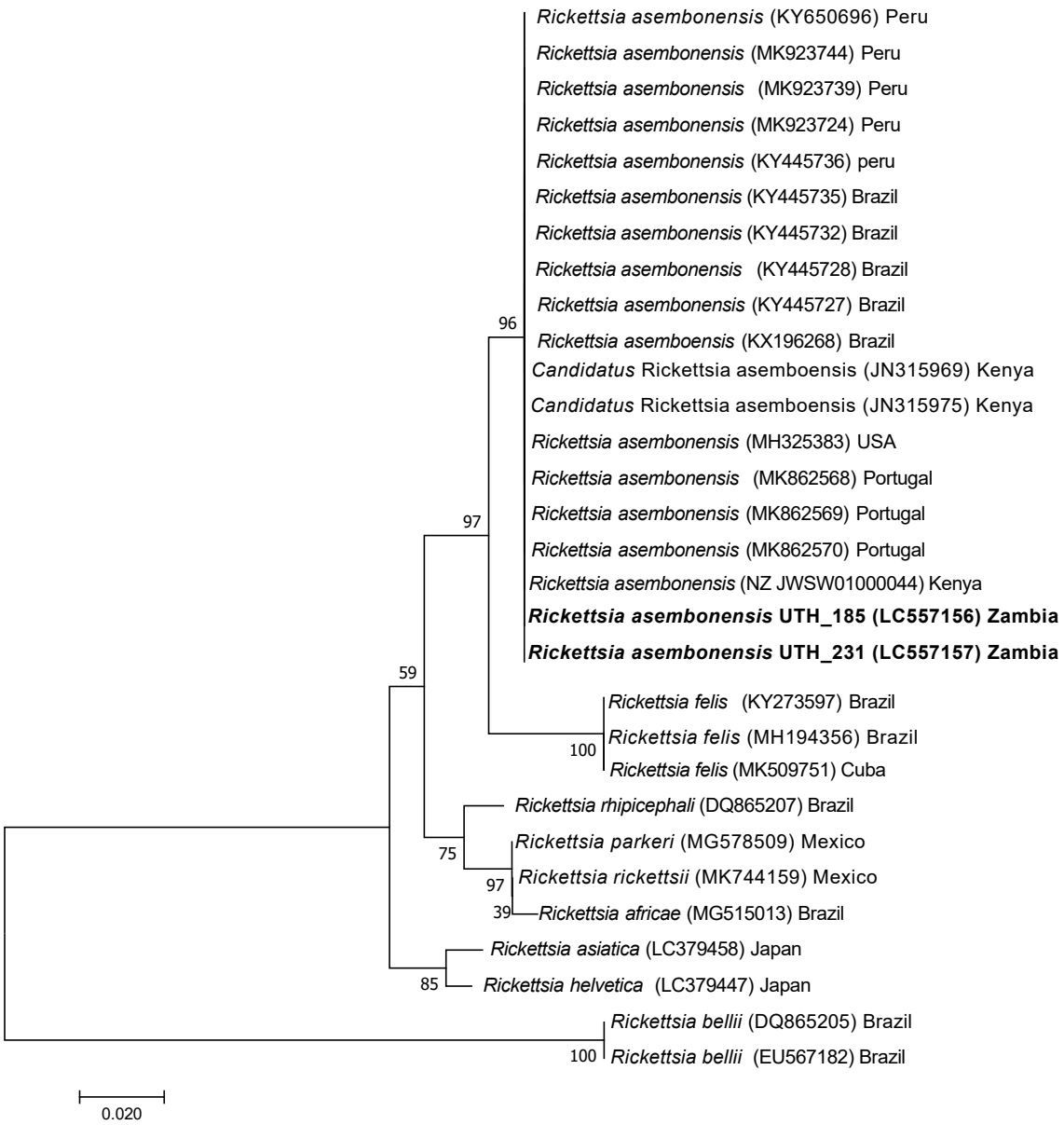

Figure 7. The phylogenetic tree showing the detected *Rickettsia* sequences (bold letters) based on the sequences of *htrA*. Unlike the *gltA* phylogeny, the *htrA* phylogeny does not show any geographical regional clustering.

3.5 Discussion

Rickettsia asembonensis was detected and characterized for the first time by multiple gene sequencing in human patients from Zambia. Two individuals (Denoted as UTH_185 and UTH_231 for descriptive purposes only) were determined to be positive for *R. asembonensis* by PCR and sequencing. Case UTH_185 had a clinical history of anemia and weight loss. No clinical information was provided for case UTH_231. Malaria is endemic in Zambia and is a common cause of anemia (Nawa *et al*., 2019); hence Malaria parasites PCR was conducted and turned negative on both samples. However, the evidence observed was limited to attribute *R. asembonensis* to be the cause of anemia and weight loss. Other than severe *R. felis* infection which was associated with severe anemia possibly due to hemorrhage resulting from vascular damage in rickettsial disease (Zavala-Castro *et al*., 2009), little evidence associates *Rickettsia* spp. infection to anemia. Earlier reports showed the presence of *R. asembonensis* in humans with febrile illness (Kho *et al*., 2016; Palacios-Salvatierra *et al*., 2018). *Rickettsia asembonensis* was detected in a 15-year-old boy with fever, myalgia, arthralgia, mild headache, and loss of appetite but showed normal hemoglobin and bilirubin values suggesting a lack of red blood cell breakdown (Kho *et al*., 2016). Moreover, *R. asembonensis* was detected in *Rhipicephalus sanguineous* (brown dog tick) after fatal cases of fever of unknown origin in human patients in Brazil (Dall'Agnol *et al*., 2017). These findings indicate the possible role of *R. asembonensis* infections in potential human illness. Therefore, the possibility of anemia and weight loss caused by *R. asembonensis* infection cannot be ruled out.

The bacterial load is one of the indexes to speculate on the severity of the illness caused by a bacterial infection. It can be conveniently shown as bacterial DNA load than direct counting by microscopy or culturing due to its rapidness and sensitivity. The bacterial load for *R. rickettsii*, the causative agent for Rocky Mountain spotted fever, was also shown to be relatively higher in fatal cases as compared to non-fatal cases (Kato *et al*., 2016). The estimated rickettsial load was 8.9×10^5 and 2.1×10^6 copies/mL of blood for case UTH_185 and UTH_231, respectively. These results are within the same range of earlier reports of *R. rickettsii,* bacterial load in non-fatal and fatal cases which ranged from 8.40×10^1 to 2.05×10^6 copies/mL of peripheral blood (Kato *et al*., 2016). Since *R. asembonensis* in the TG and *R. rickettsii* in SFG are phylogenetically closely related, similar bacterial load for the two bacteria can be assumed. Additionally, the *Orientia tsutsugamushi* burden was found to be between 1.0×10^6 to 2.8×10^6 copies/mL of whole blood from human clinical patients (Singhsilarak *et al*., 2005), which is also similar to the detected *R. asembonensis* bacterial DNA load in patients. *Orientia tsustugamushi* has similar clinical manifestations as spotted fever group rickettsia,

which is mainly fever among others. Therefore, the rickettsial load causing clinical disease could be speculated to be within similar ranges.

Sequence analysis of the identified *R. asembonensis* showed 100% identity to previously described *R. asembonensis* from sub-Sahara Africa detected from cat fleas (Figures 5 and 7). The *gltA* sequences from African origin, including those identified in Zambia, shared one single nucleotide polymorphic (Figure 6), which differentiates from Asian and American strains. Furthermore, three and five SNPs on *gltA* and *ompA*, respectively, are identified between *R. asembonensis* strain VDG7 from Peru and strain NMRCii from Kenya (Loyola *et al*., 2018). This series of evidence suggests that there are potentially two geographically distinct lineages of *R. asembonensis* circulating worldwide.

Rickettsia asembonensis has been widely reported in various flea species, including human parasitic *C. felis*, *C. canis*, *P. irritans*, *S. pallidus*, *E. gallinacean,* and *Xenopsylla* spp. (Maina *et al*., 2019). Interestingly, *R. asembonensis* has been frequently detected in cat fleas in human dwelling with a low prevalence of *R. felis,* a known human pathogen (Jiang *et al*., 2013; Palomar *et al*., 2017), despite the identification of *R. felis* in febrile patients in the same area (Jiang *et al*., 2013; Richards *et al*., 2010). *Rickettsia felis* has been identified as a cause of febrile disease in humans (Parola, 2011). However, the pathogenicity of *R. asembonensis* closely related to *R. felis* remain unknown. The detection of *R. asembonensis* genome in human blood suggests that rickettsiae of unknown pathogenicity could infect humans via flea bites as off-host fleas feed on humans (Graham *et al*., 2013). However, they remain neglected as their pathogenicity is limited so far. Our findings also demonstrated the possibility of *R. asembonensis* infection in humans. The emerging reports of detection of presumptive rickettsiae of unknown pathogenicity in human patients' blood suggest the importance of *Candidatus* Rickettsiae, including *R. asembonensis*, as possible neglected emerging infection.

3.6 Conclusion

In chapter 2, *R. asembonensis* DNA was detected in the blood of humans from Zambia. The detection of *R. asembonensis* DNA in human blood suggests the possibility of human infection with *R. asembonensis* despite its unknown pathogenicity. The genotype of *R. asembonensis* detected in human blood and cat fleas are identical, suggesting possible transmission of *R. asembonensis* from cat fleas to humans. Given the worldwide distribution of *R. asembonensis*, further studies to elucidate its pathogenicity and epidemiological cycle are warranted.

4. Chapter 3

Development of a multiplex loop-mediated isothermal amplification (LAMP) method for simultaneous detection of spotted fever group rickettsiae and malaria parasites by dipstick DNA chromatography

4.1 Background

Chapters 1 and 2 highlighted the epidemiology of flea-borne rickettsia in Zambia. Flea-borne rickettsioses manifest with febrile illness at the point of care and correct diagnosis is vital for appropriate treatment intervention. Undifferentiated Febrile Illnesses (UFI) are the most common clinical presentation at healthcare centers in low and middle-income countries (Petit and van Ginneken, 1995; Prasad *et al*., 2015). With the decline in global malaria incidence, other infectious causes of febrile illness presumptively misdiagnosed as malaria have been identified (Brah *et al*., 2015; Kamau *et al*., 2020; Reyburn *et al*., 2004). Rickettsioses and malaria are among the common cause of acute UFI, with the disease distribution overlapping (Mediannikov *et al*., 2013b; Wangdi *et al*., 2019). The acute undifferentiated febrile manifestation leads to challenges in the differential diagnosis of the definitive cause of acute UFI, mostly in diagnostic incapacitated settings. Most clinical diagnostic approaches tend to specifically target more common diseases such as malaria, hence missing out on other pathogens, including rickettsiae (Reyburn *et al*., 2004; Wangdi *et al*., 2019).

Malaria is still a common cause of febrile illness and death worldwide. The five *Plasmodium* spp. that infect humans causing malaria include *P. falciparum*, *P. vivax*, *P. ovale*, *P. malaraie*, and *P. knowlesi.* Sub-Saharan Africa carries almost 80% of the global malaria burden, with *P. falciparum* being the predominant causative agent (WHO, 2018a). Effective management of malaria depends on accurate diagnosis as misdiagnosis can result in significant morbidity and mortality (Amexo *et al*., 2004). Currently, the gold standard for malaria diagnosis is microscopy, with more feasible rapid diagnostic tests (RDTs) becoming more commonly used (WHO, 2018b). Spotted fever group (SFG) rickettsioses are neglected diseases and represent a large number of emerging and re-emerging infectious diseases with a worldwide distribution. Rickettsiosis caused by *R. africae* causes a significant proportion of acute febrile illnesses among returning travelers from sub-Saharan Africa (Raoult *et al*., 2001a). *Rickettsia felis* is also a common cause of febrile illness in sub-Saharan Africa, with

occasional co-infection with *Plasmodium* spp. (Mediannikov *et al*., 2013b). Other SFG rickettsiae associated with fever in sub-Saharan Africa include *R. conorii*, *R. sibrica*, *R. massiliae,* and *R. aeschlimannii* (Mediannikov *et al*., 2010). The rickettsial disease often results in fatal outcome if not correctly treated. Diagnosis of SFG rickettsiae is usually based on nucleic acid detection and/or serological methods, which have limitations depending on the timing of infection and sample collection (Robinson *et al*., 2019). Nucleic acid detection methods are mostly utilized at the onset of illness, while serological methods are more appropriate after antibody response, which may be late for disease management (Santibáñez *et al*., 2013). The co-infection of rickettsiosis and malaria in humans has been reported in sub-Saharan Africa (Mediannikov *et al*., 2013b). Also, many cases of non-malarial febrile illnesses have been reported to be attributed to SFG rickettsiae infection (Crump *et al*., 2013; Punjabi *et al*., 2012). As such, differential molecular tests of SFG *Rickettsia* spp. and *Plasmodium* spp. are warranted to correctly diagnose infectious causes of febrile illness in low resource settings. Therefore, the utilization of molecular methods for multiplex detection of malaria and SFG rickettsia is inevitable.

Nucleic Acid Amplification Tests (NAATs) have been widely used to detect pathogens, including malaria parasites (Cnops *et al*., 2011; Snounou *et al*., 1993) and SFG rickettsiae (Choi *et al*., 2005; Renvoisé *et al*., 2012), of which PCR-based methods have been the mainstay method. However, PCR-based methods remain unsuitable for routine diagnostic procedures because they require expensive and specialized instruments such as thermocyclers for accurate temperature fluctuation during amplification, which may not be readily available in resource-limited endemic areas. Loop-mediated isothermal amplification (LAMP) is one of the promising alternatives, which is a rapid, simple, and highly specific DNA amplification technique at a constant temperature using DNA polymerases with strand displacement activity (Notomi *et al*., 2000). LAMP methods for the detection of *Plasmodium* spp. (Han *et al*., 2007; Hayashida *et al*., 2017; Polley *et al*., 2010) and SFG rickettsiae (Noden *et al*., 2018; Pan *et al*., 2012) have been developed. Despite the high sensitivity of the developed LAMP systems, they can only detect single targets due to the use of turbidity and/or fluorescence for visualization (Mori *et al*., 2004). Since the amplified product of LAMP shows a laddering pattern, the size differentiation by the gel electrophoresis is impossible. For multiplex LAMP assay, which aims at the simultaneous detection of multiple DNA targets in a single reaction, the amplified products can be distinguished by melting curve analysis (Liu *et al*., 2017) or DNA lateral flow dipstick techniques (Takabatake *et al*., 2018).

