2021-06

Molecular detection and genetic characterization of coxiella burnetii and bartonella spp infections in small mammals from Moshi, Northern Tanzania

Theonest, Ndyetabura

NM-AIST

https://doi.org/10.58694/20.500.12479/1363

MOLECULAR DETECTION AND GENETIC CHARACTERIZATION OF C*oxiella burnetii* AND *Bartonella* spp INFECTIONS IN SMALL MAMMALS FROM MOSHI, NORTHERN TANZANIA

Ndyetabura Theonest

A dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Life Sciences of the Nelson Mandela African Institution of Science and Technology

Arusha, Tanzania

June, 2021

ABSTRACT

Zoonotic pathogens, including *Coxiella burnetii* and *Bartonella* species, are known causes of febrile illness globally; however, knowledge on their animal hosts is still limited in many countries including Tanzania. This study aimed to: 1) determine the presence and prevalence of *C. burnetii* and *Bartonella spp* in small mammal; 2) identify risk factors for *C. burnetii* and *Bartonella* infection in small mammals; and 3) characterize the *Bartonella* genotypes present in small mammals and their fleas. Spleen samples were tested for the presence of *C. burnetii* (n=382) and *Bartonella spp* (n=381) DNA. Overall, 12 (3.1%) of 382 (95% CI: 1.6-5.4) spleens from small mammal tested were positive for *C. burnetii* DNA. *Coxiella burnetii* DNA was detected in five (71.4%) of seven (95% CI: 29.0-96.3) small mammal species; *Rattus rattus* (n=7), *Mus musculus* (n=1), *Acomys wilsoni* (n=2), *Paraxerus flavovottis* (n=1) and *Atelerix albiventris* (n=1). Eleven (91.7%) of twelve (95% CI: 61.5-99.8) *C. burnetii* DNA positive were trapped within Moshi Urban District. Overall, 57 (15.0%) of 381 (95% CI: 11.3-18.5) small mammal spleens tested positive for *Bartonella* DNA. *Bartonella* DNA was detected in three species (*R. rattus* n = 54, *M. natalensis* n = 2 and *P. flavovottis* n = 1) using qPCR targeting the *ssr*A gene. Analysis of *R. rattus* species only for risk of *Bartonella* infection indicated that *Bartonella* infection was more likely in reproductively mature as compared to immature small mammal (OR = 3.42, $p<0.001$). Multiple *Bartonella* genotypes closely related to known zoonotic *Bartonella* species were identified in the tested small mammals and fleas. These findings demonstrate that small mammal in Moshi, are hosts of *C. burnetii* and *Bartonella spp* and may act as a source of these pathogens to humans and animals. Further studies covering broad range of potential hosts should be considered in order to understand the distribution of these pathogens in other animals. Efforts are needed to determine the clinical impact of *C. burnetii* and *Bartonella* infection in humans. Further studies are needed to fully characterize the prevalence, genotypes and diversity of *Bartonella* spp and *C. burnetii* in different host populations and their potential impacts on human health.

DECLARATION

I, Ndyetabura Theonest do hereby declare to the Senate of Nelson Mandela African Institution of Science and Technology that this dissertation is my own original work. Some areas of diagnostic testing, methodologies and analysis were performed in close partnership with study collaborators. Where others have contributed to this work, this has been clearly acknowledged in the text or in citation.

Finally, I declare that the work presented in this thesis has not been nor being concurrently submitted for degree award at any other institution

Ndyetabura Theonest

Candidate Name	**Signature**	**Date**

The above declaration is confirmed by

Prof. Joram Buza

Supervisor 1	**Signature**	**Date**

Dr. Jo Halliday

Supervisor 2	**Signature**	**Date**

COPYRIGHT

CERTIFICATION

The undersigned certify that have read and hereby accept the dissertation titled "Molecular detection and genetic characterization of C*oxiella burnetii* and *Bartonella spp* infections in small mammals from Moshi, northern Tanzania", in fulfillment of the requirements for the Degree of Doctor of Philosophy in Life Sciences and Bioengineering (LiSBE) at the Nelson Mandela African Institution of Science and Technology (NM-AIST).

Prof. Joram Buza

Supervisor 1	**Signature**	**Date**

Dr. Jo Halliday

Supervisor 2	**Signature**	**Date**

ACKNOWLEDGEMENT

First and foremost, I am thankful to the Almighty God and to my eternal Lord, King and savior Jesus Christ for giving me the strength, courage and ability to persevere disappointments encountered during the course of this PhD study.

I would like to give my sincere thanks to my sponsors, The University of Glasgow through Leverhulme-Royal Society African Award (grant number AA130131). I also would like to extend my appreciation to Kilimanjaro Clinical Research Institute (KCRI) and Nelson Mandela African Institution of Science and Technology (NM-AIST) for providing administration support of the project funds and suitable environment for research and study. I would like to thank my employer, the Government of Tanzania through Kilimanjaro Christian Medical Centre (KCMC) for granting me study leave and permission to pursue this PhD. My sincere thanks and appreciation go to my supervisors Professor Joram Buza and Dr. Jo Halliday for their guidance, support, patience and friendship that ensured the successful implementation of the research and preparation research papers and this dissertation. Specifically, I would like to thank you for your perseverance during time when my life was going through cycles of disappointment and illness that happened to me during this PhD study. You did not give up with me, May God reward you for that.

I would like to extend my thanks to several individuals whose contribution to this PhD study is remarkably appreciated. First, special thanks go to Dr. Kathryn Allan for providing samples from her project to be used for this study, assistance in overall laboratory work, paper writing and overall encouragement. Indeed, I can’t mention each and every help that Dr. Kathryn provided. My acknowledgment goes to Mr. Ryan Carter for providing necessary guidance in several areas of molecular detection assays set up and paper writing. My thanks go to Lorna Hume for initial training on laboratory safety and handling of samples with unknown laboratory risk. My acknowledgment goes to laboratory staff at KCRI biotechnology laboratory, specifically those working in zoonotic laboratory unity. I would like to mention few, include Kate Thomas, Elizabeth Kasagama, Nelson Amani and Ephrasia Hugho. Thanks to Professor Blandina Mmbaga, the current Director of KCRI and to the former Director Professor Gibson Kibiki for providing various supports, including establishment of zoonotic research activities at KCRI. I would like to acknowledge support obtained from several people from University of Glasgow. Specifically, Professor Daniel Haydon and Professor Barbara Mable for training in statistics using R, analysis of genetic data and writing of

research papers. I would like to thank all those I have not mentioned their names but whose contributions in this PhD are highly valued. I would like to let them know that they are also in my heart and I would like to acknowledge their support.

My sincere appreciation and love to my wife Deborah Lintu and my children Neema and Joyce for their love, prayer and support that gave me strength to successfully complete this program. I also would like to thank my parents Mr. Theonest Nyabwisho and Monica Theonest and other family members for their moral support, prayer and encouragement.

DEDICATION

This work is dedicated to my wife Deborah Lintu, children (Neema and Joyce) and to my parents Mr. Theonest and Mrs. Monica.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

µl	Microlitre
AE	Qiagen® elution buffer
AIDS	Acquired Immunodeficiency Syndrome
BHQ	Black Hole Quencher
bp	Base Pair
COSTECH	Commission for Science and Technology, Tanzania
CSD	Cat Scratch Disease
Ct	Cycle Threshold
df	Degree of Freedom
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid
EIA	Enzyme –linked Immunoassay
ELISA	Enzyme –linked Immunosorbent Assay
FAM	Fluorescein Amidites
Fg	Femtogram
FI	Febrile Illness
GLMM	Generalized Linear Mixed Model
HIV	Human Immunodeficiency virus
icd	Isocitrate Dehydrogenase
IFA	Indirect Immunofluorescence assay
IS	Insertion Sequence
KCMC	Kilimanjaro Christian Medical Centre
KCRI	Kilimanjaro Clinical Research Institute
LCV	Large Cell Variant
LiSBE	Life Science and Bio-engineering
LoD	Limit of Detection
MEGA	Molecular Evolution Genetic Analysis

MRH	Mawenzi Referral Hospital
NIMR	National Institute for Medical Research
NM-AIST	Nelson Mandela African Institution of Science and Technology
NMFI	Non-Malaria Febrile Illness
NZD	Neglected Zoonotic Diseases
^{O}C	Degree Celcius
OR	Odds Ratio
PCR	Polymerases Chain Reaction
PH	Peliosis Hepatis
PhD	Doctor of Philosophy
PTC	Peltier Thermal Cycler
qPCR	Quantitative Polymerase Chain Reaction
RML	Rocky Mountain Laboratory
rRNA	ribosomal Ribonucleic Acid
RT-PCR	Real-Time Polymerize Chain Reaction
SCV	Small Cell Variant
SOP	Standard Operating Procedure
TAWIRI	Tanzania Wild Life Research Institute
UK	United Kingdom
USA	United States of America
WHO	World Health Organization

CHAPTER ONE

INTRODUCTION

1.1 Background of the problem

More than 61% of all known pathogens to humans are zoonotic and over 75% of emerging pathogens have zoonotic origin (Taylor *et al*., 2001). Zoonotic pathogens and diseases have severe impacts on the health of people and animals, and consequently on the economy especially of poor people in developing countries (Christou, 2011; Perry *et al*., 2002; Seimenis, 2012). Majority of zoonotic diseases are often neglected as public health problem by primary healthcare systems and policy makers (Ehizibolo *et al*., 2011). Several research studies have revealed that neglected zoonotic diseases (NZD) and pathogens constitute large proportion of causes of febrile illness among people seeking health care especially in developing countries (Bouyou-Akotet *et al*., 2009; Crump *et al*., 2013; Otten *et al*., 2009; Reyburn *et al*., 2004). In northern Tanzania a retrospective study was conducted among patients admitted at either one of the two referral hospitals; Kilimanjaro Christian Medical Centre (KCMC) and Mawenzi Referral Hospital (MRH) to evaluate the etiology of febrile illness, surprisingly the study found that zoonotic pathogens were the cause of large proportion of hospital admissions due to febrile illness (Crump *et al*., 2013). In that study *C. burnetii* were responsible for 20.3% (24 of 118) cases which later were identified as zoonotic bacterial infections (Crump *et al*., 2013). In that study none of zoonotic infections were included in the admission differential diagnosis for any patient, indicating lack of awareness of public health importance of zoonotic pathogens and diseases. In recent years, an increasing number of *Bartonella* species have been identified in several animal hosts and ectoparasites (Theonest *et al*., 2019). Several of these *Bartonella* species are emerging zoonotic pathogens globally and which have been detected in patients with febrile illness and other clinical presentation (Koehler *et al*., 2003; Kosoy *et al*., 2010). It is therefore likely that *Bartonella* species could be contributing to yet unknown burden of human febrile illness in this area. Furthermore, the animal hosts for *Bartonella* species have not yet been investigated in rural and urban Moshi district. Although zoonotic pathogens were responsible for large proposition of febrile illness in this area (rural and urban Moshi district), questions still remain on where and how people acquired these infections and what are the roles of different animal hosts. To answer the questions and gain understanding on animal sources for these pathogens was conceived. Therefore this PhD was conceived to explore the epidemiology of *C. burnetii* and

Bartonella spp in different animal hosts in Moshi rural and urban districts in greater detail. The aims of this PhD study was to: a) To use molecular tools for detection of *C. burnetii* and *Bartonella* species in small mammals, b) Use these tools to determine the presence and prevalence of these infection in small mammal populations and fleas collected from them, c) To genotype and characterize these pathogens to a species level and link to global biological database to assess similarities and identities and d) To assess the risk factors for infection in these small mammal populations.

Both *C. burnetii* and *Bartonella* species have been detected in small mammals especially in rodent species across the globe and are linked to increasing burden of febrile illness and other clinical presentation mostly in vulnerable populations such as people living with HIV/AIDS, homeless, alcoholic and in immunocompromised individuals (Brouqui *et al*., 1999; Foucault *et al*., 2002; Spach *et al*., 1995). However, the epidemiology of these pathogens in small mammals from Tanzania is unknown, such understanding is important to the public health and diseases control programs. For centuries small mammals especially rodents have been linked to infectious diseases, and more recently with emerging infectious diseases as twice as any other mammal (Enria & Pinheiro, 2000; Meerburg *et al*., 2009; Mills & Childs, 1998). In this study I hypothesized that small mammals are the animal host for *C. burnetii* and *Bartonella* species in rural and urban Moshi district. These pathogens are known to contribute to the burden of febrile illness in many regions of the world.

1.2 Statement of the problem

Zoonoses, including Q fever and Bartonellosis, have severe impacts on the health of people and animals, and consequently on the economy especially of poor people in developing countries. Medical conditions due to neglected zoonotic diseases are the major causes of yet unappreciated morbidity and mortality due to febrile illness in northern Tanzania and in many other developing countries especially in Sub-Sahara Africa (Crump *et al*., 2013; Halliday *et al*., 2015). Although zoonotic pathogens represent considerable proportion of number of hospital admissions, this problems remains unappreciated due to multiple reasons, such as lack of diagnostic capacity in many laboratories and lack of awareness among clinicians (Crump & Kirk, 2015). Research into the aetiology of febrile illness conducted among 870 patients admitted at either Mawenzi Referral Hospital (MRH) or Kilimanjaro Christian Medical Centre (KCMC) hospitals in Moshi, Tanzania revealed that a diverse range of zoonotic pathogens were responsible for large proportions of hospital admissions (Crump *et*

al., 2013). On admission, in that study more than 60% of patients received a clinical diagnosis of malaria, but only 2% of study participants received an aetiological diagnosis of malaria after laboratory confirmatory testing indicating potential problem of misdiagnosis/overdiagnosis and uninformed patient management. In contrast Q fever (*C. burnetii*) was detected in 20.3% (24 of 118) of identifies bacterial zoonoses (Crump *et al*., 2013). Generally large propositions of febrile illnesses were due to bacterial or viral zoonoses which were unappreciated in the initial clinical and laboratory diagnosis. This finding demonstrated the relative importance of zoonotic pathogens in this area, but also highlighted many knowledge gaps critical for detection and control of the disease. Firstly, all diagnoses of human zoonotic pathogens were made retrospectively with little opportunity to directly inform patient management. In addition, little was known about animal hosts of these zoonotic pathogens in the study catchment area, limiting the potential to design and target effective disease control interventions. Accurate diagnostic is thus critical for treatment outcome of patients, as several zoonotic diseases that cause febrile illnesses have non-specific clinical presentation, which makes it difficult for clinician to consider them as a possible cause of illness to patients. In most cases symptoms for zoonotic diseases causes of febrile illness cannot be differentiated from that of malaria and other viral infections, therefore tools and methods that can be used to accurately detect these pathogens are needed.

Molecular detection and typing methods have been widely used for identification and characterization of several pathogens including zoonotic pathogens. For *C. burnetii* and *Bartonella* species, molecular detection and typing methods are widely used due to their greater sensitivity and ease of use in comparison to culture and serology-based approaches. In addition to that, several samples obtained from animal host are to be treated with extreme care due to the potential of unknown risk they may carry. The use nucleic acid-based methods for detections and typing are often recommended as best for handling samples with unknown risk Molecular diagnostic method capable of detecting the diverse repertoire of *Bartonella* species while maintaining genus specificity has been a challenge to find. The citrate synthase gene (*gltA*) is a common genetic target for *Bartonella* detection and is considered a reliable tool for distinguishing genotypes (Norman *et al*., 1995). However, the *glt*A gene target has some limitations, sequence from this locus has homology with sequences found in some host genomes, such as mouse, rat and human, along with other human pathogens (Diaz *et al*., 2012). Therefore experts in *Bartonella* recommend targeting second additional gene which is genus specific in order to improve the strength of findings and diagnosis. An *ssr*A sequence

has been found sufficiently able to discriminate *Bartonella* species and provides phylogenetic data consistent with that of *gltA*, a commonly used gene for differentiating *Bartonella* genotypes. The *ssr*A RNA, also known as transfer-mRNA (tmRNA), is a single-copy prokaryotic-specific molecule involved in processing of incomplete peptides and resolution of stalled ribosomes during translation.

For the detection of *C. burnetii*, several PCR-based diagnostic methods have been successfully applied for the direct detection of *C. burnetii* (Klee *et al*., 2006). However the use of quantitative real-time PCR (qPCR), targeting the IS1111 insertion element which is present in multiple copies, has been reported to be highly sensitive for the detection of *C. burnetii* DNA (Bruin & Rotterdam, 2011). Q fever is known to cause significant burden of human disease, economic losses and outbreaks in many regions of the world (Van Asseldonk *et al*., 2013). *Bartonella* species are one of emerging zoonotic pathogen being detected in various region of the world in different animal hosts, their vectors and in humans. They are both zoonotic pathogens known to cause various clinical presentations in humans, including febrile illness, a leading cause of sickness among people seeking health care in hospitals in developing countries. *Coxiella burnetii* and *Bartonella* species have been detected in several genera of small mammals including using different diagnostic methods. Several literatures support the idea that small mammals especially rodents could be playing yet unknown or poorly known role in the epidemiology of these pathogens. Therefore, this study was developed based on the hypothesis that small mammals are the animal host for *C. burnetii* and *Bartonella* species, pathogens which are known to cause febrile illness and that molecular diagnostic and typing tools could be used to effectively to detect and characterize pathogens in animals hosts. Such understanding will not only be usefully in increasing awareness on patient care and management but also provide best treatment options based on empirical laboratory evidence.

1.3 Rationale of the study

Zoonotic diseases have impacts on the health of humans and livestock with severe downstream socioeconomic impacts that contribute to perpetuating cycles of poverty. Over the last few decades several new pathogens have been identified, large proportion of them are zoonotic (Taylor *et al*., 2001). Both developed and developing countries are affected by zoonotic diseases, however in developing countries the burden is high especially due to endemic neglected zoonotic disease (Halliday *et al*., 2015). Outbreaks of zoonotic diseases

have also increased with no or little epidemiological understanding of the pathogen and diseases, including lack of knowledge on the animal sources of infection.

The high prevalence of non-malaria febrile illness (NMFI) is an increasingly important public health concern in Tanzania, and research in this area exemplifies the need. In northern Tanzania there have been dramatic declines in the incidence of malaria in the past decade (Crump *et al*., 2013). These declines have exposed the previously under-recognized health burdens of NMFI. Research into the etiology of febrile illness from Moshi, Tanzania shows that just 1.6% febrile admissions were due to malaria. In contrast, a diverse range of zoonotic pathogens are responsible for large proportions of admissions (Crump *et al*., 2013). These data raise the important questions of where and how people acquire these infections and the role of different animal hosts. Both *C. burnetii* and *Bartonella* species are known to cause fever but their epidemiology in small mammal have not been studied in Northern Tanzania. Several factors hinder the diagnostic of zoonotic pathogens including lack of standard diagnostic tool. This study used molecular diagnostic and typing tools for detection of zoonotic pathogens (*C. burnetii* and *Bartonella* species) that are known causes of NMFI. These tools were used to determine the prevalence of infections in small mammals, assess risk factors for animal infection and genotype positive samples. Genotypic data from this study is important in comparison of the pathogens present in animals host and assessment of their likely transmission route by construction of phylogenetic trees inclusive of gene sequences from GeneBank.

Findings from this study will help the clinicians/medical doctors and researchers who are interested in zoonoses to increase their awareness on zoonotic causes of NMFI through use of similar or related approaches of molecular tools used in this PhD study. Several other zoonotic pathogens can be diagnosed using protocol and standard operating procedure (SOP) generated from this study (Appendix 1 and 2) following minor revisions and validation the protocols.

1.4 Objectives

1.4.1 General objective

To determine the presence and prevalence, genetic characteristics and risk factors for *C. burnetii* and *Bartonella* species in small mammals and their fleas from Moshi, Tanzania.

1.4.2 Specific objectives

(i) To determine the presence and prevalence of *C. burnetii* and *Bartonella* spp infections in small mammals from Moshi, northern Tanzania.

(ii) To determine the genetic characteristics of *Bartonella* species infections in small mammals from Moshi, northern Tanzania.

(iii) To identify risk factors for *C. burnetii* and *Bartonella* infection in small mammals from Moshi, northern Tanzania.

1.5 Research questions

(i) Are small mammals hosts of *C. burnetii* and *Bartonella* species in northern Tanzania? What is their prevalence?

(ii) What is the genetic similarity and identity between *Bartonella* species in ectoparasites and in small mammals and how are they related to sequences from GenBank databases?

(iii) What are risk factors associated with *C. burnetii* and *Bartonella* species infections in small mammal?

1.6 Significance of the study

In addition to understanding the epidemiology and genotypes of *C. burnetii* and *Bartonella* species in these small mammal populations, this study compliments with the government priority on research on zoonotic diseases. In March, 2018 the Government of United Republic of Tanzania and partners launched the One Health Coordination Desk and the National One Health Strategic Plan which is an important milestone towards addressing zoonoses as emerging health challenges. Establishment of the One Health Coordination Desk and other elements of the National One Health Platform, underscores national commitment to strengthen mechanisms for prevention, detection and response to zoonotic diseases and pathogens inclusive of *C. burnetii* and *Bartonella* species. It is therefore inevitable that the knowledge gained and findings of this study will help to increase the understanding on *C. burnetii* and *Bartonella* species, this work will also be of interest for diagnosis and detection of many other zoonotic causes of febrile illness, hence bring impact on public health policies, improve animal productivity and human health in future.

Poor laboratory capacity for detection of zoonotic causes of febrile illness is one of the major setbacks for their management and treatment. Culture isolation of *C. burnetii* is hazardous thus should only be done in level three or four laboratory infrastructure by highly trained laboratory personnel. This study provides means for use of molecular tools for detection of these pathogens; these tools are much safer, faster and reliable. On the other hand *Bartonella* species are many and takes long period of time for culture isolation, molecular tools used for their detection in this study provides means for characterization and its quick detection in resource limited settings were its hard to keep culture for such longer duration of time. Determination of the presence, prevalence and types of these infections in small mammal is important not only to generate awareness but also in the assessment of their infection risk factors, transmission routes, prevention and control.

1.7 Delineation of the study

This study focused on application of molecular detection tools for detection of several zoonotic pathogens at Kilimanjaro Clinical Research Institute (KCRI) research laboratory with initial emphases on detection of *C. burnetii* and *Bartonella* species in small mammals. Therefore the results of this study is based on molecular detection and genetic characterization of *C. burnetii* and *Bartonella* spp using molecular assays optimized and used to detection of these pathogens in spleen tissue samples and ectoparasites from small mammals species trapped in Moshi rural and urban districts, northern Tanzania. Although I was able to report the presence and prevalence of *C. burnetii* and *Bartonella* spp, the findings from this study were subjected to some limitations which are further discussed in section 4.2.3 page 60.

CHAPTER TWO

LITERATURE REVIEW

2.1 The global problem of zoonotic diseases and non-malaria febrile illnesses

The World Health Organization warned in its 2007 report that infectious diseases are emerging at a rate that has not been seen before and that large proportional of them are zoonoses (Vinet & Zhedanov, 2010). Evaluation of a list of pathogens known to cause diseases in humans revealed that over 61% of all pathogens are of zoonotic origin (Taylor *et al*., 2001) and that over 75% of all emerging pathogens are of zoonotic origin as well (Slingenbergh *et al*., 2004; Taylor *et al*., 2001). Zoonotic diseases are diseases or infections that are naturally transmitted between vertebrate animals and humans. Zoonoses have severe impacts on the health of people and animals, and consequently on the economy especially of communities and family in developing countries (Molyneux *et al*., 2011). Unfortunately impacts of zoonotic diseases are often unnoticed due several reasons such as lack of awareness, lack of sustainable surveillance systems, scarce epidemiological studies on zoonoses and lack of developed laboratory infrastructure for their diagnostic among many other factors (Halliday *et al*., 2012). Zoonotic diseases have been recognized in different parts of the world as one of the major causes of febrile illness (Crump *et al*., 2013; Hersch & Oh, 2014; Hildenwall *et al*., 2016; Prasad *et al*., 2015; Vijayachari *et al*., 2008). Febrile disease is one of the most common reasons for patients to seek health-care in Sub-Saharan Africa (Crump & Kirk, 2015; Guracha *et al*., 2011). Malaria is the major infectious cause of fever in many countries in Africa but there is growing evidence that the burden of malaria is decreasing in many parts of the continent (D'Acremont *et al*., 2010; O'Meara *et al*., 2010) . Declines in malaria incidence are particularly prominent in East Africa over the last decade where marked reductions of up to ~ 80% have been reported in Tanzania (Bhattarai *et al*., 2007; Mmbando *et al*., 2010) and Kenya (O'Meara *et al*., 2008; Okiro *et al*., 2007). However, there is no evidence that the reduction in malaria morbidity has been accompanied by an overall reduction in the prevalence of febrile illness in Sub-Saharan Africa (D'Acremont *et al*., 2010). Over diagnosis of malaria is a serious problem in parts of Sub-Saharan Africa (Amexo *et al*., 2004; D'Acremont *et al*., 2010). Malaria continues to be diagnosed clinically and treated in a high proportion of febrile patients with little recognition or treatment of alternative causes of febrile illness. For example, in one study performed in northern Tanzania, only 46% of more than 4000 patients who received a clinical diagnosis of severe

malaria were positive for Plasmodium parasitaemia on blood- smear examination (Reyburn *et al*., 2004). In this population, case fatality was significantly higher in patients with non-malaria febrile illness (NMFI) (12.1%) than those with laboratory-confirmed malaria (6.9%; $p < 0.001$). Similar scenarios have been described in other parts of the continent including in Kenya (Ye *et al*., 2009) and Sudan (A-Elgayoum *et al*., 2009). As yet, relatively little is known about other causes of fever in this part of the world.

2.1.1 Bacterial zoonoses as the major cause of NMFI in Tanzania

In response to growing awareness of the problem of NMFI in Tanzania, a cohort study to evaluate the aetiology of febrile illness was performed in two hospitals in northern Tanzania (Crump *et al*., 2013). In total 870 patients with non-specific febrile illness were enrolled between 2007 and 2008, and tested for a range of infectious causes of fever known to occur in tropical areas (Crump *et al*., 2013). On admission, more than 60% of patients received a clinical diagnosis of malaria, but only less than 2% of study participants received an aetiological diagnosis of malaria after laboratory testing. In contrast, a diverse range of zoonotic pathogens majority being bacterial zoonotic pathogens were responsible for large proportions of admissions (Crump *et al*., 2013). Unfortunately, no zoonotic infections were included in the admission differential diagnosis for any patient in that study, indicating lack of awareness and diagnostic capacity. These findings demonstrated the relative importance of bacterial zoonotic pathogens in this area, but also highlighted many knowledge gaps critical in accurate identification of cause of febrile illness and zoonoses in this area. Firstly, all diagnoses of human zoonotic pathogens were made retrospectively with little opportunity to directly inform patient management. In addition, little was known about animal hosts of these pathogens in Moshi rural and urban districts limiting the potential to design and target effective disease control interventions. This PhD was conceived to explore the epidemiology of *C. burnetii* and *Bartonella* spp from small mammals trapped in Moshi rural and urban districts in greater detail. Furthermore this PhD was a linked study designed for the purpose using molecular approach for diagnosis and typing zoonotic pathogen (initially *C. burnetii* and *Bartonella* spp), and assessment of risk factors for *C. burnetii* and *Bartonella* species infection in small mammals trapped from Rural and Urban Moshi Districts, northern Tanzania. These tools would eventually be adopted and updated accordingly to target other zoonotic pathogens in future.

2.1.2 Rodent as reservoir and host of several zoonotic pathogens

Small mammals especially rodents are the most abundant and diversified order of living mammals in the world, comprising approximately 43% of global mammalian biodiversity (Schmid *et al*., 1993). Almost 2277 species of small mammals (rodents) are known which have nearly a worldwide distribution with exception of Antarctica and some isolated islands (Huchon *et al*., 2002). For centuries rodents among the group of small mammal are well-known reservoirs and hosts for a number of infectious diseases (e.g., plague, leptospirosis, leishmaniasis, salmonellosis and viral hemorrhagic fevers) and play an important role in their transmission and spreading. However, also in modern times rodents still form a threat for public health thus they are linked to infectious diseases, and more recently revealed to be responsible for emerging infectious diseases as twice as any other mammal (Enria & Pinheiro, 2000; Meerburg *et al*., 2009; Mills & Childs, 1998). There is little information available on the prevalence of rodent-borne diseases in Tanzania. One of the striking study on rodent borne diseases was the Ratzooman project (2003-2006) which was conducted in different countries in Africa in urban/peri-urban Tanzania and in other African countries, to determine the role of rodents and shrews in the transmission of three important zoonoses, namely plague, leptospirosis and toxoplasmosis in rodent population and in human (Machang'u, 2006). For instance, plaque which has been described to occur sporadically in different parts of Tanzania was still detected at the end of that study; the overall prevalence of leptospirosis in rodents was 17% and in human was between 0.5-10 percent in different locality. I therefore consider that studies to determine zoonotic pathogens carried by rodents are of great importance to public health in Tanzania taking into consideration of current information on their role as the main cause of febrile illness.

2.1.3 The role of vectors in transmission of rodent borne zoonoses

Rodent borne diseases including Coxiellosis and Bartonellosis are known to be transmitted to humans via two different pathways in two routes; direct or indirect route (Fig. 1). Direct route happens when rodents spread pathogens to humans through their bite or consumption of food products or water contaminated with their feces, urine or other by-products from an infected rodent (Meerburg *et al*., 2009). Another route is indirect route; this happens usually through ectoparasitic blood-sucking arthropod such as fleas, ticks and mites. Indirect route can also happen when pets/livestock such as cat or dog ingest infected rodent and consequently get infected. The pet/livestock can itself get infected or act as a vehicle to transfer pathogens to

humans (Meerburg *et al*., 2009). *Coxiella burnetii* and *Bartonella* species have been identifies in rodents in many regions across the globe (Abdel-Moein & Hamza, 2018; Li *et al*., 2007; Meerburg & Reusken, 2011; Rozental *et al*., 2017). Ectoparasites have been reported to be involved in transmission of *Bartonella* species (Li *et al*., 2007; Silaghi *et al*., 2016) and *C. burnetii* (Abbassy *et al*., 2006; Mediannikov *et al*., 2010) to animals and humans. For instance the review of rodent borne diseases in middle east (Iran) showed that 11 of 34 identified rodent borne diseases were transmitted by vectors, these diseases includes plague, tularemia, tick-borne relapsing fever, babesiosis, Lyme disease, Q fever, Crimean Congo hemorrhagic fever, leishmaniasis, babesiosis, schistosomiasis and fasciolosis (Rabiee *et al*., 2018). Almost one-third of the reported rodent-borne diseases in Iran in that study were transmitted by vectors. Based on these evidence it is therefore important to consider rodents and their ectoparasites in the epidemiology of *C. burnetii* and *Bartonella* species in this region. Assessment of risk factors for their infection would also create understanding and awareness in there epidemiology and consequently will help in informed control measures.

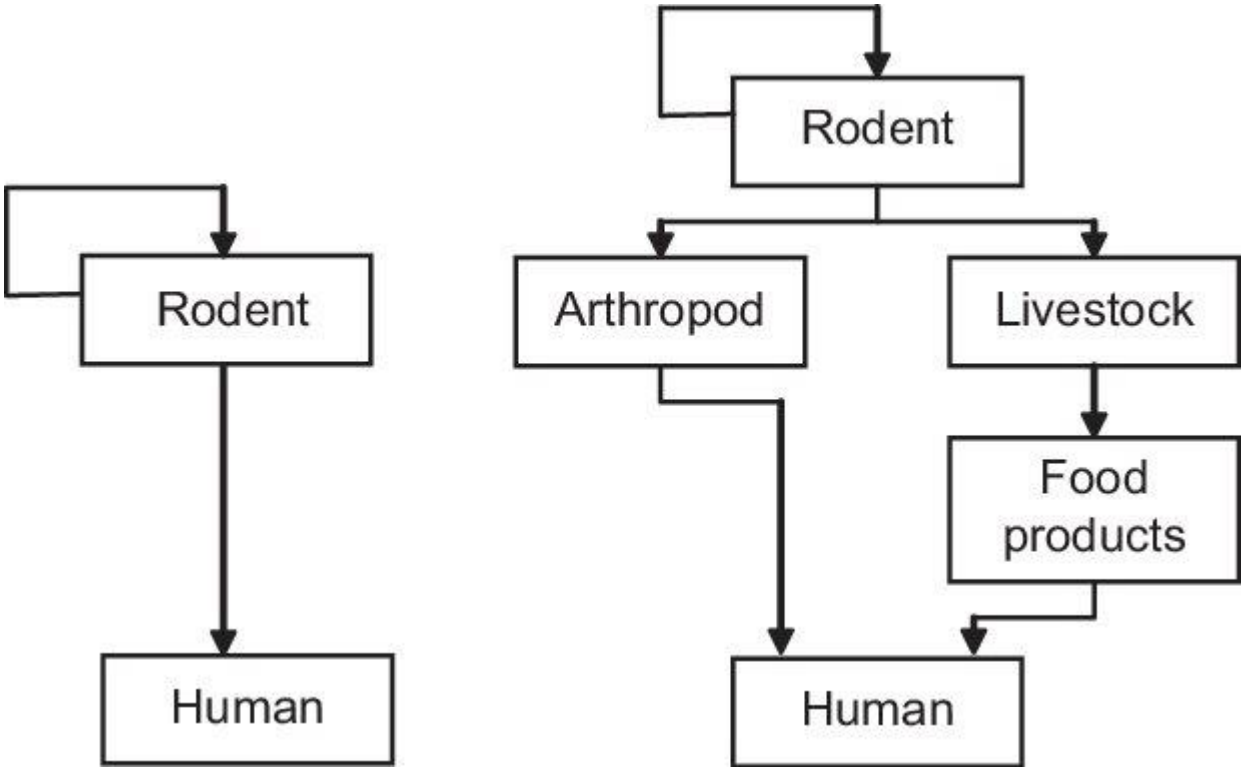


Figure 1: Two different pathogen transmission pathways for rodent borne diseases: On the left the direct route, on the right the indirect route. The pathogen is the arrow in the flowchart (Meerburg *et al*., 2009)

2.1.4 Factors leading to increases of emerging and re-emerging of rodent borne zoonoses

There are many factors which have been identified to be linked to increase in emergence or re-emergence of infectious diseases (Daszak *et al*., 2001; Jones *et al*., 2008; Morse, 1995; Taylor *et al*., 2001). Studies to evaluate factors for emergence and resurgence of infectious diseases have shown that anthropogenic factors including change of land use (e.g. deforestation, mining, oil extraction, etc.), food production changes, global trade and travel are among the leading causes of disease emergence and spreading (Daszak *et al*., 2001; Harrus & Baneth, 2005). Moreover, global climate change and changing human settlement patterns (especially in developing countries) have been found to be highly associated with increasing problems of several diseases emergence or re-emergence including rodent-borne diseases (Gubler *et al*., 2001) . Climate change is responsible for creating conditions suitable for outbreaks of diseases such as Lyme disease, Rocky Mountain spotted fever, malaria, dengue fever, and viral encephalitis by providing favorable conditions for reproduction of rodents and their associated vectors. For instance outbreaks of Hantavirus in the united the state (USA) in 1993 was preceded by a dramatic increase in rainfall (Climatic change) in 1992–1993. This led to increased rodent food resources and increase in the rodent population, followed by invasion of buildings by rodents, and an increased risk of human disease (Hjelle & Glass, 2000). In Northern Tanzania like in many other regions in developing countries increase in anthropogenic activities and climatic change is evident and consequently leading to the disturbance of biodiversity. Therefore it is imperative to speculate that rodent habitat and their ectoparasitic vectors has been affected and therefore their role in the epidemiology of diverse zoonotic pathogens needs to be understood.

Understanding of the epidemiology and factors leading to the emergence or re-emergency of rodents borne diseases is complex which requires consideration of many factors. To date there are few epidemiological studies on rodent borne diseases and pathogens from Africa including Tanzania. Research information about rodent borne diseases are often times from studies from other regions for instance a study done in Iran to determine rodent borne diseases and their public health found that some specific rodent species including *M. musculus*, *R. rattus* and *R. norvegicus* had high rank of rodent-borne diseases in comparison to other species included in that study (Rabiee *et al*., 2018). More than half of the reported diseases in that study were strongly connected to commensal rodent species , however lack

of sufficient surveys among non-commensal rodent species could have affected the findings (Khaghani, 2007). Therefore studies to determine the prevalence and assessing risk factors for rodent infection in an area that have been found to have high burden of zoonotic infections among patients seeking healthcare with febrile illness (Crump *et al*., 2013) are needed.

2.2 *Coxiella burnetii*

2.2.1 Brief overview and history of *Coxiella*

Coxiella burnetii is an obligate intracellular gram negative bacterium measuring (0.2 to 0.4µm wide and 0.4 to 1µm long), the causative agent of a zoonotic diseases Q fever in humans and Coxiellosis in animals. The bacterium has a worldwide distribution with the exception of New Zealand and Antarctica (Raoult *et al*., 2005; Voth & Heinzen, 2007). The agent of Q fever was first recognized in 1935 by Edward Holbrook Derrick in febrile livestock handlers in Australia (Derrick, 1983). The agent of Q fever was independently isolated in 1938 by Dr. Herald and colleague from *Dermacentor andersoni* ticks near Nine Mile Creek in the United States at the Rocky Mountain Laboratory (RML) in Montana and subsequently named it *Rickettsia diaporica* (Davis *et al*., 1938). The Australian and *D. andersoni* isolates were compared for virulence and infectivity and determined to be the same species. Cox re-named the pathogen *Rickettsia burnetii* after discoveries made by Dr. Burnet. Dr. Cornelius Philip solicited that the pathogen be placed in a new genus, *Coxiella*, to honor Dr. Cox's discovery (Philip, 1948). For decades only *Coxiella burnetii* was the only species in the genus *Coxiella* however recently other candidate species have now been recognized in crayfish and reptile (*C. cheraxi*) (Tan & Owens, 2000), birds (*C. avium*) (Shivaprasad *et al*., 2008) and in humans (*C. massiliensis*) (Angelakis *et al*., 2016). Additionally *Coxiella*-like bacteria are also common in ticks (Almeida *et al*., 2012), and one of these organisms was recently found in horses (Seo *et al*., 2016). Members of the genus *Coxiella* are now classified in the family Coxiellaceae, order Legionellales and gamma subdivision of the phylum Proteobacteria.

2.2.2 Structure of *C. burnetii*

Coxiella has a unique intracellular life with two distinct morphological forms, the large cell variant (LCV) and the small cell variant (SCV) (McCaul & Williams, 1981). The two distinct forms can be differentiated by size, morphology, peptidoglycan content and resistance to

physical disruption. Small cell variants are the environment extracellular form of the organism. They are 0.2 to 0.5 μm long, compact and typically rod-shaped with an electron-dense core bounded by cytoplasmic and outer membranes (McCaul & Williams, 1981). Small Cell Variant is metabolically inactive, and show a high degree of resistance to chemical agents and physical conditions, such as osmotic pressure and sonic disruption (McCaul & Williams, 1981). This confers the ability for the organism to survive for prolonged period of time in the environment. In contrast, LCVs resemble Gram-negative bacteria; they can exceed 1μm in length, but are more pleomorphic than the SCVs. They possess a thinner cell wall, with a more dispersed filamentous nucleoid region (Roest *et al.,* 2013). They are metabolically active, and are the intracellular form of the organism.

2.2.3 Epidemiology of *C. burnetii* and Q fever outbreaks

Coxiella burnetii, a zoonotic strictly intracellular bacterial pathogen has been found in all regions of the world (Klaasen *et al*., 2014; Rolain *et al*., 2005; Schimmer *et al*., 2014) except in the New Zealand and some isolated Islands (Vanderburg *et al*., 2014). *Coxiella burnetii* is the causative agent of Q fever, human being are infected through animal faeces, urine ,milk, birth products, inhalation of contaminated aerosols originating from contaminated materials from infected animals (Honarmand, 2012; Klaasen *et al*., 2014; Noden *et al*., 2014; Schimmer *et al*., 2014; Vanderburg *et al*., 2014). The bacteria have broad ranges of host including sheep, goat, cattle, birds, dogs, cats, rodents and ectoparasites. People at high risk are farmers, laboratory workers, dairy workers and Veterinarians (Honarmand, 2012; Noden *et al*., 2014). Infection by *C. burnetii* in human can be asymptomatic or symptomatic (Vanderburg *et al*., 2014), and can present as acute Q fever that have nonspecific symptoms such as chest pain with breathing, cough, fever, headache, jaundice, muscle pain and shortness of breath or as chronic Q fever that may present with chills, fatigue, night sweats, prolonged fever and shortness of breath (Bechah *et al*., 2014). Complications if not diagnosed and treated includes cirrhosis, hepatitis, encephalitis, endocarditis, myocarditis, interstitial pulmonary fibrosis, meningitis and pneumonia (Honarmand, 2012; Vanderburg *et al*., 2014). *Coxiella burnetii* infection in livestock have been associated with decreased livestock productivity which can have socioeconomic impact to livestock keeping population particularly in resource limited settings (Vanderburg *et al*., 2014). Since its discovery *C. burnetii* was not given priority until several recent outbreaks in Europe and Middle East. Q fever outbreaks have been reported in the Netherlands (Roest *et al*., 2011), Slovenia (Grilc *et*

al., 2007), the United Kingdom (Van Woerden *et al*., 2004; Wallensten *et al*., 2010), Israel (Amitai *et al*., 2010), Iraq (Faix *et al*., 2008), the United States (Bamberg *et al*., 2007), Italy (Manfredi, 1996) and France (Tissot-Dupont *et al*., 2007). Probably one of striking Q fever outbreak episode in the history of Q fever was the one that happened in The Netherlands between 2007-2010 where more than 4000 human Q fever cases was reported (De Rooij *et al*., 2016). Analysis of the Netherlands Q fever outbreak revealed that outbreak was associated with small ruminants rather than cattle (Klaasen *et al*., 2014; Schimmer *et al*., 2014). Follow up studies and laboratory experimental studies revealed that *C. burnetii* can persist in humans and rodents adipose tissues (Bechah *et al*., 2014; Comer *et al*., 2001). It has been postulated that the transmission cycle of *C. burnetii* is associated with sylvatic or domestic transmission cycles, with rodents being suspected to link the two cycles (Meerburg & Reusken, 2011).

2.2.4 Detection and prevalence of *C. burnetii* in small mammals

To date the epidemiology of *C. burnetii* in animal host or reservoir is not fully understood, however *C. burnetii* have been detected throughout the world with exception of New Zealand and Antarctica (Fournier *et al*., 1998). For the past few decades interest to find new and possible reservoir and hosts for *Coxiella* species have gained much attention especially after several recent Q fever outbreaks (Abdel-Moein & Hamza, 2018; Meerburg & Reusken, 2011).

In the United Kingdom (UK), antibodies to *C. burnetii* have been detected in wild brown rats on farms (Webster *et al*., 1995). Recent studies in the Netherlands have also indicated that wild brown rats, especially those near farms, may act as true reservoirs for *C. burnetii* (Reusken *et al*., 2011) and may be implicated in the Q fever outbreaks in livestock and humans in the Netherlands in 2007-2010. *Coxiella burnetii* have been found in rodents trapped in pristine environments that are infrequently visited by humans or livestock indicating that rodents may be a natural reservoir of *Coxiella*. In Canada *C. burnetii* was detected in rodent species trapped in forest and pristine environments, where human activities such as livestock keeping do not occur, suggesting that rodents in these livestock free areas could be acting as a host or reservoir of *C. burnetii* (Burgdorfer *et al*., 1963; Thompson *et al*., 2012). Similar findings was reported in USA where *C. burnetii* was isolated from wild rodents in western Montana (Lackman, 1963) an area that was inaccessible to livestock and

infrequently visited by humans, indicating that rodents could be possible host or reservoir of *C. burnetii* in that area.

In Africa there is paucity of studies and information on detection and prevalence of *Coxiella* in small mammals including rodents. The few studies done in Africa have reported considerable variation in prevalence of *C. burnetii* in rodent population. A study done in Zambia , a country bordering Tanzania to the Southwest; *C. burnetii* DNA was detected in 9 out of 20 (45%) rodents trapped in urban and peri-urban areas using PCR assay (Chitanga *et al*., 2018). There was evidence of variation of prevalence within individual rodent species as follows: *Saccostomus campestris* (2/2), *Gerbillinae* sp. (4/7) and *Mastomys natalensis* (3/11). Another study done in Africa was performed in Nigeria, *C. burnetii* DNA was detected by PCR assay in 4 out of 194 (2.1%) in rodents trapped in peri-urban areas (Kamani *et al*., 2018). In the Nigerian study, the sampled rodents comprised 3 out of 121 (2.5%) *Rattus norvegicus* and 1 out of 48 (2.1%) *Rattus rattus* (Kamani *et al*., 2018). In Egypt *C. burnetii* DNA was found in 2 of 55 (3.6%) *R. norvegicus* and 3 of 20 (15%) *R. rattus*, with overall prevalence of 6.7% (Abdel-Moein & Hamza, 2018) using molecular methods. In Cape Verde and Canary Islands *C. burnetii* antibodies was detected in 12.4%; 21.1% for Cape Verde and 10.2% for the Canary Islands by enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescence assay (IFA) (Foronda *et al*., 2015).

Table 1: **Review of *C. burnetii* detection in small mammals**

Author	Detection method	Rodent species	N, n (%)	Country
Lackman (1937)		Woodrat (*N. cinerea cinerea*)	20, 1 (5)	
		Golden-mantled ground squirrel (*L. escarum*)	111, 2 (1.8)	USA
	Culture	Chipmunk (*E. amoenus*)	46, 1 (2.2)	
Webster *et al.* (1995)	ELISA	Wild brown rats	127, 44 (34)	UK
Abdel-Moein & Hamza (2018)	Nested PCR	*R. norvegicus*	55, 2 (3.6)	Egypt
		R. rattus	20, 3 (15)	
Pluta *et al*. (2010)	Nested PCR	Not indicated	119, 0 (0)	Germany
Bolaños-Rivero *et al*. (2017)	PCR- IS1111	*R. norvegicus*	100, 8 (8)	Spain
Thompson *et al*. (2012)	Real-Time PCR IS1111	Woodland jumping mice, deer mice	30 (83.3), 71 (76.1)	Canada
Izquierdo-Rodríguez *et al*. (2019)		*R. rattus*	103, 9 (8.7)	France
	ELISA	*Mus musculus domesticus*	15, 2 (13.3)	
Foronda *et al*. (2015)	ELISA & IFA	Black rats (*R. rattus*)	108,12 (11.1)	Canary Islands and Cape Verde
		Mice (*M. musculus*)	77,11 (14.3)	
Kamani *et al*. (2018)	PCR,IS1111	*R. norvegicus*, *R. rattus*	194, 4 (2.1)	Nigeria
Chitanga *et al*. (2018)	PCR, icd	Gerbillinae sp, *Saccosto- mus campestris*, *Mastomys natalensis*	20, 9 (45)	Zambia
Rozental *et al*. (2017)	Conventional PCR-IS1111	Akodon cursor, *Oxymcterus dasytrichus*, Oligoryzomys	131, 6 (4.6)	Brazil

N=Total number of rodent per specie tested, n= number of *C. burnetii* positive rodents found, % = percentage of positive rodents

2.2.5 Routes of transmission of *C. burnetii*

(i) Inhalation

This is the most common route of infection in both animals and human (Russell-Lodrigue *et al*., 2006; Welsh *et al*., 1958). Under experimental conditions, inhalation of a single organism can produce infection and clinical disease in humans (Tigertt *et al*., 1961). The infectious dose for human airborne exposure to *C. burnetii* required for fifty per cent of the exposed population to become infected (infd50) is estimated at 1.5 bacteria (0.75–38.7, 95% credible interval (CI) (Brooke *et al*., 2013, 2015). Contaminated dusts and wind have been found to cause spread of *C. burnetii* at far distance from the point source (Nusinovici *et al*., 2017; Tissot-Dupont *et al*., 2004).

(ii) Other routes for *C. burnetii* transmission in humans

The means by which humans can acquire the *C. burnetii* is broad, studies have demonstrated infection through oral route mainly consumption of raw milk (Rodolakis, 2006), Human to human transmission (Watanabe & Takahashi, 2008), sexual transmission (Miceli *et al*., 2010). Transmission of *C. burnetii* has also been reported to occur through blood transfusion (Delsing & Kullberg, 2008), transplacental transmission, intradermal inoculation, and postmortem examinations have been associated with sporadic cases of Q fever (Harman, 1949).

2.2.6 Diagnosis of *C. burnetii* (Q fever)

Diagnosis of *C. burnetii* is generally difficult and poses potential risk to laboratory personnel especially in areas where laboratory infrastructures are inadequately developed. In most instances the diagnosis of Q fever in humans relies on serology (Fournier *et al*., 1998). Various other methods are also being employed, these includes: a) Microagglutination (Crăcea, 1987), b) Complement fixation (Horigan *et al*., 2011), c) Radioimmunoassay (Döller *et al*., 1984) and d) Indirect immunofluorescence antibody tests (Bouvery *et al*., 2003).

Each of these methods have some limitation, for instance the major limitations of serological assay includes the issue of cross reaction with other microorganism such as *Legionella* species (Musso *et al*., 1997), dependent on rise of antibody titre, which takes about two weeks and use of blood or blood derivative samples only (Dupont *et al*., 1994). On the other hand culture of *Coxiella* from blood or tissue is highly specific for the diagnosis of Q fever,

but is insensitive and poses a substantial infection risk to laboratory personnel without proper biosecurity measures (Omsland *et al*., 2013; Roest *et al*., 2013). *Coxiella burnetii* culture requires specialized cell or yolk sac media which are often not readily available nor commonly used in routine clinical microbiological diagnostic (Musso & Raoult, 1995). Consequently, the use of culture for the diagnosis of Q fever is rarely done outside specialized laboratories, which there are few in developing countries. The use of real-time polymerase chain reaction (qPCR) assays for detection of *C. burnetii* have been used variably successfully to detect *C. burnetii* from various sample types such as blood, milk, ticks and tissues (Piñero *et al*., 2014). Polymerase chain reaction assay targeting isocitrate dehydrogenase (*icd*), *com1* and the insertion sequence IS1111 and other genes for *C. burnetii* has been previously described (Duron, 2015; Klee *et al*., 2006). The use qPCR for detection of *C. burnetii* based on IS1111 gene target has been demonstrated as more sensitive compared to detection of other genes (Vaidya *et al*., 2008).

2.3 *Bartonella* species

2.3.1 Brief overview and history of *Bartonella* species

The bacterium was first described in Peru in 1909 by Alberto Barton of which the bacteria is named after him in recognition of his work (Maurin *et al*., 1997). Prior to 1993, the genus *Bartonella* was composed of a single species, *B. bacilliformis*, the causative agent of Oroya fever, or Carrion's disease, first described in South America in the late 1800's (Minnick *et al*., 2014). *Bartonella bacilliformis* remained the sole member of the genus *Bartonella* for nearly a century (Brenner *et al*., 1993). As a consequence of various factors, highlighted by improved detection and diagnostic capabilities, as well as reclassification of several bacterial genera on the basis of phylogenetic relatedness using 16S rRNA gene sequences, the genus has undergone a substantial expansion. In 1993, the genus was initially broadened through unification with the genus *Rochalimaea* (Brenner *et al*., 1993), which was comprised of the species *R. quintana* (formerly *Rickettsia quintana*), *R. henselae, R. Vinsonii* and *R. elizabethae*, and again in 1995 through a subsequent unification with the genus *Grahamella* (Birtles *et al*., 1995), which contained the species *G. grahamii, G. taylorii, G. talpae* and *G. doshiae*. Following these amalgamations, all of the aforementioned species were reclassified as *Bartonella* and the genus was removed from the order *Rickettsiales* (Birtles *et al*., 1995). The genus *Bartonella* has continued to grow in recent years with the isolation and characterization of numerous additional species, including multiple strains recovered from a

broad range of rodents and other wild and domestic mammals in various regions of the world (Anderson & Neuman, 1997; Breitschwerdt, 2017; Breitschwerdt & Kordick, 2000; Chomel & Kasten, 2010; Holmberg *et al*., 2003; Molin *et al*., 2011; Pérez *et al*., 2011; Rolain & Raoult, 2006). Currently, the genus has been broadened to include over 45 named species or subspecies (Table 2) (Okaro *et al*., 2017). More than half of recognized *Bartonella* species are zoonotic with varying clinical presentation (Kaewmongkol *et al*., 2011). Zoonotic *Bartonella* species are included among the list of emerging and re-emerging human pathogen (Boulouis *et al*., 2005).

Table 2: **Currently designated *Bartonella* species, their hosts and associated human disease**

Species	Host (s)	Human diseases associated	Reference
B. acomydis	Golden spiny mouse (*Acomys russatus*)	Unknown	Sato *et al*. (2013)
B. alsatica	Rabbits	Endocarditis	Heller *et al*. (1999) and Raoult *et al*. (2006)
B. ancashensis	Human patient	Verruga peruana	Mullins *et al*. (2013, 2015)
B. apis	Honeybee symbiont	Unknown	Kešnerová *et al*. (2016)
B. australis	Kangaroos	Unknown	Saisongkorh *et al*. (2009)
B. bacilliformis	Human	Oroya fever, verruga peruana	Noguchi and Battistin (1926) and Thornton *et al*. (1990)
B. birtlesii	Mice	Carrion's disease	Bermond *et al*. (2000) and Noguchi and Battistin (1926)
B. bovis	Dairy cattle	Endocarditis in cattle	Rudoler *et al*. (2014)
B. callosciuri	Plantain squirrel	Unknown	Sato *et al*. (2013)
B. capreoli	Deer	Unknown	Bermond *et al*. (2002)
B. chomelii	French cattle	Unknown	Maillard *et al*. (2004)
B. clarridgeiae	Cat	Lymphadenopathy, fever, papule, CSD	Kordick *et al*. (1997)
B. coopersplainsensis	Rat	Unknown	Kordick *et al*. (1997) and Saisongkorh *et al*. (2009)
B. doshiae	Voles	Unknown	Birtles *et al*. (1995)
B. dromedarii	Camels	Unknown	Rasis *et al*. (2014)

Species	Host (s)	Human diseases associated	Reference
B. elizabethae	Rats	Endocarditis, neuroretinitis	Brenner *et al*. (1993)
B. florenciae	Shrew, mouse	Unknown	Li *et al*. (2015)
B. fuyuanensis	Field mouse	Unknown	Li *et al*. (2015)
B. grahamii	Rodents, voles	Neuroretinitis, CSD	Inoue *et al*. (2010)
B. heixiaziensis	Vole	Unknown	Li *et al*. (2015)
B. henselae	Cat	CSD, endocarditis, bacillary	Anderson *et al*. (1992)
B. jaculi	Greater Egyptian jerboa	angiomatosis, bacteremia	Koehler *et al*. (1994) and Sato *et al*. (2013)
B. japonica	Mice	Unknown	Inoue *et al*. (2010)
B. koehlerae	Cat	Endocarditis	Avidor *et al*. (2004)
B. koehlerae subsp. bothieri	Bobcat	Unknown	Chomel *et al*. (2016)
B. koehlerae subsp. boulouisii	Mountain lion	Unknown	Chomel *et al*. (2016)
B. mayotimonensis	Bats	Endocarditis	Breitschwerdt *et al*. (2011)
B. melophagi	Sheep	Unknown	Kosoy *et al*. (2016)
B. naantaliensis	Bats	Unknown	Kosoy *et al*. (2010)
B. peromysci	Mouse	Unknown	Birtles *et al*. (1995)
B. pachyuromydis	Fat-tail gerbil	Unknown	Lydy *et al*. (2008)
B. phoceensis	Rat	Unknown	Gundi *et al*. (2004)
B. queenslandensis	Rats	Unknown	Saisongkorh *et al*. (2009)
B. quintana	Human	Trench fever, endocarditis, bacteremia,	Foucault *et al*. (2002)

Species	Host (s)	Human diseases associated	Reference
B. rattaustraliani	Rats	bacillary angiomatosis	Gundi *et al*. (2009)
B. rattimassiliensis	Rats	Unknown	Gundi *et al*. (2004)
B. rochalimae	Foxes, raccoons, coyotes	Bacteremia, splenomegaly	Eremeeva *et al*. (2007) and Henn *et al*. (2009)
B. silvatica	Mice	Unknown	Inoue *et al*. (2010)
B. schoenbuchensis	Deer	Unknown	Dehio *et al*. (2001)
B. senegalensis	Tick	Unknown	Mishra *et al*. (2012)
B. talpae	Moles	Unknown	Birtles *et al*. (1995)
B. tamiae	Rodents, humans	Fever	Kosoy *et al*. (2008) and Saisongkorh *et al*. (2009)
B. taylorii	Rats	Unknown	Birtles *et al*. (1995)
B. tribocorum	Rats	Unknown	Heller *et al*. (1998)
B. vinsonii subsp. arupensis	Mice	Endocarditis	Fenollar *et al*. (2005) and Welch *et al*. (1999)
B. vinsonii subsp. berkhoffii	Dog, coyotes	Endocarditis	Breitschwerdt and Kordick (2000) and Roux *et al*. (2000)
B. vinsonii subsp. vinsonii	Voles	Unknown	Brenner *et al*. (1993)
B. vinsonii subsp. yucatanensis	Rodents	Unknown	Fischedick *et al*. (2016)
B. weissii	Cat	Unknown	Breitschwerdt and Kordick (2000)
B. washoensis	Dog	Unknown	Chomel *et al*. (2003)

2.3.2 *Bartonella* as the cause of febrile illness and other clinical presentation in humans

Several different *Bartonella* species have been reported to cause diseases in human and have been included in the list of emerging and re-emerging pathogens. The list of potential zoonotic *Bartonella* species continues to grow (Okaro *et al*., 2017). However, the vast majority of infections in humans are attributed to *B. bacilliformis*, *B. henselae*, or *B. quintana*. The most vicious species of the *Bartonella* species historically is *B. bacilliformis*, which causes Carrion's disease with a mortality rate of up to 80% if untreated (Bass *et al*., 1997). *Bartonella quintana* the agent of trench fever, a disease that affected millions of soldiers during World War I (Bass *et al*., 1997), recently re-emerged among homeless people in Europe and in the USA (Brouqui *et al*., 1999; Foucault *et al*., 2002). *Bartonella quintana* is an opportunistic pathogen, which means that it causes much more severe symptoms in immunocompromised individuals, such as HIV infected patients (Koehler *et al*., 2003). It is likely that *Bartonella* is prevalent in Sub-Sahara African countries due to high prevalence of HIV and Tuberculosis. In the USA majority of *Bartonella* infections in humans are caused by *B. henselae* which is transmitted by a cat bite or scratch or by the cat flea, and causes cat-scratch disease (CSD). *Bartonella henselae* causes symptoms similar to *B. quintana* (trench fever) in immunocompromised individuals. Detection of pathogenic *Bartonella* species in febrile illness patients have been reported in several other countries including Thailand (Bhengsri *et al*., 2010). Several other *Bartonella* species have occasionally been associated with human disease, including *B. elizabethae* (endocarditis, neuroretinitis), *B. koehlerae* (endocarditis), *B. clarridgeae* (cat-scratch disease), *B. vinsonii arupensis* (fever, neurological symptoms) and *B. vinsonii berkhoffi* (endocarditis) (Bruno *et al*., 2006). *Bartonella rochalimae*, have been report to cause symptoms similar to *B. bacilliformis* (Eremeeva *et al*., 2007), *B. tamiae* was isolated in patient with fever and anemia in Thailand (Kosoy *et al*., 2008, 2010). It has been proposed that any *Bartonella* species found in animals may be capable of infecting humans and cause diseases as opportunistic pathogens (Lin *et al*., 2010).

Table 3: ***Bartonella*** **species known and reported to cause diseases in humans**

Species	Diseases	Brief description	Reference
B. quintana	Bacteremia, bacillary angiomatosis, chronic lymphadenopathy, Trench fever and endocarditis	Accounts for about three-fourths of *Bartonella* endocarditis cases	Huarcaya *et al*. (2006)
B. henselae	Cat Scratch Diseases	Accounts for about one-fourth of *Bartonella* endocarditis cases, has worldwide distribution	La Scola and Raoult (1999)
B. alsatica	Endocarditis	Was isolated from a patient diagnosed with BCNE who also had a preexisting valve lesion	Raoult *et al*. (2006)
B. ancashensis	Verruga peruana	Was isolated from two patients with verruga peruana in the Ancash region of Peru	Huarcaya *et al*. (2006)
B. bacilliformis	Bacteremia, bacillary angiomatosis, chronic lymphadenopathy, Trench fever and endocarditis	May result in death for more than 80% of infected patients in the absence of antibiotic treatment, and is increasing at an alarming rate in the pediatric population	Ihler (1996)
B. clarridgeiae	Severe headache, fever,lymphadenopathy,chills, sweating and malaise	Has been isolated from immunocompromised patients reported to have CSD	Kordick *et al*. (1997)
B. elizabethae	Endocarditis	Have been found in a patient with endocarditis.	Parra *et al*. (2017)
B. grahami	Neuroretinitis, fever	Has been reported as being a causative agent of CSD-like illness in an immunocompromised patient	Oksi *et al*. (2013)
B. koehlerae	Depression and anxiety, headaches, joint stiffness and hallucinations	Cat-Associated Agent of Culture-Negative Human Endocarditis	Avidor *et al*. (2004)
B. mayotimonensis	Infective endocarditis	Infective endocarditis,	Lin *et al*. (2010)
B. rochalimae	Bacteremia, fever and splenomegaly	Has genetic resemblance to *B. bacilliformis*, the causative agent of Oroya fever	Eremeeva *et al*. (2007)
B. *tamiae*	Fever, mild anemia, headache, myalgia, liver function abnormalities	Closely related to *Bartonella* species previously identifies in rats in Thailand	Kosoy *et al*. (2008)
B. vinsoni	Endocarditis, arthritis, neurological disease and vasoproliferative neoplasia	Have been isolated in cattle rancher in USA	Fenollar *et al*. (2005) and Welch *et al*. (1999)

2.3.3 *Bartonella* species in rodents and small mammal populations

Several *Bartonella* species which are found in small mammals, such as wild mice, squirrels and rats have been linked to human disease. For instance *B. grahamii* with ocular syndromes, *B. elizabethae* with endocarditis, *B. vinsonii* subsp. *arupensis* with fever and bacteremia, and *B. washoensis* with fever and myocarditis (Gil *et al*., 2010). *Bartonella* species that are able to infect rodents and small mammals include *B. elizabethae* (Ying *et al*., 2002), *B. grahamii* (Ellis *et al*., 1999), *B. vinsonii subsp. arupensis* (Welch *et al*., 1999), *B. doshiae* (Birtles *et al*., 1995), *B. taylorii* (Richard, 1995), *B. vinsonii subsp. vinsonii* (Ellis *et al*., 1999), *B. tribocorum* (Heller *et al*., 1998), *B. washoensis* (Kosoy *et al*., 2003), *B. rattimassiliensis* (Gundi *et al*., 2004), *B. phoceensis* (Gundi *et al*., 2004), *B. birtlesii* (Bermond *et al*., 2000), *B. rochalimae* (Lin *et al*., 2008), *B. japonica* (Inoue *et al*., 2010) and *B. silvatica* (Inoue *et al*., 2010).

Recent evidence from several studies on detection of *Bartonella* species in small mammals suggests that rodent-borne *Bartonella* species may play an important and previously unrecognized role as a source of infection in other animals such as stray dogs and febrile human patients (Bai *et al*., 2008; Kosoy *et al*., 2003; 2010). Rodents and several other small mammals are responsible for maintaining the highest number of *Bartonella* species and subspecies, including several Candidatus (Gundi *et al*., 2009; Billeter *et al*., 2012; Inoue *et al*., 2010). *Bartonella* species are continuing to be discovered in novel potential reservoir species of small mammal and rodent populations around the world, including Europe, Africa, Asia, North and South America (Bai *et al*., 2008; 2009; Castle *et al*., 2004; Gundi *et al*., 2012; Jardine *et al*., 2005; Kosoy *et al*., 2003; Kosoy *et al*., 1998; Pretorius *et al*., 2004; Theonest *et al*., 2019; Winoto *et al*., 2005).

Several studies support the hypothesis that *Bartonellae* have very effectively co-evolved with rodent species as reservoir hosts (Buffet *et al*., 2013; Buffet, Pisanu *et al*., 2013; Hayman *et al*., 2013; Lei & Olival, 2014; Paziewska *et al*., 2011; Telfer *et al*., 2005). It has also been described that closely related phylogenetic groups of *Bartonella* are present in populations of related species of rodents separated by considerable geographical distances and land barriers (Bai *et al*., 2009; Castle *et al*., 2004; Kosoy *et al*., 1998; Pretorius *et al*., 2004). In an experimental infection of cotton rats (*Sigmodon hispidus*) and white-footed mice (*Peromyscus leucopus*) with *Bartonella*, resulted in bacteremia only when variants were originally isolated from the same species or a close taxonomic relative (Kosoy *et al*., 2000).

However, the diversity of *Bartonella* species that can exist within a single rodent species or individual is highly variable. Multiple distinct *Bartonella* species have been documented in a single rodent species (Birtles *et al*., 2001) or within a rodent population within a single site (Kosoy *et al*., 1997).

It has been reported that the relative abundance of each *Bartonella* species within these rodent populations is thought to be dependent on host density of the rodent species, density of flea species and spill-over infection between each species of rodent (Telfer *et al*., 2007). Different means of transmissions among rodents have been reported including, transplacental transmission for some rodent species (Kosoy *et al*., 1998). Studies on detection of *Bartonella* species are expanding and occurs in different animal hosts, ectoparasites and majority of these are found in small mammals especially rodents species.

2.3.4 Transmission of *Bartonella* to humans

Species within the *Bartonella* genus are transmitted mainly by arthropod vectors, such as fleas, sand flies, lice, biting flies, potentially ticks and other vectors (Billeter *et al*., 2008). Transmission from an animal reservoir to human may also occur directly by way of skin trauma (i.e. scratch or bite), epitomized by the transmission of *B. henselae* by a cat scratch, resulting in cat scratch disease (CSD) (Zhang *et al*., 2015). Specificity in ectoparasites transmission of *Bartonella* to humans are known; the rodent flea for *B. grahamii* and *B. taylorii*, the cat flea *Ctenocephalides felis* for *B. henselae*, the human body louse *Pediculus humanus corporis* for *B. quintana* and the sandfly *Lutzomyia verrucarum* for *B. bacilliformis* (Billeter *et al*., 2008). There are however many more potential vectors in which *Bartonella* has been detected, including ticks, keds, mites and various kinds of fleas and flies (Billeter *et al*., 2008). Since some of these can feed on many different mammals, the vectors may play an important role for cross-transmission and host shifts.

2.3.5 Host diversity and specificity for *Bartonella* infection

Studies have indicated that some *Bartonella* species have preference for one or a group of mammalian hosts (reservoir host) in which they infect and multiply within erythrocytes. Occasionally, they infect another host (incidental host), in which they are not capable to infect the erythrocytes, but only vascular endothelial cells (Kosoy *et al*., 1997; Vayssier-Taussat *et al*., 2009; 2010; Withenshaw *et al*., 2016). Some *Bartonella* species infect only a single host, research and experiment studies have observed that closely related *Bartonella*

species also infect closely related hosts (Withenshaw *et al*., 2016). For instance to date, humans are the only known reservoir hosts for both *B. bacilliformis* and *B. quintana* (Drancourt *et al*., 2005; Lydy *et al*., 2008); likewise, cats have been shown to serve as the only known mammalian reservoirs for *B. henselae* and *B. koehlerae* (Chomel *et al*., 2006). Several *Bartonella* species have been associated with rodents or their ectoparasite. The underlying factors contributing to the predilection for a given species of *Bartonella* to exhibit preferential infectivity in specific host species are not fully understood.

2.3.6 Diagnoses of *Bartonella* species

Bartonella species are pathogens of emerging and re-emerging significance, causing a wide array of clinical syndromes including febrile illness. Some of the known sequelae for *Bartonella* infection include endocarditis (mostly negative on culture), neuroretinitis, chronic lymphadenopathy, peliosis hepatis (PH), fever, chronic bacteremia, bacillary angiomatosis (BA). Proper diagnostic is critical for optimal case management and treatment options of any infections including *Bartonella*. Several methods have been applied in the diagnoses of *Bartonella* species, these include:

(i) Isolation and culture methods

Bartonellae are fastidious, pleiomorphic, gram-negative bacilli that are oxidase- and catalase-negative and require a CO2-rich environment, enriched media, and prolonged periods for growth (Brenner *et al*., 1997). On blood agar, primary isolates of *Bartonella* species are obtained after 12 to 14 days, although prolonged incubation periods of up to 45 days are sometimes necessary (Maurin *et al*., 1994). On subculture of the primary isolate is also often difficult, with colony formation again taking 10 to 15 days (La Scola & Raoult, 1999). However subsequent subcultures grow more rapidly (3 to 5 days), but with varying colony morphology (Koehler *et al*., 1997). It is often difficult to evaluate the sensitivity of cultures due to the difficulty of cultivating this organism. Several studies have reported blood culture negative of samples taken from patients with endocarditis due to *Bartonella* (Lamas, 2003; Mainardi *et al*., 2010; Okaro *et al*., 2017; Roux *et al*., 2000).

(ii) Serology methods

For several decades the diagnostic of *Bartonella* infection relied on serological assays. Serologic test for diagnostic of *Bartonella* species was first developed at the Centers for

Disease Control (CDC) by Regnery (English *et al*., 1988; Regnery *et al*., 1992) for diagnostic of cat-scratch diseases (CSD) caused by *B. henselae*. This indirect fluorescent antibody (IFA) test was subsequently validated clinically in several trials (Demers *et al*., 1995; Karem *et al*., 2000). Several other IFA and enzyme-linked immunoassay (EIA) tests to diagnose *Bartonella* species especially *B. henselae* infection have been developed and several have become commercially available (Patnaik & Peter, 1995; Rolain *et al*., 2001; Zbinden *et al*., 1995). The major limitation for serological methods is that the assay is unable to differentiate different species of *Bartonella* with antigenic similarity such as *B. henselae* and *B. quintana* due to cross reactivity in IFA testing for these organisms (McGill *et al*., 1998; Sander *et al*., 2001). Serological assay for diagnostic of *Bartonella* have also shown cross-reactivity with other intracellular bacteria such as *Coxiella burnetii*, *Toxoplasma gondii*, *Francisella tularensis*, *Chlamydia*, Epstein-Bar virus, have been shown to result in false-positive test (La Scola & Raoult, 1996; Nadal & Zbinden, 1995; Sander *et al*., 1998) .