DNA lateral flow dipstick is a DNA detection technique by using DNA-DNA hybridization of a free single strand of amplicons to their complementary probes in the dipstick

without amplicon denaturation, and the reaction can be visualized by colorant accumulation (Monden *et al*., 2014; Shanmugakani *et al*., 2017). Single-stranded tag hybridization Chromatographic Printed-Array Strip (C-PAS) is one of the DNA lateral flow dipstick technology designed by Tohoku Bio-Array Company Limited (Tohoku Bio Array, Miyagi, Japan) (Niwa *et al*., 2014). The technique was originally optimized for PCR products and allows discrimination of multiple amplified products simultaneously. The multiple targets amplified with biotin-labeled primer and unique single-stranded sequence-tagged primer are developed on C-PAS with streptavidin-coated blue latex beads. The amplified DNA targets with a single-stranded unique tag sequence hybridize to the complementary tag sequence printed on the C-PAS and will show a blue line due to the accumulation of blue latex beads indicating a positive result. Multiple single-stranded unique tag sequences can be used in the multiplex reaction, and specific complementary tag sequences on the chromatographic strip will discriminate the amplified products based on the unique tag sequences (Shanmugakani *et al*., 2017; Tian *et al*., 2016). The C-PAS method enables easy differentiation and visualization of multiplex DNA signals in a single tube within a short time (15 minutes). The simplicity of the C-PAS visualization method makes it a suitable method of visualization after the multiplex LAMP reaction. Multiplex LAMP amplification and visualization with C-PAS has been applied in the food industry (Sugita-Konishi *et al*., 2019; Takabatake *et al*., 2018) but not been utilized for differential and/or simultaneous detection of infectious diseases.

This study aimed at establishing a simple and rapid diagnostic method that could simultaneously detect SFG rickettsiae and *Plasmodium* spp., using multiplex LAMP and C-PAS. The established system can be applied in point-of-care diagnostics and will improve the identification of the cause of UFI, especially in resource-limited settings. The technique is also highly versatile, which can also be utilized in other clinically or epidemiologically related infectious diseases.

4.2 Materials and methods

4.2.1 Multiplex LAMP

The LAMP primers were synthesized based on the previously reported LAMP assays targeting a single copy 17kDA protein-encoding gene of SFG rickettsiae (Noden *et al*., 2018) and the multiple copy mitochondrial cytochrome *b* gene (*cytb*) of human infective *Plasmodium* spp. (Polley *et al*., 2010). The multiplex LAMP reaction consisted of two sets of six LAMP primers (a total of 12 primers). The LAMP primer sets consisted of a 5'-terminal modified forward loop primer (LF) for each target template, which was tagged with a carbon spacer and a unique tag sequence complementary to the specific oligos on the C-PAS. The backward loop primer (LB) and backward inner primer (BIP) targeting SFG rickettsiae and *Plasmodium* species, respectively, were labeled with biotin at the 5'-terminal (Tohoku Bio-Array, Japan). All the other primers were not modified (Table 7).

Table 7. LAMP primer sequences used for multiplex LAMP. Tagged and biotin-labeled primers are shown with F1 and F4 representing a unique tag sequence. “X” indicates the position of Spacer C3 between the unique tag sequence and the LAMP primer sequence.

Target pathogen	Target gene name	Primer name (reference)	Primer name	Primer sequence
SFG rickettsiae	17kDa protein-encoding gene (*htrA*)	17kDA (Noden *et al*. 2018)	Rr17F3	5’-TGTTACAAGCCTGTAACGG-3’
			Rr17B3	5’-TCCTGTTCATCCATACCTG-3’
			Rr17FIP	5’-GAGAACCAAGTAATGCGCCGGGCGGTATGAATAAACAAGG-3’
			Rr17BIP	5’-AATTCGGTAAGGGCAAAGGACCACCGATTTGTCCACCAA-3’
			Rr17LoopF	5’-**F1**-**X**-CCGCCAAGAAGTGTTCCTGTA-3’
			Rr17LoopB	5’-Biotin-AGCTTGTTGGAGTAGGTGTAGGTG-3’
Human infective *Plasmodium* spp	Mitochondria cytochrome *b* gene (*cytb*)	PgMt19 (Polley *et al*. 2010)	PgMt19-F3	5’-TCGCTTCTAACGGTGAAC-3’
			PgMt19-B3	5’-AATTGATAGTATCAGCTATCCATAG-3’
			PgMt19-FIP	5’-GGTGGAACACATTGTTTCATTTGATCTCATTCCAATGGAACCTTG-3’
			PgMt19-BIP	5’-Biotin-GTTTGCTTCTAACATTCCACTTGCCCGTTTTGACCGGTCATT-3’
			PgMt19-LF	5’-**F4**-**X**-CACTATACCTTACCAATCTATTTGAACTTG-3’
			PgMt19-LB	5’-TGGACGTAACCTCCAGGC-3’

The LAMP mixture was basically the same as the previously reported condition (Hayashida *et al*., 2015, 2019), except for that primer concentration was a quarter for each primer set to avoid primer dimer formation. The primer concentration was determined experimentally so that the assay showed the same sensitivity with original primer sets without primer dimer formations. Each 25 µL LAMP reaction mix consisted of 0.25 µL of each primer resulting into final concentration of 0.4 µM FIP, 0.4 µM BIP, 0.2 µM LB, 0.2 µM LF, 0.05 µM F3 and 0.05 µM B3 of each LAMP system primer set. Other reagents in the reaction mix were 2.25 µL LAMP reaction buffer (consisting of 1 mM Tris-HCl (pH 10), 1 mM KCl and 1 mM $(NH_4)_2SO_4$ in 0.1% TritonX-100), 1 µL of Colori-Fluorometric Indicator (CFI; consisting of 3 mM hydroxylnaphthol blue [MP Biomedicals, Aurora, OH]) and 0.35% v/v GelGreen (10,000X solution in DMSO, Biotium, CA, USA) in distilled water) (Hayashida *et al*., 2015), 2 µL of 2 M Trehalose solution, 1.4 µL of 25 mM each deoxyribonucleotide triphosphates (dNTPs) (Nippon Gene, Tokyo, Japan), 1 µL of 8 U/µl *Bst* 2.0 WarmStart DNA polymerase (New England Bio Labs Inc., MA, USA), 1.5 µL of 100 mM $MgSO_4$ and 10.85 µL of distilled water with 0.1% Triton X-100. The reaction mix was prepared in the cleanroom and then transferred to the amplification room, where 2 µL template was added.

The LAMP reactions were performed at 62ºC for 60 minutes and monitored using the Rotor-Gene 3000 thermocycler FAM channel that detected fluorescence of the GelGreen in the LAMP reagents (Corbett Research, Sydney, Australia), and then melting curve analysis was performed.

4.2.2 Visualization of multiplex amplified products by dipstick DNA chromatography (C-PAS)

The C-PAS F4-V2 membrane strip (Tohoku Bio-Array, Japan) was inserted into a 21 µL reaction mix containing 10 µL of developing solution (300 mM NaCl) (Tohoku Bio-Array, Japan), 9 µL distilled water, 1 µL of LAMP product, and 1 µL of streptavidin-coated blue latex suspension (Tohoku Bio-Array, Japan). After 15 minutes at ambient conditions (20–25°C and 30-40% humidity), the band was observed. The blue line on the C-PAS strip test position (Figure 8) indicated a positive test result for the presence of the amplified target DNA sequence tagged with the complementary oligonucleotide and biotin.

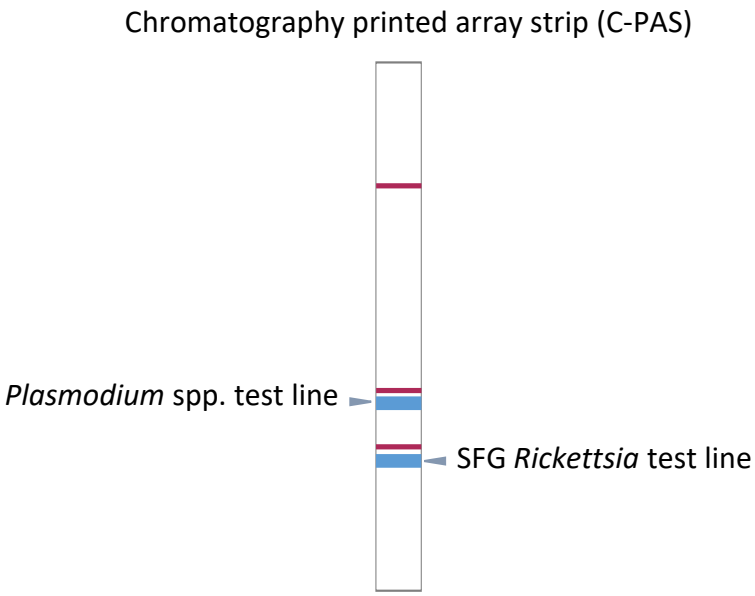

Figure 8: Schematic view of dipstick chromatography showing SFG *Rickettsia* and *Plasmodium* spp. test lines in blue color. Red lines indicate the markers for position of test lines.

4.2.3 The determination of the sensitivity of multiplex LAMP assay using synthetic gene

A partial fragment of the 17kDa protein-encoding gene of *R. felis* (1406458 bp – 1406629 bp region: accession number CP000053), which is conserved among SFG rickettsiae, was commercially synthesized (Eurofins Genomics, Tokyo, Japan) and cloned into the pEX-A2J2 plasmid. This template was referred to as *Rickettsia* gene in this chapter. Another fragment of the *P. falciparum* mitochondrial sequence (338 bp – 358 bp region: accession number AJ276844), which is conserved among genus *Plasmodium*, was synthesized and cloned into the pEX-A2J2 plasmid as well, thereby referred to as *Plasmodium* gene. The Qubit 4.0 fluorometer dsDNA HS assay kit (ThermoFischer Scientific, USA) was used to quantify the concentration of the plasmids. The templates were normalized to equal copy numbers and mixed in equal proportions. The mixed template was then serially diluted by 10-fold every time before use. The serially diluted copy number ranged from 10 to 1×10^6 gene copies per µL. The assays were performed in five replicates for each template concentration with a non-template control.

4.2.4 The determination of the sensitivity of multiplex LAMP assay using genomic DNA from in vitro cultured *R. monacensis* or *P. falciparum*

Rickettsia monacensis, one of the SFG rickettsiae, was maintained in the C6/36 cell line as described in a previous study (Thu *et al*., 2019). Genomic DNA was extracted from the cultured *R. monacensis* by Takara Simpleprep kit (Takara, Japan) and purified by ethanol precipitation. The *R. monacensis* genomic DNA copy number was then quantified by qPCR using a single copy SFG-specific gene, *ompA*, using previously described primers (Moonga *et al*., 2019, Chapter 1 in this thesis). DNA quantification was determined using the Bio-Rad CFX Manager software based on the standard curve. The determined DNA copy number was assumed to be equivalent to the genomic copy number (genome equivalents). *Plasmodium. falciparum* HB3 strain was cultured *in vitro* according to previously reported methods (Trager and Jensen, 1976) at 0.1-1.0% parasitemia. The genomic DNA was extracted using Nucleospin blood kit (Takara, Japan). The obtained DNA concentration was then measured by the dsDNA HS assay kit with Qubit 4.0 fluorometer, and the DNA quantities were converted to the genome copy numbers by calculating taking *Plasmodium* genome size to be 22.9 Mb. The genome equivalent for *in vitro* cultured *R. monacensis* and *P. falciparum* were then normalized and 10-fold serially diluted.

The multiplex LAMP was tested using the above-prepared templates. The same templates were also used for comparative sensitivity analysis of multiplex LAMP and qPCR. The previously reported *ompA* and 18S rRNA (Lefterova *et al*., 2015) gene-based qPCR was

tested on the *R. monacensis* and *P. falciparum* genomic DNA, respectively. In brief, the SYBR green-based real-time qPCR was performed in the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, CA, USA). All reaction mixtures had a final volume of 20 µL with 12.5 µL of TB Green Premix Ex Taq II (Takara, Japan), 1 µL (0.4 µM final concentration) of each primer, and 2 µL of quantified genomic DNA template. After the initial denaturation cycle at 95°C for 30 seconds, reactions were cycled 40 times as follows: 95°C for 5 seconds and 50°C for 30 seconds with a final melting curve step.

4.2.5. Detection of *R. monacensis* and *P. falciparum* spiked with human blood from DNA card using heating block and dipstick chromatography

Rickettsia monacensis from in vitro culture was spiked with human blood at 5,000 bacteria copies per µL of blood. The *Rickettsia* spiked with blood was then spotted on the classic Whatman FTA Classic card (GE Healthcare, Buckinghamshire, UK) and dried. Similarly, red blood cells infected with *P. falciparum* at 1% parasitemia were dried on the Whatman FTA Classic card (GE Healthcare, UK). A negative template control was prepared by applying uninfected blood onto the Whatman FTA Classic card (GE Healthcare, UK) and dried at room temperature. The DNA from dried blood spots was extracted by the modified boiling method (Choi *et al*., 2014). In brief, two dried blood spots of 3 mm were punched out and soaked in 40 µL of nuclease-free water in the PCR tube. The mixed template was prepared by mixing two dried blood spot punches from *R. monacensis* and *P. falciparum* in a single tube. The samples were then incubated at 60°C for 30 minutes and boiled at 99°C for 10 minutes. The multiplex LAMP was performed on a heating block, powered by a portable rechargeable battery, at 62°C for 60 minutes. The amplicon was then visualized using a portable fluorometer (Hayashida *et al*., 2015), and dipstick chromatography.

4.3 Results

4.3.1 Multiplex LAMP using single synthetic templates of *Rickettsia* or *Plasmodium* genes in plasmids

The reported two LAMP primer sets targeting *Rickettsia* 17kDa protein-encoding gene (Noden *et al*., 2018) and *Plasmodium* mitochondrial DNA (Polley *et al*., 2010) were utilized for multiplex LAMP reaction. The multiplex LAMP reaction was first tested by mixing two primer sets at 25% quantity from the original condition. The target loci for the two LAMP systems were synthesized and inserted in the plasmids. Using this condition, the multiplex LAMP detected up to 100 template copies per reaction using either *Rickettsia* or *Plasmodium* gene in individual reaction tubes (Figures 9B and 9E). The amplification was monitored by a real-time PCR machine, and its specificity was confirmed by melting curve analysis (Rolando *et al*., 2020). The melting temperature (Tm) for *Rickettsia* and *Plasmodium* gene was 87ºC and 83ºC, respectively (Figures 9C and 9F, respectively). Based on this result, it was concluded that the SFG rickettsiae and *Plasmodium* spp. LAMP systems were compatible for multiplexing.