(iii) Molecular methods

The development of molecular identification and typing methods has markedly advanced the diagnosis and epidemiologic investigation of several diseases and causative pathogens including *Bartonella* species. In the study of Bartonellae, the use of molecular techniques has provided evidence of the bacterium from several sample sources and has aided in the demonstration of the diversity of human disease caused by these organisms, has allowed epidemiologic investigation both in individual cases and in populations, and has provided important phylogenetic information regarding to reclassification of *Bartonella* species (Goral *et al*., 1994; Mouritsen *et al*., 1997). Several different molecular methods have been used for the identification and differentiation of *Bartonella* species including southern blot, gel and capillary electrophoresis, polymerase chain reaction (PCR), DNA-hybridization, restriction fragment length polymorphism (RFLP), real-time PCR (RT-PCR) and genetic sequence analysis (Bereswill *et al*., 1999; Diaz *et al*., 2012; Handley & Regnery, 2000; Matar *et al*., 1999; Renesto *et al*., 2001; Tay *et al*., 2014). Polymerase chain react isn't just one of the mainstays for diagnosing *Bartonella* infections, but it's played a critical role in fulfilling molecular Koch's postulates to associate *Bartonella* with new disease syndromes. The detection of *Bartonella* 16S rRNA gene sequences within the lesions of patients with bacillary angiomatosis provided the primary link of *Bartonella* with this condition (Relman *et al*., 1990). Similarly, the detection of *B. henselae* 16S rRNA gene sequences in skin test

antigens used to diagnose CSD helped resolve a longstanding mystery about the etiology of CSD (Regnery *et al*., 1992). Multiple PCR or coupled with restriction fragment digestion to detect polymorphisms, enrichment broth culture, or DNA sequencing has all been used to identify *Bartonella* isolates (Cook, 2006). Many different specific primer pairs and techniques have been described to detect *Bartonella* DNA in clinical specimens by PCR (When specifically applied to *Bartonella* endocarditis, amplification of *Bartonella* DNA from valvular tissue by PCR has been shown in multiple case series to have higher sensitivity and specificity, ranging from 72 to 98% (Bosshard *et al*., 2003; Fournier *et al*., 2001). The use of PCR assay for detection of *Bartonella* species can be performed on range of sample sources including whole blood, plasma, or serum samples, with studies reporting sensitivity of 58% and specificity of 100% (Sanogo *et al*., 2003).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Brief description of study site

The samples for this study were collected from small mammals trapped from two districts in Kilimanjaro Region of northern Tanzania. Trapping of the small mammals was conducted in two of seven districts of Kilimanjaro Region in a previous study (Allan *et al*., 2018). The two districts, Moshi Municipal (Urban) and Moshi Rural (Fig. 2), were chosen as the site of the study due to the high reported incidence of febrile illness due to zoonotic pathogens (Crump *et al*., 2013). The climate of the two the district for this area is tropical, with two rain seasons in the year. Subsistence farming is common; agriculture which is mainly mixed cropping and livestock farming is the main activity in the study area.

3.2 Sampling and sample size determination

Spleen samples from difference small mammal species and ectoparasites were collected during field work from a collaborative cross-sectional study conducted to explore the role of small mammals in the epidemiology of leptospirosis and other zoonoses in the Kilimanjaro region of northern Tanzania (Allan *et al*., 2018), for which I was part of the field staff (Figs. 3 and 4). Small mammals were trapped in five villages within Moshi Urban District and seven villages within Moshi Rural District, as previously described (Allan *et al*., 2018) and shown in Fig. 2. The target sample size for detection of *C. burnetii* and *Bartonella* spp for this study were 50 small mammals per village to give sufficient power ($\alpha = 0.95$, $\beta = 0.8$) to detect a minimum infection prevalence of 10% (Allan *et al*., 2018). Villages for sampling were randomly selected from a list, home to people that had sought care, and had been enrolled in previous febrile illness surveillance studies at local hospitals (Crump *et al*., 2013). Small mammal trapping was performed in three sessions: a) May-June 2013 (wet season); b) May-June 2014 (wet season); and c) August-September 2014 (dry season). Small mammal were trapped in households in a total of 12 villages through cross-sectional visits, with one additional round of repeat sampling conducted in one village (based on high trap success in the initial visit) (Allan *et al*., 2018). Trapped small mammals were euthanized by terminal halothane anaesthesia and cervical dislocation. Data gathered for every trapped small mammal included: species (determined by observation of phenotypic characteristics and measurement of morphological features), season (Wet or dry), location of sampling (Urban vs

Rural District), sex and reproductive maturity status (mature or immature determined based on external sexual characteristics (Cunningham & Moors, 1993). A full necropsy and tissue sampling were performed for each small mammal sampled (Figs. 3 and 4). A fresh sterile scalpel blade was used for each small mammal and all other necropsy equipment was washed using 5% Virkon and dried between usages to avoid cross-contamination. Spleen tissue samples were collected into sterile Eppendorf tubes and stored at -80°C prior to DNA extraction. Ectoparasites observed on trapped small mammal were collected and stored in 70-96% ethanol; all ectoparasites from the same small mammal were stored together (Allan *et al*., 2018). Collected fleas were identified to species level using a dissecting microscope and a pictorial flea identification guide (Mathison & Pritt, 2014). Selection of fleas and their testing for the presence of *Bartonella* was performed in collaboration with colleague in UK, sequence editing, analysis and phylogenetic construction. The work has been included in a co-authored published paper (Theonest *et al*., 2019). *Xenopsylla cheopis* fleas were selected for DNA extraction and *Bartonella* testing based on their known contribution to *Bartonella* transmission (Billeter *et al*., 2011). For each small mammal with at least one *X. cheopis* collected, DNA was extracted from one (if only one *X. cheopis* present on that host) or two (if more than one *X. cheopis* present on that host). Where multiple *X. cheopis* were collected from the same small mammal, selection of individual fleas for DNA extraction was opportunistic.

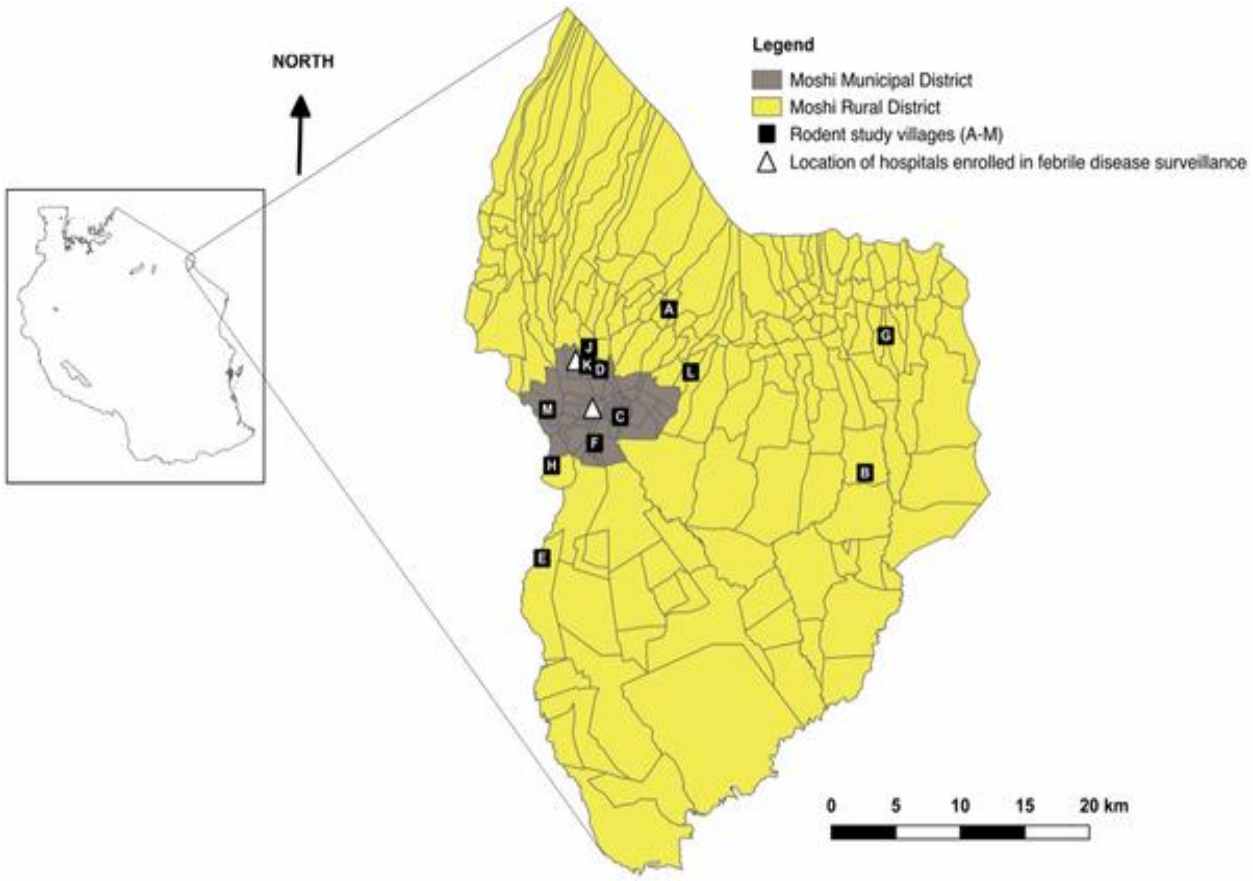


Figure 2: Map indicating Moshi Urban and Moshi Rural Districts, showing locations of rodent study villages in relation to the two hospitals (Kilimanjaro Christian Medical Centre and Mawenzi Regional Referral Hospital) at which febrile illness surveillance has been conducted in previous studies (Credit to Kathryn Allan in designing and editing the Map) (Allan *et al*., 2018)

Figure 3: Necropsy and tissue sampling for each sampled rodent (Credit to Rigobert Tarimo)

Figure 4: Determination of weight and other demographic characteristic for each trapped rodent (Credit to Rigobert Tarimo)

3.3 Laboratory analysis

3.3.1 Deoxyribonucleic acid extraction from spleens of small mammals

Deoxyribonucleic acid was extracted from approximately 10 milligrams (mg) of spleen tissue (after initial processing (Appendix 1: SOP 1) using the DNeasy Blood and Tissue Kit spin-column protocol for DNA purification from tissues (Qiagen, Hilden, Germany). Deoxyribonucleic acid from spleen tissues was eluted in 100µl of AE buffer and quantified using a Nano-Drop spectrophotometer (Thermo Scientific, Waltham, MA, USA). DNA from individual whole fleas were also extracted using the DNeasy Blood and Tissue Kit, following the protocol for purification of total DNA from ticks and eluted in a final volume of 65 µl AE buffer. For all extractions, a no-template extraction control (PCR-grade water) was included for every 20 samples. Deoxyribonucleic acid extracts were stored at -20 ^{o}C prior to testing. Prior to screening all DNA extracts were diluted in 20 µl of AE buffer to a standard DNA concentration of 10-50 ng/µl for PCR testing, to minimize the potential for PCR inhibition due to the high concentration of host DNA in the small mammal spleen extracts.

3.4 Laboratory analysis for detection of *Bartonella* species

3.4.1 Quantitative PCR for detection of *Bartonella* species from spleens of small mammal and fleas samples targeting *ssr*A gene

The work for this section was conducted in the UK with exception of extraction of DNA from spleens of small mammal. Deoxyribonucleic acid extraction was conducted at Kilimanjaro Clinical Research Institute biotechnology laboratory (KCRI BL), the data generated was included as part of co-authored research paper (Theonest *et al*., 2019). Deoxyribonucleic acid extracts from spleens of small mammals and fleas were screened for the presence of *Bartonella* species using a *Bartonella* genus-specific real-time PCR assay (qPCR) targeting the transfer-mRNA *ssr*A gene, using a previously published protocol (Diaz *et al*., 2012). Lower limit of detection of < 5 fg of *Bartonella* DNA, equivalent to < 3 genomic copies per reaction when tested against four *Bartonella* species (*B. quintana*, *B. henselae*, *B. bovis*, and *B. elizabethae*) was adopted for this work from paper describing this assay by (Diaz *et al*., 2012). The primers *ssr*A-F(5'-GCTATGGTAATAAATGGACAATGAAATAA-3'), *ssr*A-R(5'GCTTCTGTTGCCAGGTG-3') and 6-carboxyfluorescein FAM-labelled probe (5'-ACCCCGCTTAAACCTGCGACG-3'-BHQ1) were used to amplify an *ssr*A gene fragment of approximately 300bp. qPCR reactions were carried out in total of 20 µl volumes comprised

of 10 µl QuantiNova Probe PCR mix (Qiagen), 0.8 µl of each primer (10 µM) and probe (5 µM), 2.6 µl nuclease-free water and 5 µl DNA template. Positive control (rodent tissue DNA extract obtained from a previous study (Laudisoit *et al*., 2014) positive for *Bartonella* with closest similarity to *B. tribocorum*), extraction controls and no-template controls were included in each qPCR run. Assays were performed on a Rotor-Gene Q/6000 (Qiagen) with optimized thermocycling conditions as follows: 95 °C for 2 minutes, followed by 45 cycles of 95 °C for 5 seconds and 60 °C for 5 seconds. A qPCR run was considered valid when all negative controls showed no evidence of amplification and the positive controls amplified with a Ct value of < 40. Extracts were tested in duplicate and considered positive when amplification was recorded in one or more test wells with a Ct value ≤40.

3.4.2 Conventional PCR for detection of *Bartonella* species from rodent spleen samples

Conventional PCR amplification of the *glt*A gene was performed on all rodent spleen DNA extracts, based on a previously published protocol (Norman *et al*., 1995). Each PCR reaction (25 µl) comprised 12.5 µl of PCR 2X master mix (Promega, Madison, WI, USA), 1.25 µl of 5% dimethyl sulphoxide (DMSO) (Sigma-Aldrich, St. Louis, USA), 1.25 µl molecular grade water (Qiagen), 2.5 µl of each oligonucleotide primer (10 µM), BhCS781.p(5'-GGGGACCAGCTCATGGTGG3') and BhCS1137.n(5'AATGCAAAAAGAACAGTAAAC A-3') (Norman *et al*., 1995) and 5 µl DNA template. Amplifications were performed on a PTC-240 DNA-Engine (MJ Research/BioRad Technologies, USA) with the following thermocycling conditions: 94 °C for 2 minutes, followed by 40 cycles of 94 °C for 30 seconds, 54.3 °C for 30 seconds, and 72 °C for 2 minutes, then a final step of 72 °C for 7 minutes. Positive and negative controls were included in each PCR run. PCR products were visualized by electrophoresis in a 1.5% agarose gel stained with ethidium bromide (Invitrogen, USA). A sample was considered positive if a clearly defined DNA band of approximately 379 bp for *Bartonella glt*A was visible in the gel and confirmed as *Bartonella* by sequencing of the product. Purification, sequencing and BLAST analyses were conducted as for the *ssr*A gene.

3.4.3 Sequencing and phylogenetic analysis of *ssr*A and *glt*A PCR products

Sequencing work was performed in the UK. In addition to the sequences of *glt*A and *ssr*A products obtained from spleens of small mammals as described above, sequences of the *ssr*A target obtained from fleas collected from the same rodents were also available for analysis.

Phylogenetic analyses were performed as part of this thesis work. Generated sequences were shared, after receiving raw sequences, all sequences were initially edited using BioEdit sequence editor software (Hall, 1999). Incomplete and poor quality sequences (e.g. sequences with ambiguous peaks and short length) were excluded from phylogenetic analysis for both *ssr*A and *glt*A gene fragments. For each gene, sequences were aligned using the ClustalW algorithm, implemented in MEGA 7.0 (Kumar *et al*., 2016). The model test function in MEGA 7.0 was used to select the best-fitting nucleotide substitution models, which were then incorporated into a phylogenetic analysis based on a maximum likelihood optimality criterion for tree reconstruction, with 1000 bootstrap pseudoreplicates. For the *ssr*A sequence analysis, rodent spleen and flea sequences from this study were aligned with reference *ssr*A sequences from cultured *Bartonella* species downloaded from GenBank (see GenBank accession numbers in Fig. 5). A *Brucella melitensis* sequence was used as the outgroup (Frank *et al*., 2018). For analysis of the *glt*A sequence data, sequences from study rodent spleens were aligned with those from *Bartonella* reference strains obtained from GenBank and also with representative sequences from previous studies conducted in East Africa (see GenBank accession numbers in Fig. 6). Reference sequences in the alignment included *glt*A sequences from previous studies of *Bartonella* in rodents from Tanzania (Gundi *et al*., 2012)), Kenya (Halliday *et al*., 2015), the Democratic Republic of Congo (Gundi *et al*., 2012; Laudisoit *et al*., 2014) and Uganda (Billeter *et al*., 2014) that included either similar rodent species or *Bartonella glt*A sequences similar to those obtained in this study. A *B. tamiae glt*A sequence obtained from an African bat was used as the outgroup (Ying *et al*., 2015).

3.5 Laboratory analysis for detection of *C. burnetii*

3.5.1 Determination of sensitivity and limit of detection (LoD) for IS1111 qPCR assay

Initial set-up and verification of the assay was performed on the Rotor-Gene Q/6000 System (Qiagen). The approximate sensitivity and limit of detection (LoD) of the IS1111 qPCR assay for this study was determined using a 10-fold dilution series of DNA from *C. burnetii* Nine Mile RSA493 reference strain. The primer and probe for the IS1111 detection for reference strain and samples was as follows:-Forward primer (5'-CATCACATTGCCGCGTTTAC-3'), Reverse primer (5'-GGTTGGTCCCTCGACAACAT-3'), and 6-carboxyfluorescein FAM-labelled probe (5'-AATCCCCAACAACACCTCCTTATTCCCAC-BHQ1-3') as described in previous study (Klee *et al*., 2006).

3.5.2 IS1111 qPCR for detection of *C. burnetii* DNA from rodent spleens

DNA extracts from rodent spleens were also screened for the presence of *C. burnetii* by qPCR using the same primers, probe and target (IS1111) used for the determination sensitivity and LoD of the assay as described above (Appendix 2, SOP 2). The qPCR reactions were carried out in a total volume of 20 µl comprising of 10 µl QuantiNova qPCR mix (Qiagen), 0.8 µl of each primer (10 µM) and probe (5 µM), 2.6 µl nuclease-free water and 5 µl DNA template. Positive controls, extractions controls and no template controls were included in each qPCR run. Assays were performed on a Rotor-Gene Q/6000 with thermocycling conditions as follows: 1 cycle of 95 °C for 2 minutes followed by 45 cycles of 95 °C for 5 seconds then 60 °C for 5 seconds. Fluorescence readings were acquired via the green (510 nm) detection channel at the end of each annealing/extension phase. A qPCR run was considered valid when the negative controls (PCR-grade water) showed no amplification and the positive controls (Nine Mile RSA493 strain) amplified with Ct value < 40. Samples were tested in duplicate initially and then in an additional three wells if amplification (Ct < 40) was seen in just one of two initial duplicate wells. A sample was considered positive for *C. burnetii* if at least two test wells, out of the maximum five, produced amplification with Ct <40 and all other assay conditions above were fulfilled.

3.6 Data analyses

3.6.1 Data analysis for detection of *Bartonella* species

Statistical analyses were performed in R (R Development Core Team, 2018). Exact binomial proportions and confidence intervals for prevalence estimates were calculated using the package 'binom' (Dorai-Raj, 2014). Generalized linear mixed models (GLMM), with binomial family and logit link function, were used to examine variables associated with small mammal and flea *ssr*A qPCR test status (qPCR positive vs negative) and implemented using the package 'lme4' (Bates *et al.*, 2015). For small mammal, the dataset for these analyses was limited to *R. rattus* only, given the dominance of this species. Explanatory variables considered in the GLMM for small mammal *ssr*A qPCR status included host sex and reproductive maturity (mature or immature), which were determined based on external sexual characteristics (Cunningham & Moors, 1993). Trapping season (wet or dry), trapping district (Moshi Urban or Moshi Rural) and small mammal abundance were also included as explanatory variables for analysis. Adjusted trap success data were calculated by dividing the

total number (n) of small mammal caught per village by the corrected number of trap nights, which is calculated as: total number of trap nights (number of traps x number of nights) minus lost trap nights (sum of number of closed, damaged or lost traps / 2) and expressed as a percentage (Allan *et al*., 2018). The village identification variable was included as a random effect to account for the clustered sampling strategy. For fleas, small mamma *ssr*A qPCR test status was the only explanatory variable evaluated in the GLMM and the village identification variable was included as a random effect. Initial maximal multivariable models were created including all candidate explanatory variables and likelihood ratio tests were used to compare candidate models and guide model selection.

3.6.2 Data analysis for detection of *Coxiella burnetii*

Due to small number (12/382) of positive samples obtained with Ct values greater than 33, statistical analysis and genotyping were difficult to perform. Where appropriate for some selected variables statistical analyses were performed in R (R Development Core Team, 2018), binomial proportions and confidence intervals (95%) for prevalence estimates were calculated using the package 'binom' version 1.0-5 (Dorai-Raj, 2014).

3.7 Ethical consideration

Ethical approval for the study was granted by the Tanzania Commission for Science and Technology (COSTECH 2012-471-ER-2005-141 and 2015-71-NA-2011-199); Kilimanjaro Christian Medical Centre (KCMC) Ethics Committee (535 & 537); National Institute of Medical Research ethics certificate (NIMR), Tanzania (NIMR/HQ/R.8a/Vol.IX/1499 and NIMR/HQ/R.8a/Vol.IX/1522); Tanzania Wildlife Research Institute (TAWIRI) (Certificate number could not be retried); University of Glasgow College of Medical, Veterinary and Life Sciences Ethics Committee (200120020), and University of Glasgow Faculty of Veterinary Medicine Ethics and Welfare Committee (01a/13 & 02a/13). Written consent for study participation was obtained for each participating household, using forms translated into Swahili. Small mammal sampling was performed in accordance with the UK Guidance on the Operation of the Animals (Scientific Procedures) Act 1986 and American Veterinary Medical Association Guidelines for the Euthanasia of Animals (Home Office, 2014; Leary *et al*., 2013).

CHAPTER FOUR

RESULTS AND DISCUSSIONS

4.1 Results

Sections and contents of results mirrors work which has been presented in papers published in peer review journals. The first paper (attached as research output) is about molecular detection and genetic characterizations of *Bartonella* species in small mammals from Moshi, northern Tanzania. In this paper I was able to present the detection of *Bartonella* species in small mammal using both conventional and real time PCR. I was also able to present *Bartonella* sequences (deposited in Genbank). Comparison of *Bartonella ssr*A and *glt*A sequences similarities and identities from this work to *Bartonella ssr*A and *glt*A reference sequences found in Genbank were performed. In the second paper (attached as research output) titled molecular detection of *C. burnetii* in small mammals in Moshi, northern Tanzania, I was able to present detection of *C. burnetii* in 3.1% (12/382) of small mammals screened. Risk factors for *Bartonella* and *C. burnetii* infection in rodents and others small mammals were assessed. Further analysis for *Coxiella burnetii* was not possible due to small number of positive samples and high Ct values at above 33 which does not give best outcome for genotyping using multilocus sequence typing (MLST), a common method for genotyping of *C. burnetii* (Tilburg *et al*., 2010).

4.1.1 General sample characteristics

A total of 381 and 382 spleen samples from small mammals were available for *Bartonella* species and *C. burnetii* testing respectively. The majority (n=317, 83. %) were black rats (*Rattus rattus*). Other small mammal species tested included: house mice (*Mus musculus*, n=44, 11.5%); African pygmy mice (*Mus minutoides*, n=3, 0.8%); multimammate rat (*Mastomys natalensis*, n=8; 2.10%), spiny mice (*Acomys wilsoni*, n=6, 1.6%), the four-toed hedgehog (*Atelerix albiventris*=1; 0.2% and striped bush squirrels (*Paraxerus flavovittis*, n=3; 0.8). Of the tested host population, 219 individuals (57.5%) were female and 224 (58.8%) were classified as sexually mature based on external sexual characteristics. The majority of small mammal (n=265, 69.6%) were trapped during the wet season and 227 (59.4%) were trapped from Moshi Urban District while the rest were trapped from Moshi Rural District. Flea species collected and identified from the trapped small mammals were *Xenopsylla cheopis* (n=306), *Echidnophaga gallinacea* (n=204) and *Ctenocephalides felis* (n=3) (work

was performed in collaboration with research fellow based in UK). Deoxyribonucleic acid extracts from a total of 193 *Xenopsylla cheopis* collected from 124 small mammals (*Rattus rattus* n=118, *Mus musculus* n=4 and *Mastomys natalensis* n=2) were used for *Bartonella* species testing by targeting the *ssr*A gene fragment.

4.1.2 *Bartonella* detection in spleens of small mammal and risk factors for their infection

Bartonella DNA was detected by *ssr*A qPCR in a total of 57 of 381 (15.0%, 95% CI 11.5 – 18.9%) small mammals screened (Table 4). Samples derived from three out of seven species; *Mastomys natalensis*, *Paraxerus flavovittis* and *R. rattus* (Table 5) were all classified as *Bartonella* positive by this test. The positive control used in *ssr*A qPCR runs to test small mammals spleen extracts had a mean Ct value of 32. The assay showed a 100% lower limit of detection of 1.8 fg of *Bartonella quintana* DNA control. *Rattus rattus* species on was the only species considered for logistic regression analysis due to their dominance and abundance. Further analysis on *Bartonella* species only data indicate that rodent reproductive maturity status was the only significant risk factor (LRT: χ_2= 13.30, df = 1, $p < 0.0003$) for their infection, with reproductively mature individuals more likely to be *ssr*A qPCR positive (OR 3.42, 95% CI 1.69-6.89, $p < 0.001$) than immature. None of the other candidate variables evaluated (small mammals sex, trapping season, trapping district or rodent abundance at trapping village) were significantly associated with *R. rattus ssr*A qPCR test status. The breakdown of small mammal trapped by village, test and sequencing outcome for *Bartonella* species only is given in Table 5.

Table 4: Summary of small mammal species and their *Bartonella* detection status defined by *ssr*A qPCR and sequence confirmed by *glt*A PCR testing of spleen samples

Rodent species	Number of spleen samples tested	*Bartonella ssr*A qPCR positive n (%)	*Bartonella* ssrA qPCR Ct value for positive	*Bartonella glt*A product sequence positive n (%)
Acomys wilsonii	6	0 (0)	Not available	0 (0)
Mastomys natalensis	8	2 (25.0)	33.23 & 33.54	1 (25.0)
Mus minutoides	3	0 (0.0)	Not available	0 (0)
Mus musculus	44	0 (0.0)	Not available	0 (0)
Paraxerus flavovittis	3	1 (33.3)	36.49	0 (0)
Rattus rattus	317	54 (17.0)	Median value of 33.07, range 24.25-39.56	16 (5.0)
Total	381	57 (15.0)		17 (4.4)

Table 5: Summary of small mammal trapping data and the *Bartonell*a *ssr*A and *glt*A genotypes detected in trapped small mammal by village around Moshi, Tanzania. Village codes correspond to village locations indicated in Fig. 2. Distinct *ssr*A and *glt*A genotypes are identified by a sequential number and "s" or "g" respectively

Village code	District	Total number of rodents tested for *Bartonella*	Adjusted trap success	*Bartonella* genotypes identified, with data on the number of individuals and small mammal species each genotype was detected in	
				***ssr*A genotypes**	***gltA* genotypes**
A	Rural	12	9.79	1s – 2 * *Rattus rattus*	3g – 2 * *Rattus rattus*
B	Rural	13	4.28	7s - 1 * *Rattus rattus*	None
				10s - 1 * *Rattus rattus*	None
C	Municipal	31	4.77	10s - 1 * *Rattus rattus*	1g – 1 * *Rattus rattus*
D	Municipal	25	2.68	10s - 1 * *Rattus rattus*	1g – 1 * *Rattus rattus*
				2s - 1 * *Paraxerus flavovittis*	None
E	Rural	39	5.28	4s – 1 * *Rattus rattus*	1g – 1 * *Rattus rattus*
				10s - 2 * *Rattus rattus*	3g – 1 * *Rattus rattus*
F	Municipal	76	10.8	1s - 5 * *Rattus rattus*	1g – 1 * *Rattus rattus*
				9s – 1 * *Rattus rattus*	3g – 3 * *Rattus rattus*
				10s – 1 * *Rattus rattus*	None
				11s - 5 * *Rattus rattus*	None
F (visit 2)	Municipal	33	4.42	1s - 1 * *Rattus rattus*	None
				11s - 3 * *Rattus rattus*	None
G	Rural	15	1.94	6s - 1 * *M. natalensis*	3g - 1 * *M. natalensis*
H	Rural	35	4.69	1s - 1 * *Rattus rattus*	1g – 2 * *Rattus rattus*
				5s - 5 * *Rattus rattus*	3g – 1 * *Rattus rattus*
				8s – 1 * *Rattus rattus*	None
				10s - 3 * *Rattus rattus*	None
J	Rural	19	2.70	10s - 4 * *Rattus rattus*	2g – 1 * *Rattus rattus*
K	Municipal	23	3.19	10s - 1 * *Rattus rattus*	None
L	Rural	22	2.93	1s - 2 * *Rattus rattus*10s - 3 * *Rattus rattus*	1g - 2 * *Rattus rattus*
M	Municipal	38	5.06	3s - 1 * *Rattus rattus*	None

4.1.3 *Bartonella* species detection in fleas

Detection of *Bartonella* species was performed in collaboration with research fellow in the UK, positive samples were sequenced and raw sequence data were shared for further analysis and phylogenetic tree construction. *Bartonella* DNA was detected by *ssr*A qPCR in 53 of 193 (27.5%, 95% CI 21.3 – 34.3%) *X. cheopis* flea extracts. All *ssr*A qPCR positive flea extracts were collected from *R. rattus* individuals. The positive control used in *ssr*A qPCR runs to test flea extracts had a mean Ct value of 32. Logistic regression analysis identified a relationship between flea *ssr*A qPCR test status and the *ssr*A qPCR test status of the small mammal that each flea was collected from (LRT: χ_2= 20.73, df = 1, $p < 0.001$). *Xenopsylla cheopis* fleas collected from *ssr*A qPCR positive small mammal were more likely to themselves be *ssr*A qPCR positive (OR 7.23, 95% CI 2.90-17.97, $p < 0.001$).

4.1.4 Genetic characterization of *Bartonella* in Tanzanian rodents and fleas by *ssr*A sequencing

Full length sequences of the *ssr*A gene target were obtained from 45 of 57 small mammal spleen samples (*Rattus rattus* n=43, *Mastomys natalensis* n=1 and *Paraxerus flavovottis* n=1) and 39 of 53 *ssr*A positive *X. cheopis* fleas. From the 237 bp trimmed *ssr*A sequences, 12 unique genotypes (1s to 12s) were identified in the sequences from small mammal and flea populations combined (Table 5; Fig. 5). The 12 genotypes were grouped into monophyletic groups with ≥97% similarity within the group. The following groupings were identified: a) group As: Genotypes 1s to 5s clustering with a *B. tribocorum* reference sequence from strain GDHL73 (GenBank accession number MF765681); b) group Bs: genotype 6s was unique but clustered with a *B. elizabethae* reference sequence (GenBank accession number MF765617); c) group Cs: genotypes 7s to 10s clustering with a sequence from *B. tribocorum* strain GDHL25 (GenBank accession number MF765675); d) group Ds: genotype 11s clustering with a *B. rochalimae* reference sequence (GenBank accession number MF7651); and e) group Es: genotype 12s from *P. flavovittis*, which was mostly closely related to a *B. quintana* reference sequence (GenBank accession number HG518998). The distribution of *ssr*A genotypes between trapping villages (Table 6) show that more frequently detected *ssr*A genotypes (1s, 10s and 11s) were present in small mammals trapped at multiple villages and provides no evidence of spatial segregation of the genotypes in this rodent population. Data on *ssr*A genotypes were available for fourteen pairs of *X. cheopis* fleas and *R. rattus* hosts (n = 11 *R. rattus* including three from which two *X. cheopis* were collected and tested). For seven pairs

the *ssr*A genotype detected in fleas and small mammal hosts were identical, but in the other seven pairs the genotypes differed. At the group level, 11 flea and host pairs had sequences from the same *ssr*A group and three pairs differed.

Table 6: Summary of *Bartonella ssr*A genotypes identified in small mammal spleens and fleas from Moshi, Tanzania

***ssr*A genotypes**	***ssr*A Group**	**Small mammal species and number of positive samples**	**Flea species and number of positive samples**	**Closest *Bartonella* species (GenBank ID)***	**% similarity to closest *Bartonella* spp. (number of base pair identities/ total base pair length)**
1s	As	11 * *R. rattus* (MN25672)	11 * *X. cheopis* (MN25673)	*B.tribocorum* (MF765681)	99 (240/244)
2s	As	-	1 * *X. cheopis* (MN25674)	*B.tribocorum* (MF765681)	99 (217/222)
3s	As	1 * *R. rattus* (MN25675)	1 * *X. cheopis* (MN25676)	*B.tribocorum* (MF765681)	99 (228/233)
4s	As	1 * *R. rattus* (MN25677)	-	*B.tribocorum* (MF765681)	99 (239/244)
5s	As	1 * *R. rattus* (MN25678)	1 * *X. cheopis* (MN25679)	*B.tribocorum* (MF765681)	98 (237/244)
6s	Bs	1* *M. natalensis* (MN25680)	-	*B.elizabethae* (MF765617)	99 (222/224)
7s	Cs	1 * *R. rattus* (MN25681)	1 * *X. cheopis* (MN25682)	*B.tribocorum* (MF765675)	99 (242/244)
8s	Cs	1 * *R. rattus* (MN25683)	-	*B.tribocorum* (MF765675)	99 (239/244)
9s	Cs	1 * *R. rattus* (MN25684)	-	*B.tribocorum* (MF765675)	99 (236/237)
10s	Cs	18 * *R. rattus* (MN25685)	9 * *X. cheopis* (MN25686)	*B.tribocorum* (MF765675)	100 (244/244)
11s	Ds	8 * *R. rattus* (MN25687)	15 * *X. cheopis* (MN25688)	*B.rochalimae* (MF765651)	100 (246/246)
12s	Es	1* *P. flavovottis* (MN25689)	-	*B.quintana* (HG518998)	98 (233/239)

The number of individuals of each small mammal (n=45) and fleas (n=39) species from which each genotype was obtained are shown, as well as data on % similarity to reference *Bartonella* species sequences, with the number of base pair identities indicated in parentheses. The Genbank accession numbers for each genotype are also indicated in parentheses in columns 2 and 3. * The closest reference sequences to the study sequences were selected from fully characterized sequences in Genbank obtained from cultures.

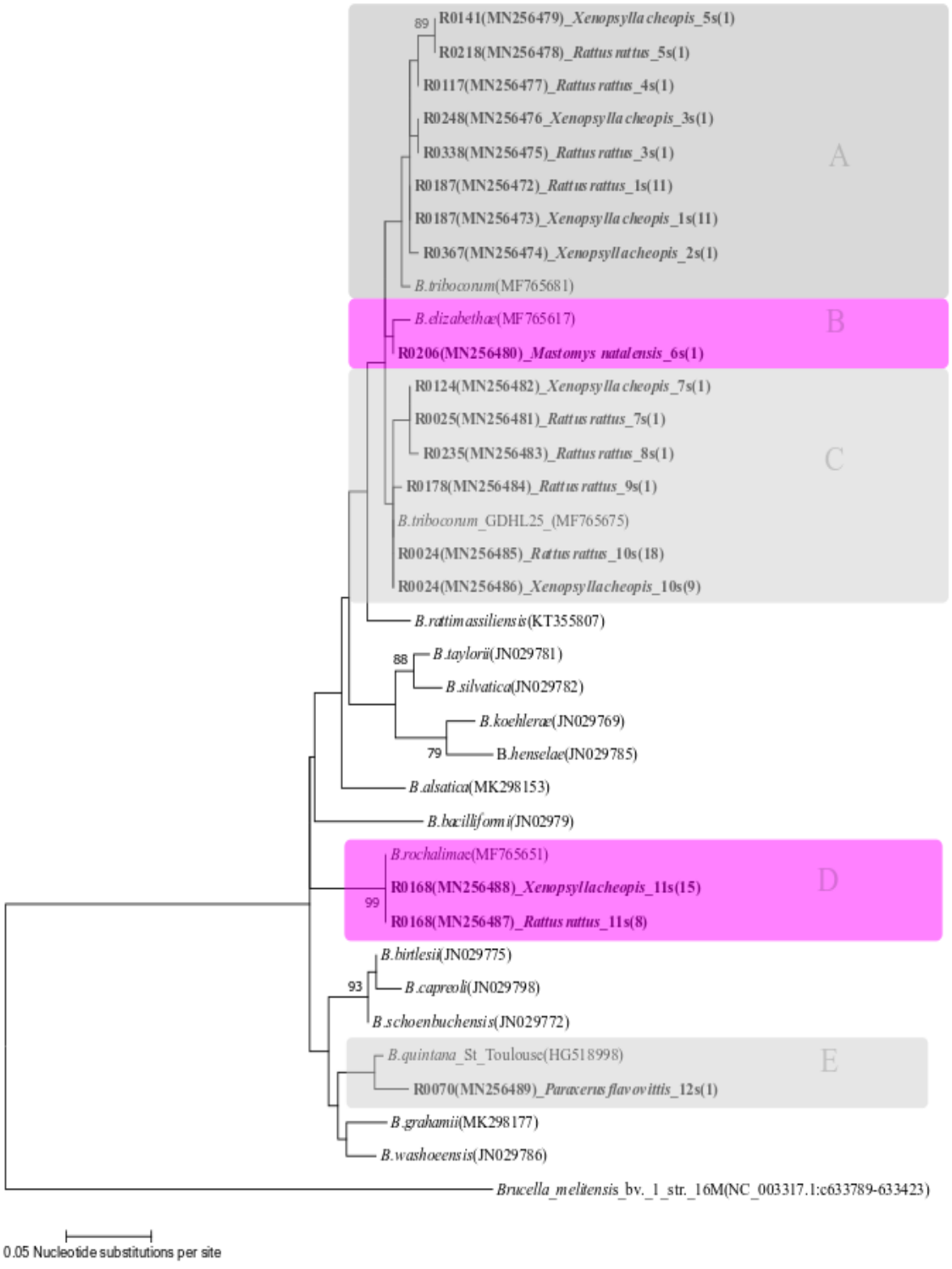


Figure 5: Phylogenetic tree showing the relatedness of the *Bartonella ssr*A gene Sequences (237bp gene fragments) derived from 45 small mammal spleen tissue samples (43 *R. rattus,* 1 *M. natalensis* and 1 *P. flavovottis) and* 39 *X. cheopis fleas* collected in northern Tanzania

A single representative sample sequence is included for each combination of *Bartonella* genotype identified in this study and host of origin. Genotypes (1s - 12s) and groups (As – Es) are indicated by lettering. Groups A, C and E are shaded grey, with groups B and D in pink. The phylogenetic tree was constructed using the maximum likelihood method based on a Kimura 2-parameter substitution model (Tamura, 1992), as determined by Model test as implemented in MEGA 7.0 (Kumar *et al*., 2016). The tree with the highest log likelihood is shown and drawn to scale, with branch lengths shown in terms of the number of substitutions per site. Vertical branches indicate identical sequences. The numbers at the nodes correspond to bootstrap values higher than 70% after 1000 replicates. Sequences from this study are labelled with unique identifiers, with prefix "R" followed by sample identifier numbers, Genbank accession number, the small mammal or flea host species, the genotype code and the number of samples yielding each genotype (in parentheses). Sequences from reference strains of *Bartonella* are included with the *Bartonella* species name and GenBank accession numbers given in parentheses. *Brucella melitensis* was included as an outgroup.

4.1.5 Characterisation of *Bartonella* in Tanzanian small mammals by *glt*A sequencing

Full length sequences of the *glt*A gene were obtained from 17 small mammal spleen DNA extracts (*R. rattus* n=16 and *M. natalensis* n=1). To be conservative, the alignment was pruned to the length of the shortest sequence (283 bp out of 379 bp expected) and three unique genotypes were identified in this fragment (Fig. 6). The correspondence between *glt*A and *ssr*A genotypes and groups is shown in Table 7. Only one *R. rattus* individual had a *glt*A sequence without a corresponding *ssr*A sequence. For the 16 remaining *glt*A genotypes, the same individuals all yielded *ssr*A sequences falling into groups As to Cs (Table 5). Eight sequences (genotype 1g, GenBank accession number MK906044) collected from *R. rattus* were identical to a *B. elizabethae* sequence obtained from a *R. rattus* sampled previously at a rural site in Kenya (strain B29297 (Halliday *et al*., 2015), GenBank accession number KM233489). A second genotype (2g, GenBank accession number MK906046) was identified in one *R. rattus* and showed similarity of 97% (278 of 284 base pair matches) to a *B. elizabethae* reference sequence (GQ225710) found in GenBank. Eight sequences (genotype 3g) collected from *M. natalensis* (GenBank accession number MK906045) and from *R. rattus* (GenBank accession number MK906043) were identical to a sample identified in a *R. rattus* from an urban site in Kenya (strain B28391 (Halliday *et al*., 2015), GenBank accession

number: KC763968) and to a cultured reference strain of *B. elizabethae* (strain BR02, GenBank accession number: GU056192).

Table 7: Summary of *glt*A genotypes identified in spleens of small mammal(n=17) from Moshi, Tanzania and the correspondence with *ssr*A genotypes identified in the same species and individuals

***glt*A Genotype**	**GenBank accession number**	**Rodent species and number of positive samples**	**Closest *Bartonella* species (GenBank ID)*.**	**% similarity to closest *Bartonella* spp. (number of base pair identities/ total base pair length)**	***ssr*A group and genotype**
1g	MK906044	8 * *R. rattus*	*B.elizabethae*(GQ225710)	99.65 (282/283)	Group C:10s
2g	MK906046	1 * *R. rattus*	*B.elizabethae* (GQ225710)	97 (278/284)	Group C:10s
3g	MK906043	5* *R. rattus*	*B.elizabethae*(GU056192)	100 (283/283)	Group A: 1s
3g	MK906043	1 * *R. rattus*	*B.elizabethae*(GU056192)	100 (283/283)	Group A: 4s
3g	MK906045	1 * *M. natalensis*	*B.elizabethae*(GU056192)	100 (283/283)	Group B: 6s
3g	MK906043	1 * *R. rattus*	*B.elizabethae*(GU056192)	100 (283/283)	None

Data on the % similarity to reference *Bartonella* species sequences, and the number of base pair identities are indicated. The Genbank accession numbers for each genotype identified in the study are indicated.* the closest reference sequences to the study sequences were selected from fully characterized sequences in Genbank obtained from cultures.

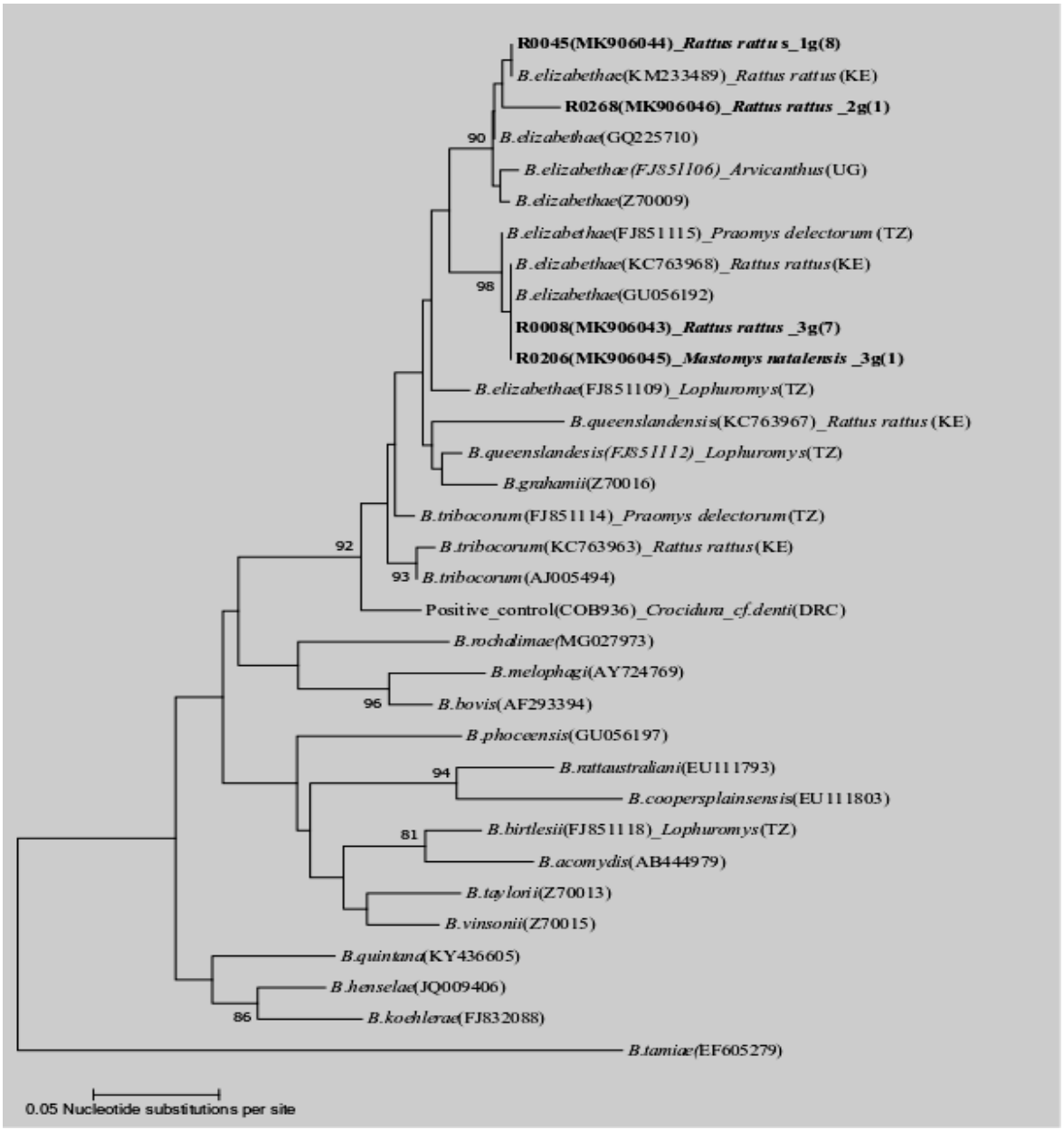


Figure 6: Phylogenetic tree showing the relatedness of the *Bartonella glt*A gene sequences (283bp fragments) derived from 17 spleen tissue samples from small mammals (16 *R. rattus* and 1 *Mastomys natalensis)* trapped in northern Tanzania

A single representative sample sequence is included for each genotype identified in this study, with the exception of genotype 3 g to illustrate the identical sequences obtained from *R. rattus* and *M. natalensis*. Sequences from this study are labelled with unique identifiers, with prefix "R" followed by sample identifier numbers, Genbank accession number, the small mammal or flea host species, the genotype code and the number of samples yielding each genotype (in parentheses). Reference *Bartonella* sequences from small mammal trapped elsewhere in East Africa obtained from GenBank are indicated by GenBank accession numbers in parentheses, small mammal species and country code (Kenya (KE) (Halliday *et al*., 2015), Uganda (UG) (Billeter *et al*., 2014) Tanzania (TZ) (Gundi *et al*., 2012), Democratic Republic of Congo (DRC) (Gundi *et al*., 2012). The sequence obtained for the known *Bartonella* positive control sample provided by a colleague from a previous study is included and indicated with a unique identification number (COB936) (Gundi *et al*., 2012).

4.1.6 IS1111 detection and prevalence of *C. burnetii* in spleen samples from small mammal

Quantitative real time PCR for detection of *C. burnetii* IS1111, with 100% reproducibility was estimated at approximately 10 genome copies per µl. *Coxiella burnetii* DNA was detected by IS1111 qPCR in a total of 12 (3.1%) of 382 (95% CI: 1.6-5.4) spleen samples screened and in five (71.4%) of seven (95% CI: 29.0-96.3) individual small mammal species tested; *Rattus rattus* (n=7), *Mus musculus* (n=1), *Acomys wilsonii* (n=2), *Paraxerus flavovottis* (n=1) and *Atelerix albiventris* (n=1). Eleven (91.7%) of twelve (95% CI: 61.5-99.8) *C. burnetii* positive small mammals were trapped within Moshi Urban District. Five (2.3%) of 219 females (95% CI: 0.7-5.4) and seven (4.3%) of 163 males (95% CI: 1.7-8.6) were positive for *C. burnetii*. Nine (3.4%) of 266 (95% CI: 1.6- 6.3) small mammal sampled during the wet season were *C. burnetii* positive and three (2.6%) of 116 (95% CI: 0.5-7.4) tested positive from the batch of small mammal trapped during dry season (Table 8).

Table 8: Distribution and abundance of trapped small mammals per village and their *C. burnetii* IS1111 qPCR test outcome

Variable		Number of rodents tested for *C. burnetii*	*C. burnetii* positive n (%)	95% CI
Village Code	A	12	0 (0.0)	0.0-26.5
	B	13	1 (7.7)	0.2-36
	C	31	0 (0.0)	0.0-11.2
	D	26	7 (26.9)	11.6-47.8
	E	39	0 (0.0)	0.0-9.0
	F	109	2 (1.8)	0.2-6.5
	G	15	0 (0.0)	0.0-21.8
	H	35	0 (0.0)	0.0-10.0
	J	19	0 (0.0)	0.0-17.6
	K	23	0 (0.0)	0.0-14.8
	L	22	0 (0.0)	0.0-15.4
	M	38	2 (5.3)	0.6-17.7
District	Rural	155	1 (0.6)	0.02-3.5
	Urban	227	11 (4.8)	2.4-8.5
Sex	Male	163	7 (4.3)	1.7-8.6
	Female	219	5 (2.3)	0.7-5.2
Age	Mature	225	10 (4.4)	2.2-8.0
	Immature	157	2 (1.3)	0.2-4.5
Season	Wet	266	9 (3.4)	1.6-6.3
	Dry	116	3 (2.6)	0.5-7.4
Overall	NA	382	12 (3.1)	1.6-5.4

4.2 Discussion

The detection of these pathogens in small mammal population sampled in northern Tanzania indicated that small mammals could be playing yet unknown role in transmission of *C. burnetii* and *Bartonella* spp to humans and other animals. Small mammals especially rodents are well known for centuries to be host and reservoir of several zoonotic pathogens. Recently several studies have demonstrated that rodents may be important hosts or reservoir of emerging and re-emerging pathogens including *Coxiella* and *Bartonella* spp. *Coxiella burnetii* is zoonotic pathogen that is known to cause a substantial proportion of cases of human febrile illness in northern Tanzania and *Bartonella* spp. are likely to contribute to the burden of human febrile illness, but have not yet been investigated in this part of Africa. Below I provide the discussion for each of the two pathogens separately.

4.2.1 *Bartonella* species

This study revealed substantial variation in *Bartonella* genotypes among small mammals and their fleas in a previously unstudied region of northern Tanzania (the Moshi Municipal and Moshi Rural Districts). *Rattus rattus* was the most common small mammal species trapped and showed a high *Bartonella* prevalence defined by *ssr*A qPCR, which is consistent with the global distribution of *Bartonella* species in *Rattus* (Buffet *et al*., 2013). Within *R. rattus*, the probability of qPCR positivity was higher in reproductively mature species as compared to immature species, consistent with other studies (performed in the USA) that have found an association with age (Firth *et al*., 2014; Peterson *et al*., 2017). Sequencing of *ssr*A and *glt*A gene fragments revealed a variety of genotypes and the majority of sequences obtained showed greatest similarity to *B. tribocorum* and *B. elizabethae* reference sequences, both of which have been isolated in humans with febrile illness (Kosoy *et al*., 2010). Sequences similar to *B. rochalimae* and *B. quintana* were also identified based on *ssr*A sequencing. These species were not detected by sequencing of the *glt*A, indicating reduced sensitivity of the *glt*A conventional PCR for detection of *Bartonella* species. This is consistent with the findings of a previous study (Diaz *et al*., 2012).

The *ssr*A qPCR was used to estimate prevalence in small mammals and fleas, and the sequencing of *ssr*A and *glt*A PCR products to assess genetic variation and characterize the *Bartonella* detected. The overall prevalence of *Bartonella* (15%) detected in small mammals using the *ssr*A qPCR was lower than that has been detected in many comparable studies of global small mammal populations (Gundi *et al*., 2010; Meheretu *et al*., 2013), including studies that have used a less sensitive *glt*A assay for prevalence determination (Kamani *et al*., 2013; Lipatova *et al*., 2015). In a previous Tanzanian study on small mammal species (*Grammomys sp, Lophuromys sp, Mus minutoides* and *Praomys detectorum*) an overall *glt*A prevalence of 41% was detected (Gundi *et al*., 2012). A Ugandan survey using *glt*A to test invasive and indigenous small mammal populations found variable prevalence across species, with higher prevalence in indigenous species (60% in *Arvicanthis niloticus* and 61% in *Cricetomys gambianus*), but low prevalence (1.4%) was recorded in invasive *R. rattus* (Billeter *et al*., 2014). Similarly, in Kenya the *Bartonella* prevalence determined by culture varied by species of small mammal (Halliday *et al*., 2015). Considering the data from *R. rattus* only, the prevalence seen in this study and previous African studies reveals consistently lower prevalence in comparison to *R. rattus* sampled in Asia and tested using *ssr*A qPCR

methods (e.g. 32.5% (R Development Core Team, 2018). The prevalence of *Bartonella* detection in *X. cheopis* fleas in this study using the *ssr*A qPCR was also lower than what has been recorded in this species in the USA, where 190 of 200 (95%) *X. cheopis* tested were positive for *Bartonella* DNA (Billeter *et al*., 2011). The low prevalence of *Bartonella* in *R. rattus* and *X. cheopis* observed in this study are consistent with several other studies conducted in Africa. It has been argued that this pattern of lower *Bartonella* prevalence in African *R. rattus* populations could be attributed to host escape during colonization (Torchin *et al*., 2003). Further investigation of native and invasive small mammal (rodent) populations across Africa would be needed to investigate this further, and also evaluate the possible implications for human disease risk on the continent.

Phylogenetic analysis of sequences from small mammal and their fleas revealed high concordance of sequences between hosts and ectoparasites. Overall, 10 distinct *ssr*A genotypes were identified that were most similar to reference sequences of *B. tribocorum* and *B. elizabethae* (Groups As to Cs), with only one genotype (10s) showing an identical match to a published *B. tribocorum* sequence in GenBank. However, since all of the reference sequences that were most similar were from a single study in China (Ying *et al*., 2002) it is important to recognize the limited reference data available currently and need for future comparison to datasets from other geographic areas to further evaluate these data on the diversity and types of *Bartonella* found in Tanzania. Moreover, *B. elizabethae* and *B. tribocorum* share identical published *ssr*A sequences in Genbank and our results showed clustering in the phylogenetic tree (Fig. 5), so the two species cannot be distinguished by this *ssr*A fragment. The other two *Bartonella* species identified were: a) a single sequence (11s) with greatest similarity (98%) to *B. quintana* obtained from a sample from a *P. flavovittis* host; and b) a sequence obtained from multiple samples of *R. rattus* and *X. cheopis* that showed an identical match to *B. rochalimae,* emphasizing the diversity of *Bartonella* present in rodents in Tanzania. To the best of my knowledge, this is the first report of molecular detection and characterization of *Bartonella* species in small mammals and their associated ectoparasite in Africa using the *ssr*A gene target. The scope for comparison with other sequences is thus limited, as there is currently little reference material on *ssr*A sequences from *Bartonella* sampled elsewhere, particularly in Africa.

In contrast, the *glt*A gene has been widely used to study *Bartonella* globally. Phylogenetic analysis of sequences from 17 *glt*A PCR products from this Tanzanian small mammal

population (16 *R. rattus* and 1 *M. natalensis*) showed the highest similarity to reference sequences of *B. elizabethae,* which has multiple published sequences in Genbank, including many from east Africa. The association of sequences similar to *B. elizabethae* with *Rattus* spp. in this study is consistent with similar findings from Malaysia (Tay *et al*., 2014) and Thailand (Klangthong *et al*., 2015). *Bartonella elizabethae* has also been identified in different small mammal species in Africa (Billeter *et al*., 2014; Gundi *et al*., 2012). Identical *glt*A sequences were amplified from *R. rattus* and *M. natalensis* in my study, suggesting possible transmission between different small mammal species in the Tanzania site or a shared common source of infection. Identical sequences were also identified previously in *R. rattus* sampled at both rural (strain B28297, accession number KM233489, from Asembo, Kenya) and urban (strain B28391, accession number KC763968, from Kibera, Nairobi Kenya) sites in Kenya (Halliday *et al*., 2015). This suggests that similar *Bartonella* could be found in rodents in Kenya and Tanzania. However, these comparisons are based on short sequences of a single gene target only. Longer sequences from multiple genes and greater sampling effort across the region would be required to robustly confirm sharing of genotypes to trace source populations or determine patterns of host connectivity.

Several studies of zoonotic disease have shown that a variety of pathogens account for high proportions of febrile illness in northern Tanzania but that considerable proportions remain unexplained (Chipwaza *et al*., 2015; Crump *et al*., 2013). *Bartonella* species have been identified as important causes of human febrile illness in several global settings but there has been little investigation of the impact of bartonellosis upon human health, in Africa particularly. The finding of *Bartonella* genotypes that are most similar to *B. elizabethae, B. rochalimae* and *B. quintana* reference sequences in rodents trapped in and around households in Moshi, Tanzania, and the fleas collected from these rodents, indicates the possibility that *Bartonella* infection may be responsible for an as yet unknown proportion of febrile illnesses in this region. Efforts are needed to determine the clinical impact of bartonellosis in this region and increase awareness about *Bartonella* and other zoonotic pathogens among physicians and health care workers, especially where the cause of large proportions of febrile illness remains unknown. These results also demonstrate that molecular detection tools can be effectively used for surveillance and diagnostic of zoonotic pathogens in resource limited settings especially in Africa where the application of molecular approaches has remaining low.

4.2.2 *Coxiella burnetii*

Coxiella burnetii DNA was detected in 3.1% of small mammal trapped from Moshi Rural and Urban Districts in northern Tanzania between May 2013 and September 2014. To the best of my knowledge this is the first study to demonstrate the presence and prevalence of *C. burnetii* in small mammals from Tanzania. Infected small mammals may act as a source of *C. burnetii* infection for both humans and other animals. This detection of *C. burnetii* infection in small mammals and the known clinical impact of *C. burnetii* infections in humans indicate that further studies into the contribution of small mammals to the transmission of *C. burnetii* to livestock and humans are warranted. The outcome of such studies would be beneficial by generating data on the epidemiology of *C. burnetii* and providing evidence to inform Q fever and Coxiellosis control programs. Genetical comparison of data generated with data obtained from GeneBank would enable understanding of source of pathogens.

There is significant variation in the prevalence of *C. burnetii* reported in different small mammal populations sampled within Africa. A study done in Zambia, a country bordering Tanzania to the Southwest *C. burnetii* DNA was detected by conventional PCR on 16SrRNA gene target in 9 out of 20 (45%) of overall small mammals trapped in urban and peri-urban (Chitanga *et al*., 2018). *Coxiella burnetii* detection within individual small mammal species varied as well: *Saccostomus campestris* (2/2), *Gerbillinae* sp. (4/7) and *Mastomys natalensis* (3/11). Another study done in Nigeria, *C. burnetii* DNA was detected by conventional PCR on IS1111 gene target in 4 out of 194 (2.1%) of overall rodents trapped in peri-urban (Kamani *et al*., 2018). Considering specie prevalence; 3 out of 121 (2.5%) were *Rattus norvegicus* and 1 out of 48 (2.1%) was *Rattus rattus* (Kamani *et al*., 2018). In Egypt *C. burnetii* DNA was found in 2 out of 55 (3.6%) *R. norvegicus* and 3 out of 20 (15%) *R. rattus*, with overall prevalence of 6.7% (Abdel-Moein & Hamza, 2018). Knowledge of the prevalence of *C. burnetii* in different small mammal populations and improved understanding of the factors that drive this variation could be taken into consideration during designing for *C. burnetii* control programs.

In this study there are indications of variation in the proportion of small mammal that are *C. burnetii* positive across different small mammal species, season of sampling, age, species, sex and location of sampling (rural vs urban districts and villages) (Table 7). The small number of positive individuals identified in this study limits the scope for statistical analyses of these patterns but the factors that determine *C. burnetii* prevalence in small mammal

populations should be investigated further. In this study the majority of *C. burnetii* positive small mammals were trapped from Moshi Urban District. It has been documented that emerging and re-emerging zoonotic diseases and pathogens are linked with increasing globalization and urbanization (Amitai *et al*., 2010; Buzan *et al*., 2017; Comer *et al*., 2001; Liu *et al*., 2013). Furthermore, the number of rodent-borne diseases in urban areas have been reported to be higher than those in rural areas (Bordes *et al*., 2015; Gratz, 1999) this could as well be possible explanations to the findings for this study. Since the small mammals sampled were from in or around household, the risk of *C. burnetii* infection could potentially extend to a wider community due to close proximity of houses in urban areas.

Observations from this study indicate that specific small mammal species are more likely to carry *C. burnetii* bacteria than others. But due to the low number of positive cases it was not powered for statistical analyses of these findings. However, findings from other studies have also shown that some small mammals species are responsible for maintenance of endemic circulating *Coxiella* species in the environment in comparison to others species (Burgdorfer *et al*., 1963; Reusken *et al*., 2011; Rozental *et al*., 2017) indicating that similar trend could be happening in northern Tanzania.

From this study it was also observed that there were more *C. burnetii* positive in mature small mammals as compared to immature. In a laboratory based experimental model study on mice (Leone *et al*., 2007) revealed that mature mice at 14 months had increased *C. burnetii* load and granuloma in the spleens than younger mice at 1 month after intraperitoneal injection of *C. burnetii* organisms indicating that findings from the present study are in concordance with other studies. In this study we observed more *C. burnetii* positive male small mammals as compared to females. Also other studies have reported similar trend. A study done in Canada found that woodland jumping mice, adult males had higher rates of *C. burnetii* infection than adult females ($v2 = 13.588$, $P < 0.001$). The possible explanations are that males tend to move around in search for females mates. *Coxiella burnetii* can also be transmitted sexually through sperm cells transferred to females (Kruszewska & Tylewska-Wierzbanowska, 1993). Thus, species with more promiscuous mating systems could have higher a prevalence of infection, as infected individuals are more likely to come into contact with and infect previously uninfected individuals under this scenario (Lockhart *et al*., 1996).

In Tanzania febrile illnesses caused by zoonotic pathogens, including *C. burnetii* are of public health importance but often underappreciated or misdiagnosed (Crump *et al*., 2013). The

detection of *C. burnetii* in small mammals trapped in and around household premises from the same area where the previous study (Crump *et al*., 2013) has reported high prevalence of Q fever in humans provides a clear rationale for further investigation of the epidemiology of *C. burnetti* in this setting and role of small mammals. In the Netherlands, small mammal (rodents) were found to be responsible for maintaining the cycle of *C. burnetii* infection between wildlife and domestic animals, and consequently transmission to humans (Reusken *et al*., 2011). Related or similar *C. burnetii* transmission scenario may be happening in Tanzania, where the main source for human *C. burnetii* infection is poorly known. In other region *C. burnetii* have been detected in small mammal especially in rodent species trapped in forest and natural environments, where human activities such as livestock keeping do not occur, and therefore suggesting that small mammals could be a reservoir of *C. burnetii* (Burgdorfer *et al*., 1963; Thompson *et al*., 2012)

Additional work is needed to understand the role of small mammals in the maintenance and transmission of *C. burnetii* infection in this region and to examine linkages between human, livestock and small mammal infections. *Coxiella burnetii* strains circulating in small mammals should be typed and compared with isolates from human, other animals and environmental sources. This will provide information on the role of small mammals in *C. burnetii* transmission through analysis of phylogenetic tree and assessment of evolutionary relationship of animal source of sequences.

4.2.3 Limitations of the study

This study had some limitations, firstly detection of *Bartonella* species and *C. burnetii* was performed using DNA extracted from tissues where the host DNA would likely become PCR inhibiting factor. However, efforts were made to minimize the effect of host DNA by diluting the DNA concentration to approximately 10-50 ng/µl.

Secondly, while there are many hosts of *Bartonella* species and *C. burnetii*, this study was performed in rodents only. Detection of these pathogens in other possible hosts/reservoirs such as cats, dogs, goats, sheep and others would have strengthened analysis and interpretation of findings, making it possible to assess the best possible likely route of human infection with these pathogens.

Thirdly, due to low number of positive samples for *C. burnetii* and high threshold cycle (>33Ct), analysis and interpretation could not performed to give power of statistical

significance. This also limited other downstream molecular analysis such as genotyping using multilocus sequence typing which is necessary for identification circulating *C. burnetii* strains.

Finally, molecular detection of these pathogens is not 100% accurate; therefore there is possibility of false positive or false negative detections. For instance Coxiella-like organisms have been reported because false positive test when IS1111 is used for *C. burnetii* detection. Although I used highly specific primers and probes and optimized the assay, I cannot infer by 100% that the detection was specifically limited to the targeted pathogens only (*Bartonella* spp and *C. burnetii*) and exclude possibilities of missing or testing wrong pathogens.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The *ssr*A and *glt*A sequences obtained from rodent spleens and *ssr*A sequences obtained from fleas reveal the presence of a diverse set of *Bartonella* genotypes and increase our understanding of the bartonellae present in Tanzanian. Further studies are needed to fully characterise the prevalence, genotypes and diversity of *Bartonella* in different host populations and their potential impacts on human health.

On the other hand, the detection of *C. burnetii* in different species of small mammal from Moshi, northern Tanzania demonstrates the relative importance of small mammals as host of *C. burnetii* in the region. Infected small mammals may act as a source of *C. burnetii* infection to humans and other animals in the study area. This detection of *C. burnetii* infections in small mammals should motivate further studies into the contribution of small mammals in transmission of *C. burnetii* to humans and other animals and to assess risk factors for small mammal *C. burnetii* infection.

5.2 Recommendations

Efforts are needed to determine the clinical impact of bartonellosis and coxiellosis in this region and increase awareness about *Bartonella* and *C. burnetii* and other zoonotic pathogens among physicians and health care workers, especially where the cause of large proportions of febrile illness remains unknown.

Further studies are needed to fully characterize the prevalence, genotypes and diversity of *Bartonella* spp and *C. burnetii* in different host populations and their potential impacts on human health.

Sustainable surveillance of several other zoonotic pathogens using molecular methods in humans and animal population should do. Phylogenetic and genotyping of pathogens identifies should be performed in future in order to improve assessment of likely source of these pathogens to humans. Since zoonotic pathogens including the one studied under this PhD work continue to pose substantial risk to humans, their livestock and economic a more

holistic dealing and surveyance should be considered which implements one health concept should be strengthened in Tanzanian health systems.