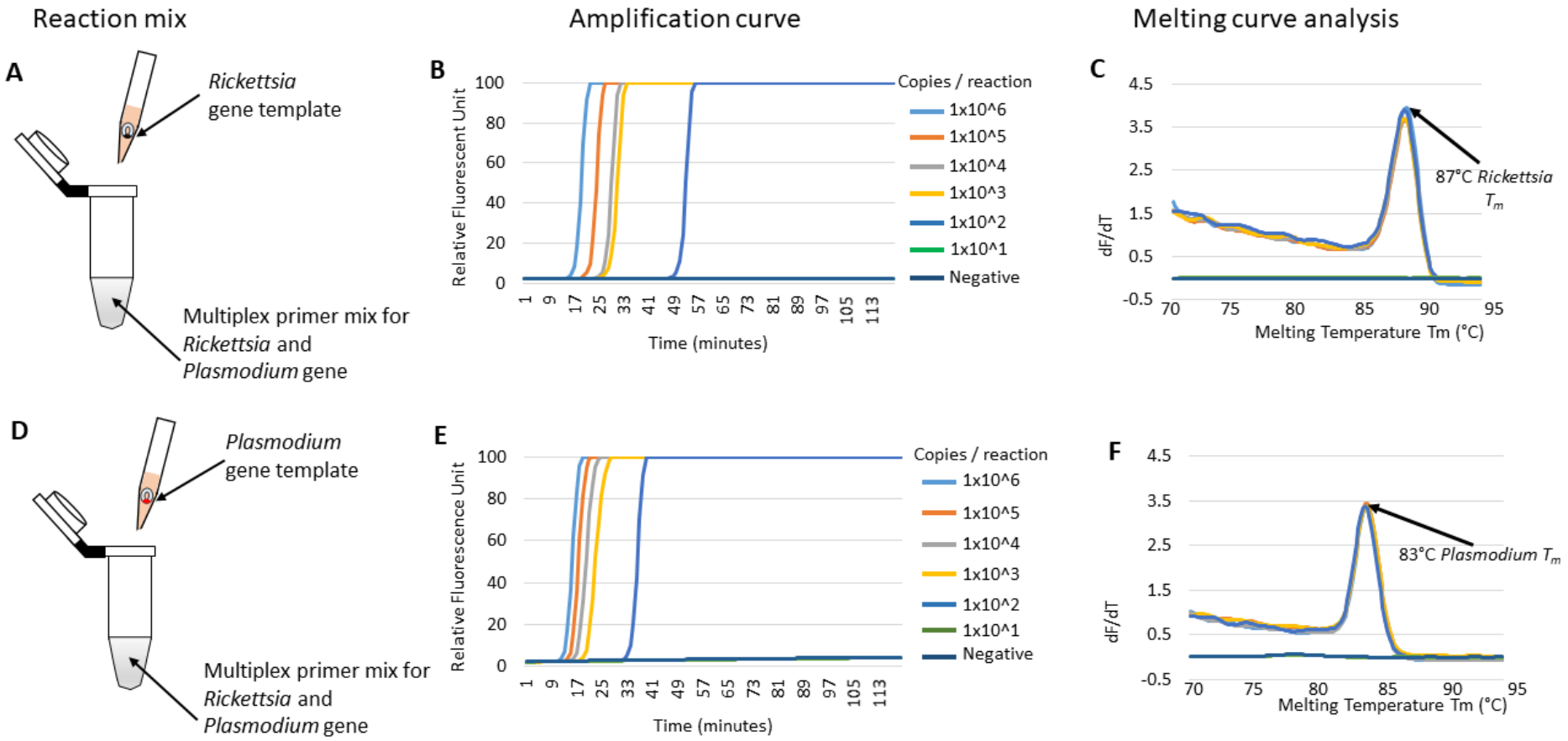

Figure 9. Multiplex LAMP amplification using a synthetic single target gene template. The multiplex LAMP reaction mix was set up with a single target template of the *Rickettsia* or *Plasmodium* gene (Fig. 9A and 9D). The amplification curve was observed by fluorescence with a detection limit of 100 copies for both templates (Fig. 9B and 9E). The amplified products were identified by melting temperature showing 87ºC for *Rickettsia* gene (Fig. 9C) and 83ºC for *Plasmodium* gene in figure 9E.

4.3.2 Optimization of multiplex LAMP using mixed synthetic templates of *Rickettsia* and *Plasmodium* gene, and discrimination by C-PAS

To test if the multiplex LAMP was able to amplify multiple targets simultaneously in a single reaction, equally mixed templates of *Rickettsia* and *Plasmodium* genes were amplified, and discrimination by C-PAS was tested. The amplification efficiency was monitored by fluorescence on the real-time PCR machine, but this cannot distinguish amplified products based on amplification fluorescence (Figure 10B). The simultaneously amplified products of *Rickettsia* and *Plasmodium* target gene were first discriminated by melting curve analysis. Two melting temperatures were observed at 87ºC and 83ºC, indicating successful amplification of both *Rickettsia* and *Plasmodium* target genes (Figure 10C). The simultaneously amplified products were further discriminated by C-PAS, which showed a consistent result as the melting temperature analysis. According to the results, the multiplex LAMP amplified both targets, and the simultaneously amplified products were distinguishable by C-PAS (Figure 10D). The multiplex LAMP for the simultaneous detection of both *Rickettsia* and *Plasmodium* genes detected up to 1,000 copies of each template per reaction. The discrimination of mixed *Rickettsia* and *Plasmodium* genes amplified by multiplex LAMP was consistently comparable for C-PAS and melting curve analysis in five independent experiments (Table 8). This result showed the potential of C-PAS to be applied for distinguishing multiplex amplified products without using a thermocycler.

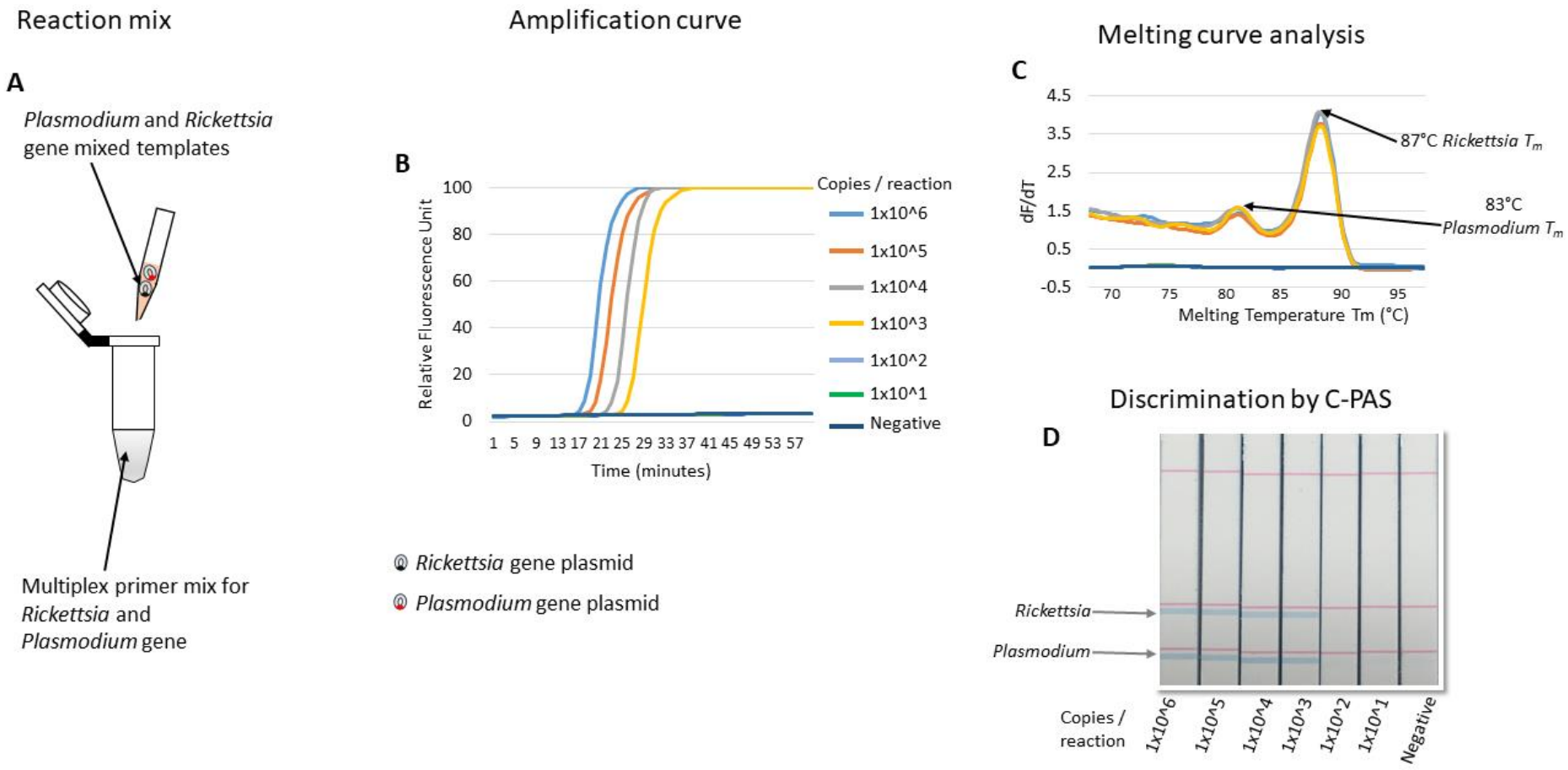

Figure 10. Multiplex LAMP amplification and discrimination by dipstick DNA chromatography (C-PAS) using mixed target gene templates. The reaction mix contained multiplex LAMP primer sets and two templates mix for Rickettsia and Plasmodium gene target templates (Figure 10A). The amplification curve detected up to 1,000 indistinguishable gene copies per reaction (Figure 10B). Figure 10C shows the C-PAS panel indicating Rickettsia and Plasmodium genes amplified products distinguished as two bands (Figure 10C). The C-PAS result is consistent with the melting curve analysis panel showing two melting temperatures for Rickettsia and Plasmodium genes at 87ºC and 83ºC, respectively (Figure 10D).

Table 8. The sensitivity of the multiplex LAMP system based on melting curve analysis and C-PAS detection technique. Melting curve analysis and C-PAS showed the same sensitivities for detecting synthetic genes of *Plasmodium* and *Rickettsia* in five independent experiments. Number shows positive numbers of five experiments. NTC: no template control.

Copy number per reaction		**10^6**	**10^5**	**10^4**	**10^3**	**10^2**	**10^1**	**NTC**
Melting peak analysis result	*Plasmodium* gene	5/5 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)	3/5 (60%)	1/5 (20%)	0/5 (0%)
	Rickettsia gene	5/5 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)	0/5 (0%)	0/5 (0%)	0/5 (0%)
C-PAS result	*Plasmodium* gene	5/5 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)	3/5 (60%)	1/5 (20%)	0/5 (0%)
	Rickettsia gene	5/5 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)	0/5 (0%)	0/5 (0%)	0/5 (0%)

4.3.3 Validation of multiplex LAMP using genomic DNA from in vitro cultured *R. monacensis* or *P. falciparum* organisms

The multiplex LAMP amplification was further tested with genomic DNA from individual targets of *in vitro* cultured *R. monacensis* or *P. falciparum* organisms as reaction templates (Figures 11A and 11D). Multiplex LAMP detected up to 100 genome copies of *R. monacensis* per reaction and was identifiable on C-PAS (Figures 11B and 11C). When genomic DNA from *P. falciparum* was used, multiplex LAMP detected up to 0.26 pg, which was equivalent to 10 genome copies per reaction, and was also identifiable on C-PAS (Figures 11E and 11F).

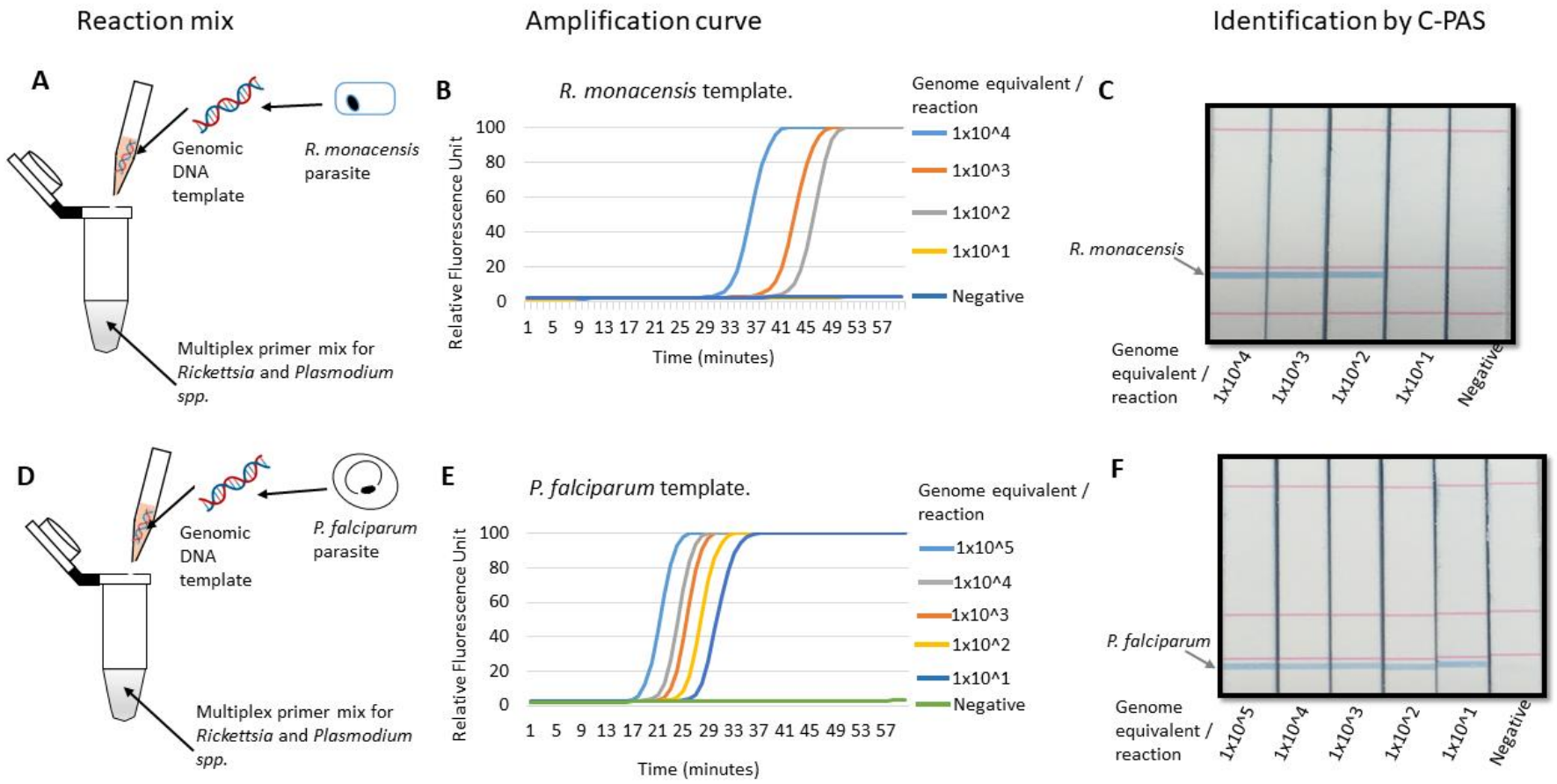

Figure 11. Multiplex LAMP amplification using genomic DNA from *in vitro* cultured organisms. The multiplex LAMP reaction was performed using *R. monacensis* genomic DNA as the template (Fig. 11A). The amplification curve detected up to 100 genome copies per reaction of *R. monacensis* (Figure 11B) and identification of amplicons as *Rickettsia* by C-PAS (Figure 11C). The reaction mix contained multiplex LAMP primer sets with *P. falciparum* genomic DNA (Figure 11D). Figure 11E shows the amplification curve of *P. falciparum* detecting up to 10 genomic DNA copies per reaction. The amplified product was identified by C-PAS as *Plasmodium* (Figure 11F).