REFERENCES

A-Elgayoum, S. M. E., El-Feki, A. E. K. A., Mahgoub, B. A., El-Rayah, E. A., & Giha, H. A. (2009). Malaria overdiagnosis and burden of malaria misdiagnosis in the suburbs of central Sudan: special emphasis on artemisinin-based combination therapy era. *Diagnostic Microbiology and Infectious Disease*, 64(1), 20–26. https://doi.org/10.1016/j.diagmicrobio.2009.01.029

Abbassy, M. M., Loftis, A. D., Dasch, G. A., Szumlas, D. E., Helmy, I. M., Moriarity, J. R., & Reeves, W. K. (2006). Surveillance of Egyptian fleas for agents of public health significance: *Anaplasma*, *Bartonella*, *Coxiella*, *Ehrlichia*, *Rickettsia* and *Yersinai Pestis*. *The American Journal of Tropical Medicine and Hygiene*, 75(1), 41–48. https://doi.org/10.4269/ajtmh.2006.75.41

Abdel-Moein, K. A., & Hamza, D. A. (2018). Rat as an overlooked reservoir for *Coxiella burnetii*: A public health implication. *Comparative Immunology, Microbiology and Infectious Diseases*, 61(1), 30–33. https://doi.org/10.1016/j.cimid.2018.11. 002

Allan, K. J., Halliday, J. E. B., Moseley, M., Carter, R. W., Ahmed, A., Goris, M. G. A., Hartskeerl, R. A., Keyyu, J., Kibona, T., Maro, V. P., Maze, M. J., Mmbaga, B. T., Tarimo, R., Crump, J. A., & Cleaveland, S. (2018). Assessment of animal hosts of pathogenic *Leptospira* in Northern Tanzania. *PLoS Neglected Tropical Diseases*, 12(6), e0006444. https://doi.org/10.1371/journal.pntd.0006444

Almeida, A. P., Marcili, A., Leite, R. C., Nieri-Bastos, F. A., Domingues, L. N., Martins, J. R., & Labruna, M. B. (2012). *Coxiella* symbiont in the tick Ornithodoros rostratus (Acari: Argasidae). *Ticks and Tick-Borne Diseases*, 3(4), 203–206. https://doi.org/10.1016/j.ttbdis.2012.02.003

Amexo, M., Tolhurst, R., Barnish, G., & Bates, I. (2004). Malaria misdiagnosis: Effects on the poor and vulnerable. *Lancet*, 364(944), 1896-1898. https://doi.org/10.1016/S0140-6736(04)17446-1

Amitai, Z., Bromberg, M., Bernstein, M., Raveh, D., Keysary, A., David, D., Pitlik, S., Swerdlow, D., Massung, R., Rzotkiewicz, S., Halutz, O., & Shohat, T. (2010). A Large Q Fever Outbreak in an Urban School in Central Israel. *Clinical Infectious Diseases*, 50(11), 1433–1438. https://doi.org/10.1086/652442

Anderson, B. E., & Neuman, M. A. (1997). *Bartonella* spp. as emerging human pathogens. *Clinical Microbiology Reviews*, 10(2), 203–219. https://doi.org/10.1128/cmr.10.2.203

Angelakis, E., Mediannikov, O., Jos, S. L., Berenger, J. M., Parola, P., & Raoult, D. (2016). Candidatus *Coxiella massiliensis* infection. *Emerging Infectious Diseases*, 22(2), 285-288. https://doi.org/10.3201/eid2202.150106

Avidor, B., Graidy, M., Efrat, G., Leibowitz, C., Shapira, G., Schattner, A., Zimhony, O., & Giladi, M. (2004). *Bartonella koehlerae*, a new cat-associated agent of culture-negative human endocarditis. *Journal of Clinical Microbiology*, 42(8), 3462–3468. https://doi.org/10.1128/JCM.42.8.3462-3468.2004

Bai, Y., Kosoy, M. Y., Calisher, C. H., Cully, J. F., & Collinge, S. K. (2009). Effects of rodent community diversity and composition on prevalence of an endemic bacterial pathogen - *Bartonella*. *Biodiversity*, *10*(4), 3–11. https://doi.org/10.1080/14888386. 2009. 9712856

Bai, Y., Hayman, D., McKee, C., & Kosoy, M. Y. (2015). Classification of *Bartonella* Strains Associated with Straw-Colored Fruit Bats (Eidolon helvum) across Africa Using a Multi-locus Sequence Typing Platform. *PLoS Neglected Tropical Diseases*, 9(1), 1–16. https://doi.org/10.1371/journal.pntd.0003478

Bai, Y., Kosoy, M. Y., Lerdthusnee, K., Peruski, L. F., & Richardson, J. H. (2009). Prevalence and genetic heterogeneity of *Bartonella* strains cultured from rodents from 17 provinces in Thailand. *American Journal of Tropical Medicine and Hygiene*, 81(5), 811–816. https://doi.org/10.4269/ajtmh.2009.09-0294

Bai, Y., Kosoy, M. Y., Ray, C., Brinkerhoff, R. J., & Collinge, S. K. (2008). Temporal and spatial patterns of *Bartonella* infection in black-tailed prairie dogs (Cynomys ludovicianus). *Microbial Ecology*, 56(2), 373-382. https://doi.org/10.1007/s00248-007-9355-6

Bamberg, W. M., Pape, W. J., Beebe, J. L., Nevin-Woods, C., Ray, W., Maguire, H., Nucci, J., Massung, R. F., & Gershman, K. (2007). Outbreak of Q fever associated with a horse-boarding ranch, Colorado, 2005. *Vector-Borne and Zoonotic Diseases*, 7(3), 394-402. https://doi.org/10.1089/vbz.2007.0104

Bass, J. W., Vincent, J. M., & Person, D. A. (1997). The expanding spectrum of *Bartonella* infections: I. Bartonellosis and trench fever. *Pediatric Infectious Disease Journal*, 16(1), 2–10. https://doi.org/10.1097/00006454-199701000-00003

Bates, D., Mächler, M., Bolker, B., & Walker, S. (2015). Fitting Linear Mixed-Effects Models Using lme4. *Journal of Statistical Software*, 67(1). https://doi.org/10.18637/jss.v067.i01

Bechah, Y., Verneau, J., Ben Amara, A., Barry, A. O., Lépolard, C., Achard, V., Panicot-Dubois, L., Textoris, J., Capo, C., Ghigo, E., & Mege, J. (2014). Persistence of *Coxiella burnetii*, the agent of Q fever, in murine adipose tissue. *PloS One*, 9(5), e97503. https://doi.org/10.1371/journal.pone.0097503

Bereswill, S., Hinkelmann, S., Kist, M., & Sander, A. (1999). Molecular analysis of riboflavin synthesis genes in *Bartonella henselae* and use of the ribC gene for differentiation of *Bartonella* species by PCR. *Journal of Clinical Microbiology*, 37(10), 3159–3166. https://doi.org/10.1128/jcm.37.10.3159-3166.1999

Bermond, D., Heller, R., Barrat, F., Delacour, G., Dehio, C., Alliot, A., Monteil, H., Chomel, B., Boulouis, H. J., & Piémont, Y. (2000). *Bartonella birtlesii* sp. nov., isolated from small mammals (Apodemus spp.). *International Journal of Systematic and Evolutionary Microbiology*, 50(6), 1973–1979. https://doi.org/10.1099/00207713-50-6-1973

Bermond, D. B. H. J., Heller, R., Van Laere, G., Monteil, H., Chomel, B. B., Sander, A., Dehio, C., & Piémont, Y. (2002). *Bartonella bovis* Bermond et al. sp. nov. and *Bartonella capreoli* sp. nov., isolated from European ruminants. *International Journal of Systematic and Evolutionary Microbiology*, 52(2), 383–390. https://doi.org/10.1099/00207713-52-2-383

Bhattarai, A., Ali, A. S., Kachur, S. P., Mårtensson, A., Abbas, A. K., Khatib, R., Al-mafazy, A. W., Ramsan, M., Rotllant, G., Gerstenmaier, J. F., Molteni, F., Abdulla, S., Montgomery, S. M., Kaneko, A., & Björkman, A. (2007). Impact of artemisinin-based combination therapy and insecticide-treated nets on malaria burden in Zanzibar. *PLoS Medicine*, 4(11), 1784-1790. https://doi.org/10.1371/journal.pmed.0040309

Bhengsri, S., Baggett, H. C., Peruski, L. F., Morway, C., Bai, Y., Fisk, T. L., Sitdhirasdr, A., Maloney, S. A., Dowell, S. F., & Kosoy, M. (2010). *Bartonella* spp. infections, Thailand. *Emerging Infectious Diseases*, 16(4), 743–745. https://doi.org/10.3201/eid1604.090699

Billeter, S. A., Levy, M. G., Chomel, B. B., & Breitschwerdt, E. B. (2008). Vector transmission of *Bartonella* species with emphasis on the potential for tick transmission. *Medical and Veterinary Entomology*, 22(1), 1–15. https://doi.org/10.1111/j.1365-2915.2008.00713.x

Billeter, S. A., Borchert, J. N., Atiku, L. A., Mpanga, J. T., Gage, K. L., & Kosoy, M. Y. (2014). *Bartonella* species in invasive rats and indigenous rodents from Uganda. *Vector-Borne and Zoonotic Diseases*, 14(3), 182–188. https://doi.org/10.1089/vbz.2013.1375

Billeter, S. A., Gundi, V. A. K. B., Rood, M. P., & Kosoy, M. Y. (2011). Molecular detection and identification of *Bartonella* species in *Xenopsylla cheopis* fleas (Siphonaptera: Pulicidae) collected from *Rattus norvegicus* rats in Los Angeles, California. *Applied and Environmental Microbiology*, 77(21), 7850–7852. https://doi.org/10.1128/AEM.06012-11

Birtles, R. J., Harrison, T. G., Saunders, N. A., & Molyneux, D. H. (1995). Proposals to unify the genera *Grahamella* and *Bartonella*, with descriptions of *Bartonella talpae* comb. nov., *Bartonella peromysci* comb. nov., and three new species, *Bartonella grahamii* sp. nov., *Bartonella taylorii*. *International Journal of Systematic Bacteriology*, 45(1), 1–8. https://doi.org/10.1099/00207713-45-1-1

Birtles, R. J., Hazel, S. M., Bennett, M., Bown, K., Raoult, D., & Begon, M. (2001). Longitudinal monitoring of the dynamics of infections due to *Bartonella* species in UK woodland rodents. *Epidemiology and Infection*, 126(2), 323–329. https://doi.org/10.1017/S095026880100526X

Birtles, R. J. (1995). Differentiation of *Bartonella* species using restriction endonuclease analysis of PCR-amplified 16S rRNA genes. *FEMS Microbiology Letters*, 129(2–3), 261–265. https://doi.org/10.1016/0378-1097(95)00169-6

Bolaños-Rivero, M., Carranza-Rodríguez, C., Rodríguez, N. F., Gutiérrez, C., & Pérez-Arellano, J. L. (2017). Detection of *Coxiella burnetii* DNA in Peridomestic and Wild Animals and Ticks in an Endemic Region (Canary Islands, Spain). *Vector-Borne and Zoonotic Diseases*, 17(9), 630–634. https://doi.org/10.1089/vbz.2017.2120

Bordes, F., Blasdell, K., & Morand, S. (2015). Transmission ecology of rodent-borne diseases: New frontiers. *Integrative Zoology*, 10(5), 424-435. https://doi.org/10. 1111/1749-4877.12149

Bosshard, P. P., Kronenberg, A., Zbinden, R., Ruef, C., Bottger, E. C., & Altwegg, M. (2003). Etiologic Diagnosis of Infective Endocarditis by Broad-Range Polymerase Chain Reaction: A 3-Year Experience. *Clinical Infectious Diseases*, 37(2), 167-172. https://doi.org/10. 1086/375592

Boulouis, H. J., Chang, C. C., Henn, J. B., Kasten, R. W., & Chomel, B. B. (2005). Factors associated with the rapid emergence of zoonotic *Bartonella* infections. *Veterinary Research*, 36(3), 383–410. https://doi.org/10.1051/vetres:2005009

Bouvery, N. A., Souriau, A., Lechopier, P., & Rodolakis, A. (2003). Experimental *Coxiella burnetii* infection in pregnant goats: Excretion routes. *Veterinary Research*, 34(4), 423–433. https://doi.org/10.1051/vetres:2003017

Bouyou-Akotet, M. K., Mawili-Mboumba, D. P., Kendjo, E., Mabika-Mamfoumbi, M., Ngoungou, E. B., Dzeing-Ella, A., Pemba-Mihindou, M., Ibinga, E., Efame-Eya, E., Planche, T., Kremsner, P. G., & Kombila, M. (2009). Evidence of decline of malaria in the general hospital of Libreville, Gabon from 2000 to 2008. *Malaria Journal*, 8(1), 300. https://doi.org/10.1186/1475-2875-8-300

Breitschwerdt, E. B. (2017). Bartonellosis, One Health and all creatures great and small. *Veterinary Dermatology*, 28(1), 96-e21. https://doi.org/10.1111/vde.12413

Breitschwerdt, E. B., & Kordick, D. L. (2000). *Bartonella* infection in animals: Carriership, reservoir potential, pathogenicity, and zoonotic potential for human infection. *Clinical Microbiology Reviews*, 13(3), 428–438. https://doi.org/10.1128/CMR.13.3.428-438. 2000

Breitschwerdt, E. B., Mascarelli, P. E., Schweickert, L. A., Maggi, R. G., Hegarty, B. C., Bradley, J. M., & Woods, C. W. (2011). Hallucinations, sensory neuropathy, and peripheral visual deficits in a young woman infected with *Bartonella koehlerae*. *Journal of Clinical Microbiology*, 49(9), 3415–3417. https://doi.org/10.1128/JCM.00833-11

Brenner, D. J., O'Connor, S. P., Winkler, H. H., & Steigerwalt, A. G. (1993). Proposals to unify the genera *Bartonella* and Rochalimaea, with descriptions of *Bartonella quintana* comb. nov., *Bartonella vinsonii* comb. nov., *Bartonella henselae* comb. nov., and *Bartonella elizabethae* comb. nov., and to. *International Journal of Systematic Bacteriology*, 43(4), 777–786. https://doi.org/10.1099/00207713-43-4-777

Brenner, S. A., Rooney, J. A., Manzewitsch, P., & Regnery, R. L. (1997). Isolation of *Bartonella (Rochalimaea) henselae*: Effects of methods of blood collection and handling. *Journal of Clinical Microbiology*, 35(3), 544–547. https://doi.org/10.1128/jcm.35.3.544-547.1997

Brooke, R. J., Kretzschmar, M. E. E., Mutters, N. T., & Teunis, P. F. (2013). Human dose response relation for airborne exposure to *Coxiella burnetii*. *BMC Infectious Diseases*, 13(1), 488. https://doi.org/10.1186/1471-2334-13-488

Brooke, R. J., Mutters, N. T., Péter, O., Kretzschmar, M. E. E., & Teunis, P. F. M. (2015). Exposure to low doses of *Coxiella burnetii* caused high illness attack rates: Insights from combining human challenge and outbreak data. *Epidemics*, 11, 1–6. https://doi.org/10.1016/j.epidem.2014.12.004

Brouqui, P., Lascola, B., Roux, V., & Raoult, D. (1999). Chronic *Bartonella quintana* bacteremia in homeless patients. *New England Journal of Medicine*, 340(3), 184–189. https://doi.org/10.1056/NEJM199901213400303

Buffet, J. P., Kosoy, M., & Vayssier-Taussat, M. (2013). Natural history of *Bartonella*-infecting rodents in light of new knowledge on genomics, diversity and evolution. *Future Microbiology*, 8(9), 1117–1128. https://doi.org/10.2217/fmb.13.77

Buffet, J. P., Pisanu, B., Brisse, S., Roussel, S., Félix, B., Halos, L., Chapuis, J. L., & Vayssier-Taussat, M. (2013). Deciphering *Bartonella* Diversity, Recombination, and Host Specificity in a Rodent Community. *PLoS ONE*, 8(7), e68956. https://doi.org/10.1371/journal.pone.0068956

Burgdorfer, W., Pickens, E. G., Newhouse, V. F., & Lackman, D. B. (1963). Isolation of *Coxiella burnetii* from rodents in western montana. *Journal of Infectious Diseases*, 112(2), 181–186. https://doi.org/10.1093/infdis/112.2.181

Buzan, E., Zupan, S., & Jugovic, J. (2017). Changes in rodent communities as consequence of urbanization and inappropriate waste management. *Applied Ecology and Environmental Research*, 15(1), 573–588. https://doi.org/10.15666/aeer/1501_573588

Castle, K. T., Kosoy, M., Lerdthusnee, K., Phelan, L., Bai, Y., Gage, K. L., Leepitakrat, W., Monkanna, T., Khlaimanee, N., Chandranoi, K., Jones, J. W., & Coleman, R. E. (2004). Prevalence and diversity of *Bartonella* in rodents of northern Thailand: A comparison with *Bartonella* in rodents from southern China. *American Journal of Tropical Medicine and Hygiene*, 70(4), 429–433. https://doi.org/10.4269/ajtmh.2004.70.429

Chipwaza, B., Mhamphi, G. G., Ngatunga, S. D., Selemani, M., Amuri, M., Mugasa, J. P., & Gwakisa, P. S. (2015). Prevalence of Bacterial Febrile Illnesses in Children in Kilosa District, Tanzania. *PLoS Neglected Tropical Diseases*, 9(5), e0003750. https://doi.org/10.1371/ journal.pntd.0003750

Chitanga, S., Simulundu, E., Simuunza, M. C., Changula, K., Qiu, Y., Kajihara, M., Nakao, R., Syakalima, M., Takada, A., Mweene, A. S., Mukaratirwa, S., & Hang'ombe, B. M. (2018). First molecular detection and genetic characterization of *Coxiella burnetii* in Zambian dogs and rodents. *Parasites and Vectors*, 11(1), 40. https://doi.org/10.1186/s13071-018-2629-7

Chomel, B. B., & Kasten, R. W. (2010). Bartonellosis, an increasingly recognized zoonosis. *Journal of Applied Microbiology*, 109(3), 743–750. https://doi.org/10.1111/j.1365-2672.2010.04679.x

Chomel, B. B., Boulouis, H. J., Maruyama, S., & Breitschwerdt, E. B. (2006). *Bartonella* spp. in pets and effect on human health. *Emerging Infectious Diseases*, 12(3), 389–394. https://doi.org/10.3201/eid1203.050931

Chomel, B. B., Molia, S., Kasten, R. W., Borgo, G. M., Stuckey, M. J., Maruyama, S., Chang, C. C., Haddad, N., & Koehler, J. E. (2016). Isolation of *Bartonella henselae* and Two New *Bartonella* Subspecies, *Bartonella koehlerae* Subspecies boulouisii subsp. nov. and *Bartonella koehlerae* Subspecies bothieri subsp. nov. from Free-Ranging Californian Mountain Lions an. *PloS One*, 11(3), e0148299. https://doi.org/10.1371/journal.pone.0148299

Chomel, B. B., Wey, A. C., & Kasten, R. W. (2003). Isolation of *Bartonella washoensis* from a Dog with Mitral Valve Endocarditis. *Journal of Clinical Microbiology*, 41(11), 5327–5332. https://doi.org/10.1128/JCM.41.11.5327-5332.2003

Christou, L. (2011). The global burden of bacterial and viral zoonotic infections. *Clinical Microbiology and Infection*, 17(3), 326–330. https://doi.org/10.1111/j.1469-0691.2010.03441.x

Comer, J. A., Paddock, C. D., & Childs, J. E. (2001). Urban zoonoses caused by *Bartonella*, *Coxiella*, *Ehrlichia*, and *Rickettsia* species. *Vector Borne and Zoonotic Diseases (Larchmont, N. Y.)*, 1(2), 91–118. https://doi.org/10.1089/153036601316977714

Cook, M. (2006). Manual of Molecular and Clinical Laboratory Immunology. *Immunology and Cell Biology*, 84(6), 563–563. https://doi.org/10.1111/j.1440-1711.2006.01474.x

Crăcea, E. (1987). Q fever laboratory diagnostic methods used in Roumania. *Zentralblatt Für Bakteriologie, Mikrobiologie Und Hygiene. Series A: Medical Microbiology, Infectious Diseases, Virology, Parasitology*, 267(1), 64–66. https://doi.org/10.1016/S0176-6724(87)80188-8

Crump, J. A., & Kirk, M. D. (2015). Estimating the Burden of Febrile Illnesses. *PLOS Neglected Tropical Diseases*, 9(12), e0004040. https://doi.org/10.1371/journal.pntd.0004040

Crump, J. A., Morrissey, A. B., Nicholson, W. L., Massung, R. F., Stoddard, R. A., Galloway, R. L., Ooi, E. E., Maro, V. P., Saganda, W., Kinabo, G. D., Muiruri, C., & Bartlett, J. A. (2013). Etiology of Severe Non-malaria Febrile Illness in Northern Tanzania: A Prospective Cohort Study. *PLoS Neglected Tropical Diseases*, 7(7), e2324. https://doi.org/10.1371/journal.pntd.0002324

Cunningham, D. M., & Moors, P. J. (1993). A guide to the identification and collection of New Zealand rodents. *New Zealand Wildlife Service Occasional Publication*, 4, 1–19.

D'Acremont, V., Lengeler, C., & Genton, B. (2010). Reduction in the proportion of fevers associated with Plasmodium falciparum parasitaemia in Africa: A systematic review. *Malaria Journal*, 9(1), 240. https://doi.org/10.1186/1475-2875-9-240

Daszak, P., Cunningham, A. A., & Hyatt, A. D. (2001). Anthropogenic environmental change and the emergence of infectious diseases in wildlife. *Acta Tropica*, 78(2), 103–116. https://doi.org/10.1016/S0001-706X(00)00179-0

Davis, G. E., Cox, H. R., Parker, R. R., & Dyer, R. E. (1938). A Filter-Passing Infectious Agent Isolated from Ticks. *Public Health Reports* (1896-1970), 53(52), 2259. https://doi.org/10.2307/4582746

De Rooij, M. M. T., Borlée, F., Smit, L. A. M., De Bruin, A., Janse, I., Heederik, D. J. J., & Wouters, I. M. (2016). Detection of *Coxiella burnetii* in ambient air after a large Q fever outbreak. *PLoS ONE*, 11(3), e0151281. https://doi.org/10.1371/journal.pone.0151281

Dehio, C., Lanz, C., Pohl, R., Behrens, P., Bermond, D., Piémont, Y., Pelz, K., & Sander, A. (2001). *Bartonella schoenbuchii* sp. nov., isolated from the blood of wild roe deer. *International Journal of Systematic and Evolutionary Microbiology*, 51(4), 1557–1565. https://doi.org/10.1099/00207713-51-4-1557

Delsing, C. E., & Kullberg, B. J. (2008). Q fever in the Netherlands: A concise overview and implications of the largest ongoing outbreak. *Netherlands Journal of Medicine*, 66(9), 365-367.

Demers, D. M., Bass, J. W., Vincent, J. M., Person, D. A., Noyes, D. K., Staege, C. M., Samlaska, C. P., Lockwood, N. H., Regnery, R. L., & Anderson, B. E. (1995). Cat-scratch disease in Hawaii: Etiology and seroepidemiology. *The Journal of Pediatrics*, 127(1), 23-26. https://doi.org/10.1016/S0022-3476(95)70251-2

Derrick, E. H. (1983). "Q" fever, a new fever entity: clinical features, diagnosis and laboratory investigation. *Reviews of Infectious Diseases*, 5(4), 790–800. https://doi.org/10.5694/j.1326-5377.1937.tb43743.x

Diaz, M. H., Bai, Y., Malania, L., Winchell, J. M., & Kosoy, M. Y. (2012). Development of a novel genus-specific real-time PCR assay for detection and differentiation of *Bartonella* species and genotypes. *Journal of Clinical Microbiology*, 50(5), 1645–1649. https://doi.org/10.1128/JCM.06621-11

Döller, G., Döller, P. C., & Gerth, H. J. (1984). Early diagnosis of Q fever: Detection of immunoglobulin M by radioimmunoassay and enzyme immunoassay. *European Journal of Clinical Microbiology*, 3(6), 550–553. https://doi.org/10.1007/BF02013617

Dorai-Raj, S. (2014). binom: Binomial confidence intervals for several parameterizations. *R Package Version*, 1, 1.

Drancourt, M., Tran-Hung, L., Courtin, J., Lumley, H. de, & Raoult, D. (2005). *Bartonella quintana* in a 4000-Year-Old Human Tooth. *The Journal of Infectious Diseases*, 191(4), 607–611. https://doi.org/10.1086/427041

Duron, O. (2015). The IS1111 insertion sequence used for detection of *Coxiella burnetii* is widespread in Coxiella-like endosymbionts of ticks. *FEMS Microbiology Letters*, 362(17), fnv132. https://doi.org/10.1093/femsle/fnv132

Ehizibolo, D. O., Ehizibolo, P. O., Ehizibolo, E. E., Sugun, M. Y., & Idachaba, S. E. (2011). The control of neglected zoonotic diseases in Nigeria through animal intervention. *African Journal of Biomedical Research*, 14(2), 81–88.

Ellis, B. A., Regnery, R. L., Beati, L., Bacellar, F., Rood, M., Glass, G. G., Marston, E., Ksiazek, T. G., Jones, D., & Childs, J. E. (1999). Rats of the Genus *Rattus* are Reservoir Hosts for Pathogenic *Bartonella* Species: An Old World Origin for a New World Disease? *The Journal of Infectious Diseases*, 180(1), 220–224. https://doi.org/10.1086/314824

English, C. K., Wear, D. J., Margileth, A. M., Lissner, C. R., & Walsh, G. P. (1988). Cat-Scratch Disease: Isolation and Culture of the Bacterial Agent. *The Journal of the American Medical Association*, 259(9), 1347–1352. https://doi.org/10.1001/jama.1988.03720090037031

Enria, D. A., & Pinheiro, F. (2000). Rodent-Borne emerging viral zoonosis: Hemorrhagic fevers and hantavirus infections in South America. *Infectious Disease Clinics of North America*, 14(1), 167–184. https://doi.org/10.1016/S0891-5520(05)70223-3

Eremeeva, M. E., Gerns, H. L., Lydy, S. L., Goo, J. S., Ryan, E. T., Mathew, S. S., Ferraro, M. J., Holden, J. M., Nicholson, W. L., Dasch, G. A., & Koehler, J. E. (2007). Bacteremia, fever, and splenomegaly caused by a newly recognized *Bartonella* species. *New England Journal of Medicine*, 356(23), 2381–2387. https://doi.org/10.1056/NEJMoa065987

Faix, D. J., Harrison, D. J., Riddle, M. S., Vaughn, A. F., Yingst, S. L., Earhart, K., & Thibault, G. (2008). Outbreak of Q Fever among US Military in Western Iraq, June–July 2005. *Clinical Infectious Diseases*, 46(7), e65–e68. https://doi.org/10.1086/528866

Fenollar, F., Sire, S., & Raoult, D. (2005). *Bartonella vinsonii* subsp. arupensis as an agent of blood culture-negative endocarditis in a human. *Journal of Clinical Microbiology*, 43(2), 945–947. https://doi.org/10.1128/JCM.43.2.945-947.2005

Firth, C., Bhat, M., Firth, M. A., Williams, S. H., Frye, M. J., Simmonds, P., Conte, J. M., Ng, J., Garcia, J., Bhuva, N. P., Lee, B., Che, X., Quan, P. L., & Ian Lipkin, W. (2014). Detection of zoonotic pathogens and characterization of novel viruses carried by commensal *Rattus norvegicus* in New York city. *MBio*, 5(5). https://doi.org/10.1128/mBio.01933-14

Foronda, P., Plata-Luis, J., del Castillo-Figueruelo, B., Fernández-Álvarez, Á., Martín-Alonso, A., Feliu, C., Cabral, M. D., & Valladares, B. (2015). Serological survey of antibodies to *Toxoplasma gondii* and *Coxiella burnetii* in rodents in north-Western African islands (Canary Islands and Cape Verde). *Onderstepoort Journal of Veterinary Research*, 82(1), 4–7. https://doi.org/10.4102/ojvr.v82i1.899

Foucault, C., Barrau, K., Brouqui, P., & Raoult, D. (2002). *Bartonella quintana* Bacteremia among Homeless People. *Clinical Infectious Diseases*, 35(6), 684–689. https://doi.org/10.1086/342065

Fournier, P. E., Lelievre, H., Eykyn, S. J., Mainardi, J. L., Marrie, T. J., Bruneel, F., Roure, C., Nash, J., Clave, D., James, E., Benoit-Lemercier, C., Deforges, L., Tissot-Dupont, H., & Raoult, D. (2001). Epidemiologic and clinical characteristics of *Bartonella quintana* and *Bartonella henselae* endocarditis: A study of 48 patients. *Medicine*, 80(4), 245–251. https://doi.org/10.1097/00005792-200107000-00003

Fournier, P. E., Marrie, T. J., & Raoult, D. (1998). Minireview Diagnosis of Q Fever. *Journal of Clinical Microbiology*, 36(7), 1823–1834.

Frank, H. K., Boyd, S. D., & Hadly, E. A. (2018). Global fingerprint of humans on the distribution of *Bartonella* bacteria in mammals. *PLoS Neglected Tropical Diseases*, 12(11), 1–17. https://doi.org/10.1371/journal.pntd.0006865

Gil, H., Garcia-Esteban, C., Barandika, J. F., Peig, J., Toledo, A., Escudero, R., Jado, I., Rodriguez-Vargas, M., Garcia-Amil, C., Lobo, B., Roales, P., Rodriguez-Moreno, I., Olmeda, A. S., Garcia-Perez, A. L., & Anda, P. (2010). Variability of *Bartonella* Genotypes among Small Mammals in Spain. *Applied and Environmental Microbiology*, 76(24), 8062–8070. https://doi.org/10.1128/AEM.01963-10

Goral, S., Anderson, B., Hager, C., & Edwards, K. M. (1994). Detection of *Rochalimoea henselae* DNA by polymerase chain reaction from suppurative nodes of children with cat-scratch disease. *Pediatric Infectious Disease Journal*, 13(11), 994–997. https://doi.org/10.1097/00006454-199411000-00011

Gratz, N. G. (1999). Urbanization, arthropod and rodent pests and human health. *Proceedings of the 3rd International Conference on Urban Pests*, 51–58.

Grilc, E., Socan, M., Koren, N., Ucakar, V., Avsic, T., Pogacnik, M., & Kraigher, A. (2007). Outbreak of Q fever among a group of high school students in Slovenia, March-April 2007. *Euro Surveillance: Bulletin Européen Sur Les Maladies Transmissibles = European Communicable Disease Bulletin*, 12(7). https://doi.org/10.2807/esw.12.29.03237-en

Gubler, D. J., Reiter, P., Ebi, K. L., Yap, W., Nasci, R., & Patz, J. A. (2001). Climate variability and change in the United States: Potential impacts on vector- and Rodent-Borne diseases. *Environmental Health Perspectives*, 109(2), 223–233. https://doi.org/10.2307/3435012

Gundi, V. A. K. B., Billeter, S. A., Rood, M. P., & Kosoy, M. Y. (2012). *Bartonella* spp. in rats and Zoonoses, Los Angeles, California, USA. *Emerging Infectious Diseases*, 18(4), 631–633. https://doi.org/10.3201/eid1804.110816

Gundi, V. A. K. B., Davoust, B., Khamis, A., Boni, M., Raoult, D., & La Scola, B. (2004). Isolation of *Bartonella rattimassiliensis* sp. nov. and *Bartonella phoceensis* sp. nov. from European *Rattus norvegicus*. *Journal of Clinical Microbiology*, 42(8), 3816–3818. https://doi.org/10.1128/JCM.42.8.3816-3818.2004

Gundi, V. A. K. B., Kosoy, M. Y., Makundi, R. H., & Laudisoit, A. (2012). Identification of diverse *Bartonella* genotypes among small mammals from democratic Republic of Congo and Tanzania. *American Journal of Tropical Medicine and Hygiene*, 87(2), 319–326. https://doi.org/10.4269/ajtmh.2012.11-0555

Gundi, V. A. K. B., Kosoy, M. Y., Myint, K. S. A., Shrestha, S. K., Shrestha, M. P., Pavlin, J. A., & Gibbons, R. V. (2010). Prevalence and genetic diversity of *Bartonella* species detected in different tissues of small mammals in Nepal. *Applied and Environmental Microbiology*, 76(24), 8247–8254. https://doi.org/10.1128/AEM.01180-10

Gundi, V. A. K. B., Taylor, C., Raoult, D., & La Scola, B. (2009). *Bartonella rattaustraliani* sp. nov., *Bartonella queenslandensis* sp. nov. and *Bartonella coopersplainsensis* sp. nov., identified in Australian rats. *International Journal of Systematic and Evolutionary Microbiology*, 59(12), 2956–2961. https://doi.org/10.1099/ijs.0.002865-0

Guracha, A., Kalani, R., Njenga, M. K., Njuguna, C., Feikin, D. R., Ari, M. D., Pimentel, G., Breiman, R. F., Omballa, V., Tetteh, C., Warfu, O., Fadeel, M. A., & Abdi, H. (2011). Challenges of Establishing the Correct Diagnosis of Outbreaks of Acute Febrile Illnesses in Africa: The Case of a Likely Brucella Outbreak among Nomadic Pastoralists, Northeast Kenya, March–July 2005. *The American Journal of Tropical Medicine and Hygiene*, 85(5), 909–912. https://doi.org/10.4269/ajtmh.2011.11-0030

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor. *Nucleic Acids Symposium Series*, 41, 95-98.

Halliday, J., Daborn, C., Auty, H., Mtema, Z., Lembo, T., Bronsvoort, B. M. D., Handel, I., Knobel, D., Hampson, K., & Cleaveland, S. (2012). Bringing together emerging and endemic zoonoses surveillance: Shared challenges and a common solution. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, 367(1604), 2872–2880. https://doi.org/10.1098/rstb.2011.0362

Halliday, J. E. B., Allan, K. J., Ekwem, D., Cleaveland, S., Kazwala, R. R., & Crump, J. A. (2015). Endemic zoonoses in the tropics: a public health problem hiding in plain sight. *Veterinary Record*, 176(9), 220–225. https://doi.org/10.1136/vr.h798

Halliday, J. E. B., Knobel, D. L., Agwanda, B., Bai, Y., Breiman, R. F., Cleaveland, S., Njenga, M. K., & Kosoy, M. (2015). Prevalence and Diversity of Small Mammal-Associated *Bartonella* Species in Rural and Urban Kenya. *PLoS Neglected Tropical Diseases*, 9(3), e0003608. https://doi.org/10.1371/journal.pntd.0003608

Handley, S. A., & Regnery, R. L. (2000). Differentiation of pathogenic *Bartonella* species by infrequent restriction site PCR. *Journal of Clinical Microbiology*, 38(8), 3010–3015. https://doi.org/10.1128/jcm.38.8.3010-3015.2000

Harman, J. B. (1949). Q Fever in Great Britain Clinical Account of Eight Cases. *The Lancet*, 254(6588), 1028–1030. https://doi.org/10.1016/S0140-6736(49)91600-1

Harrus, S., & Baneth, G. (2005). Drivers for the emergence and re-emergence of vector-borne protozoal and bacterial diseases. *International Journal for Parasitology*, 35(11-12), 1309-1318. https://doi.org/10.1016/j.ijpara.2005.06.005

Hayman, D. T. S., McDonald, K. D., & Kosoy, M. Y. (2013). Evolutionary history of rat-borne *Bartonella*: The importance of commensal rats in the dissemination of bacterial infections globally. *Ecology and Evolution*, 3(10), 3195–3203. https://doi.org/10.1002/ece3.702

Heller, R., Kubina, M., Mariet, P., Riegel, P., Delacour, G., Dehio, C., Lamarque, F., Kasten, R., Boulouis, H. J., Monteil, H., Chomel, B., & Piémont, Y. (1999). *Bartonella alsatica* sp. nov., a new *Bartonella* species isolated from the blood of wild rabbits. *International Journal of Systematic Bacteriology*, 49(1), 283–288. https://doi.org/10.1099/00207713-49-1-283

Heller, R., Riegel, P., Hansmann, Y., Delacour, G., Bermond, D., Dehio, C., Lamarque, F., Monteil, H., Chomel, B., & Piémont, Y. (1998). *Bartonella tribocorum* sp. nov., a new *Bartonella* species isolated from the blood of wild rats. *International Journal of Systematic Bacteriology*, 48(4), 1333–1339. https://doi.org/10.1099/00207713-48-4-1333

Henn, J. B., Chomel, B. B., Boulouis, H. J., Kasten, R. W., Murray, W. J., Bar-Gal, G. K., King, R., Courreau, J. F., & Baneth, G. (2009). *Bartonella rochalimae* in raccoons, coyotes, and red foxes. *Emerging Infectious Diseases*, 15(12), 1984–1987. https://doi.org/10.3201/eid1512.081692

Hersch, E. C., & Oh, R. C. (2014). Prolonged febrile illness and fever of unknown origin in adults. *American Family Physician*, 90(2), 91–96.

Hildenwall, H., Amos, B., Mtove, G., Muro, F., Cederlund, K., & Reyburn, H. (2016). Causes of non-malarial febrile illness in outpatients in Tanzania. *Tropical Medicine & International Health*, 21(1), 149–156. https://doi.org/10.1111/tmi.12635

Hjelle, B., & Glass, G. E. (2000). Outbreak of Hantavirus Infection in the Four Corners Region of the United States in the Wake of the 1997–1998 El Niño–Southern Oscillation. *The Journal of Infectious Diseases*, 181(5), 1569–1573. https://doi.org/10.1086/315467

Holmberg, M., Mills, J. N., McGill, S., Benjamin, G., & Ellis, B. A. (2003). *Bartonella* infection in sylvatic small mammals of central Sweden. *Epidemiology and Infection*, 130(1), 149–157. https://doi.org/10.1017/S0950268802008075

Home Office. (2014). Guidance on the operation of the Animals (Scientific Procedures) Act 1986. In *Publications GOV.UK*. https://doi.org/ISBN 1474100287- 9781474100281

Honarmand, H. (2012). Q Fever: An old but still a poorly understood disease. *Interdisciplinary Perspectives on Infectious Diseases*, *2012*, 131932. https://doi.org/10.1155/2012/131932

Horigan, M. W., Bell, M. M., Pollard, T. R., Sayers, A. R., & Pritchard, G. C. (2011). Q fever diagnosis in domestic ruminants: Comparison between complement fixation and commercial enzyme-linked immunosorbent assays. *Journal of Veterinary Diagnostic Investigation*, 23(5), 924–931. https://doi.org/10.1177/1040638711416971

Huarcaya, E., Maguiña, C., Torres, R., Rupay, J., & Fuentes, L. (2006). Bartonelosis (Carrion's Disease) in the pediatric population of Peru: An overview and update. *Brazilian Journal of Infectious Diseases*, 8(5), 331–339. https://doi.org/10.1590/s1413-86702004000500001

Huchon, D., Madsen, O., Sibbald, M. J. J. B., Ament, K., Stanhope, M. J., Catzeflis, F., De Jong, W. W., & Douzery, E. J. P. (2002). Rodent phylogeny and a timescale for the evolution of glires: Evidence from an extensive taxon sampling using three nuclear genes. *Molecular Biology and Evolution, 19(7), 1053-1065*. https://doi.org/10.1093/oxfordjournals. molbev.a004164

Ihler, G. M. (1996). *Bartonella bacilliformis*: Dangerous pathogen slowly emerging from deep background. *FEMS Microbiology Letters*, 144(1), 1–11. https://doi.org/10.1016/0378-1097(96)00307-2

Inoue, K., Kabeya, H., Shiratori, H., Ueda, K., Kosoy, M. Y., Chomel, B. B., Boulouis, H. J., & Maruyama, S. (2010). *Bartonella japonica* sp. nov. and *Bartonella silvatica* sp. nov., isolated from Apodemus mice. *International Journal of Systematic and Evolutionary Microbiology*, 60(4), 759–763. https://doi.org/10.1099/ijs.0.011528-0

Izquierdo-Rodríguez, E., Fernández-Álvarez, Á., Martín-Carrilo, N., Feliu, C., Marchand, B., Quilichini, Y., & Foronda, P. (2019). Rodents as Reservoirs of the Zoonotic Pathogens *Coxiella burnetii* and *Toxoplasma gondii* in Corsica (France). *Vector-Borne and Zoonotic Diseases*, 19(12), 879–883. https://doi.org/10.1089/vbz.2019. 2485

Jardine, C., Appleyard, G., Kosoy, M. Y., McColl, D., Chirino-Trejo, M., Wobeser, G., & Leighton, F. A. (2005). Rodent-Associated *Bartonella* in Saskatchewan, Canada. *Vector-Borne and Zoonotic Diseases*, 5(4), 402–409. https://doi.org/10.1089/vbz.2005.5.402

Jones, K. E., Patel, N. G., Levy, M. A., Storeygard, A., Balk, D., Gittleman, J. L., & Daszak, P. (2008). Global trends in emerging infectious diseases. *Nature*, 451(7181), 990–993. https://doi.org/10.1038/nature06536

Kaewmongkol, G., Kaewmongkol, S., Fleming, P. A., Adams, P. J., Ryan, U., Irwin, P. J., & Fenwick, S. G. (2011). Zoonotic *Bartonella* species in fleas and blood from red foxes in australia. *Vector-Borne and Zoonotic Diseases*, 11(12), 1549–1553. https://doi.org/10.1089/vbz.2011.0646

Kamani, J., Baneth, G., Gutiérrez, R., Nachum-Biala, Y., Mumcuoglu, K. Y., & Harrus, S. (2018). *Coxiella burnetii* and *Rickettsia conorii*: Two zoonotic pathogens in peridomestic rodents and their ectoparasites in Nigeria. *Ticks and Tick-Borne Diseases*, 9(1), 86–92. https://doi.org/10.1016/j.ttbdis.2017.10.004

Kamani, J., Morick, D., Mumcuoglu, K. Y., & Harrus, S. (2013). Prevalence and Diversity of *Bartonella* Species in Commensal Rodents and Ectoparasites from Nigeria, West Africa. *PLoS Neglected Tropical Diseases*, 7(5), e2246. https://doi.org/10.1371/journal.pntd.0002246

Karem, K. L., Paddock, C. D., & Regnery, R. L. (2000). *Bartonella henselae*, *B. quintana*, and *B. bacilliformis*: Historical pathogens of emerging significance. *Microbes and Infection*, 2(10), 1193–1205. https://doi.org/10.1016/S1286-4579(00)01273-9

Kešnerová, L., Moritz, R., & Engel, P. (2016). *Bartonella apis* sp. nov., a honey bee gut symbiont of the class Alphaproteobacteria. *International Journal of Systematic and Evolutionary Microbiology*, 66(1), 414–421. https://doi.org/10.1099/ijsem.0.000736

Khaghani, R. (2007). The economic and health impact of rodent in urban zone and harbours and their control method. *Annals of Military and Health Sciences Research*, 4(4), 1071–1078.

Klaasen, M., Roest, H. J., Van Der Hoek, W., Goossens, B., Secka, A., & Stegeman, A. (2014). *Coxiella burnetii* seroprevalence in small ruminants in the Gambia. *PLoS ONE*, 9(1), e85424. https://doi.org/10.1371/journal.pone.0085424

Klangthong, K., Promsthaporn, S., Leepitakrat, S., Schuster, A. L., McCardle, P. W., Kosoy, M., & Takhampunya, R. (2015). The distribution and diversity of *Bartonella* species in rodents and their ectoparasites across Thailand. *PLoS ONE*, 10(10), 1–16. https://doi.org/10.1371/journal.pone.0140856

Klee, S. R., Tyczka, J., Ellerbrok, H., Franz, T., Linke, S., Baljer, G., & Appel, B. (2006). Highly sensitive real-time PCR for specific detection and quantification of *Coxiella burnetii*. *BMC Microbiology*, 6, 1–8. https://doi.org/10.1186/1471-2180-6-2

Koehler, J. E., Glaser, C. A., & Tappero, J. W. (1994). *Rochalimaea henselae* Infection: A New Zoonosis With the Domestic Cat as Reservoir. *The Journal of the American Medical Association*, 271(7), 531–535. https://doi.org/10.1001/jama.1994.03510310061039

Koehler, J. E., Sanchez, M. A., Garrido, C. S., Whitfeld, M. J., Chen, F. M., Berger, T. G., Rodriguez-Barradas, M. C., LeBoit, P. E., & Tappero, J. W. (1997). Molecular epidemiology of *Bartonella* infections in patients with bacillary angiomatosis-peliosis. *New England Journal of Medicine*, 337(26), 1876–1883. https://doi.org/10.1056/NEJM199712253372603

Koehler, J. E., Sanchez, M. A., Tye, S., Garrido-Rowland, C. S., Chen, F. M., Maurer, T., Cooper, J. L., Olson, J. G., Reingold, A. L., Hadley, W. K., Regnery, R. R., & Tappero, J. W. (2003). Prevalence of *Bartonella* Infection among Human Immunodeficiency Virus—Infected Patients with Fever. *Clinical Infectious Diseases*, 37(4), 559–566. https://doi.org/10.1086/375586

Kordick, D. L., Hilyard, E. J., Hadfield, T. L., Wilson, K. H., Steigerwalt, A. G., Brenner, D. J., & Breitschwerdt, E. B. (1997). *Bartonella clarridgeiae*, a newly recognized zoonotic pathogen causing inoculation papules, fever, and lymphadenopathy (cat scratch disease). *Journal of Clinical Microbiology*, 35(7), 1813–1818. https://doi.org/10.1128/jcm.35.7.1813-1818.1997

Kosoy, M., Bai, Y., Enscore, R., Rizzo, M. R., Bender, S., Popov, V., Albayrak, L., Fofanov, Y., & Chomel, B. (2016). *Bartonella melophagi* in blood of domestic sheep (Ovis aries) and sheep keds (Melophagus ovinus) from the southwestern US: Cultures, genetic characterization, and ecological connections. *Veterinary Microbiology*, 190, 43–49. https://doi.org/10.1016/j.vetmic.2016.05.009

Kosoy, M., Bai, Y., Sheff, K., Morway, C., Baggett, H., Maloney, S. A., Boonmar, S., Bhengsri, S., Dowell, S. F., Sitdhirasdr, A., Lerdthusnee, K., Richardson, J., & Peruski, L. F. (2010). Identification of *Bartonella* infections in febrile human patients from Thailand and their potential animal reservoirs. *American Journal of Tropical Medicine and Hygiene*, 82(6), 1140–1145. https://doi.org/10.4269/ajtmh.2010.09-0778

Kosoy, M., Morway, C., Sheff, K. W., Bai, Y., Colborn, J., Chalcraft, L., Dowell, S. F., Peruski, L. F., Maloney, S. A., Baggett, H., Sutthirattana, S., Sidhirat, A., Maruyama, S., Kabeya, H., Chomel, B. B., Kasten, R., Popov, V., Robinson, J., Kruglov, A., & Petersen, L. R. (2008). *Bartonella tamiae* sp. nov., a Newly Recognized Pathogen Isolated from Three Human Patients from Thailand. *Journal of Clinical Microbiology*, 46(2), 772–775. https://doi.org/10.1128/JCM.02120-07

Kosoy, M., Murray, M., Gilmore, R. D., Bai, Y., & Gage, K. L. (2003). *Bartonella* strains from ground squirrels are identical to *Bartonella washoensis* isolated from a human patient. *Journal of Clinical Microbiology*, 41(2), 645–650. https://doi.org/10.1128/JCM.41.2.645-650.2003

Kosoy, M. Y., Saito, E. K., Green, D., Marston, E. L., Jones, D. C., & Childs, J. E. (2000). Experimental evidence of host specificity of *Bartonella* infection in rodents. *Comparative Immunology, Microbiology and Infectious Diseases*, 23(4), 221–238. https://doi.org/10.1016/S0147-9571(99)00075-2

Kosoy, Michael Y., Regnery, R. L., Kosaya, O. I., Jones, D. C., Marston, E. L., & Childs, J. E. (1998). Isolation of *Bartonella* spp. from embryos and neonates of naturally infected rodents. *Journal of Wildlife Diseases*, 34(2), 305–309. https://doi.org/10.7589/0090-3558-34.2.305

Kosoy, Michael Y., Regnery, R. L., Tzianabos, T., Marston, E. L., Jones, D. C., Green, D., Maupin, G. O., Olson, J. G., & Childs, J. E. (1997). Distribution, diversity, and host specificity of *Bartonella* in rodents from the southeastern United States. *American Journal of Tropical Medicine and Hygiene*, 57(5), 578–588. https://doi.org/10.4269/ajtmh.1997.57.578

Kruszewska, D., & Tylewska-Wierzbanowska, S. K. (1993). *Coxiella burnetii* penetration into the reproductive system of male mice, promoting sexual transmission of infection. *Infection and Immunity,* 61(10), 4188-4195.https://doi.org/10.1128/iai.61.10.4188-4195.1993.

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Molecular Biology and Evolution*, *33*(7), 1870–1874. https://doi.org/10.1093/molbev/msw054

La Scola, B., & Raoult, D. (1996). Serological cross-reactions between *Bartonella quintana*, *Bartonella henselae*, and *Coxiella burnetii*. *Journal of Clinical Microbiology*, 34(9), 2270–2274. https://doi.org/10.1128/jcm.34.9.2270-2274.1996

La Scola, B., & Raoult, D. (1999). Culture of *Bartonella quintana* and *Bartonella henselae* from human samples: A 5-year experience (1993 to 1998). *Journal of Clinical Microbiology*, 37(6), 1899–1905. https://doi.org/10.1128/jcm.37.6.1899-1905.1999

Lackman, D. B. (1937). Isolation of *Coxiella burnetii* from rodents a chipmunk (Eutamias amoenus). *From the Rocky Mountain Laboratory, National Institute of Allergy and Infectious Diseases, U. S. Public Health Service, Hamilton, Montana*, 181–186.

Lamas, C. C. (2003). Blood culture negative endocarditis: analysis of 63 cases presenting over 25 years. *Heart*, 89(3), 258–262. https://doi.org/10.1136/heart.89.3.258

Laudisoit, A., Falay, D., Amundala, N., Akaibe, D., De Bellocq, J. G., Van Houtte, N., Breno, M., Verheyen, E., Wilschut, L., Parola, P., Raoult, D., & Socolovschi, C. (2014). High prevalence of *Rickettsia typhi* and *Bartonella* species in rats and fleas, Kisangani, Democratic Republic of the Congo. *American Journal of Tropical Medicine and Hygiene*, 90(3), 463–468. https://doi.org/10.4269/ajtmh.13-0216

Leary, S., Underwood, W., Anthony, R., Cartner, S., Lilly, E., Anthony, R., Cartner, S., Corey, D., Clinic, A. V., Walla, W., Grandin, T., Collins, F., Greenacre, C., Gwaltney-brant, S., Mccrackin, M. A., Polytechnic, V., Meyer, R., State, M., Miller, D., Division, A. W. (2013). AVMA Guidelines for the Euthanasia of Animals: In *American Veterinary Medical Association*, 2013 Edition, 199-217. https://doi.org/10.1016/B978-0120884489-0.50009-1

Lei, B. R., & Olival, K. J. (2014). Contrasting Patterns in Mammal–Bacteria Coevolution: *Bartonella* and *Leptospira* in Bats and Rodents. *PLoS Neglected Tropical Diseases*, 8(3), e2738. https://doi.org/10.1371/journal.pntd.0002738

Leone, M., Bechah, Y., Meghari, S., Lepidi, H., Capo, C., Raoult, D., & Mege, J. L. (2007). *Coxiella burnetii* infection in C57BL/6 mice aged 1 or 14 months. *FEMS Immunology and Medical Microbiology*, 50(3), 396–400. https://doi.org/10.1111/j.1574-695X.2007.00272.x

Li, D. M., Hou, Y., Song, X. P., Fu, Y. Q., Li, G. C., Li, M., Eremeev, M. E., Wu, H. X., Pang, B., Yue, Y. J., Huang, Y., Lu, L., Wang, J., & Liu, Q. Y. (2015). High prevalence and genetic heterogeneity of rodent-borne *Bartonella* species on Heixiazi Island, China. *Applied and Environmental Microbiology*, 81(23), 7981–7992. https://doi.org/10.1128/AEM.02041-15

Li, D. M., Liu, Q. Y., Yu, D. Z., Zhang, J. Z., Gong, Z. Da, & Song, X. P. (2007). Phylogenetic analysis of *Bartonella* detected in rodent fleas in Yunnan, China. *Journal of Wildlife Diseases*, 43(4), 609–617. https://doi.org/10.7589/0090-3558-43.4.609

Lin, E. Y., Tsigrelis, C., Baddour, L. M., Lepidi, H., Rolain, J.-M., Patel, R., & Raoult, D. (2010). Candidatus *Bartonella mayotimonensis* and Endocarditis. *Emerging Infectious Diseases*, 16(3), 500–503. https://doi.org/10.3201/eid1603.081673

Lin, J. W., Chen, C. Y., Chen, W. C., Chomel, B. B., & Chang, C. C. (2008). Isolation of *Bartonella* species from rodents in Taiwan including a strain closely related to "*Bartonella rochalimae*" from *Rattus norvegicus*. *Journal of Medical Microbiology*, 57(12), 1496–1501. https://doi.org/10.1099/jmm.0.2008/004671-0

Lipatova, I., Paulauskas, A., Puraite, I., Radzijevskaja, J., Balciauskas, L., & Gedminas, V. (2015). *Bartonella* infection in small mammals and their ectoparasites in Lithuania. *Microbes and Infection*, 17(11–12), 884–888. https://doi.org/10.1016/j.micinf.2015.08.013

Liu, L., Xu, B., Fu, Y., Li, M., Yang, Y., Hou, Y., Wang, S., Hu, M., Guo, T., Jiang, C., Sun, X., & Wang, J. (2013). *Coxiella burnetii* in rodents on Heixiazi Island at the Sino-Russian border. *American Journal of Tropical Medicine and Hygiene*, 88(4), 770–773. https://doi.org/10.4269/ajtmh.12-0580

Lockhart, A. B., Thrall, P. H., & Antonovics, J. (1996). Sexually transmitted diseases in animals: Ecological and evolutionary implications. *Biological Reviews,* 71(3), 415-471. https://doi.org/10.1111/j.1469-185X.1996.tb01281.x

Lydy, S. L., Eremeeva, M. E., Asnis, D., Paddock, C. D., Nicholson, W. L., Silverman, D. J., & Dasch, G. A. (2008). Isolation and characterization of *Bartonella bacilliformis* from an expatriate Ecuadorian. *Journal of Clinical Microbiology*, 46(2), 627–637. https://doi.org/10.1128/JCM.01207-07

Machang'u, R. (2006). Rodent and human disease in Tanzania. *Rats and Human Health in Africa: Proceedings of an International Workshop on Rodent-Borne Diseases and the RatZooMan Research Project*, 1, 26-27

Maillard, R., Riegel, P., Barrat, F., Boullin, C., Thibault, D., Gandoin, C., Halos, L., Demanche, C., Alliot, A., Guillot, J., Piémont, Y., Boulouis, H. J., & Vayssier-Taussat, M. (2004). *Bartonella chomelii* sp. nov., isolated from French domestic cattle (Bos taurus). *International Journal of Systematic and Evolutionary Microbiology*, 54(1), 215–220. https://doi.org/10.1099/ijs.0.02770-0

Mainardi, J., Maurin, M., Lepidi, H., Thuny, F., Collart, F., Raoult, D., Fournier, P., Caus, T., Casalta, J., Célard, M., Richet, H., Habib, G., & Arzouni, J. (2010). Comprehensive Diagnostic Strategy for Blood Culture–Negative Endocarditis: A Prospective Study of 819 New Cases. *Clinical Infectious Diseases, 51(2), 131-140*. https://doi.org/10.1086/653675

Manfredi Selvaggi, T. (1996). Investigation of a Q-fever outbreak in Northern Italy. *European Journal of Epidemiology,* 12(4), 403-408. https://doi.org/10.1007/BF00145305

Matar, G. M., Koehler, J. E., Malcolm, G., Lambert-Fair, M. A., Tappero, J., Hunter, S. B., & Swaminathan, B. (1999). Identification of *Bartonella* species directly in clinical specimens by PCR-restriction fragment length polymorphism analysis of a 16S rRNA gene fragment. *Journal of Clinical Microbiology*, 37(12), 4045–4047. https://doi.org/10.1128/jcm.37.12.4045-4047.1999

Mathison, B. A., & Pritt, B. S. (2014). Laboratory identification of arthropod ectoparasites. *Clinical Microbiology Reviews*, 27(1), 48-67. https://doi.org/10.1128/CMR.00008-13

Maurin, M., Birtles, R., & Raoult, D. (1997). Current knowledge of *Bartonella* species. *European Journal of Clinical Microbiology and Infectious Diseases*, 16(7), 487–506. https://doi.org/10.1007/BF01708232

Maurin, M., Roux, V., Stein, A., Ferrier, F., Viraben, R., & Raoult, D. (1994). Isolation and characterization by immunofluorescence, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, Western blot, restriction fragment length polymorphism-PCR, 16S rRNA gene sequencing, and pulsed-field gel electrophoresis of Rochalimaea quin. *Journal of Clinical Microbiology*, 32(5), 1166-1171. http://doi.org/10.1128/jcm.32.5.1166-1171.1994

McCaul, T. F., & Williams, J. C. (1981). Developmental cycle of *Coxiella burnetii*: Structure and morphogenesis of vegetative and sporogenic differentiations. *Journal of Bacteriology*, 147(3), 1063–1076. https://doi.org/10.1128/jb.147.3.1063-1076.1981

McGill, S. L., Regnery, R. L., & Karem, K. L. (1998). Characterization of human immunoglobulin (Ig) isotype and IgG subclass response to *Bartonella henselae* infection. *Infection and Immunity*, 66(12), 5915–5920. https://doi.org/10.1128/iai.66.12.5915-5920.1998

Mediannikov, O., Fenollar, F., Socolovschi, C., Diatta, G., Bassene, H., Molez, J. F., Sokhna, C., Trape, J. F., & Raoult, D. (2010). *Coxiella burnetii* in Humans and Ticks in Rural Senegal. *PLoS Neglected Tropical Diseases*, 4(4), e654. https://doi.org/10.1371/journal.pntd.0000654

Medić, A., Dželalija, B., Polićo, V. P., Margan, I. G., Turković, B., & Gilić, V. (2005). Q fever epidemic among employees in a factory in the suburb of Zadar, Croatia. *Croatian Medical Journal*, 46(2), 315-319.

Meerburg, B. G., & Reusken, C. B. E. M. (2011). The role of wild rodents in spread and transmission of *Coxiella burnetii* needs further elucidation. *Wildlife Research*, 38(7), 617. https://doi.org/10.1071/WR10129

Meerburg, B. G., Singleton, G. R., & Kijlstra, A. (2009). Rodent-borne diseases and their risks for public health Rodent-borne diseases and their risks for public health. *Critical Reviews in Microbiology*, 35(3), 221-270. https://doi.org/10.1080/10408410902989837

Meheretu, Y., Leirs, H., Welegerima, K., Breno, M., Tomas, Z., Kidane, D., Girmay, K., & De Bellocq, J. G. (2013). *Bartonella* prevalence and genetic diversity in small mammals from Ethiopia. *Vector-Borne and Zoonotic Diseases*, 13(3), 164–175. https://doi.org/10.1089/vbz.2012.1004

Miceli, M. H., Veryser, A. K., Anderson, A. D., Hofinger, D., Lee, S. A., & Tancik, C. (2010). A case of person-to-person transmission of Q fever from an active duty serviceman to his spouse. *Vector-Borne and Zoonotic Diseases*, 10(5), 539-591. https://doi.org/10.1089/vbz.2009.0101

Mills, J. N., & Childs, J. E. (1998). Ecologic studies of rodent reservoirs: Their relevance for human health. *Emerging Infectious Diseases*, 4(4), 529–537. https://doi.org/10.3201/eid0404.980403

Minnick, M. F., Anderson, B. E., Lima, A., Battisti, J. M., Lawyer, P. G., & Birtles, R. J. (2014). Oroya Fever and Verruga Peruana: Bartonelloses Unique to South America. *PLoS Neglected Tropical Diseases*, 8(7). https://doi.org/10.1371/journal.pntd.0002919

Mishra, A. K., Lagier, J. C., Rivet, R., Raoult, D., & Fournier, P. E. (2012). Non-contiguous finished genome sequence and description of *Paenibacillus senegalensis* sp. nov. *Standards in Genomic Sciences*, 7(1), 70-81. https://doi.org/10.4056/sigs.3056450

Mmbando, B. P., Vestergaard, L. S., Kitua, A. Y., Lemnge, M. M., Theander, T. G., & Lusingu, J. P. A. (2010). A progressive declining in the burden of malaria in north-eastern Tanzania. *Malaria Journal*, 9(1), 216. https://doi.org/10.1186/1475-2875-9-216

Molin, Y., Lindeborg, M., Nyström, F., Madder, M., Hjelm, E., Olsen, B., Jaenson, T. T., & Ehrenborg, C. (2011). Migratory birds, ticks, and *Bartonella*. *Infection Ecology and Epidemiology*, 1(1), 5997. https://doi.org/10.3402/iee.v1i0.5997

Molyneux, D., Hallaj, Z., Keusch, G. T., McManus, D. P., Ngowi, H., Cleaveland, S., Ramos-Jimenez, P., Gotuzzo, E., Kar, K., Sanchez, A., Garba, A., Carabin, H., Bassili, A., Chaignat, C. L., Meslin, F. X., Abushama, H. M., Willingham, A. L., & Kioy, D. (2011). Zoonoses and marginalised infectious diseases of poverty: where do we stand? *Parasites and Vectors*, 4(1), 106. https://doi.org/10.1186/1756-3305-4-106

Morse, S. S. (1995). Factors in the emergence of infectious diseases. *Emerging Infectious Diseases*, 1(1), 7–15. https://doi.org/10.3201/eid0101.950102

Mouritsen, C. L., Litwin, C. M., Maiese, R. L., Segal, S. M., & Segal, G. H. (1997). Rapid polymerase chain reaction-based detection of the causative agent of cat scratch disease (*Bartonella henselae*) in formalin-fixed, paraffin- embedded samples. *Human Pathology*, 28(7), 820–826. https://doi.org/10.1016/S0046-8177(97)90156-8

Mullins, K. E., Hang, J., Jiang, J., Leguia, M., Kasper, M. R., Maguiña, C., Jarman, R. G., Blazes, D. L., & Richards, A. L. (2013). Molecular Typing of "Candidatus *Bartonella ancashi*," a New Human Pathogen Causing Verruga Peruana. *Journal of Clinical Microbiology*, 51(11), 3865–3868. https://doi.org/10.1128/JCM.01226-13

Mullins, K. E., Hang, J., Jiang, J., Leguia, M., Kasper, M. R., Ventosilla, P., Maguiña, C., Jarman, R. G., Blazes, D., & Richards, A. L. (2015). Description of *Bartonella ancashensis* sp. nov., isolated from the blood of two patients with verruga peruana. *International Journal of Systematic and Evolutionary Microbiology*, 65(10), 3339–3343. https://doi.org/10.1099/ijsem.0.000416

Musso, D., & Raoult, D. (1995). *Coxiella burnetii* blood cultures from acute and chronic Q-fever patients. *Journal of Clinical Microbiology*, 33(12), 3129–3132.

Musso, D, R, D., Musso D., & Raoult D. (1997). Serological cross-reactions between *Coxiella burnetii* and *Legionella micdadei*. *Clinical and Diagnostic Laboratory Immunology*, 4(2), 208–212.

Nadal, D., & Zbinden, R. (1995). Serology to *Bartonella* (Rochalimaea) henselae may replace traditional diagnostic criteria for cat-scratch disease. *European Journal of Pediatrics*, 154(11), 906–908. https://doi.org/10.1007/BF01957503

Noden, B. H., Tshavuka, F. I., Van Der Colf, B. E., Chipare, I., & Wilkinson, R. (2014). Exposure and risk factors to *Coxiella burnetii*, spotted fever group and typhus group rickettsiae, and *Bartonella henselae* among volunteer blood donors in Namibia. *PLoS ONE*, 9(9), e108674. https://doi.org/10.1371/journal.pone.0108674

Noguchi, H., & Battistin, T. S. (1926). Etiology of oroya fever. *Journal of Experimental Medicine*, 43(6), 851–864. https://doi.org/10.1084/jem.43.6.851

Norman, A. F., Regnery, R., Jameson, P., Greene, C., & Krause, D. C. (1995). Differentiation of Bartonella-like isolates at the species level by PCR-restriction fragment length polymorphism in the citrate synthase gene. *Journal of Clinical Microbiology*, 33(7), 1797–1803.

Nusinovici, S., Hoch, T., Brahim, M. L., Joly, A., & Beaudeau, F. (2017). The Effect of Wind on *Coxiella burnetii* Transmission Between Cattle Herds: A mechanistic approach. *Transboundary and Emerging Diseases*, 64(2), 585–592. https://doi.org/10.1111/tbed.12423

O'Meara, W, P., Bejon, P., Mwangi, T. W., Okiro, E. A., Peshu, N., Snow, R. W., Newton, C. R., & Marsh, K. (2008). Effect of a fall in malaria transmission on morbidity and mortality in Kilifi, Kenya. *The Lancet*, 372(9649), 1555–1562. https://doi.org/10.1016/S0140-6736(08)61655-4

O'Meara, W, P, J. N., Steketee, R., & Greenwood, B. (2010). Changes in the burden of malaria in sub-Saharan Africa. *The Lancet Infectious Diseases*, 10(8), 545–555. https://doi.org/10.1016/S1473-3099(10)70096-7

Okaro, U., Addisu, A., Casanas, B., & Anderson, B. (2017). *Bartonella* Species, an Emerging Cause of Blood-Culture-Negative Endocarditis. *Clinical Microbiology Reviews*, 30(3), 709–746. https://doi.org/10.1128/CMR.00013-17

Okiro, E. A., Hay, S. I., Gikandi, P. W., Sharif, S. K., Noor, A. M., Peshu, N., Marsh, K., & Snow, R. W. (2007). The decline in paediatric malaria admissions on the coast of Kenya. *Malaria Journal*, 6(1), 151. https://doi.org/10.1186/1475-2875-6-151

Oksi, J., Rantala, S., Kilpinen, S., Silvennoinen, R., Vornanen, M., Veikkolainen, V., Eerola, E., & Pulliainen, A. T. (2013). Cat scratch disease caused by *Bartonella grahamii* in an immunocompromised patient. *Journal of Clinical Microbiology*, 51(8), 2781–2784. https://doi.org/10.1128/JCM.00910-13

Omsland, A., Hackstadt, T., & Heinzen, R. A. (2013). Bringing Culture to the Uncultured: *Coxiella burnetii* and Lessons for Obligate Intracellular Bacterial Pathogens. *PLoS Pathogens*, 9(9), e1003540. https://doi.org/10.1371/journal.ppat.1003540

Oporto, B., Barandika, J. F., Hurtado, A., Aduriz, G., Moreno, B., & Garcia-Perez, A. L. (2006). Incidence of ovine abortion by *Coxiella burnetii* in Northern Spain. *Annals of the New York Academy of Sciences*, 1078, 498–501. https://doi.org/10.1196/annals.1374.095

Otten, M., Aregawi, M., Were, W., Karema, C., Medin, A., Bekele, W., Jima, D., Gausi, K., Komatsu, R., Korenromp, E., Low-Beer, D., & Grabowsky, M. (2009). Initial evidence of reduction of malaria cases and deaths in Rwanda and Ethiopia due to rapid scale-up of malaria prevention and treatment. *Malaria Journal*, 8(1), 14. https://doi.org/10.1186/1475-2875-8-14

Paretsky, D., & Stueckemann, J. (1970). Chemical and biochemical changes in subcellular fractions of guinea pig liver during infection with *Coxiella burneti*. *Journal of Bacteriology*, *102*(2), 334–340. https://doi.org/10.1128/jb.102.2.334-340.1970

Parra, E., Segura, F., Tijero, J., Pons, I., & Nogueras, M. M. (2017). Development of a real-time PCR for *Bartonella* spp. detection, a current emerging microorganism. *Molecular and Cellular Probes*, 32, 55–59. https://doi.org/10.1016/j.mcp.2016.10.006

Patnaik, M., & Peter, J. B. (1995). Cat-scratch disease, *Bartonella henselae*, and the usefulness of routine serological testing for afipia felis. *Clinical Infectious Diseases*, 21(4), 1064–1065. https://doi.org/10.1093/clinids/21.4.1064

Paziewska, A., Harris, P. D., Zwoli, L., Bajer, A., & Si, E. (2011). *Recombination Within and Between Species of the Alpha Proteobacterium Bartonella Infecting Rodents*. 134–145. https://doi.org/10.1007/s00248-010-9735-1

Pérez, C., Maggi, R. G., Diniz, P. P. V. P., & Breitschwerdt, E. B. (2011). Molecular and serological diagnosis of *Bartonella* infection in 61 dogs from the United States. *Journal of Veterinary Internal Medicine*, 25(4), 805-810. https://doi.org/10.1111/j.1939-1676.2011.0736.x

Perry, B. D., Randolph, T. F., Mcdermott, J. J., Sones, K. R., & Thornton, P. K. (2002). Animal diseases and their impact on the poor. *Investing in Animal Health Research to Alleviate Poverty*, 49–61. Nairobi Kenya: International Livestock Research Institute

Peterson, A. C., Ghersi, B. M., Alda, F., Firth, C., Frye, M. J., Bai, Y., Osikowicz, L. M., Riegel, C., Lipkin, W. I., Kosoy, M. Y., & Blum, M. J. (2017). Rodent-Borne *Bartonella* Infection Varies According to Host Species Within and Among Cities. *EcoHealth*, 14(4), 771–782. https://doi.org/10.1007/s10393-017-1291-4

Philip, C. B. (1948). Comments on the Name of the Q Fever Organism. *Public Health Reports* (1896-1970), 63(2), 58. https://doi.org/10.2307/4586402

Piñero, A., Barandika, J. F., Hurtado, A., & García-Pérez, A. L. (2014). Evaluation of *Coxiella burnetii* Status in Dairy Cattle Herds with Bulk-tank Milk Positive by ELISA and PCR. *Transboundary and Emerging Diseases*, 61(2), 163–168. https://doi.org/10.1111/tbed.12013

Pluta, S., Hartelt, K., Oehme, R., Mackenstedt, U., & Kimmig, P. (2010). Prevalence of *Coxiella burnetii* and Rickettsia spp. in ticks and rodents in southern Germany. *Ticks and Tick-Borne Diseases*, 1(3), 145–147. https://doi.org/10.1016/j.ttbdis.2010.04.001

Prasad, N., Murdoch, D. R., Reyburn, H., & Crump, J. A. (2015). Etiology of severe febrile illness in low- and middle-income countries: A systematic review. *PLoS Neglected Tropical Diseases*, 10(6), 1–25. https://doi.org/10.1371/journal.pone.0127962

Pretorius, A. M., Beati, L., & Birtles, R. J. (2004). Diversity of bartonellae associated with small mammals inhabiting Free State province, South Africa. *International Journal of Systematic and Evolutionary Microbiology*, 54(6), 1959–1967. https://doi.org/10.1099/ijs.0.03033-0

R Development Core Team. (2018). A Language and Environment for Statistical Computing. In *R Foundation for Statistical Computing* (Vol. 2, p. https://www.R-project.org). https://doi.org/R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org.