4.3.4 Comparative sensitivity of the multiplex LAMP system against real-time qPCR system

The performance of the multiplex LAMP was compared to the reported real-time qPCR system using a single target genomic DNA of either *in vitro* cultured *R. monacensis* or *P. falciparum*. The real-time qPCR targeting *Rickettsia* outer membrane protein *A* gene (*ompA*) detected up to 100 genomic DNA copies per reaction, indicating that the developed multiplex LAMP was comparable to the real-time qPCR method (Figures 11B and 12A). Similarly, real-time qPCR targeting pan-*Plasmodium* 18S rRNA detected up to 10 genome copies (0.26 pg), showing the same sensitivity with the developed multiplex LAMP system (Figures 11E and 12B).

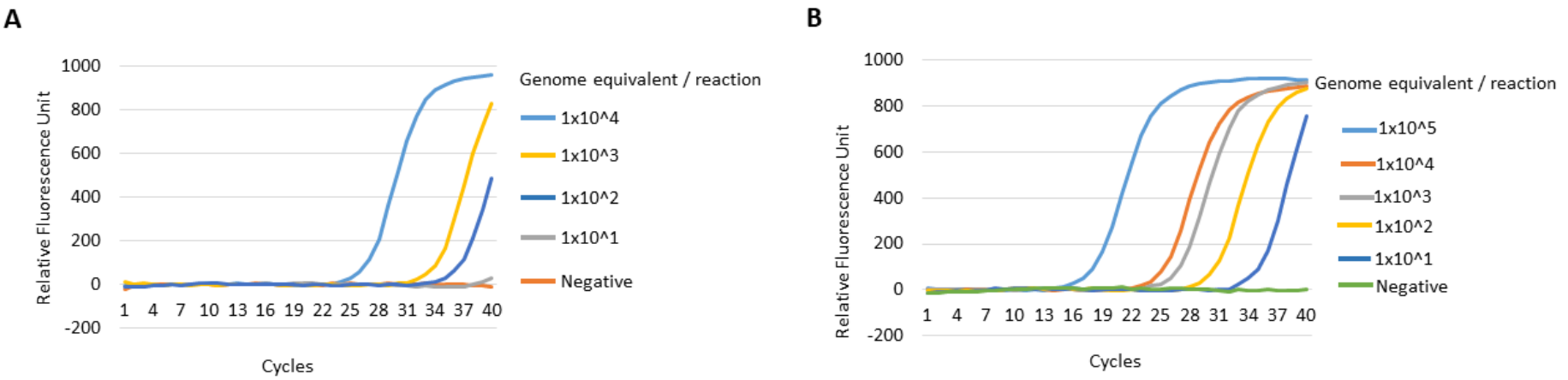

Figure 12. The sensitivity of real-time qPCR using genomic DNA from *in vitro* cultured organisms. Figure 12A shows the amplification curve of *R. monacensis* by qPCR based on the *Rickettsia ompA* gene. Figure 12B shows the *Plasmodium* 18S rRNA gene amplification curve using *P. falciparum* template. The qPCR showed detection up to 100 (Figure 12A) and 10 (Figure 12B) genome copies for *in vitro* cultured *R. monacensis* and *P. falciparum,* respectively.

4.3.5. Simplified LAMP reaction using human blood spiked samples and field-deployable amplification methods

To test the applicability of the developed method in a simple field condition, the multiplex LAMP was performed on the heating block powered by a portable battery using cultured parasites spiked with normal human blood. The multiplex LAMP successfully amplified the *R. monacensis* and *P. falciparum* (Figure 13A and 13B). The amplified product was visualized by fluorescence detection method using a portable fluorometer (Figure 13C). The amplified products were further confirmed to the detected species by dipstick chromatography. The test clearly distinguished the amplified *Rickettsia* spp. and *Plasmodium* spp. as well as simultaneous amplification. The negative control using genomic DNA extracted from known negative blood was negative indicating no nonspecific amplification (Figure 13D).

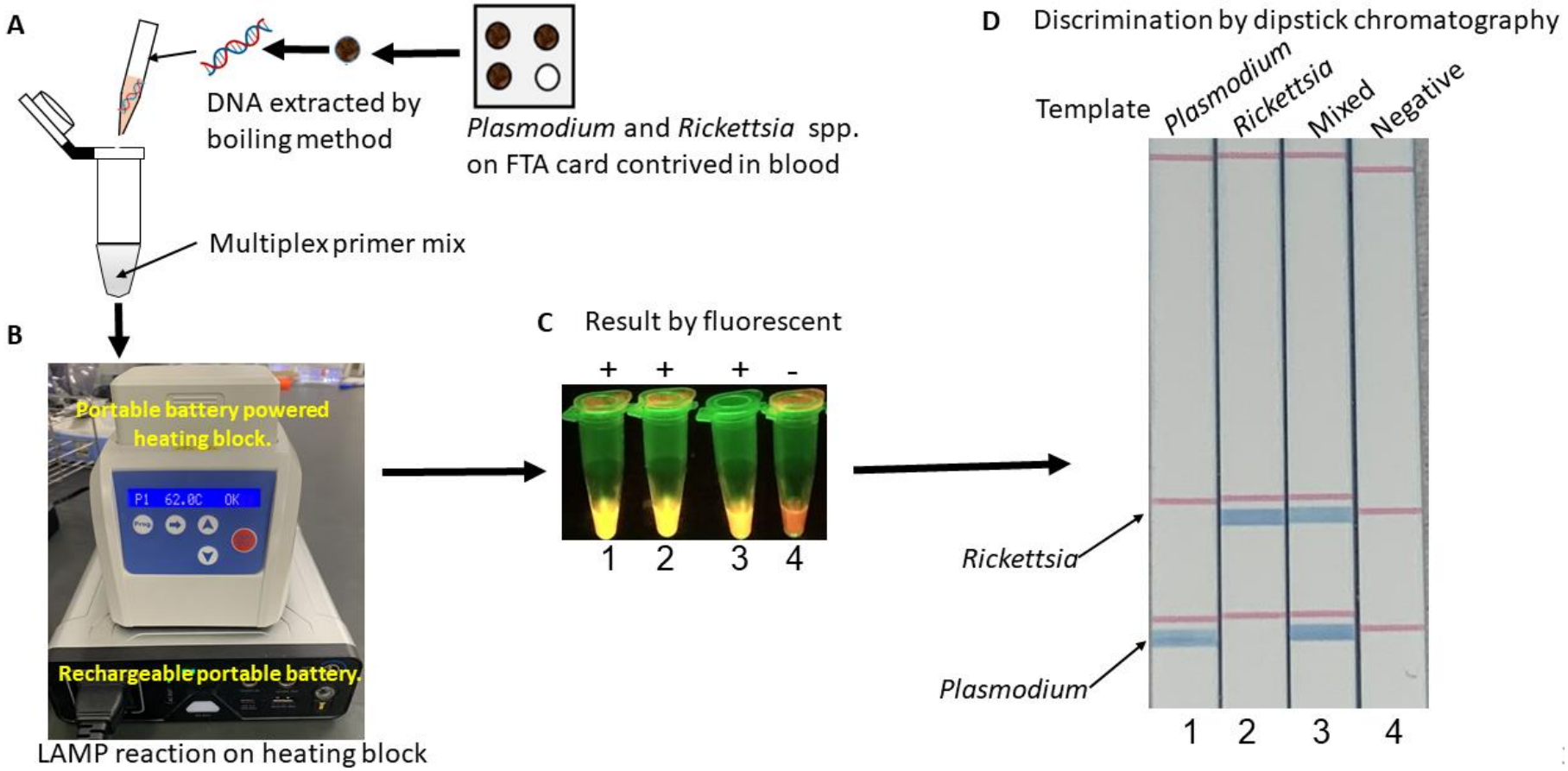

Figure 13. Simplified multiplex LAMP for detection of *R. monacensis* and *P. falciparum* spiked with human blood. The multiplex LAMP reaction was set up using *R. monacensis* and *P. falciparum* spiked with blood, dried on Whatman FTA Classic card, and genomic DNA extracted by boiling as the template (Fig. 13A). The reaction was performed using a rechargeable battery-powered heating block (Fig. 13B). Figure 13C shows the detection result by fluorescence and multiple amplicon discrimination by dipstick chromatography (Fig. 13D).

4.4 Discussion

Differential diagnosis of the diseases with similar clinical manifestations is critical for successful case management outcomes. Spotted fever group rickettsia such as *R. felis* has been reported to often co-infect febrile patients with *Plasmodium* spp. in endemic areas (Mediannikov *et al*., 2013b). Co-infection of *Plasmodium* spp. with other pathogens such as dengue virus and *Leptospira* spp. are associated with severe malaria (Mandage *et al*., 2020). Other rickettsial infections have been reported mixed with either *Plasmodium*, *Leptospira,* or *Coxiella burnetii*, resulting in complicated clinical management and poor prognosis (McGready *et al*., 2010; Phimda *et al*., 2007; Rolain *et al*., 2005; Watt and Parola, 2003). Therefore, the development of multiplex differential diagnostic tools is essential for better diagnosis and case management of patients with undifferentiated fevers.

The established multiplex LAMP system in this study can be applied in both differential and simultaneous detection of SFG rickettsiae and *Plasmodium* spp. This multiplex LAMP primer design was applied from the previously described LAMP systems (Polley *et al*., 2010; Noden *et al*., 2018), suggesting that already developed LAMP systems could be improved by multiplexing. This proof of concept shows that LAMP methods can be multiplexed for the detection of infectious diseases and discriminate by dipstick DNA chromatography (C-PAS) visualization, hence easing their application as a point of care differential diagnostic tool. The use of C-PAS makes it possible to differentiate multiple amplified targets and visualize without any sophisticated equipment such as thermocycler for melting curve analysis. The current C-PAS is designed to detect four to eight different amplified PCR products. However, the report of multiplexed LAMP systems is limited mostly due to the formation of primer dimer with an increase in the number of primer sets in a reaction. Since LAMP can also be performed on a heating block or water bath for amplification without a thermocycler (Gandasegui *et al*., 2018; Oriero *et al*., 2015), the whole procedure of the established multiplex LAMP and C-PAS requires minimal sophisticated materials. The robustness of the *Bst* polymerase allows the multiplexing of LAMP systems. The differentiation and visualization of multiple amplified products by C-PAS, which shows multiple blue lines for multiple amplified products, allows easy differential diagnosis in clinical settings. However, it is recommended to use different rooms for the preparation of multiplex LAMP master mix and performing dipstick chromatography where the LAMP reaction tube is opened, to avoid cross-contamination. The developed multiplex LAMP demonstrated the multiplexing of SFG rickettsia LAMP and *Plasmodium* spp. LAMP, and distinguish the specific amplicons by C-PAS. This technique can also be applied in various differential diagnoses. For example, multiplexing of *P. falciparum*

and non-*P. falciparum* LAMP could be beneficial for their differentiation as artemisinin tend to fail to clear non-*P. falciparum* malaria parasites (Dinko *et al*., 2013).

Multiplex LAMP was a combination of two LAMP primer sets in the same reaction. The target template was either mixed (two) or single (one) target templates. The sensitivity of the multiplex LAMP on the mixed targets was ten times (1,000 copies/reaction) lower than the single target template (100 copies/reaction). The discrepancy could be attributed to the competitiveness of multiplex reactions for reagents in mixed target template reactions. However, the multiplex LAMP is potentially applicable in clinical diagnosis as the malaria parasitemia was reported to be approximately 1,000 parasites per µL (Gill *et al*., 2019; Imwong *et al*., 2015), translating into detectable quantities by multiplex LAMP system with dipstick DNA chromatography. Spotted fever group rickettsia pathogens such as *R. rickettsii* have shown bacteremia of more than 100 copies per µL in fatal cases (Kato *et al*., 2016), which can be detected by our multiplex LAMP. Nevertheless, the sensitivity of the SFG LAMP system still needs to be improved to detect non-fatal cases that have lower bacteremia for its applicability in diagnosis. Additionally, an internal positive control targeting the host genome needs to be included in the multiplex LAMP system to validate the success of sample preparation methods.

4.5 Conclusion

In summary, a multiplex LAMP system to simultaneously detect SFG rickettsiae and *Plasmodium* spp. was developed. The differentiation of the multiple amplified targets was accomplished by dipstick DNA chromatography (C-PAS). These results showed that LAMP can be multiplexed for the simultaneous detection of infectious diseases and easily visualized by dipstick chromatography with minimum sophisticated materials.