Rabiee, M. H., Mahmoudi, A., Siahsarvie, R., Kryštufek, B., & Mostafavi, E. (2018). Rodent-borne diseases and their public health importance in Iran. *PLoS Neglected Tropical Diseases*, 12(4), 1–20. https://doi.org/10.1371/journal.pntd.0006256

Raoult, D., Marrie, T., & Mege, J. (2005). Natural history and pathophysiology of Q fever. *The Lancet Infectious Diseases*, 5(4), 219–226. https://doi.org/10.1016/S1473-3099(05)70052-9

Raoult, D, R, F., Rolain, J.-M., Besnier, J.-M., Loulergue, J., Bastides, F., & Choutet, P. (2006). First Isolation of *Bartonella alsatica* from a Valve of a Patient with Endocarditis. *Journal of Clinical Microbiology*, 44(1), 278–279. https://doi.org/10.1128/JCM.44.1.278-279.2006

Rasis, M., Rudoler, N., Schwartz, D., & Giladi, M. (2014). *Bartonella dromedarii* sp. nov. Isolated from Domesticated Camels (*Camelus dromedarius*) in Israel. *Vector-Borne and Zoonotic Diseases*, 14(11), 775–782. https://doi.org/10.1089/vbz.2014.1663

Regnery, R., Olson, J., Perkins, B., & Bibb, W. (1992). Serological response to "*Rochalimaea henselae*" antigen in suspected cat-scratch disease. *The Lancet*, 339(8807), 1443–1445. https://doi.org/10.1016/0140-6736(92)92032-B

Regnery, R. L., Anderson, B. E., Clarridge, J. E., Rodriguez-Barradas, M. C., Jones, D. C., & Carr, J. H. (1992). Characterization of a novel *Rochalimaea* species, *R. henselae* sp. nov., isolated from blood of a febrile, human immunodeficiency virus-positive patient. *Journal of Clinical Microbiology*, 30(2), 265–274. https://doi.org/10.1128/ jcm.30.2.265-274.1992

Relman, D. A., Falkow, S., Tompkins, L. S., Loutit, J. S., & Schmidt, T. M. (1990). The agent of bacillary angiomatosis: An Approach to the Identification of Uncultured Pathogens. *New England Journal of Medicine*, 323(23), 1573–1580. https://doi.org/10.1056/NEJM199012063232301

Renesto, P., Gouvernet, J., Drancourt, M., Roux, V., & Raoult, D. (2001). Use of rpoB gene analysis for detection and identification of *Bartonella* species. *Journal of Clinical Microbiology*, 39(2), 430–437. https://doi.org/10.1128/JCM.39.2.430-437.2001

Reusken, C., van der Plaats, R., Opsteegh, M., de Bruin, A., & Swart, A. (2011). *Coxiella burnetii* (Q fever) in *Rattus norvegicus* and *Rattus rattus* at livestock farms and urban locations in the Netherlands; could *Rattus* spp. represent reservoirs for (re)introduction? *Preventive Veterinary Medicine*, *101*(1–2), 124–130. https://doi. org /10.1016/j.prevetmed.2011.05.003

Reyburn, H., Mbatia, R., Drakeley, C., Carneiro, I., Mwakasungula, E., Mwerinde, O., Saganda, K., Shao, J., Kitua, A., Olomi, R., Greenwood, B. M., & Whitty, C. J. M. (2004). Overdiagnosis of malaria in patients with severe febrile illness in Tanzania: A prospective study. *British Medical Journal*, 329(7476), 1212–1215. https://doi. org/10.1136/bmj.38251.658229.55

Rodolakis, A. (2006). Q fever, state of art: Epidemiology, diagnosis and prophylaxis. *Small Ruminant Research*, 62(1-2), 121-124. https://doi.org/10.1016/j.smallrumres.2005. 07. 038

Roest, H. I. J., Tilburg, J. J. H. C., Van Der Hoek, W., Vellema, P., Van Zijderveld, F. G., Klaassen, C. H. W., & Raoult, D. (2011). The Q fever epidemic in the Netherlands: History, onset, response and reflection. *Epidemiology and Infection*, 139(1), 1–12. https://doi.org/10.1017/S0950268810002268

Roest, H. I. J., Bossers, A., van Zijderveld, F. G., & Rebel, J. M. L. (2013). Clinical microbiology of coxiella burnetii and relevant aspects for the diagnosis and control of the zoonotic disease Q fever. *Veterinary Quarterly*, 33(3), 148–160. https://doi.org/ 10.1080/01652176.2013.843809

Rolain, J. M., Gouriet, F., Brouqui, P., Larrey, D., Janbon, F., Vene, S., Jarnestrom, V., & Raoult, D. (2005). Concomitant or consecutive infection with *Coxiella burnetii* and tickborne diseases. *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America*, 40(1), 82–88. https://doi.org/10.1086/426440

Rolain, J. M., La Scola, B., Liang, Z., Davoust, B., & Raoult, D. (2001). Immunofluorescent detection of intraerythrocytic *Bartonella henselae* in naturally infected cats. *Journal of Clinical Microbiology*, 39(8), 2978-2980. https://doi.org/10.1128/JCM.39.8.2978-2980.2001

Rolain, J. M., & Raoult, D. (2006). *Bartonella* spp. In *Principles and Practice of Clinical Bacteriology*, John Wiley & Sons, Ltd, 2nd Edition, 295-304. https://doi.org/10.1002/9780470017968.ch23

Roux, V., Eykyn, S. J., Wyllie, S., & Raoult, D. (2000). *Bartonella vinsonii* subsp. berkhoffii as an agent of afebrile blood culture-negative endocarditis in a human. *Journal of Clinical Microbiology, 43(2), 945-947*.https://doi.org/10.1128/JMC.43.2.945-947.2005

Rozental, T., Ferreira, M. S., Guterres, A., Mares-Guia, M. A., Teixeira, B. R., Gonçalves, J., Bonvicino, C. R., D'Andrea, P. S., & de Lemos, E. R. S. (2017). Zoonotic pathogens in Atlantic Forest wild rodents in Brazil: *Bartonella* and *Coxiella* infections. *Acta Tropica*, 168, 64–73. https://doi.org/10.1016/j.actatropica.2017.01.003

Rudoler, N., Rasis, M., Sharir, B., Novikov, A., Shapira, G., & Giladi, M. (2014). First description of *Bartonella bovis* in cattle herds in Israel. *Veterinary Microbiology*, 173(1–2), 110–117. https://doi.org/10.1016/j.vetmic.2014.07.006

Russell-Lodrigue, K. E., Zhang, G. Q., McMurray, D. N., & Samuel, J. E. (2006). Clinical and pathologic changes in a guinea pig aerosol challenge model of acute Q fever. *Infection and Immunity*. https://doi.org/10.1128/IAI.00763-06

Saisongkorh, W., Rolain, J. M., Suputtamongkol, Y., & Raoult, D. (2009). Emerging *Bartonella* in humans and animals in Asia and Australia. *Journal of the Medical Association of Thailand*, 92(5), 707–731.

Sander, A., Berner, R., & Ruess, M. (2001). Serodiagnosis of cat scratch disease: Response to *Bartonella henselae* in children and a review of diagnostic methods. *European Journal of Clinical Microbiology and Infectious Diseases*, 20(6), 392-401. https://doi.org/10. 1007/PL00011280

Sander, A, P, M., Oberle, K., & Bredt, W. (1998). Seroprevalence of antibodies to *Bartonella henselae* in patients with cat scratch disease and in healthy controls: evaluation and comparison of two commercial serological tests. *Clinical and Diagnostic Laboratory Immunology*, 5(4), 486–490.

Sanogo, Y. O., Zeaiter, Z., Caruso, G., Merola, F., Shpynov, S., Brouqui, P., & Raoult, D. (2003). *Bartonella henselae* in Ixodes ricinus ticks (Acari: Ixodida) removed from humans, Belluno Province, Italy. *Emerging Infectious Diseases*, 9(3), 329-332. https://doi.org/10. 3201/eid0903.020133

Sato, S., Kabeya, H., Fujinaga, Y., Inoue, K., Une, Y., Yoshikawa, Y., & Maruyama, S. (2013). *Bartonella jaculi* sp. nov., *Bartonella callosciuri* sp. nov., *Bartonella pachyuromydis* sp. nov. and *Bartonella acomydis* sp. nov., isolated from wild Rodentia. *International Journal of Systematic and Evolutionary Microbiology*, 63(PART 5), 1734-1740. https://doi.org/10.1099/ijs.0.041939-0

Schimmer, B., Schotten, N., van Engelen, E., Hautvast, J. L. A., Schneeberger, P. M., & van Duijnhoven, Y. T. H. P. (2014). *Coxiella burnetii* seroprevalence and risk for humans on dairy cattle farms, the Netherlands, 2010-2011. *Emerging Infectious Diseases*, 20(3), 417–425. https://doi.org/10.3201/eid2003.131111

Schmid, R., Wilson, D. E., & Reeder, D. M. (1993). Mammal Species of the World: A Taxonomic and Geographic Reference. *Taxon*, 42(2), 512. https://doi.org/10.2307/1223169

Schulte F. F. B., Stuckey, M. J., Aguilar-Setién, A., Moreno-Sandoval, H., Galvez-Romero, G., Salas-Rojas, M., Arechiga-Ceballos, N., Overgaauw, P. A. M., Kasten, R. W., & Chomel, B. B. (2016). Identification of *Bartonella* Species Isolated from Rodents from Yucatan, Mexico and Isolation of *Bartonella vinsonii* subsp. yucatanensis subsp. nov. *Vector-Borne and Zoonotic Diseases*, 16(10), 636–642. https://doi.org/10.1089/vbz.2016.1981

Seimenis, A. (2012). Zoonoses and poverty: A long road to the alleviation of suffering. *Veterinaria Italiana*, 48(1), 5–13.

Seo, M. G. X., Lee, S. H., Ouh, I. O., Lee, G. H., Goo, Y. K., Kim, S., Kwon, O. D., & Kwak, D. (2016). Molecular detection and genotyping of coxiella-like endosymbionts in ticks that infest horses in South Korea. *PLoS ONE*, 11(10), e0165784. https://doi.org/10.1371/journal.pone.0165784

Shivaprasad, H. L., Cadenas, M. B., Diab, S. S., Nordhausen, R., Bradway, D., Crespo, R., & Breitschwerdt, E. B. (2008). Coxiella-Like Infection in Psittacines and a Toucan. *Avian Diseases*, 52(3), 426–432. https://doi.org/10.1637/8192-120707-Reg

Silaghi, C., Pfeffer, M., Kiefer, D., Kiefer, M., & Obiegala, A. (2016). *Bartonella*, Rodents, Fleas and Ticks: a Molecular Field Study on Host-Vector-Pathogen Associations in Saxony, Eastern Germany. *Microbial Ecology*, 72(4), 965–974. https://doi.org/10.1007/s00248-016-0787-8

Slingenbergh, J., Gilbert, M., Balogh, K. De, & Wint, W. (2004). Ecological sources of zoonotic diseases Factors affecting the emergence. *OIE Revue Scientifique et Technique*, 23(2), 467–484. https://doi.org/ 10.20506/rst.23.2.1492

Spach, D. H., Root, R. K., Stamm, W. E., Dougherty, M. J., Larson, A. M., Coyle, M. B., Kanter, A. S., Brenner, D. J., Swaminathan, B., Matar, G. M., & Welch, D. F. (1995). *Bartonella* (rochalimaea) *quintana* bacteremia in inner-city patients with chronic alcoholism. *New England Journal of Medicine*, 332(7), 424–428. https://doi.org/10.1056/NEJM199502163320703

Tamura, K. (1992). Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G+C-content biases. *Molecular Biology and Evolution*, 9(4), 678–687. https://doi.org/10.1093/oxfordjournals.molbev.a040752

Tan, C. K., & Owens, L. (2000). Infectivity, transmission and 16S rRNA sequencing of a *Rickettsia*, *Coxiella cheraxi* sp. nov., from the freshwater crayfish *Cherax quadricarinatus*. *Diseases of Aquatic Organisms*, 41(2), 115–122. https://doi.org/10.3354/dao041115

Tay, S. T., Mokhtar, A. S., Zain, S. N. M., & Low, K. C. (2014). Isolation and molecular identification of Bartonellae from wild rats (*Rattus* species) in Malaysia. *The American Journal of Tropical Medicine and Hygiene*, 90(6), 1039–1042. https://doi.org/10.4269/ajtmh.13-0273

Taylor, L. H., Latham, S. M., & Woolhouse, M. E. J. (2001). Risk factors for human disease emergence. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 356(1411), 983–989. https://doi.org/10.1098/rstb.2001.0888

Telfer, S., Bown, K. J., Sekules, R., Begon, M., Hayden, T., & Birtles, R. (2005). Disruption of a host-parasite system following the introduction of an exotic host species. *Parasitology*, 130(6), 661–668. https://doi.org/10.1017/S0031182005007250

Telfer, S, C, H. E., Birtles, R. J., Bennett, M., Carslake, D., Helyar, S., & Begon, M. (2007). Ecological differences and coexistence in a guild of microparasites: *Bartonella* in wild rodents. *Ecology*, 88(7), 1841–1849. https://doi.org/10.1890/06-1004.1

Theonest, N. O., Carter, R. W., Amani, N., Doherty, S. L., Hugho, E., Keyyu, J. D., Mable, B. K., Shirima, G. M., Tarimo, R., Thomas, K. M., Haydon, D. T., Buza, J. J., Allan, K. J., & Halliday, J. E. B. (2019). Molecular detection and genetic characterization of *Bartonella* species from rodents and their associated ectoparasites from northern Tanzania. *PLoS ONE*, 14(10), e0223667. https://doi.org/10.1371/journal. pone. 0223667

Thompson, M., Mykytczuk, N., Gooderham, K., & Schulte-Hostedde, A. (2012). Prevalence of the bacterium *Coxiella burnetii* in wild rodents from a canadian natural environment park. *Zoonoses and Public Health*, *59*(8), 553–560. https://doi.org/10. 1111/j.1863-2378.2012.01493.x

Thornton, S. A., Wignall, F. S., Knobloch, J., Smith, W. A., Kelley, P. W., Escudero, L. O., Huayda, M. A., Gray, G. C., & Johnson, A. A. (1990). An Epidemic of Oroya Fever in the Peruvian Andes. *The American Journal of Tropical Medicine and Hygiene*, 42(3), 215–221. https://doi.org/10.4269/ajtmh.1990.42.215

Tigertt, W. D., Benenson, A. S., & Gochenour, W. S. (1961). Airborne Q fever. *Bacteriological Reviews*, *25*, 285–293. https://doi.org/10.1128/mmbr.25.3.285-293. 1961

Tilburg, J. J. H. C., Melchers, W. J. G., Pettersson, A. M., Rossen, J. W. A., Hermans, M. H. A., van Hannen, E. J., Nabuurs-Franssen, M. H., de Vries, M. C., Horrevorts, A. M., & Klaassen, C. H. W. (2010). Interlaboratory Evaluation of Different Extraction and Real-Time PCR Methods for Detection of Coxiella burnetii DNA in Serum. *Journal of Clinical Microbiology*, 48(11), 3923–3927. https://doi.org/10.1128/JCM.01006-10

Tissot-Dupont, H., Vaillant, V., Rey, S., & Raoult, D. (2007). Role of Sex, Age, Previous Valve Lesion, and Pregnancy in the Clinical Expression and Outcome of Q Fever after a Large Outbreak. *Clinical Infectious Diseases*, 44(2), 232-237. https://doi.org/10. 1086/510389

Tissot-Dupont, H. A. M. A., Nezri, M., & Raoult, D. (2004). Wind in November, Q Fever in December. *Emerging Infectious Diseases*, 10(7), 1264–1269. https://doi.org/10.3201/ eid1007.030724

Tissot Dupont, T., Thirion, X., & Raoult, D. (1994). Q fever serology: Cutoff determination for microimmunofluorescence. *Clinical and Diagnostic Laboratory Immunology*, 1(2), 189–196.

Torchin, M. E., Lafferty, K. D., Dobson, A. P., McKenzie, V. J., & Kuris, A. M. (2003). Introduced species and their missing parasites. *Nature*, 421(6923), 628-630 . https://doi.org/10.1038/ nature01346

Vaidya, V. M., Malik, S. V. S., Kaur, S., Kumar, S., & Barbuddhe, S. B. (2008). Comparison of PCR, Immunofluorescence Assay, and Pathogen Isolation for Diagnosis of Q Fever in Humans with Spontaneous Abortions. *Journal of Clinical Microbiology*, 46(6), 2038–2044. https://doi.org/10.1128/JCM.01874-07

Van Asseldonk, M. A. P. M., Prins, J., & Bergevoet, R. H. M. (2013). Economic assessment of Q fever in the Netherlands. *Preventive Veterinary Medicine*, 112(1–2), 27–34. https://doi.org/10.1016/j.prevetmed.2013.06.002

Van Woerden, H. C., Mason, B. W., Nehaul, L. K., Smith, R., Salmon, R. L., Healy, B., Valappil, M., Westmoreland, D., De Martin, S., Evans, M. R., Lloyd, G., Hamilton-Kirkwood, M., & Williams, N. S. (2004). Q fever outbreak in industrial setting. *Emerging Infectious Diseases, 10(7), 1282-1289*. https://doi.org/10. 3201/eid1007. 030536

Vanderburg, S., Rubach, M. P., Halliday, J. E. B., Cleaveland, S., Reddy, E. A., & Crump, J. A. (2014). Epidemiology of *Coxiella burnetii* Infection in Africa: A OneHealth Systematic Review. *PLoS Neglected Tropical Diseases*, 8(4), 1-10. https://doi.org/10.1371/journal.pntd.0002787

Vayssier-Taussat, M., Le Rhun, D., Bonnet, S., & Cotté, V. (2009). Insights in *Bartonella* Host Specificity. *Annals of the New York Academy of Sciences*, 1166(1), 127–132. https://doi.org/10.1111/j.1749-6632.2009.04531.x

Vayssier-Taussat, M., Le Rhun, D., Deng, H. K., Biville, F., Cescau, S., Danchin, A., Marignac, G., Lenaour, E., Boulouis, H. J., Mavris, M., Arnaud, L., Yang, H., Wang, J., Quebatte, M., Engel, P., Saenz, H., & Dehio, C. (2010). The Trw Type IV Secretion System of *Bartonella* Mediates Host-Specific Adhesion to Erythrocytes. *PLoS Pathogens*, 6(6), 1-10. https://doi.org/10.1371/journal.ppat.1000946

Vijayachari, P., Sugunan, A. P., & Shriram, A. N. (2008). Leptospirosis: an emerging global public health problem. *Journal of Biosciences*, 33(4), 557–569. https://doi.org/10.1007/s12038-008-0074-z

Vinet, L., & Zhedanov, A. (2010). A “missing” family of classical orthogonal polynomials. *Global Public Health*, 44(8), 085201. https://doi.org/10.1088/1751-8113/44/8/085201

Voth, D. E., & Heinzen, R. A. (2007). Lounging in a lysosome: The intracellular lifestyle of *Coxiella burnetii*. *Cellular Microbiology*, 9(4), 829–840. https://doi.org/10.1111/j.1462-5822.2007.00901.x

Wallensten, A., Moore, P., Webster, H., Johnson, C., van der Burgt, G., Pritchard, G., Ellis-Iversen, J., & Oliver, I. (2010). Q fever outbreak in Cheltenham, United Kingdom, in 2007 and the use of dispersion modelling to investigate the possibility of airborne spread. *Eurosurveillance*, 15(12), 2807. https://doi.org/10.2807/ese.15.12.19521-en

Watanabe, A., & Takahashi, H. (2008). Diagnosis and treatment of Q fever: Attempts to clarify current problems in Japan. *Journal of Infection and Chemotherapy*, 14(1), 1-7. https://doi.org/10.1007/s10156-007-0566-z

Webster, J. P., Lloyd, G., & Macdonald, D. W. (1995). Q fever (Coxiella burnetii) reservoir in wild brown rat (Rattus norvegicus) populations in the UK. *Parasitology*, 110(1), 31–35. https://doi.org/10.1017/S0031182000081014

Welch, D. F., Carroll, K. C., Hofmeister, E. K., Persing, D. H., Robison, D. A., Steigerwalt, A. G., & Brenner, D. J. (1999). Isolation of a new subspecies, *Bartonella vinsonii* subsp. arupensis, from a cattle rancher: Identity with isolates found in conjunction with *Borrelia burgdorferi* and *Babesia microti* among naturally infected mice. *Journal of Clinical Microbiology*, 37(8), 2598–2601.

Welsh, H. H., Lennette, E. H., Abinanti, F. R., & Winn, J. F. (1958). Air-borne transmission of q fever: The role of parturition in the generation of infective aerosols. *Annals of the New York Academy of Sciences*, 70(3), 528–540. https://doi.org/10.1111/j.1749-6632.1958.tb35409.x

Winoto, I. L., Goethert, H., Ibrahim, I. N., Yuniherlina, I., Stoops, C., Susanti, I., Kania, W., Maguire, J. D., Bangs, M. J., Telford, S. R., & Wongsrichanalai, C. (2005). *Bartonella* species in rodents and shrews in the Greater Jakarta area. *Southeast Asian Journal of Tropical Medicine and Public Health*, 36(6), 1523-1529.

Withenshaw, S. M., Devevey, G., Pedersen, A. B., & Fenton, A. (2016). Multihost *Bartonella* parasites display covert host specificity even when transmitted by generalist vectors. *Journal of Animal Ecology*, 85(6), 1442–1452. https://doi.org/10.1111/1365-2656.12568

Ye, Y., Madise, N., Ndugwa, R., Ochola, S., & Snow, R. W. (2009). Fever treatment in the absence of malaria transmission in an urban informal settlement in Nairobi, Kenya. *Malaria Journal*, 8(1), 1–7. https://doi.org/10.1186/1475-2875-8-160

Ying, B., Kosoy, M. Y., Maupin, G. O., Tsuchiya, K. R., & Gage, K. L. (2002). Genetic and ecologic characteristics of *Bartonella* communities in rodents in Southern China. *American Journal of Tropical Medicine and Hygiene*, 66(5), 622–627. https://doi.org/10.4269/ajtmh.2002.66.622

Zbinden, R., Hochli, M., & Nadal, D. (1995). Intracellular location of *Bartonella henselae* cocultivated with Vero cells and used for an indirect fluorescent-antibody test. *Clinical and Diagnostic Laboratory Immunology*, 2(6), 693-695.https://doi.org/10.1128/cdli.2.6.693-695

Zhang, Q., Li, H., & Zhang, X. (2015). Cat Scratch Disease. In *Radiology of Infectious Diseases* Springer Netherlands, 2, 63-68. https://doi.org/10.1007/978-94-017-9876-1_4

APPENDICES

Appendix 1: Standard operation procedure (SOP) for laboratory handling of animal samples and detection of Zoonotic bacterial pathogens (*Bartonella* spp)

KCRI BIOTECHNOLOGY LABORATORY	Document No: KCRI /SOP/5.4-06	
SOP TITLE: **DNA Extraction from animal tissues samples**	Copy No: 0	Version No: BZ-1 SOP #: 3
AREA: Zoonoses Lab	Page: 1 of 6	Effective Date: April 2016
AUTHOR: Theonest Ndyetabura, Kathryn Allan		Revision Date: April 2016

I. SCOPE

- The purpose of this standard operating procedure is to describe procedure for handling and extraction DNA from tissues using Qiagen's DNeasy Blood and Tissue kit (cat # 69506, 69504, or 69582). This extraction procedure is used in the detection of diverse range of pathogens from animal samples, the range of pathogens includes but not limited to:

- *Coxiella burnetii*
- *Leptospira spp*
- *Brucella spp*
- *Rickettsia spp*
- *Toxoplasma gondi*
- *Bartonella spp*
- etc

NB: The Quality Control samples should be treated in the exact same manner as the test samples and are used to validate the test run.

II. Procedure

Following the guidelines described in the general laboratory good practice and KCRI Laboratory safety and equipment operation procedure. Procedure for Aseptic Techniques in the zoonotic Laboratory" proceed to perform the SOP described here.

Verify that you have all the materials and equipment necessary to perform this procedure.

III. Equipment

- Micropipettes calibrated to the following volumes: 0.5-10 µl, 2-20 µl, 20- 200 µl, and 100-1000 µl
- Microcentrifuge
- Balance
- Refrigerator (2 to 8 ºC)
- Freezer (-10 to -25 ºC)
- Freezer (-75 to -85 ºC)
- Biological Safety Cabinet
- Mini Vortexer
- Heat block or water bath (56-70°C)

Page 1 of 6

IV. Materials

- Disposable gloves (VWR cat # 32916-502 or equivalent)
- DNAse- and RNAse-free 1.5 ml microcentrifuge tubes (VWR 53550-698) or equivalent
- Aerosol resistant micropipette tips, 0.5-10 µl, 20-200 µl, and 100-1000 µl (USA Scientific 1121-3810, 1120-8810, 1126-7810 respectively) or equivalent
- Biohazard Bags (Lilly stores cat # 44-003360) or equivalent
- DNeasy Kit (Qiagen 69506) or equivalent
- DNAse- and RNAse-free water (Invitrogen cat # 10977-015) or equivalent
- 100% Ethanol (Sigma cat # E 7023) or equivalent
- Razor Blades or scalpel blade
- Nunc Petri Dishes, Polystyrene with lid, 100x 15mm (Fisher Scientific cat # 08-757-099) or equivalent
- Sterile Phosphate Buffered Saline (PBS)
- Disposable HSW FINE-JECT® needles (suitable for Luer and Luer Lock)

I. SAFETY CONSIDERATION

A. This process shall be performed in the zoonoses lab unit, wearing lab coat and disposable gloves.
B. Clean the work area within the biosafety cabinet, DNA hood, and table tops, using 70% ethanol or 5-10 % bleach (NaOCl) (refer KCRI safety SOP/Manual).
C. Turn on the UV light in the biosafety cabinet/hood for a minimum of 20 minutes before starting work in the hood.
D. Remove gloves and lab coat before leaving the room
E. All steps involving open tubes should be performed in the biosafety cabinet/hood
F. Take extra precaution when handling and using sharps

1. Heat inactivation

   i. All samples from freezer should be disinfected by wiping the outside of cryovial with Virkon.This should be done in the biosafety cabinet

   ii. Add 1ml of sterile 1x PBS to sample containing tubes, then subject to heat treatment at 67°C for an hour. Allow the sample cool to room temperature, proceed with protocol step 3

2. **DNA extraction**

   DNA extraction from animal specimens will be performed following published Qiagen's DNeasy Blood and Tissue kit and associated modification described in this SOP. All reagents referred to in the following protocol are part of the Qiagen's DNeasy Blood and Tissue kit

3. Protocol for DNA extraction heat inactivated tissues

1) Pipette 180 µl buffer ATL into pre-labeled 1.5ml or 2ml microcentrifuge tube.
2) Transfer heat inactivated tissue samples to the laminar flow cabinet (BSL2). Using a sterile petridish and sterile scalpel blade, cut up to 10mg of tissues into small pieces.
3) Using sterile needle place diced tissue into the 1.5 ml or 2ml microcentrifuge tube containing 180µl buffer ATL and add 20µl proteinase K to each sample.
4) Mix by pulse-vortexing for 15 seconds and incubate at 56°C in a heat block or water bath until the tissue has completely lysed at the solution is clear (1-3 hours; additional vortexing will accelerate tissue lysis).
5) Briefly centrifuge to remove drops from the lid of the tube.

NB: After proteinase K digestion, tissue samples can also be stored in Buffer ATL for up 6 months at ambient temperature without any reduction in DNA quality

6) Add 200µl Buffer AL. Pulse-vortex to mix for 15 seconds then incubate at 70°C for 10 minutes. Briefly centrifuge again.
7) Add 200µl ethanol (96-100%) and mix by pulse vortexing for 15s and briefly centrifuge.

---

8) Add the mixture to the DNeasy spin column without wetting the rim. Close the lid and centrifuge at 8000 rpm for 1 minute.
9) Place the spin column in a clean 2 ml collecting tube and discard the tube containing the filtrate into standard laboratory biohazardous waste.
10) Add 500 µl buffer AW1 without wetting the rim and centrifuge at 8000 rpm for 1 minute.
11) Place the spin column into a clean 2 ml collection tube and discard the tube containing the filtrate.
12) Add 500 µl buffer AW2 without wetting the rim and centrifuge at 13000 rpm for 1 minute.
13) Place the spin column in a 1.5 ml microcentrifuge tube from which the cap has been cut off and discard the tube containing the filtrate.
14) Repeat step 8 by applying the remaining mixture to the DNeasy spin column.
15) Centrifuge at 13000rpm for 4 minutes to eliminate buffer AW2 carry over. Make sure the spin column is dry. If not, open the lid and leave for at least 5 minutes to air-dry.
16) Place the spin column into a new 1.5 ml microcentrifuge tube from which the cap has been cut off and discard the tube containing the filtrate.
17) Add 100 µl DNAse free water and incubate at room temperature (RT) for 3 minutes; then centrifuge for 1 minute at 13000rpm.
18) Using the same collecting tube, add again 100ul DNAse free water and incubate at RT for 1 minute; then centrifuge again for 1 minute at 13000rpm.
19) Transfer the DNA product from **steps 16 & 17** to a new 1.5 ml microcentrifuge tube with cap, and briefly vortex and spin down.
20) DNA is ready to be used for PCR or can be stored at -20°C.

References:

Health Protection Agency (2010) Guidelines for action in the event of a deliberate release: Q Fever. Version 1.7. http://www.hpa.org.uk/webc/HPAwebFile/HPAweb_C/1194947387885

Holsinger VH, Kajkowski KT & Stabel JR (1997) Milk pasteurisation and safety: a brief history and update. *Rev Sci Tech Off Int Epiz.* 16(2) 441-451

Rutala WA, Weber DJ & HIPAC (2008) Guidelines for Disinfection and Sterilisation in Health Care facilities. Centers for Disease Control (CDC)
http://www.cdc.gov/hicpac/disinfection_sterilization/6_0disinfection.html
KCRIBL/ALS/F5.3-02f .Biosafety cabinet Usage, Cleaning and Disinfection Log References

Biological Safety Manual located on the shelf in unit one, immunology section
Qiagen - DNeasy®Blood & Tissue Handbook

Name	Signature and Date

Revised by: Jo Halliday (BZ study coordinator) **Date:** ——, ———, ———

Quality manager: BZ lab co-coordinator (Kate Thomas) **Date:**——,———,———

Declaration

I have read & understand the Bacterial Zoonoses – Laboratory Preparations for Field Sample Collection, and I agree to abide by the procedures described

Name	Signature and Date
.....	.....
.....	.....
.....	.....
.....	.....
.....	.....
.....	.....
.....	.....

Appendix 2: Standard operation procedure (SOP) for real time qPCR quantification of *Coxiella burnetii*

KCRI BIOTECHNOLOGY LABORATORY		Document No: KCRI /SOP/5.4-06	
SOP TITLE: IS1111 qPCR To Detect *Coxiella spp.* DNA (Qiagen Rotor-Gene Platform)		Copy No: 0	Version No: BZ-4 SOP #: 4
AREA: Zoonoses Lab		Page: 1 of 7	Effective Date: Sept 2016
AUTHOR: Theonest Ndyetabura, Ryan Carter			Revision Date: Sept 2017

1. Summary/Aim

This SOP concerns the process of conducting diagnostic quantitative PCR (qPCR) on DNA extracts for the purpose of detecting the presence of DNA deriving from *Coxiella* species. The procedure detailed here uses the Qiagen Rotor-Gene Q/6000 qPCR platform and associated Rotor-Gene Q Series Software for analysis. The document describes the laboratory procedure to be followed to arrive at a decision as to whether *Coxiella* organisms were present in the animal from which the samples were taken, at the time of sampling.

2. Safety

Standard laboratory safety practices should be adhered to throughout the procedure detailed below. All appropriate safety SOPs should be read and signed before commencing work.
Personal Protective Equipment (PPE) – lab coat and gloves - should be worn when conducting any procedures in the laboratory.

3. Samples

The DNA extraction process is described in an accompanying SOP. DNA samples should be eluted in either nuclease-free H_2O or a suitable elution buffer (e.g. Buffer AE). Ideally, the concentration of DNA extracts being analysed should be approximately 10ng/ul prior to adding to qPCR mastermix – samples of greater concentration should be diluted to meet this requirement where possible.

4. Equipment/Consumables/Reagents

- Rotor-Gene Q/6000 qPCR machine
- 72-Well Rotor or 36-Well Rotor plus locking ring
- PCR laminar flow cabinet
- Vortex
- Micro-centrifuge
- 10ul, 20ul, 200ul and 1,000ul pipettes
- 10ul, 20ul, 200ul and 1,000ul sterile, filter pipette tips
- 1.5ml sterile microcentrifuge tubes
- Strip tubes (4-tube strips) and caps 0.1ml; x250 (Cat. No. 981103, Qiagen)
- QuantiNova Probe PCR Kit; x500 (Cat. No. 208254, Qiagen)
- Nuclease-free water

Page 1 of 7

- Primers and dual-labelled probe (for IS1111 target sequence); (IDT – Integrated DNA Technologies). Sequences detailed below:

Primer/Probe Name	Sequence (5' – 3')
Forward Primer (IS1111 For)	CAT CAC ATT GCC GCG TTT AC
Reverse Primer (IS1111 Rev)	GGT TGG TCC CTC GAC AAC AT
Probe (IS1111 Probe)	*FAM* - AA TCC CCA ACA ACA CCT CCT TAT TCC CAC – *BHQ1*

5. qPCR Mastermix

The qPCR mastermix should be prepared in a designated DNA-free room. Any items posing a high risk of contaminating the mastermix with foreign DNA (such as unprocessed samples and/or DNA extracts to be used as a positive control) should not be handled immediately prior to preparing the mastermix. Any such tasks should be conducted either after preparing the mastermix or, preferably, on a different day. ***Note that no reference dye (e.g. ROX) is required on this platform.***

Reagent	Concentration	ul per reaction	Final concentration	Mix for 72 (36 in duplicate) reactions (µl)
QuantiNova Mastermix	2x	10	1x	720
Primer IS1111 For	10uM	0.8	0.4uM	57.6
Primer IS1111 Rev	10uM	0.8	0.4uM	57.6
IS1111 Probe	5uM	0.8	0.2uM	57.6
H_2O	-	2.6	-	187.2
Total volume mix before DNA	-	15	-	1080
DNA	~10ng/ul	5	~50ng/reaction	-
Total/ reaction	-	20	-	-

To minimise risk of contaminating stock reagents, create aliquots of appropriate volumes, storing working aliquots at ~3°C, and freeze the stock reagents at -20°C until further aliquots are required. Homogenise reagents before preparing mastermix by briefly pulse-vortexing and centrifuging.

6. (a) Set-Up – qPCR Reagents and Samples

Remove DNA from freezer and thaw at room temperature, then briefly pulse-vortex and centrifuge to homogenise. Similarly, ensure the mastermix is homogenised by briefly pulse-vortexing then centrifuging. Transfer 15ul of the mastermix into each of the strip-tube wells using sterile filter pipette tips, while inside the laminar flow hood. The solution may remain near the top of the tube - this is not a cause for concern due to the centrifugal force the samples will experience in the Rotor-Gene; simply ensure that the full 15ul is inside the tube. Subsequently transfer 5ul of DNA to each well to obtain a final reaction volume of 20ul, using a new filter tip for every reaction. As the set-up of each four-tube strip is completed, firmly secure strip caps in place to minimise the opportunity for contamination of reaction mixtures. Finally, load the negative extraction control well (if not determined in a previous run), the negative PCR (NTC) well, and then positive control well. Ensure all caps are secure before proceeding.
All available spaces in the Rotor-Gene rotor must be occupied before starting a run to ensure the rotor is balanced - use empty tubes to fill vacant spaces if necessary.
The template blow can be used to record the samples being subjected to qPCR (in duplicate) and the resulting Ct values of each reaction. *(See page 6 for 4 copies per page - easier printing).*

ID	Ct	ID	Ct	ID	Ct	ID	Ct	ID	Ct	ID	Ct	ID	Ct	ID	Ct	ID	Ct
1.		5.		9.		13.		17.		21.		25.		29.		33.	
2.		6.		10.		14.		18.		22.		26.		30.		34./-ve ext.	
3.		7.		11.		15.		19.		23.		27.		31.		+ve	
4.		8.		12.		16.		20.		24.		28.		32.		NTC	

(b) Set-up – Standardised Sample and File Naming Formats
For ease of subsequent interpretation and analysis of results, it would be best ensure that the name of all samples being screened are recorded in a standard format, as follows:

- **'Project or owner of sample_sample type_sample ID number.'**

- For example: **BZA_M1_1351**. *This indicates that the sample is from the BacZoo project, has been extracted from milk, and is sample number 1351 in the series.*

For the same reasons noted above, it would also be sensible to ensure that the name of all RotorGene run files (.rex) and output files (.csv, .docx etc.) follow a standard naming format, as follows:

- **'Target Locus / type of assay / project or owner of sample_sample type_sample ID range / Date.'**
- For example: **IS1111 qPCR BZA_M1_0007-0133 23May16**. *This indicates that the run file is from a qPCR reaction targetting the IS1111 locus, that the samples being screened originate from the BacZoo project and are milk samples with IDs ranging from 0007 to 0133, and that the run was carried out on the 23rd May 2016.*
- **NB** When generating a results output file, use the same file name as that used for the Run file (above), with the addition of the word "report" preceding the date.