5. General Summary

Chapter 1 focused on the neglected flea-borne disease caused by *R. felis;* hence, animals and arthropods samples were screened for SFG rickettsiae. *Rickettsia felis* was identified in cat fleas which are blood-sucking arthropod vectors associated with domestic dogs and peridomestic rodents. *Rickettsia felis* was also detected in domestic dogs and peridomestic rodents in Zambia. In cat fleas, *R. asembonensis* was also frequently detected. These observations suggest that domestic dogs sharing habitat with humans, peridomestic rodents, and cat fleas which are known indiscriminate feeders potentially circulate *R. felis* in Zambia. This poses a potential public health risk to humans as *R. felis*, a known human pathogen is circulating in mammalian hosts and arthropod vectors in Zambia.

In chapter 2, to investigate human exposure to SFG rickettsiae in Zambia; human blood samples from various parts of Zambia were screened. *Rickettsia asembonensis* was detected in two human blood samples from Zambia. The sequences of *R. asembonensis* detected in human patients phylogenetically clustered together with previously isolates detected in cat fleas from Zambia. These results signify the possible role of flea-borne *R. asembonensis* as a cause of human illness. *Rickettsia felis* was not detected in all the samples tested despite its possible circulation in dogs, rodents and cat fleas.

In chapter 3, to improve differential diagnosis of rickettsial illness, a novel, simple and rapid multiple pathogen detection loop-mediated isothermal amplification (LAMP) method was developed for easy simultaneous detection of both spotted fever group *Rickettsia* spp. and *Plasmodium* spp. The technique was based on the combination of LAMP methods and dipstick DNA chromatography technology. The developed simultaneous rapid diagnosis technique would contribute to the differential diagnosis of undifferentiated febrile illness caused by either spotted fever group *Rickettsia* spp. or *Plasmodium* spp. in resource-limited endemic areas.

Taken together, this thesis highlights the circulation of zoonotic *R. felis* in domestic dogs, peridomestic rodents, and cat fleas in Zambia. Also, *R. asembonensis* of similar genotype detected in cat fleas and humans is an important finding as it highlights possible human infection by *R. asembonensis*. *Rickettsia* infections could be underdiagnosed in Zambia due to the endemicity of malaria. Thus, with the potential significance of *Rickettsia* infections, the novel developed multiplex LAMP technique could be essential for the differential detection of *Rickettsia* spp. to better understand the epidemiology of rickettsial diseases in Zambia. Furthermore the simultaneous detection of SFG *Rickettsia* and *Plasmodium* spp. which both presents with fever could contribute to better treatment outcomes in endemic areas.

6. Acknowledgements

The success of this work would have hardly seen the light of the day without the many people and collaborators who immensely contributed to my studies. I thank all the people who took part in any way in the realization of this thesis.

I wish to thank Professor Chihiro Sugimoto (Division of Collaboration and Education, Research Center for Zoonosis Control, Hokkaido University, Sapporo, Japan), for giving me an opportunity to pursue my studies in his laboratory. In the same vein, I would like to thank Dr. Martin Simuunza (Department of Disease Control, School of Veterinary Medicine, University of Zambia, Lusaka, Zambia) and Dr. Chummy Sikasunge (Department of Paraclinical Studies, School of Veterinary Medicine, University of Zambia, Lusaka, Zambia) for recommending me to study under Professor Sugimoto at Hokkaido University. I greatly appreciate the opportunity provided.

I would like to enormously thank my supervisors Associate Professor Junya Yamagishi and Dr. Kyoko Hayashida (both from Division of Collaboration and Education, Research Center for Zoonosis Control, Hokkaido University, Sapporo, Japan) for their valuable support, supervision, research training, quality research conceptualization and thorough proofreading of all my research contents throughout my study. I convey my heartfelt thanks to Professor Boniface Namangala (Department of Paraclinical Studies, School of Veterinary Medicine, University of Zambia, Lusaka, Zambia) for the critical advice and supervision during the period of study. I also thank Ms. Naoko Kawai (Division of Collaboration and Education, Research Center for Zoonosis Control, Hokkaido University, Sapporo, Japan) for support and guidance during my laboratory experiments.

I am greatly thankful to our collaborator Dr. Mable Mutengo (Department of Biomedical Sciences, Levy Mwanawasa Medical University, Lusaka, Zambia) for her tireless contributions to all my studies in Zambia. I am greatly indebted to her efforts, without which my study would not have succeeded. I also thank all the members of the laboratory of Parasitology at the University Teaching Hospital, Zambia.

I sincerely appreciate the valuable advice of my quality examiners, Professor Satoru Konnai (Laboratory of Infectious Disease, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Japan), Professor Yasuhiko Suzuki (Division of Bioresources and Education, Research Center for Zoonosis Control, Hokkaido University, Sapporo, Japan) Associate Professor Ryo Nakao and Professor Nariaki Nonaka (both from Laboratory of

Parasitology, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Japan). I deeply thank them for refining and improving my thesis through their critical advice.

Last but not the least, I would like to acknowledge all the people who were involved in sample collection. I thank all of them for undertaking such challenging activities. Additionally, I would like to thank Professor Ryo Nakao again for providing me with *in vitro* cultured *Rickettsia* sp. for validation of all protocols.

I am grateful to the Japanese scholarship from the Ministry of Education, Culture, Sports, Science and Technology MEXT for granting me the scholarship to study at Hokkaido University and the program of Fostering Global Leaders in Veterinary Science toward Contributing to 'One Health' (Leading Program) for the funding of my overseas activities and internship during my doctoral course.

7. References

Abarca K, López J, Acosta-Jamett G, Martínez-Valdebenito C. 2013. *Rickettsia felis* in *Rhipicephalus sanguineus* from two distant Chilean cities. *Vector-Borne Zoonotic Dis* **13**:607–609.

Abdad MY, Abou Abdallah R, Fournier PE, Stenos J, Vasoo S. 2018. A concise review of the epidemiology and diagnostics of rickettsioses: *Rickettsia* and *Orientia* spp. *J Clin Microbiol* **56**:e01728-17.

Adams JR, Azad AF, Schmidtmann ET. 1990. Infection of colonized cat fleas, *Ctenocephalides Felis* (Bouché), with a *Rickettsia*-like microorganism. *Am J Trop Med Hyg* **43**:400–409.

Amexo M, Tolhurst R, Barnish G, Bates I. 2004. Malaria misdiagnosis: Effects on the poor and vulnerable. *Lancet* **364**:1896–1898.

Angelakis E, Mediannikov O, Parola P, Raoult D. 2016. *Rickettsia felis*: The complex journey of an emergent human pathogen. *Trends Parasitol* **32**:554–564.

Bai Y, Osikowicz LM, Kosoy MY, Eisen RJ, Atiku LA, Mpanga JT, Boegler KA, Enscore RE, Gage KL. 2017. Comparison of zoonotic bacterial agents in fleas collected from small mammals or host-seeking fleas from a Ugandan region where plague is endemic. *mSphere* **2**:e00402-17.

Bartlett JG. 2004. *Rickettsia parkeri*: A newly recognized cause of spotted fever rickettsiosis in the United States. *Infect Dis Clin Pract* **12**:261–262.

Barua S, Hoque MM, Kelly PJ, Poudel A, Adekanmbi F, Kalalah A, Yang Y, Wang C. 2020. First report of *Rickettsia felis* in mosquitoes, USA. *Emerg Microbes Infect* **9**:1008–1010

Bechah Y, Capo C, Mege JL, Raoult D. 2008. Epidemic typhus. *Lancet Infect Dis* **8**:417–426.

Berrelha J, Briolant S, Muller F, Rolain JM, Marie JL, Pagés F, Raoult D, Parola P. 2009. *Rickettsia felis* and *Rickettsia massiliae* in Ivory Coast, Africa. *Clin Microbiol Infect* **15**:251–252.

Betancourt-Ruiz P, Martínez-Díaz HC, Gil-Mora J, Ospina C, Olaya-M LA, Benavides E, Bolaños E, Cuervo C, Blanton L, Hidalgo M. 2020. *Candidatus* Rickettsia senegalensis in cat fleas (Siphonaptera: Pulicidae) collected from dogs and cats in Cauca, Colombia. *J Med Entomol* **57**:382–387.

Billeter SA, Diniz PP, Jett LA, Wournell AL, Kjemtrup AM, Padgett KA, Yoshimizu MH, Metzger ME, Barr MC. 2016. Detection of *Rickettsia* species in fleas collected from cats in regions

endemic and nonendemic for flea-borne rickettsioses in California. *Vector Borne Zoonotic Dis* **16**:151–156.

Blanton LS, Walker DH. 2017. Flea-borne rickettsioses and Rickettsiae. *Am J Trop Med Hyg* **96**:53–56.

Bonwitt J, Sáez AM, Lamin Joseph, Ansumana R, Dawson M, Buanie J, Lamin J, Sondufu D, Borchert M, Sahr F, Fichet-Calvet E, Brown H. 2017. At home with *Mastomys* and *Rattus*: Human-rodent interactions and potential for primary transmission of lassa virus in domestic spaces. *Am J Trop Med Hyg* **96**:935–943.

Boostrom A, Beier MS, Macaluso JA, Macaluso KR, Hayes DS, Radulovic S, Azad AF. 2002. Geographic association of *Rickettsia felis*-infected opossums with human murine typhus, Texas. *Emerg Infect Dis* **8**:549–554.

Bouyer DH, Stenos J, Crocquet-Valdes P, Moron CG, Popov VL, Zavala-Velazquez JE, Foil LD, Stothard DR, Azad AF, Walker DH. 2001. *Rickettsia felis*: Molecular characterization of a new member of the spotted fever group. *Int J Syst Evol Microbiol* **51**:339–347.

Boyd AS. 1997. Rickettsialpox. *Dermatol Clin* **15**:313–318.

Brah S, Daou M, Salissou L, Mahaman S, Alhousseini D, Moussa S, Malam-Abdou B, Adamou H, Adehossi E. 2015. Fever of unknown origin in Africa: The causes are often determined! *Heal Sci Dis* **16**:1–8.

Carrol ED, Guiver M, Nkhoma S, Mankhambo LA, Marsh J, Balmer P, Banda DL, Jeffers G, White SA, Molyneux EM, Molyneux ME, Smyth RL, Hart CA. 2007. High pneumococcal DNA loads are associated with mortality in Malawian children with invasive pneumococcal disease. *Pediatr Infect Dis J* **26**:416–422.

Chen S, Huang T, Zhou Y, Han Y, Xu M, Gu J. 2017. AfterQC: automatic filtering, trimming, error removing, and quality control for fastq data. *BMC Bioinformatics* **18**:80.

Choi E, Kwang S, Ihm C, Sohn Y. 2014. Rapid DNA extraction from dried blood spots on filter paper : Potential applications in Biobanking. *Osong Public Heal Res Perspect* **5**:351–357.

Choi YJ, Lee SH, Park KH, Koh YS, Lee KH, Baik HS, Choi MS, Kim IS, Jang WJ. 2005. Evaluation of PCR-based assay for diagnosis of spotted fever group rickettsiosis in human serum samples. *Clin Diagn Lab Immunol* **12**:759–763.

Sexton DJ, Corey GR. 1992. Rocky Mountain "spotless" and "almost spotless" fever : A wolf in sheep's clothing. *Clin Infect Dis* **15**:439–448.

Cnops L, Jacobs J, Van Esbroeck M. 2011. Validation of a four-primer real-time PCR as a diagnostic tool for single and mixed *Plasmodium* infections. *Clin Microbiol Infect*

17:1101–1107.

Colomba C, Saporito L, Polara VF, Rubino R, Titone L. 2006. Mediterranean spotted fever: Clinical and laboratory characteristics of 415 Sicilian children. *BMC Infect Dis* **6**:1–5.

Crump JA, Morrissey AB, Nicholson WL, Massung RF, Stoddard RA, Galloway RL, Ooi EE, Maro VP, Saganda W, Kinabo GD, Muiruri C, Bartlett JA. 2013. Etiology of severe non-malaria febrile illness in northern Tanzania: A prospective cohort study. *PLoS Negl Trop Dis* **7**:e0002324

Dall'Agnol B, Souza U, Webster A, Weck B, Stenzel B, Labruna M, Klafke G, Martins JR, Ferreira CAS, Reck J. 2017. "*Candidatus* Rickettsia asemboensis" in *Rhipicephalus sanguineus* ticks, Brazil. *Acta Trop* **167**:18–20.

Darton T, Guiver M, Naylor S, Jack DL, Kaczmarski EB, Borrow R, Read RC. 2009. Severity of meningococcal disease associated with genomic bacterial load. *Clin Infect Dis* **48**:587–594.

Dieme C, Bechah Y, Socolovschi C, Audoly G, Berenger JM, Faye O, Raoult D, Parola P. 2015. Transmission potential of *Rickettsia felis* infection by *Anopheles gambiae* mosquitoes. *Proc Natl Acad Sci* **112**:8088–8093

Dinko B, Oguike MC, Larbi JA, Bousema T, Sutherland CJ. 2013. Persistent detection of *Plasmodium falciparum*, *P. malariae*, *P. ovale curtisi* and *P. ovale wallikeri* after ACT treatment of asymptomatic Ghanaian school-children. *Int J Parasitol Drugs Drug Resist* **3**:45–50.

Eremeeva ME, Warashina WR, Sturgeon MM, Buchholz AE, Olmsted GK, Park SY, Effler PV, Karpathy SE. 2008. *Rickettsia typhi* and *R. felis* in rat fleas (*Xenopsylla cheopis*), Oahu, Hawaii. *Emerg Infect Dis* **14**:1613–1615.

Foongladda S, Inthawong D, Kositanont U, Gaywee J. 2011. *Rickettsia, Ehrlichia, Anaplasma*, and *Bartonella* in ticks and fleas from dogs and cats in Bangkok. *Vector Borne Zoonotic Dis* **11**:1335–1341.

Fournier PE, Dumler JS, Greub G, Zhang J, Wu Y, Raoult D. 2003. Gene sequence-based criteria for identification of new *Rickettsia* isolates and description of *Rickettsia heilongjiangensis* sp. nov. *J Clin Microbiol* **41**:5456–5465.

Gajda E, Hildebrand J, Sprong H, Buńkowska-Gawlik K, Perec-Matysiak A, Coipan EC. 2017. Spotted fever rickettsiae in wild-living rodents from south-western Poland. *Parasit Vectors* **10**:4–9.

Galay RL, Manalo AAL, Dolores SLD, Aguilar IPM, Sandalo KAC, Cruz KB, Divina BP, Andoh

M, Masatani T, Tanaka T. 2018. Molecular detection of tick-borne pathogens in canine population and *Rhipicephalus sanguineus* (sensu lato) ticks from southern Metro Manila and Laguna, Philippines. *Parasit Vectors* **11**:1–8.

Gandasegui J, Fernández-Soto P, Muro A, Barbosa CS, Melo FL, Loyo R, Gomes SEC. 2018. A field survey using LAMP assay for detection of *Schistosoma mansoni* in a low-transmission area of schistosomiasis in Umbuzeiro, Brazil: Assessment in human and snail samples. *PLoS Negl Trop Dis* **12**: e0006314.

Gilles J, Silaghi C, Just FT, Pradel I, Pfister K. 2009. Polymerase chain reaction detection of *Rickettsia felis*-like organism in *Archaeopsylla erinacei* (Siphonaptera: Pulicidae) from Bavaria, Germany. *J Med Entomol* **46**:703–707.

Gracia MJ, Marcén JM, Pinal R, Calvete C, Rodes D. 2015. Prevalence of *Rickettsia* and *Bartonella* species in Spanish cats and their fleas. *J Vector Ecol* **40**:233–239.

Graham CB, Borchert JN, Black WC, Atiku LA, Mpanga JT, Boegler KA, Moore SM, Gage KL, Eisen RJ. 2013. Blood meal identification in off-host cat fleas (*Ctenocephalides felis*) from a plague-endemic region of Uganda. *Am J Trop Med Hyg* **88**:381–389.

Haanshuus CG, Mohn SC, Mørch K, Langeland N, Blomberg B, Hanevik K. 2013. A novel, single-amplification PCR targeting mitochondrial genome highly sensitive and specific in diagnosing malaria among returned travelers in Bergen, Norway. *Malar J* **12**:1–8.

Haanshuus CG, Mørch K, Blomberg B, Strøm GEA, Langeland N, Hanevik K, Mohn SC. 2019. Assessment of malaria real-time PCR methods and application with focus on low-level parasitemia. *PLoS ONE* **14**: e0218982.

Hamzaoui B, Zurita A, Cutillas C, Parola P. 2020. Fleas and flea-borne diseases of North Africa. *Acta Trop* **211**:105627.

Han BA, Schmidt JP, Bowden SE, Drake JM. 2015. Rodent reservoirs of future zoonotic diseases. *Proc Natl Acad Sci* **112**:7039–7044.

Han ET, Watanabe R, Sattabongkot J, Khuntirat B, Sirichaisinthop J, Iriko H, Jin L, Takeo S, Tsuboi T. 2007. Detection of four *Plasmodium* species by genus- and species-specific loop-mediated isothermal amplification for clinical diagnosis. *J Clin Microbiol* **45**:2521–2528.

Hanaoka N, Matsutani M, Satoh M, Ogawa M, Shirai M, Ando S. 2017. Development of a novel loop-mediated isothermal amplification (LAMP) assay for the detection of *Rickettsia* spp. *Jpn J Infect Dis* **70**:119–123.

Hawley JR, Shaw SE, Lappin MR. 2007. Prevalence of *Rickettsia felis* DNA in the blood of

cats and their fleas in the United States. *J Feline Med Surg* **9**:258–262.

Hayashida K, Kajino K, Hachaambwa L, Namangala B, Sugimoto C. 2015. Direct blood dry LAMP: A rapid, stable, and easy diagnostic tool for human African trypanosomiasis. *PLoS Negl Trop Dis* **9**:1–14.

Hayashida K, Kajino K, Simukoko H, Simuunza M, Ndebe J, Chota A, Namangala B, Sugimoto C. 2017. Direct detection of falciparum and non-falciparum malaria DNA from a drop of blood with high sensitivity by the dried-LAMP system. *Parasit Vectors* **10**:1–9.

Hayashida K, Orba Y, Sequeira PC, Sugimoto C, Hall WW, Eshita Y, Suzuki Y, Runtuwene L, Brasil P, Calvet G, Rodrigues CDS, Santos CC, Mares-Guia MAM, Yamagishi J, Filippis AMB, Sawa H. 2019. Field diagnosis and genotyping of chikungunya virus using a dried reverse transcription loop-mediated isothermal amplification (LAMP) assay and MinION sequencing. *PLoS Negl Trop Dis* **13**:e0007480.

Hebert PDN, Cywinska A, Ball SL, DeWaard JR. 2003. Biological identifications through DNA barcodes. *Proc R Soc B Biol Sci* **270**:313–321.

Helmick CG, Bernard KW, D'Angelo LJ. 1984. Rocky Mountain spotted fever: Clinical, laboratory, and epidemiological features of 262 cases. *J Infect Dis* **150**:480–488.

Hii SF, Kopp SR, Abdad MY, Thompson MF, O'Leary CA, Rees RL, Traub RJ. 2011. Molecular evidence supports the role of dogs as potential reservoirs for *Rickettsia felis*. *Vector-Borne Zoonotic Dis* **11**:1007–1012.

Hornok S, Meli ML, Perreten A, Farkas R, Willi B, Beugnet F, Lutz H, Hofmann-Lehmann R. 2010. Molecular investigation of hard ticks (Acari: Ixodidae) and fleas (Siphonaptera: Pulicidae) as potential vectors of rickettsial and mycoplasmal agents. *Vet Microbiol* **140**:98–104.

Imwong M, Woodrow CJ, Hendriksen ICE, Veenemans J, Verhoef H, Faiz MA, Mohanty S, Mishra S, Mtove G, Gesase S, Seni A, Chhaganlal KD, Day NPJ, Dondorp AM, White NJ. 2015. Plasma concentration of parasite DNA as a measure of disease severity in falciparum malaria. *J Infect Dis* **211**:1128–1133.

Isaac R, Varghese GM, Mathai E, Manjula J, Joseph I. 2004. Scrub typhus: Prevalence and diagnostic issues in rural southern India. *Clin Infect Dis* **39**:1395–1396.

Islam AS, Chizyuka HB. 1983. Prevalence of helminth parasites of dogs in Lusaka, Zambia. *Trop Anim Hlth Prod* **15**:234–236.

Jado I, Oteo JA, Aldámiz M, Gil H, Escudero R, Ibarra V, Portu J, Portillo A, Lezaun MJ, García-Amil C, Rodríguez-Moreno I, Anda P. 2007. *Rickettsia monacensis* and human

disease, Spain. *Emerg Infect Dis* **13**:1405–1407.

Jang WJ, Choi YJ, Kim JH, Jung KD, Ryu JS, Lee SH, Yoo CK, Paik HS, Choi MS, Park KH, Kim IS. 2005. Seroepidemiology of spotted fever group and typhus group rickettsioses in humans, South Korea. *Microbiol Immunol* **49**:17–24.

Jensenius M, Fournier PE, Kelly P, Myrvang B, Raoult D. 2003. African tick-bite fever. *Lancet Infect Dis* **3**:557–564.

Jiang J, Maina AN, Knobel DL, Cleaveland S, Laudisoit A, Wamburu K, Ogola E, Parola P, Breiman RF, Njenga MK, Richards AL. 2013. Molecular detection of *Rickettsia felis and Candidatus* Rickettsia asembonensis in fleas from human habitats, Asembo, Kenya. *Vector-Borne Zoonotic Dis* **13**:550–558.

Jiang J, Sangkasuwan V, Lerdthusnee K, Sukwit S, Chuenchitra T, Rozmajzl PJ, Eamsila C, Jones JW, Richards AL. 2005. Human infection with *Rickettsia honei*, Thailand. *Emerg Infect Dis* **11**:1473–1475.

Kamau A, Mogeni P, Okiro EA, Snow RW, Bejon P. 2020. A systematic review of changing malaria disease burden in sub-Saharan Africa since 2000: Comparing model predictions and empirical observations. *BMC Med* **18**:1–11.

Kato C, Chung I, Paddock C. 2016. Estimation of *Rickettsia rickettsii* copy number in the blood of patients with Rocky Mountain spotted fever suggests cyclic diurnal trends in bacteremia. *Clin Microbiol Infect* **22**:394–396.

Kho KL, Koh FX, Singh HKL, Zan HAM, Kukreja A, Ponnampalavanar S, Tay ST. 2016. Case report: Spotted fever group rickettsioses and murine typhus in a Malaysian teaching hospital. *Am J Trop Med Hyg* **95**:765–768.

Kolo AO, Sibeko-Matjila KP, Maina AN, Richards AL, Knobel DL, Matjila PT. 2016. Molecular detection of zoonotic *Rickettsiae* and *Anaplasma* spp. in domestic dogs and their ectoparasites in Bushbuckridge, South Africa. *Vector Borne Zoonotic Dis* **16**:245–252.

Kumar S, Stecher G, Tamura K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Mol Biol Evol* **33**:1870–1874.

La Scola B, Raoult D. 1997. Laboratory diagnosis of rickettsioses: Current approaches to the diagnosis of old and new rickettsial diseases. *J Clin Microbiol* **35**:2715–2727.

Labruna MB, Whitworth T, Bouyer DH, McBride J, Camargo LM, Camargo EP, Popov V, Walker DH, Horta MC, McBride JW, Pinter A, Gennari SM. 2004. *Rickettsia bellii* and *Rickettsia amblyommii* in *Amblyomma* ticks from the State of Rondonia, Western Amazon, Brazil. *J Med Entomol* **41**:1073–1081.

Langmead B, Salzberg SL. 2012. Fast gapped-read alignment with Bowtie 2. *Nat Methods* **9**:357–359.

Lawrence AL, Brown GK, Peters B, Spielman DS, Morin-Adeline V, Šlapeta J. 2014. High phylogenetic diversity of the cat flea (*Ctenocephalides felis*) at two mitochondrial DNA markers. *Med Vet Entomol* **28**:330–336.

Lefterova MI, Budvytiene I, Sandlund J, Färnert A, Banaei N. 2015. Simple real-time PCR and amplicon sequencing method for identification of *Plasmodium* species in human whole blood. *J Clin Microbiol* **53**:2251–2257.

Liu N, Zou D, Dong D, Yang Z, Ao D, Liu W, Huang L. 2017. Development of a multiplex loop-mediated isothermal amplification method for the simultaneous detection of *Salmonella* spp. and *Vibrio parahaemolyticus*. *Sci Rep* **7**:1–7.

Loyola S, Flores-Mendoza C, Torre A, Kocher C, Melendrez M, Luce-Fedrow A, Maina AN, Richards AL, Leguia M. 2018. *Rickettsia asembonensis* characterization by multilocus sequence typing of complete genes, Peru. *Emerg Infect Dis*. **24**:931–933.

Maina AN, Fogarty C, Krueger L, Macaluso KR, Odhiambo A, Nguyen K, Farris CM, Luce-Fedrow A, Bennett S, Jiang J, Sun S, Cummings RF, Richards AL. 2016a. Rickettsial infections among *Ctenocephalides felis* and host animals during a flea-borne rickettsiosis outbreak in Orange County, California. *PLoS One* **11**:1–13.

Maina AN, Jiang J, Luce-Fedrow A, St. John HK, Farris CM, Richards AL. 2019. Worldwide presence and features of flea-borne *Rickettsia asembonensis*. *Front Vet Sci* **5**:334.

Maina AN, Knobel DL, Jiang J, Halliday J, Feikin DR, Cleaveland S, Ng'ang'a Z, Junghae M, Breiman RF, Richards AL, Njenga MK. 2012. *Rickettsia felis* infection in febrile patients, western Kenya, 2007-2010. *Emerg Infect Dis* **18**:328–331.

Maina AN, Luce-Fedrow A, Omulo S, Hang J, Chan TC, Ade F, Jima DD, Ogola E, Ge H, Breiman RF, Njenga MK, Richards AL. 2016b. Isolation and characterization of a novel *Rickettsia* species (*Rickettsia asembonensis* sp. nov.) obtained from cat fleas (*Ctenocephalides felis*). *Int J Syst Evol Microbiol* **66**:4512–4517.

Mandage R, Kaur C, Pramanik A, Kumar V, Kodan P, Singh A, Saha S, Pandey S, Wig N, Pandey RM, Soneja M, Acharya P. 2020. Association of dengue virus and *Leptospira* co-Infections with malaria severity. *Emerg Infect Dis* **26**:1645–1653.

Marcos P, Costa J. 2012. *Ctenocephalides felis felis* vs. *Ctenocephalides canis* (Siphonaptera: Pulicidae): Some issues in correctly identifying these species. *Rev Bras Parasitol Vet* **21**:3456–354.

Masaninga F, Chanda E, Chanda-Kapata P, Hamainza B, Masendu HT, Kamuliwo M, Kapelwa W, Chimumbwa J, Govere J, Otten M, Fall IS, Babaniyi O. 2013. Review of the malaria epidemiology and trends in Zambia. *Asian Pac J Trop Biomed* **3**:89–94.

Mawuntu AHP, Johar E, Anggraeni R, Feliana F, Bernadus JBB, Safari D, Yudhaputri FA, Dhenni R, Dewi YP, Kato C, Powers AM, Rosenberg R, Soebandrio A, Myint KSA. 2020. *Rickettsia felis* identified in two fatal cases of acute meningoencephalitis. *PLoS Negl Trop Dis* **14**:1–10.

McGready R, Blacksell SD, Luksameetanasan R, Wuthiekanun V, Jedsadapanpong W, Day NPJ, Nosten F. 2010. First report of an *Orientia tsutsugamushi* type TA716-related scrub typhus infection in Thailand. *Vector-Borne Zoonotic Dis* **10**:191–193.

Mediannikov O, Diatta G, Fenollar F, Sokhna C, Trape JF, Raoult D. 2010. Tick-borne rickettsioses, neglected emerging diseases in rural Senegal. *PLoS Negl Trop Dis* **4**:e821.

Mediannikov O, Fenollar F, Bassene H, Tall A, Sokhna C, Trape JF, Raoult D. 2013a. Description of “yaaf”, the vesicular fever caused by acute *Rickettsia felis* infection in Senegal. *J Infect* **66**:536–540.

Mediannikov O, Socolovschi C, Edouard S, Fenollar F, Mouffok N, Bassene H, Diatta G, Tall A, Niangaly H, Doumbo O, Lekana-Douki JB, Znazen A, Sarih M, Ratmanov P, Richet H, Ndiath MO, Sokhna C, Parola P, Raoult D. 2013b. Common epidemiology of *Rickettsia felis* infection and malaria, Africa. *Emerg Infect Dis* **19**:1775–1783.

Merhej V, Angelakis E, Socolovschi C, Raoult D. 2014. Genotyping, evolution, and epidemiological findings of *Rickettsia* species. *Infect Genet Evol* **25**:122–137.

Minichová L, Hamšíková Z, Mahríková L, Slovák M, Kocianová E, Kazimírová M, Škultéty Ľ, Štefanidesová K, Špitalská E. 2017. Molecular evidence of *Rickettsia* spp. in ixodid ticks and rodents in suburban, natural and rural habitats in Slovakia. *Parasit Vectors* **10**:158.

Monden Y, Takasaki K, Futo S, Niwa K, Kawase M, Akitake H, Tahara M. 2014. A rapid and enhanced DNA detection method for crop cultivar discrimination. *J Biotechnol* **185**:57–62.

Moonga LC, Hayashida K, Nakao R, Lisulo M, Kaneko C, Nakamura I, Eshita Y, Mweene AS, Namangala B, Sugimoto C, Yamagishi J. 2019. Molecular detection of *Rickettsia felis* in dogs, rodents and cat fleas in Zambia. *Parasit Vectors* **12**:168.

Mori Y, Kitao M, Tomita N, Notomi T. 2004. Real-time turbidimetry of LAMP reaction for quantifying template DNA. *J Biochem Biophys Methods* **59**:145–157.

Nakamura I, Hang’ombe BM, Sawa H, Kobayashi S, Orba Y, Ishii A, Thomas Y, Isozumi R,

Yoshimatsu K, Mweene AS, Takada A, Sugimoto C, Arikawa J. 2013. Cross-reactivity of secondary antibodies against African rodents and application for sero-surveillance. *J Vet Med Sci* **75**:819–825.

Nakayima J, Hayashida K, Nakao R, Ishii A, Ogawa H, Nakamura I, Moonga L, Hang'ombe BM, Mweene AS, Thomas Y, Orba Y, Sawa H, Sugimoto C. 2014. Detection and characterization of zoonotic pathogens of free-ranging non-human primates from Zambia. *Parasit Vectors* **7**:490.

Nawa M, Hangoma P, Morse AP, Michelo C. 2019. Investigating the upsurge of malaria prevalence in Zambia between 2010 and 2015: A decomposition of determinants. *Malar J* **18**:1–10.

Ng-Nguyen D, Hii SF, Hoang MT, Nguyen VT, Rees R, Stenos J, Traub RJ. 2020. Domestic dogs are mammalian reservoirs for the emerging zoonosis flea-borne spotted fever, caused by *Rickettsia felis*. *Sci Rep* **10**:1–10.

Niwa K, Oribe A, Okumura H, Shimono M, Nagai K, Hirota T, Yasue H, Kawase M. 2014. Tag/hybridization-based sensitive detection of polymerase chain reaction products. *Anal Biochem* **464**:12–16.

Noden BH, Martin J, Carrillo Y, Talley JL, Ochoa-Corona FM. 2018. Development of a loop-mediated isothermal amplification (LAMP) assay for rapid screening of ticks and fleas for spotted fever group rickettsia. *PLoS One* **13**:1–11.

Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T. Loop-mediated isothermal amplification of DNA. 2000. *Nucleic Acids Res* **28**:e63.

Nziza J, Tumushime JC, Cranfield M, Ntwari AE, Modrý D, Mudakikwa A, Gilardi K, Šlapeta J. 2019. Fleas from domestic dogs and rodents in Rwanda carry *Rickettsia asembonensis* and *Bartonella tribocorum*. *Med Vet Entomol* **33**:177–184.

Odhiambo AM, Maina AN, Taylor ML, Jiang J, Richards AL. 2014. Development and validation of a quantitative real-time polymerase chain reaction assay specific for the detection of *Rickettsia felis* and not *Rickettsia felis*-like organisms. *Vector-Borne Zoonotic Dis* **14**:476–481.

Okabayashi T, Hasebe F, Samui KL, Mweene AS, Pandey SG, Yanase T, Muramatsu Y, Ueno H, Morita C. 1999. Short report: Prevalence of antibodies against spotted fever, murine typhus, and Q fever rickettsiae in humans living in Zambia. *Am J Trop Med Hyg* **61**:70–72.

Oriero EC, Geertruyden JP, Nwakanma DC, Dalessandro U, Jacobs J. 2015. Novel

techniques and future directions in molecular diagnosis of malaria in resource-limited settings. *Expert Rev Mol Diagn* **15**:1419–1426.

Oteo JA, Portillo A, Portero F, Zavala-Castro J, Venzal JM, Labruna MB. 2014. "*Candidatus* Rickettsia asemboensis" and *Wolbachia* spp. in *Ctenocephalides felis* and *Pulex irritans* fleas removed from dogs in Ecuador. *Parasit Vectors* **7**:1–5.

Oteo JA, Portillo A, Santibáñez S, Blanco JR, Pérez-Martínez L, Ibarra V. 2006. Cluster of cases of human *Rickettsia felis* infection from southern Europe (Spain) diagnosed by PCR. *J Clin Microbiol* **44**:2669–2671.

Palacios-Salvatierra R, Cáceres-Rey O, Vásquez-Domínguez A, Mosquera-Visaloth P, Anaya-Ramírez E. 2018. Rickettsial species in human cases with non-specific acute febrile syndrome in Peru. *Rev Peru Med Exp Salud Publica* **35**:630–635.

Palomar AM, Cevidanes A, Portillo A, Kalema-Zikusoka G, Chirife AD, Romero L, Muro J, Mugisha L, Oteo JA, Millán J. 2017. High prevalence of *Rickettsia* spp. in dog fleas (Siphonaptera: Pulicidae) in rural Uganda. *J Med Entomol* **54**:1076–1079.

Pan L, Zhang L, Wang G, Liu Q. 2012. Rapid, simple, and sensitive detection of the *ompB* of spotted fever group rickettsiae by loop-mediated isothermal amplification. *BMC Infect Dis* **12**:254.

Panti-May JA, Torres-Castro M, Hernández-Betancourt S, Dzul-Rosado K, Zavala-Castro J, López-Avila K, Tello-Martín R. 2015. Detection of *Rickettsia felis* in wild mammals from three municipalities in Yucatan, Mexico. *Ecohealth* **12**:523–527.

Paris DH, Dumler JS. 2016. State of the art of diagnosis of rickettsial diseases: The use of blood specimens for diagnosis of scrub typhus, spotted fever group rickettsiosis, and murine typhus. *Curr Opin Infect Dis* **29**:433–439.

Parola P. 2011. *Rickettsia felis*: From a rare disease in the USA to a common cause of fever in sub-Saharan Africa. *Clin Microbiol Infect* **17**:996–1000.

Parola P, Sanogo OY, Lerdthusnee K, Zeaiter Z, Chauvancy G, Gonzalez JP, Miller RS, Telford SR, Wongsrichanalai C, Raoult D. 2003. Identification of *Rickettsia* spp. and *Bartonella* spp. from the Thai-Myanmar border. *Ann N Y Acad Sci* **990**:173–181.

Pérez-Osorio CE, Zavala-Velázquez JE, León JJA, Zavala-Castro JE. 2008. *Rickettsia felis* as emergent global threat for humans. *Emerg Infect Dis* **14**:1019–1023.

Perotti MA, Clarke HK, Turner BD, Braig HR. 2006. *Rickettsia* as an obligate and mycetomic bacteria. *FASEB J* **20**:2372–2374.

Petit PL, Ginneken JK. 1995. Analysis of hospital records in four African countries, 1975-1990,

with an emphasis on infectious diseases. *J Trop Med Hyg* **98**:217–227.

Philip RN, Casper EA, Anacker RL. 1983. *Rickettsia bellii* sp. nov.: A tick-borne *Rickettsia*, widely distributed in the United States, that is distinct from the spotted fever and typhus biogroups. *Int J Syst Bacteriol* **33**:94–106.

Phimda K, Hoontrakul S, Suttinont C, Chareonwat S, Losuwanaluk K, Chueasuwanchai S, Chierakul W, Suwancharoen D, Silpasakorn S, Saisongkorh W, Peacock SJ, Day NPJ, Suputtamongkol Y. 2007. Doxycycline versus azithromycin for treatment of leptospirosis and scrub typhus. *Antimicrob Agents Chemother* **51**:3259–3263.

Phoosangwalthong P, Hii SF, Kamyingkird K, Kengradomkij C, Pinyopanuwat N, Chimnoi W, Traub RJ, Inpankaew T. 2018. Cats as potential mammalian reservoirs for *Rickettsia* sp. genotype RF2125 in Bangkok, Thailand. *Vet Parasitol Reg Stud Reports* **13**:188–192.

Polley SD, Mori Y, Watson J, Perkins MD, González IJ, Notomi T, Chiodini PL, Sutherland CJ. 2010. Mitochondrial DNA targets increase the sensitivity of malaria detection using loop-mediated isothermal amplification. *J Clin Microbiol* **48**:2866–2871.

Prasad N, Murdoch DR, Reyburn H, Crump JA. 2015. Etiology of severe febrile illness in low- and middle-income countries: A systematic review. *PLoS One* **10**:1–25.

Pretorius AM, Birtles RJ. 2002. *Rickettsia aeschlimannii*: A new pathogenic spotted fever group *Rickettsia*, South Africa. *Emerg Infect Dis* **8**:874.

Punjabi NH, Taylor WRJ, Murphy GS, Purwaningsih S, Picarima H, Sisson J, Olson JG, Baso S, Wangsasaputra F, Lesmana M, Oyofo BA, Simanjuntak CH, Subekti D, Corwin AL, Richie TL. 2012. Etiology of acute, non-malaria, febrile illnesses in Jayapura, Northeastern Papua, Indonesia. *Am J Trop Med Hyg* **86**:46–51.

Rakotonanahary RJL, Harrison A, Maina AN, Jiang J, Richards AL, Rajerison M, Telfer S. 2017. Molecular and serological evidence of flea-associated typhus group and spotted fever group rickettsial infections in Madagascar. *Parasit Vectors* **10**:125.

Raoult D, Fournier PE, Fenollar F, Jensenius M, Prioe T, Pina JJ, Caruso G, Jones N, Laferl H, Rosenblatt JE, Marrie TJ. 2001a. *Rickettsia africae*, a tick-borne pathogen in travelers to sub-Saharan Africa. *N Engl J Med* **344**:1504–1510.

Raoult D, La Scola B, Enea M, Fournier PE, Roux V, Fenollar F, Galvao MAM, De Lamballerie X. 2001b. A flea-associated *Rickettsia* pathogenic for humans. *Emerg Infect Dis* **7**:73–81.

Rauch J, Sothmann P, Aldrich C, Hogan B, Owusu-Dabo E, May J, Eibach D, Tappe D. 2018. Serum cytokine responses in *Rickettsia felis* infected febrile children, Ghana. *Med Microbiol Immunol* **207**:243–248.

Reeves WK, Nelder MP, Korecki JA. 2005. *Bartonella* and *Rickettsia* in fleas and lice from mammals in South Carolina, USA. *J Vector Ecol* **30**:310–315.

Reif KE, Macaluso KR. 2009. Ecology of *Rickettsia felis*: A Review. *J Med Entomol* **46**:723–736.

Renvoisé A, Mediannikov O, Raoult D. 2009. Old and new tick-borne rickettsioses. *Int Health* **1**:17–25.

Renvoisé A, Rolain JM, Socolovschi C, Raoult D. 2012. Widespread use of real-time PCR for rickettsial diagnosis. *FEMS Immunol Med Microbiol* **64**:126–129.

Reyburn H, Mbatia R, Drakeley C, Carneiro I, Mwakasungula E, Mwerinde O, Saganda K, Shao J, Kitua A, Olomi R, Greenwood BM, Whitty CMJ. 2004. Overdiagnosis of malaria in patients with severe febrile illness in Tanzania: A prospective study. *Br Med J* **329**:1212–1215.

Richards AL, Jiang J, Omulo S, Dare R, Abdirahman K, Ali A, Sharif SK, Feikin DR, Breiman RF, Njenga MK. 2010. Human infection with *Rickettsia felis*, Kenya. *Emerg Infect Dis* **16**:1081–1086.

Robinson MT, Satjanadumrong J, Hughes T, Stenos J, Blacksell SD. 2019. Diagnosis of spotted fever group *Rickettsia* infections: the Asian perspective. *Epidemiol Infect* **147**:e286.

Rolain JM, Gouriet F, Brouqui P, Larrey D, Janbon F, Vene S, Jarnestrom V, Raoult D. 2005. Concomitant or consecutive infection with *Coxiella burnetti*, tickborne diseases. *Clin Infect Dis* **40**:82–88.

Rolain JM, Maurin M, Vestris G, Raoult D. 1998. In vitro susceptibilities of 27 rickettsiae to 13 antimicrobials. *Antimicrob Agents Chemother* **42**:1537–1541.

Rolando JC, Jue E, Barlow JT, Ismagilov RF. 2020. Real-time kinetics and high-resolution melt curves in single-molecule digital LAMP to differentiate and study specific and non-specific amplification. *Nucleic Acids Res* **48**:e42.

Roucher C, Mediannikov O, Diatta G, Trape JFF, Raoult D. 2012. A new *Rickettsia* species found in fleas collected from human dwellings and domestic cats and dogs in Senegal. *Vector-Borne Zoonotic Dis* **12**:360–365.

Rust MK. 2017. The biology and ecology of cat fleas and advancements in their pest management: A review. *Insects* **8**:118.

Rzotkiewicz S, Gutiérrez R, Krasnov BR, Morick D, Khokhlova IS, Nachum-Biala Y, Baneth G, Harrus S. 2015. Novel evidence suggests that a "*Rickettsia felis*-like" organism is an

endosymbiont of the desert flea, *Xenopsylla ramesis*. *Mol Ecol* **24**:1364–1373.

Salva I, De Sousa R, Gouveia C. 2014. Rickettsial meningitis. *BMJ Case Rep* **2014**: e2013203283.

Sansyzbayev Y, Nurmakhanov T, Berdibekov A, Vilkova A, Yeskhodzhayev O, John HK, Jiang J, Farris CM, Richards AL. 2016. Survey for Rickettsiae within fleas of great Gerbils, Almaty Oblast, Kazakhstan. *Vector-Borne Zoonotic Dis* **17**:172–178.

Santibáñez S, Portillo A, Santibáñez P, Palomar AM, Oteo JA. 2013. Usefulness of rickettsial PCR assays for the molecular diagnosis of human rickettsioses. *Enferm Infecc Microbiol Clin* **31**:283–288.

Sashika M, Abe G, Matsumoto K, Inokuma H. 2010. Molecular survey of rickettsial agents in feral raccoons (*Procyon lotor*) in Hokkaido, Japan. *Jpn J Infect Dis* **63**:353–354.

Schriefer ME, Sacci JB, Dumler JS, Bullen MG, Azad AF. 1994. Identification of a novel rickettsial infection in a patient diagnosed with murine typhus. *J Clin Microbiol* **32**:949–954.

Sekeyová Z, Danchenko M, Filipčík P, Fournier PE. 2019. Rickettsial infections of the central nervous system. *PLoS Negl Trop Dis* **13**:e0007469.

Sexton DJ, Dwyer B, Kemp R, Graves S. 1991. Spotted fever group rickettsial infections in Australia. *Rev Infect Dis* **13**:876–886.

Sfar N, M'Ghirbi Y, Letaïef A, Parola P, Bouattour A, Raoult D. 2008. First report of *Rickettsia monacensis* and *Rickettsia helvetica* from Tunisia. *Ann Trop Med Parasitol* **102**:561–564.

Shanmugakani RK, Akeda Y, Yamamoto N, Sakamoto N, Hagiya H, Yoshida H, Takeuchi D, Sugawara Y, Kodera T, Kawase M, Laolerd W, Chaihongsa N, Santanirand P, Ishii Y, Hamada S, Tomono K. 2017. PCR-dipstick chromatography for differential detection of carbapenemase genes directly in stool specimens. *Antimicrob Agents Chemother* **61**:1–4.

Silva AB, Vizzoni VF, Costa AP, Costa FB, Moraes-Filho J, Labruna MB, Gazêta GS, Nogueira RMS. 2017. First report of a *Rickettsia asembonensis* related infecting fleas in Brazil. *Acta Trop* **172**:44–49.

Singh B, Bobogare A, Cox-Singh J, Snounou G, Abdullah MS, Rahman HA. 1999. A genus- and species-specific nested polymerase chain reaction malaria detection assay for epidemiologic studies. *Am J Trop Med Hyg* **60**:687–692.

Singhsilarak T, Leowattana W, Areesuwan S, Wongchotigul V, Jiang J, Richards AL, Watt G. 2005. Short report: Detection of *Orientia tsutsugamushi* in clinical samples by quantitative

real-time polymerase chain reaction. *Am J Trop Med Hyg* **72**:640–641.

Snounou G, Viriyakosol S, Zhu XP, Jarra W, Pinheiro L, Rosario VE, Thaithong S, Brown KN. 1993. High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. *Mol Biochem Parasitol* **61**:315–320.

Socolovschi C, Mediannikov O, Sokhna C, Tall A, Diatta G, Bassene H, Trape JF, Raoult D. 2010. *Rickettsia felis*-associated uneruptive fever, Senegal. *Emerg Infect Dis* **16**:1140–1142.

Socolovschi C, Pagés F, Raoult D. 2012. *Rickettsia felis* in *Aedes albopictus* mosquitoes, Libreville, Gabon. *Emerg Infect Dis* **18**:1687–1689.

Stenos J, Graves S, Popov VL, Walker DH. 2003. *Aponomma hydrosauri*, the reptile-associated tick reservoir of *Rickettsia honei* on Flinders Island, Australia. *Am J Trop Med Hyg* **69**:314–317.

Sugita-Konishi Y, Kobayashi N, Takasaki K, Kanno T, Itoh M, Riztyan, Futo S, Asakura H, Taira K, Kawakami Y. 2019. Detection of *Sarcocystis* spp. and Shiga toxin-producing *Escherichia coli* in Japanese sika deer meat using a loop-mediated isothermal amplification-lateral flow strip. *J Vet Med Sci* **81**:586–592.

Takabatake R, Kagiya Y, Minegishi Y, Futo S, Soga K, Nakamura K, Kondo K, Mano J, Kitta K. 2018. Rapid screening detection of genetically modified crops by loop-mediated isothermal amplification with a lateral flow dipstick. *J Agric Food Chem* **66**:7839–7845.

Tay ST, Kho KL, Vythilingam I, Ooi CH, Lau YL. 2019. Investigation of possible rickettsial infection in patients with malaria. *Trop Biomed* **36**:257–262.

Tay ST, Koh FX, Kho KL, Sitam FT. 2015. Rickettsial infections in monkeys, Malaysia. *Emerg Infect Dis* **21**:545–547.

Tay ST, Mokhtar AS, Low KC, Mohd ZSN, Jeffery J, Abdul AN, Kho KL. 2014. Identification of rickettsiae from wild rats and cat fleas in Malaysia. *Med Vet Entomol* **28**:104–108.

Thepparit C, Sunyakumthorn P, Guillotte ML, Popov VL, Foil LD, Macaluso KR. 2011. Isolation of a rickettsial pathogen from a non-hematophagous arthropod. *PLoS One* **6**:e16396

Thu MJ, Qiu Y, Kataoka-Nakamura C, Sugimoto C, Katakura K, Isoda N, Nakao R. 2019. Isolation of *Rickettsia*, *Rickettsiella*, and *Spiroplasma* from questing ticks in Japan using arthropod cells. *Vector-Borne Zoonotic Dis* **19**:474–485.

Tian L, Sato T, Niwa K, Kawase M, Mayanagi G, Washio J, Takahashi N. 2016. PCR-dipstick DNA chromatography for profiling of a subgroup of caries-associated bacterial species in plaque from healthy coronal surfaces and periodontal pockets. *Biomed Res* **37**:29–36.

Trager W, Jensen JB. 1976. Human malaria parasites in continuous culture. *Science* **193**:673–675.

Tsui PY, Tsai KH, Weng MH, Hung YW, Liu YT, Hu KY, Lien JC, Lin PR, Shaio MF, Wang HC, Ji DD. 2007. Molecular detection and characterization of spotted fever group rickettsiae in Taiwan. *Am J Trop Med Hyg* **77**:883–890.

Vitale G, Mansueto S, Rolain JM, Raoult D. 2006. *Rickettsia massiliae* human isolation. *Emerg Infect Dis* **12**:174–175.

Wa G, Ohashi N, Aochi M, Ritu W, Wu D, Yoshikawa Y, Kawamori F, Honda T, Fujita H, Takada N, Oikawa Y, Kawabata H, Ando S, Kishimoto T. 2013. Rickettsiae in ticks, Japan. *Emerg Infect Dis* **19**:338–340.

Walker DH, Fishbein DB. 1991. Epidemiology of rickettsial diseases. *Eur J Epidemiol* **7**:237–245.

Wangdi K, Kasturiaratchi K, Nery SV, Lau CL, Gray DJ, Clements ACA. 2019. Diversity of infectious aetiologies of acute undifferentiated febrile illnesses in south and Southeast Asia: A systematic review. *BMC Infect Dis* **19**:1–17.

Watt G, Parola P. 2003. Scrub typhus and tropical rickettsioses. *Curr Opin Infect Dis* **16**:429–436.

Wedincamp J, Foil LD. 2002. Vertical transmission of *Rickettsia felis* in the cat flea (*Ctenocephalides felis* Bouché). *J Vector Ecol* **27**:96–101.

WHO. 2018a. The World malaria report 2018. WHO website. (Accessed from url: https://www.who.int/malaria/publications/world-malaria-report-2018/report/en/).

WHO. 2018b. Rapid diagnostic tests. WHO website. (Accessed from url: https://www.who.int/malaria/areas/diagnosis/rapid_diagnostic_tests/en/).

Williams SG, Sacci JB, Schriefer ME, Andersen EM, Fujioka KK, Sorvillo FJ, Barr AR, Azad AF. 1992. Typhus and typhus-like rickettsiae associated with opossums and their fleas in Los Angeles County, California. *J Clin Microbiol* **30**:1758–1762.

Wood H, Artsob H. 2012. Spotted fever group rickettsiae: A brief review and a Canadian perspective. *Zoonoses Public Health* **59**:65–79.

Zavala-Castro J, Zavala-Velázquez J, Walker D, Pérez-Osorio J, Peniche-Lara G. 2009. Severe human infection with *Rickettsia felis* associated with hepatitis in Yucatan, Mexico. *Int J Med Microbiol* **299**:529–533.

Zavala-Velázquez JE, Ruiz-Sosa JA, Zavala-Castro J, Jiménez-Delgadillo B, Vado-Solis IE,

Sánchez-Elias RA, Becerra-Carmona G, Moron C, Bouyer DH, Walker DH. 2000. *Rickettsia felis* rickettsiosis in Yucatan. *Lancet* **356**:1079–1080.

Zhang J, Lu G, Kelly PJ, Wang C. 2019. Seasonal and gender differences in presence of *Rickettsia felis* and blood meals provide additional evidence of a vector role for mosquitoes. *Can J Infect Dis Med Microbiol* **2019**:8543460.

Zhu Y, Zhang R, Zhang B, Zhao T, Wang P, Liang G, Cheng G. 2017. Blood meal acquisition enhances arbovirus replication in mosquitoes through activation of the GABAergic system. *Nat Commun* **8**:1–12.

Znazen A, Rolain JM, Hammami N, Hammami A, Jemaa BM , Raoult D. 2006. *Rickettsia felis* infection, Tunisia. *Emerg Infect Dis* **12**:138–140.

8. Summary in Japanese

紅斑熱群リケッチアは吸血性節足動物が保有する細菌群であり、そのうち一部の種がヒトに感染し、発熱や発疹などの症状を引き起こす。これまでにマダニによって媒介される疾病がいくつか報告されてきたが、近年ノミが保有する*Rickettsia felis*や*R. asembonensis*のヒトからの遺伝子の検出例や分離例が相次いで報告され、これらノミ媒介性紅斑熱リケッチアの新興感染症起因菌としての可能性が議論されつつある。しかし、その分布域や自然界における存続様式、ヒトへの病原性についてはほとんど明らかにされていない。特にサブサハラアフリカにおける知見は乏しく、これら地方における感染の実態調査が急務であった。そこで本研究では分子遺伝学的手法を用いて、ザンビア共和国の動物と節足動物、ヒトにおける感染率調査を行い、ノミ媒介性紅斑熱群リケッチア症の流行疫学の基礎的知見を得ることを目的とした。加えてリケッチア症はマラリアと類似した症状を示すため、マラリアと紅斑熱群リケッチアを鑑別診断可能な迅速診断法の開発を目指した。

第一章では、ザンビア共和国各地より収集したノミ・蚊・犬血液・げっ歯類臓器より抽出したDNAを試料として、紅斑熱群リケッチア特異的なPCRを行った。陽性検体について、サンガーシーケンス法、リアルタイムPCR法、および次世代シーケンサーを用いたアンプリコン解析によって種の同定を行った。その結果、犬血液150検体中7検体（4.6%）、ネコノミ48検体中７検体（14.5%）、マストミス属ネズミ77検体中12検体（15.5%）から*R. felis*の遺伝子が検出された。一方で、*R. felis*の媒介昆虫として報告のある蚊について172検体調査したが、本研究では紅斑熱群リケッチアは検出されなかった。さらに、ネコノミでは22検体（45.8%）から*R. asembonensis*の遺伝子が検出され、*R. felis*が陽性であった７検体は全て*R. asembonensis*との共感染が認められた。これらの結果から、ザンビア共和国では犬やネズミとネコノミの間で*R. felis*が、ネコノミ間で*R. asembonensis*の生活環が維持されている可能性が示唆された。これは同国において*R. felis*と*R. asembonensis*が分布していることを示した初めての報告であり、ヒトへの感染の有無についての調査が急務であると考えられた。

第二章ではノミ媒介性紅斑熱群リケッチアのヒトにおける感染実態調査を行った。調査試料として、ザンビアの首都ルサカとその近郊都市の２箇所で採取した血液検体、および地方部における健常人の血液濾紙検体の合計1,153検体の既存試料を使用した。これらからDNAを抽出した後に、紅斑熱群リケッチア特異的PCRによる検出を行い、陽性検体についてサンガーシーケンス法による遺伝子配列解析を行った。その結果、ルサカの病院で採取さ

れた２名の検体が紅斑熱群リケッチア特異的PCRで陽性を示した。これら２検体からそれぞれ４遺伝子の増幅と解析を行ったところ、いずれも*R. asembonensis*の既報配列と100%の相同性を示した。うち１遺伝子（*gltA*）は第一章でのネコノミからの配列を含む、アフリカ大陸に固有の一塩基変異を有する配列であった。２名の*R. asembonensis*陽性患者のうち1名では貧血と体重減少が認められ、もう１名の臨床症状は不明であった。貧血と*R. asembonensis*感染との相関は不明であり、*R. asembonensis*のヒトへの感染性およびその病原性について、さらなる探索が必要であると考えられた。

第三章では、紅斑熱群リケッチアとマラリア原虫を同時に検出可能な迅速診断法の開発を行った。紅斑熱群リケッチアとヒト感染性マラリア原虫を特異的に検出する２つのLAMP増幅プライマーを混合し、増幅後の反応液を核酸クロマトグラフィーに展開した。本反応系ではマラリア原虫とリケッチアのそれぞれ各100コピー相当の遺伝子を60分以内に検出し、その反応性は培養細菌・原虫を用いた試験によっても確認できた。ザンビアを含むサブサハラ地方は高度なマラリア流行地域であり、症状の類似する紅斑熱群リケッチア症が広く見過ごされている可能性がある。本法はこれら地域において使用可能な、迅速診断法としての普及が期待される。

以上のように本研究によって、ザンビア共和国にノミ媒介性の紅斑熱群リケッチアが動物と節足動物内に存在することを初めて明らかにした。そのうち*R. asembonensis*はヒトにも感染し、疾病を起こしている可能性があることを明らかにした。また、マラリアと同時検出可能な紅斑熱群リケッチアの迅速診断法を開発した。これらの研究成果は、これまで顧みられてこなかったノミ媒介性の紅斑熱群リケッチアのサブサハラにおける存続様式の解明と、その診断治療に貢献するものである。