7. Cycling Conditions

The reactions should be conducted using a Rotor-Gene Q/6000.
Place strip-tubes into slots of appropriate rotor, being careful to insert the tubes in the correct order and orientation, ensuring the order matches the slot numbers on the rotor itself. Attach the locking disc to secure the tubes in place, and insert into the Rotor-Gene machine; the rotor will click into place. Close the lid.
For a standard run, the following procedure may be followed. Choose the two-step cycle, then select the appropriate size of rotor and check the 'Locking Ring Attached' box before continuing. Choose appropriate sample volume and layout. Choose appropriate channel for detection purposes ('Green' channel chosen in this case due to FAM labelled probe). Opt to Edit Profile and choose the following settings:

Cycle Profile:

Stage	Temperature (°C)	Time	
Initial Heat Activation	95	2 minutes	
Denaturation	95	5 seconds	*X45 cycles*
Annealing/Extension	60	5 seconds	

Ensure the fluorescence will be acquired via the appropriate channel (here, 'Green'), at the Annealing/Extension phase, then click OK to return to the previous window. Highlight the Green fluorescence option then open the Gain Optimisation window. Choose 'Optimise Acquiring' and select OK before checking the box to 'Perform Optimisation at 60 Degrees at Beginning of Run', then close. Start run.

Once run has started, use Edit Samples to input the sample information, choosing a colour to represent the curve produced for each sample, then a sample name, type and concentration if known. Lastly, select whether the sample is to be analysed ('selected') or not, which units the measurements should be taken in and the format for the concentrations provided. When the run is complete, sample analysis can be conducted using the tab of same name and a report of the results can be generated.

8. Diagnostic criteria

First, ascertain whether all negative control wells (NTC and negative extraction control if included) are negative and do not show a Ct reading. Then progress to checking that both positive control wells are positive and exhibit a Ct value within the expected range. If these requirements are satisfied, proceed.

Before reading the Ct values, the following steps may be followed: choose to view the curves produced in 'linear scale', choose 'slope correct' if necessary and choose whether it is beneficial to ignore a certain number of early steps (up to 5). Now adjust the threshold to the most appropriate level. Read the Ct values reported for samples that show amplification, and make a decision as to whether sufficient evidence exists to determine whether there was or was not Coxiella in the sample. Use the following table to inform diagnosis and subsequent steps:

Result	Interpretation	Next step?
1. Duplicate samples Ct < 40	1. Sample is positive for *Coxiella* spp. infection	1. Record as positive (1) -> select samples with Ct < 36
2. Single sample Ct < 40	2. Inconclusive result	2. Repeat reaction in triplicate (see 4 a-c)
3. Both samples Ct > 40 (or no reading)	3. Negative	3. Record as negative (0)
4. Repeat testing in triplicate		
a. 2-3 tests Ct < 40	a. Sample is positive for *Coxiella* spp. infection (Total = 3-4/5 Ct < 40)	See 1. Above
b. 1 test Ct < 40	b. Sample is probable for *Coxiella* spp. infection (Total = 2/5 Ct < 40)	Record as probable (3)

c. 0 test Ct < 40	c. Sample is negative for *Coxiella* spp. infection (Total = 1/5 Ct < 40)	See 3. Above

Save results before exiting the programme.

Assay Target Locus: Sample types: Date:

ID	Ct	ID	Ct	ID	Ct	ID	Ct	ID	Ct	ID	Ct	ID	Ct	ID	Ct	ID	Ct
1.		5.		9.		13.		17.		21.		25.		29.		33.	
2.		6.		10.		14.		18.		22.		26.		30.		34.	
3.		7.		11.		15.		19.		23.		27.		31.		+ve	
4.		8.		12.		16.		20.		24.		28.		32.		NTC	

Assay Target Locus: Sample types: Date:

ID	Ct	ID	Ct	ID	Ct	ID	Ct	ID	Ct	ID	Ct	ID	Ct	ID	Ct	ID	Ct
1.		5.		9.		13.		17.		21.		25.		29.		33.	
2.		6.		10.		14.		18.		22.		26.		30.		34.	
3.		7.		11.		15.		19.		23.		27.		31.		+ve	
4.		8.		12.		16.		20.		24.		28.		32.		NTC	

Assay Target Locus: Sample types: Date:

ID	Ct	ID	Ct	ID	Ct	ID	Ct	ID	Ct	ID	Ct	ID	Ct	ID	Ct	ID	Ct
1.		5.		9.		13.		17.		21.		25.		29.		33.	
2.		6.		10.		14.		18.		22.		26.		30.		34./-	

																		ve ext.	
3.		7.		11.		15.		19.		23.		27.		31.		+ve			
4.		8.		12.		16.		20.		24.		28.		32.		NTC			

RESEARCH OUTPUTS

Journal papers

Theonest, N. O., Carter, R. W., Amani, N., Doherty, S. L., Hugho, E., Keyyu, J. D., Mable, B. K., Shirima, G. M., Tarimo, R., Thomas, K. M., Haydon, D. T., Buza, J. J., Allan, K. J., & Halliday, J. E. B. (2019). Molecular detection and genetic characterization of *Bartonella* species from rodents and their associated ectoparasites from northern Tanzania. *PLoS ONE, 14*(10), e0223667. https://doi.org/10.1371/journal.pone.0223667

Theonest, N. O., Carter, R. W., Elizabeth, K., Keyyu, J. D., Shirima, G. M., Tarimo, R., Thomas, K. M., Wheelhouse, N., Maro, P. V., Hydon, T. D., Buza, J. J., Allan J. K., & Halliday B. E. J. (2019). Molecular detection of *Coxiella burnetii* infection in small mammals from Moshi Rural and Urban Districts, northern Tanzania. *Veterinary Medicine and Science*, 2020 (00), 1–8. https://doi.org/10.1002/vms3.401

Poster presentation

Ndyetabura, T., Allan, K., Keyyu, J., Hugo, E., Buza, J., Shirima, G., Haydon, D., & Halliday, J. (2020). Molecular detection of rodent borne zoonoses in the Northern zone of Tanzania: *A one health approach towards understanding of zoonotic cause of febrile illness. In the 38th TVA Scientific Conference at Arusha International Conference Centre.*

PLOS ONE

RESEARCH ARTICLE

Molecular detection and genetic characterization of *Bartonella* species from rodents and their associated ectoparasites from northern Tanzania

Ndyetabura O. Theonest[1,2], Ryan W. Carter[3], Nelson Amani[2], Siân L. Doherty[3], Ephrasia Hugho[2], Julius D. Keyyu[4], Barbara K. Mable[3], Gabriel M. Shirima[1], Rigobert Tarimo[1,2], Kate M. Thomas[2,5], Daniel T. Haydon[3], Joram J. Buza[1], Kathryn J. Allan[3], Jo E. B. Halliday[3]*

1 School of Life Sciences and Bioengineering, Nelson Mandela African Institution of Science and Technology, Arusha, Tanzania, 2 Kilimanjaro Clinical Research Institute, Moshi, Tanzania, 3 The Boyd Orr Centre for Population and Ecosystem Health, Institute of Biodiversity Animal Health and Comparative Medicine, College of Medical Veterinary and Life Sciences, University of Glasgow, Glasgow, Scotland, United Kingdom, 4 Tanzania Wildlife Research Institute, Arusha, Tanzania, 5 Centre for International Health, Dunedin School of Medicine, University of Otago, Dunedin, New Zealand

* Jo.Halliday@glasgow.ac.uk

OPEN ACCESS

Citation: Theonest NO, Carter RW, Amani N, Doherty SL, Hugho E, Keyyu JD, et al. (2019) Molecular detection and genetic characterization of *Bartonella* species from rodents and their associated ectoparasites from northern Tanzania. PLoS ONE 14(10): e0223667. https://doi.org/10.1371/journal.pone.0223667

Editor: Baochuan Lin, Defense Threat Reduction Agency, UNITED STATES

Received: March 4, 2019

Accepted: September 25, 2019

Published: October 15, 2019

Data Availability Statement: Datasets supporting this manuscript are available through: http://dx.doi.org/10.5525/gla.research.data.859. Unique sequences generated through this study are available through GenBank (accession numbers MK906043 to MK906046 and MN256472 to MN256489).

Funding: This study was supported by a Leverhulme - Royal Society Africa Award (grant number AA130131; https://www.leverhulme.ac.uk,

Abstract

Background

Bartonellae are intracellular bacteria, which can cause persistent bacteraemia in humans and a variety of animals. Several rodent-associated *Bartonella* species are human pathogens but data on their global distribution and epidemiology are limited. The aims of the study were to: 1) determine the prevalence of *Bartonella* infection in rodents and fleas; 2) identify risk factors for *Bartonella* infection in rodents; and 3) characterize the *Bartonella* genotypes present in these rodent and flea populations.

Methods and results

Spleen samples collected from 381 rodents representing six different species were tested for the presence of *Bartonella* DNA, which was detected in 57 individuals (15.0%; 95% CI 11.3–18.5), of three rodent species (*Rattus rattus* n = 54, *Mastomys natalensis* n = 2 and *Paraxerus flavovittis* n = 1) using a qPCR targeting the *ssr*A gene. Considering *R. rattus* individuals only, risk factor analysis indicated that *Bartonella* infection was more likely in reproductively mature as compared to immature individuals (OR = 3.42, $p < 0.001$). *Bartonella* DNA was also detected in 53 of 193 *Xenopsylla cheopis* fleas (27.5%: 95% CI 21.3–34.3) collected from *R. rattus* individuals. Analysis of *ssr*A and *glt*A sequences from rodent spleens and *ssr*A sequences from fleas identified multiple genotypes closely related (≥ 97% similar) to several known or suspected zoonotic *Bartonella* species, including *B. tribocorum*, *B. rochalimae*, *B. elizabethae* and *B. quintana*.

https://royalsociety.org). KJA was supported by the Wellcome Trust (grant number 098400/Z/11/Z; https://wellcome.ac.uk/). JEBH, GMS and DTH received support from the Research Councils UK, UK Department for International Development, and UK Biotechnology and Biological Sciences Research Council (BBSRC) (grant numbers BB/J010367/1, BB/L018845; http://www.bbsrc.ac.uk/). KMT received support from the Research Councils UK, UK Department for International Development, and UK Biotechnology and Biological Sciences Research Council (BBSRC) (grant number BB/L017679; http://www.bbsrc.ac.uk/). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

Conclusions

The *ssr*A and *glt*A sequences obtained from rodent spleens and *ssr*A sequences obtained from fleas reveal the presence of a diverse set of *Bartonella* genotypes and increase our understanding of the bartonellae present in Tanzanian. Further studies are needed to fully characterise the prevalence, genotypes and diversity of *Bartonella* in different host populations and their potential impacts on human health.

Introduction

Bartonella are fastidious, Gram-negative, vector-borne bacteria with worldwide distribution. *Bartonella* species are known to infect mainly erythrocytes and endothelial cells of various mammals, such as humans, cats, dogs, ruminants, wild rabbits and rodents [1]. Epidemiological studies have demonstrated that rodents and other small mammals are important hosts of *Bartonella* species and that ectoparasites such as fleas, ticks, sand flies, and lice are key vectors of *Bartonella* infection [2,3].

In recent years, an increasing number of *Bartonella* species have been identified as zoonotic pathogens. To date there are roughly 45 *Bartonella* species and subspecies that have been designated [4], of which at least 20 are rodent-associated [1]. Several studies indicate that rodent-associated *Bartonella* are the cause of human infections in various regions of the world, particularly in areas where humans are in close contact with rodents [5–10]. However, knowledge of the distribution and epidemiology of *Bartonella* in rodents and of the role of *Bartonella* species in human disease in Tanzania is limited.

Clinical manifestations of *Bartonella* infection in humans can range from mild [7] to life threatening disease and can present as acute or chronic [5,11]. Known sequelae attributed to *Bartonella* species include endocarditis [8], myocarditis [12], fever and neurologic disorders [13], intraocular neuroretinitis [14], meningitis, splenomegaly and lymphadenopathy [15]. This range of non-specific and variable symptoms makes *Bartonella* infections hard to diagnose clinically. This contributes to a poor understanding of the current distribution and relative importance of infections caused by this pathogen. The challenges of identifying the causes of non-specific febrile illness are demonstrated by previous research conducted in Moshi, northern Tanzania, where a study of patients admitted to hospital with febrile illness revealed that a range of zoonotic pathogens were responsible for roughly a quarter of the hospital admissions [16]. However, no zoonotic infections were included in the admission differential diagnosis for any patient in that study, indicating lack of awareness and diagnostic capacity for many zoonotic pathogens. Over the past few decades, numerous reports of bartonellosis in febrile humans have been made globally [6,8–10,17,18]. However, in developing countries bartonellosis is often not considered as a potential diagnosis.

Molecular detection and typing methods for *Bartonella* are widely used due to their greater sensitivity and ease of use in comparison to culture and serology based approaches. Real-time PCR assays are recommended for primary screening of *Bartonella* species followed by confirmatory assays, using either conventional or real-time PCR and sequencing [19,20]. In Africa, studies conducted in Ethiopia [21], Kenya [22], South Africa [23], the Democratic Republic of Congo [24,25] and Uganda [26] have previously confirmed detection of *Bartonella* species in rodents using conventional PCR detection methods for multiple gene targets. In northern Tanzania, a study performed in Mbulu, a rural district in northern Tanzania, detected *Bartonella* in 41% of indigenous rodents using *glt*A and *rpo*B PCR targets [25]. Analyses of *glt*A

sequences from these samples revealed the presence of multiple genotypes similar to known *Bartonella* species, including *B. elizabethae*, *B. tribocorum*, *B. birtlesii* and *B. queenslandensis* [25].

Bartonella species are present in Tanzania and may contribute to the burden of human febrile illness in northern Tanzania. However, the distribution and epidemiology of *Bartonella* in Tanzania is largely unknown. The aims of this study were to use molecular diagnostic tools to estimate the prevalence of *Bartonella* infection in rodents and their fleas sampled in northern Tanzania. Specifically, we aimed to: 1) determine the prevalence of *Bartonella* infection in rodents and fleas using quantitative real-time polymerase chain reaction (qPCR); 2) identify risk factors for *Bartonella* infection in rodents; and 3) use sequencing of the *gltA* and *ssrA* genes to characterize the *Bartonella* genotypes present in these rodent and flea populations.

Methods

Ethics statement

Ethical approval for the study was granted by the Tanzania Commission for Science and Technology (COSTECH 2012-471-ER-2005-141 & 2015-71-NA-2011-199); Kilimanjaro Christian Medical Centre (KCMC) Ethics Committee (535 & 537); National Institute of Medical Research (NIMR), Tanzania (NIMR/HQ/R.8a/Vol.IX/1499 & NIMR/HQ/R.8a/Vol.IX/1522); Tanzania Wildlife Research Institute (TAWIRI); University of Glasgow College of Medical, Veterinary and Life Sciences Ethics Committee (200120020), and University of Glasgow Faculty of Veterinary Medicine Ethics and Welfare Committee (01a/13 & 02a/13). Written consent for study participation was obtained for each participating household, using forms translated into Swahili. Rodent sampling was performed in accordance with the UK Guidance on the Operation of the Animals (Scientific Procedures) Act 1986 and American Veterinary Medical Association Guidelines for the Euthanasia of Animals [27,28].

Rodent trapping and sampling

Rodent spleen samples and ectoparasites were obtained from a cross-sectional study conducted to explore the role of rodents in the epidemiology of leptospirosis and other zoonoses in the Kilimanjaro region of northern Tanzania [29]. Rodents were trapped in five villages within Moshi Municipal District and seven villages within Moshi Rural District, as previously described [29] (Fig 1). The target sample size was 50 rodents per village to give sufficient power ($\alpha = 0.95$, $\beta = 0.8$) to detect a minimum infection prevalence of 10% [29]. Villages for sampling were randomly selected from a list, home to people that had sought care, and had been enrolled in previous febrile illness surveillance studies at local hospitals [16]. Rodent trapping was performed in three sessions: 1) May-June 2013 (wet season); 2) May-June 2014 (wet season); and 3) August-September 2014 (dry season). Rodents were trapped in households in a total of 12 villages through cross-sectional visits, with one additional round of repeat sampling conducted in one village (based on high trap success in the initial visit) [29]. Trapped rodents were euthanized by terminal halothane anaesthesia and cervical dislocation. Data gathered for every trapped rodent included: species (determined by observation of phenotypic characteristics and measurement of morphometric features), sex and reproductive maturity status (mature or immature determined based on external sexual characteristics [30]). A full necropsy and tissue sampling were performed for each rodent sampled. A fresh sterile scalpel blade was used for each rodent and all other necropsy equipment was washed using 5% Virkon and dried between usages to avoid cross-contamination. Spleen tissue samples were collected into sterile Eppendorf tubes and stored at -80˚C prior to DNA extraction. Ectoparasites

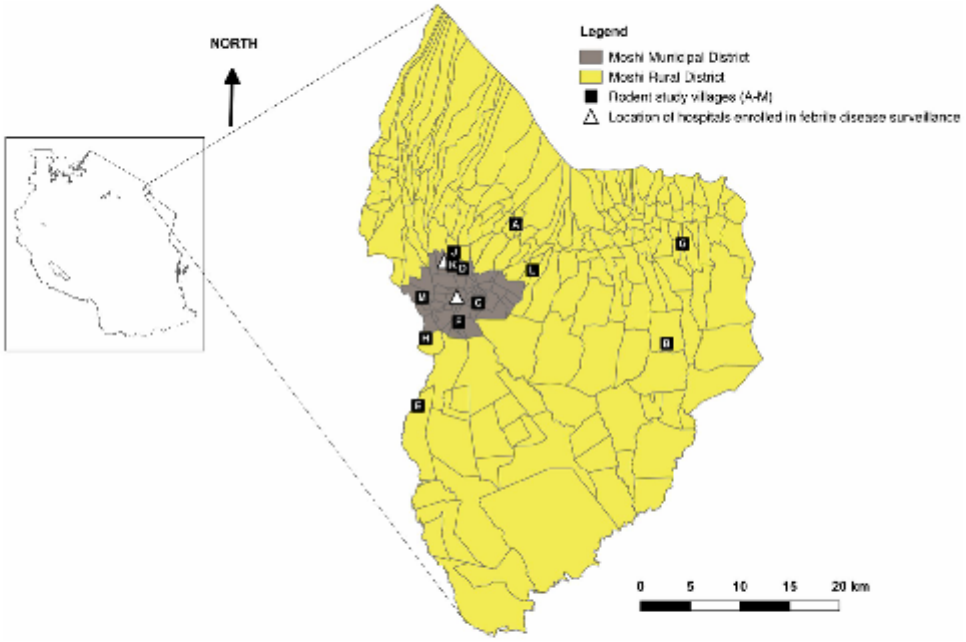


Fig 1. Map of Moshi Municipal and Moshi Rural Districts, showing representative locations of rodent study villages in relation to the two hospitals (Kilimanjaro Christian Medical Centre and Mawenzi Regional Referral Hospital) at which febrile illness surveillance has been conducted in previous studies. Letters indicate the different villages in which rodent trapping was conducted. Polygons in the main image show local administrative boundaries. Inset map on left shows outline of Tanzania and location of study districts within the country. This figure is adapted from a version published previously [29].

https://doi.org/10.1371/journal.pone.0223667.g001

observed on trapped rodents were collected and stored in 70–96% ethanol; all ectoparasites from the same rodent were stored together. Collected fleas were identified to species level using a dissecting microscope and a pictorial flea identification guide [31]. *Xenopsylla cheopis* fleas were selected for DNA extraction and *Bartonella* testing based on their known contribution to *Bartonella* transmission [32]. For each rodent with at least one *X. cheopis* collected, DNA was extracted from one (if only one *X. cheopis* present on that host) or two (if more than one *X. cheopis* present on that host). Where multiple *X. cheopis* were collected from the same rodent, selection of individual fleas for DNA extraction was opportunistic.

DNA extraction

DNA was extracted from approximately 10 milligrams (mg) of spleen tissue using the DNeasy Blood and Tissue Kit spin-column protocol for DNA purification from tissues (Qiagen, Hilden, Germany). DNA from spleen tissues was eluted in 100μl of AE buffer and quantified using a Nano-Drop spectrophotometer (Thermo Scientific, Waltham, MA, USA). DNA from individual whole fleas was also extracted using the DNeasy Blood and Tissue Kit, following the protocol for purification of total DNA from ticks and eluted in a final volume of 65μl AE buffer. For all extractions, a no-template extraction control (PCR-grade water) was included

for every 20 samples and DNA extracts were stored at -20˚C prior to testing. DNA extracts from spleens were diluted in 20μl of AE buffer to a standard DNA concentration of 10–50 ng/μl for PCR testing, to minimize the potential for PCR inhibition due to the high concentration of host DNA in the rodent spleen extracts. Due to the lower concentration of DNA in extracts from fleas, these were tested directly from extraction concentrations.

Quantitative real-time PCR for detection of *Bartonella* species *ssr*A gene

DNA extracts from rodent spleens and fleas were screened using a *Bartonella* genus-specific real-time PCR assay (qPCR) targeting the transfer-mRNA *ssr*A gene, using a previously published protocol [20]. The original paper describing this assay reports a lower limit of detection of < 5 fg of *Bartonella* DNA, equivalent to < 3 genomic copies per reaction when tested against four *Bartonella* species (*B. quintana*, *B. henselae*, *B. bovis*, and *B. elizabethae*) [20]. The primers *ssr*A-F (5'–GCTATGGTAATAAATGGACAATGAAATAA–3'), *ssr*A-R (5' GCTTCTGTTGCCAGGTG–3') and 6-carboxyfluorescein FAM-labelled probe (5'–ACCCCGCTTAAACCTGCGACG–3'–BHQ1) were used to amplify an *ssr*A gene fragment of approximately 300bp. qPCR reactions were carried out in 20 μl volumes comprised of 10 μl QuantiNova Probe PCR mix (Qiagen), 0.8 μl of each primer (10 μM) and probe (5 μM), 2.6 μl nuclease-free water and 5 μl DNA template. Positive control (rodent tissue DNA extract obtained from a previous study [24] positive for *Bartonella* with closest similarity to *B. tribocorum*), extraction controls and no-template controls were included in each qPCR run. Assays were performed on a Rotor-Gene Q/6000 (Qiagen) with manufacturer recommended thermocycling conditions as follows: 95 ˚C for 2 minutes, followed by 45 cycles of 95 ˚C for 5 seconds and 60 ˚C for 5 seconds. A qPCR run was considered valid when all negative controls showed no evidence of amplification and the positive controls amplified with a Ct value of < 40. Extracts were tested in duplicate and considered positive when amplification was recorded in one or more test wells with a Ct value ≤40.

PCR amplification and sequencing of *Bartonella ssr*A gene products

For sequencing, conventional PCR amplification of the *ssr*A gene was performed on all DNA extracts from both rodent spleens and fleas that were positive in the *ssr*A qPCR, based on a previously published protocol [20]. Each PCR reaction (25 μl) comprised 2.5 μl of PCR 10X buffer, 0.1μL Platinum Taq polymerase, 0.75μL $MgCl_2$, 0.5 μl dNTPs (10 μM) (Invitrogen, USA) and 0.5 μl of each primer *ssr*A-F and *ssr*A-R at 10 μM [20]. Template DNA volume varied from 5–10 μl depending on *ssr*A assay Ct value. Nuclease-free water was used to make up the total reaction volumes as needed. Amplifications were performed with the following conditions: 94 ˚C for 2 minutes, followed by 40 cycles of 94 ˚C for 15 seconds, 60 ˚C for 60 seconds, and 72 ˚C for 30 seconds, and then a final extension step of 72 ˚C for 3 minutes. Positive and negative controls were included in each PCR run. PCR products were visualized by electrophoresis in a 1.5% agarose gel stained with GelRed (Cambridge Bioscience, Cambridge, UK). A sample was considered positive if a clearly defined DNA band of approximately 300 bp was visible in the gel and confirmed as *Bartonella* by sequencing of the product. To confirm and characterize the genotypes of *Bartonella* detected, amplicons with the expected size were purified using either a QIAquick PCR or Gel Extraction Purification Kit (Qiagen). Sequencing was performed at Source Biosciences (Nottingham, UK) using the same primers as for the detection PCR [33]. Sequence identity was confirmed using BLASTn, as implemented in the National Centre for Biotechnology Information (NCBI) web portal.

PCR amplification and sequencing of *Bartonella gltA* gene products

Conventional PCR amplification of the *glt*A gene was performed on all rodent spleen DNA extracts, based on a previously published protocol [34]. Each PCR reaction (25 μl) comprised 12.5 μl of PCR 2X master mix (Promega, Madison, WI, USA), 1.25 μl 5% dimethyl sulphoxide (DMSO) (Sigma-Aldrich, St. Louis, USA), 1.25 μl molecular grade water (Qiagen), 2.5 μl of each oligonucleotide primer (10 μM), BhCS781.p (5'-GGGGACCAGCTCATGGTGG-3') and BhCS1137.n (5'- AATGCAAAAAGAACAGTAAACA-3') [34], and 5 μl DNA template. Amplifications were performed on a PTC-240 DNA-Engine (MJ Research/BioRad Technologies, USA) with the following conditions: 94 ˚C for 2 minutes, followed by 40 cycles of 94 ˚C for 30 seconds, 54.3 ˚C for 30 seconds, and 72 ˚C for 2 minutes, then a final step of 72 ˚C for 7 minutes. Positive and negative controls were included in each PCR run. PCR products were visualized by electrophoresis in a 1.5% agarose gel stained with ethidium bromide (Invitrogen, USA). A sample was considered positive if a clearly defined DNA band of approximately 379 bp was visible in the gel and confirmed as *Bartonella* by sequencing of the product. Purification, sequencing and BLAST analyses were conducted as for the *ssr*A gene.

Phylogenetic analyses for *ssr*A and *glt*A gene products

Incomplete and poor quality sequences (e.g. with ambiguous peaks) were excluded from phylogenetic analysis for both *ssr*A and *glt*A gene fragments. For each gene, sequences were aligned using the ClustalW algorithm, implemented in MEGA 7.0 [35]. The model test function in MEGA 7.0 was used to select the best-fitting nucleotide substitution models, which were then incorporated into a phylogenetic analysis based on a maximum likelihood optimality criterion for tree reconstruction, with 1000 bootstrap pseudoreplicates. For the *ssr*A analysis, rodent spleen and flea sequences from this study were aligned with reference *ssr*A sequences from cultured *Bartonella* species downloaded from GenBank (see GenBank accession numbers in Fig 2). A *Brucella melitensis* sequence was used as the outgroup [36]. For analysis of the *glt*A data, sequences from study rodent spleens were aligned with those from *Bartonella* reference strains obtained from GenBank and also with representative sequences from previous studies conducted in East Africa (see GenBank accession numbers in Fig 3). Reference sequences in the alignment included *glt*A sequences from previous studies of *Bartonella* in rodents from Tanzania [25], Kenya [22], the Democratic Republic of Congo [24,25] and Uganda [26] that included either similar rodent species or *Bartonella glt*A sequences similar to those obtained in this study. A *B. tamiae glt*A sequence obtained from an African bat was used as the outgroup [37].

Statistical analyses

Statistical analyses were performed in R [38]. Exact binomial proportions and confidence intervals for prevalence estimates were calculated using the package 'binom' [39]. Generalized linear mixed models (GLMM), with binomial family and logit link function, were used to examine variables associated with rodent and flea *ssr*A qPCR test status (qPCR positive vs negative) and implemented using the package 'lme4' [40]. For rodents, the dataset for these analyses was limited to *R. rattus* only, given the dominance of this species. Explanatory variables considered in the GLMM for rodent *ssr*A qPCR status included host sex and reproductive maturity (mature or immature), which were determined based on external sexual characteristics [30]. Trapping season (wet or dry), trapping district (Moshi Municipal or Moshi Rural) and rodent abundance were also included as explanatory variables for analysis. Adjusted trap

Fig 2. Phylogenetic tree showing the relatedness of the *Bartonella ssrA* gene sequences (237bp gene fragments) derived from 45 rodent spleen tissue samples (43 *R. rattus*, 1 *M. natalensis* and 1 *P. flavovottis)* and 39 *X. cheopis fleas* collected in northern Tanzania. A single representative sample sequence is included for each combination of *Bartonella* genotype identified in this study and host of origin. Genotypes (1s–12s) and groups (As–Es) are indicated by lettering. Groups A, C and E are shaded grey, with groups B and D in pink. The phylogenetic tree was constructed using the maximum likelihood method based on a Kimura 2-parameter substitution model [41], as determined by Modeltest as implemented in MEGA 7.0 [35]. The tree with the highest log likelihood is shown and drawn to scale, with branch lengths shown in terms of the number of substitutions per site. Vertical branches indicate identical sequences. The numbers at the nodes correspond to bootstrap values higher than 70% after 1000 replicates. Sequences from this study are labelled with unique identifiers, with prefix "R" followed by sample identifier numbers, Genbank accession number, the

rodent or flea host species, the genotype code and the number of samples yielding each genotype (in parentheses). Sequences from reference strains of *Bartonella* are included with the *Bartonella* species name and GenBank accession numbers given in parentheses. *Brucella melitensis* was included as an outgroup.

https://doi.org/10.1371/journal.pone.0223667.g002

success data were calculated by dividing the total number (n) of rodents caught per village by the corrected number of trap nights, which is calculated as: total number of trap nights (number of traps x number of nights) minus lost trap nights (sum of number of closed, damaged or lost traps / 2) and expressed as a percentage [29]. The village identification variable was included as a random effect to account for the clustered sampling strategy. For fleas, rodent *ssr*A qPCR test status was the only explanatory variable evaluated in the GLMM and the village identification variable was included as a random effect. Initial maximal multivariable models were created including all candidate explanatory variables and likelihood ratio tests were used to compare candidate models and guide model selection.

Results

Sample characteristics

Spleen samples from a total of 381 rodents were available for testing. The majority (n = 317, 83.2%), were from black rats (*R. rattus*) (Table 1). Other rodent species tested included: house mice (*Mus musculus*, n = 44, 11.5%); African pygmy mice (*Mus minutoides*, n = 3, 0.8%); multimammate mice (*Mastomys natalensis*, n = 8; 2.1%), spiny mice (*Acomys wilsonii*, n = 6, 1.6%) and striped bush squirrels (*Paraxerus flavovittis*, n = 3, 0.8%). Of the rodents tested, 219 individuals (57.5%) were female. The majority of all sampled rodents (n = 224, 58.8%) were classified as sexually mature based on external sexual characteristics. A total of 265 of the 381 rodents (69.6%) were trapped during wet season sampling. A total of 513 fleas were collected from 153 of the 381 (40.2%) rodents included in the study. Flea species identified were *Xenopsylla cheopis* (n = 306), *Echidnophaga gallinacea* (n = 204) and *Ctenocephalides felis* (n = 3). DNA extracts from a total of 193 *Xenopsylla cheopis* collected from 124 rodents (*Rattus rattus* n = 118, *Mus musculus* n = 4 and *Mastomys natalensis* n = 2) were available for *Bartonella* testing using *ssr*A.

Bartonella detection in rodent spleens and risk factors for rodent infection

Bartonella DNA was detected by *ssr*A qPCR in a total of 57 of 381 (15.0%, 95% CI 11.5–18.9%) rodents screened (Table 1). Samples derived from *Mastomys natalensis*, *Paraxerus flavovittis* and *R. rattus* (Table 1) were all classified as *Bartonella* positive by this test. The positive control used in *ssr*A qPCR runs to test rodent spleen extracts had a mean Ct value of 32. The assay showed a 100% lower limit of detection of 1.8 fg of *Bartonella quintana* DNA control. For the logistic regression analysis considering data from *R. rattus* only, rodent reproductive maturity status was the only significant risk factor (LRT: $\chi_2 = 13.30$, df = 1, $p < 0.0003$), with reproductively mature individuals more likely to be *ssr*A qPCR positive (OR 3.42, 95% CI 1.69–6.89, $p < 0.001$). None of the other candidate variables evaluated (rodent sex, trapping season, trapping district or rodent abundance at trapping village) were significantly associated with *R. rattus ssr*A qPCR test status. The breakdown of rodents trapped by village is given in Table 2.

Bartonella detection in fleas

Bartonella DNA was detected by *ssr*A qPCR in 53 of 193 (27.5%, 95% CI 21.3–34.3%) *X. cheopis* flea extracts. All *ssr*A qPCR positive flea extracts were collected from *R. rattus* individuals. The positive control used in *ssr*A qPCR runs to test flea extracts had a mean Ct value of 32.

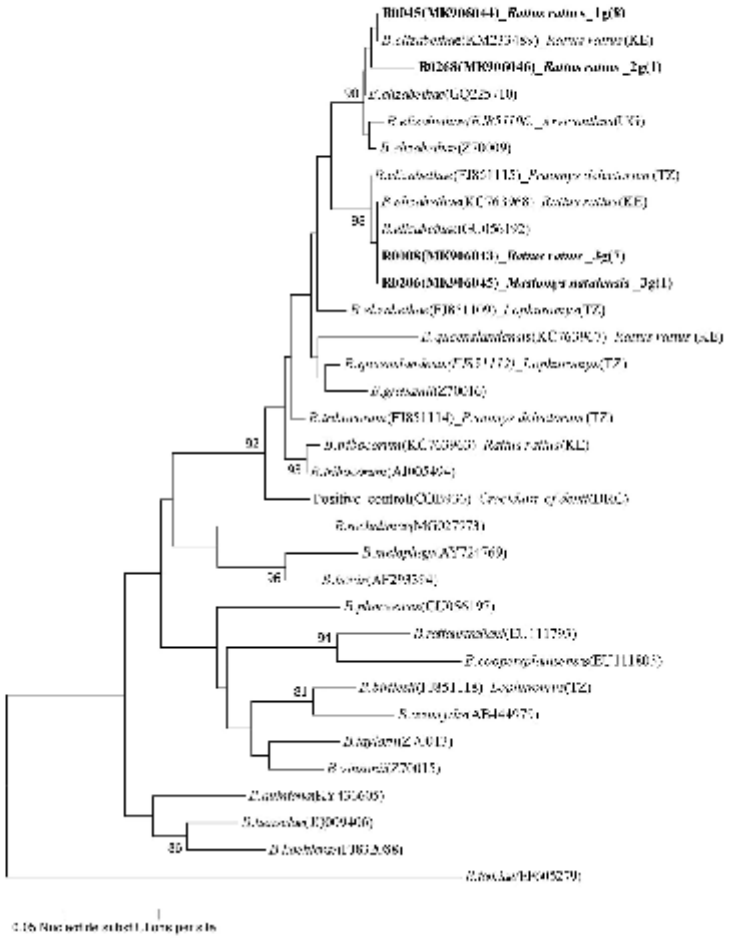


Fig 3. Phylogenetic tree showing the relatedness of the *Bartonella gltA* gene sequences (283bp fragments) derived from 17 spleen tissue samples from rodents (16 *R. rattus* and 1 *Mastomys natalensis*) trapped in northern Tanzania. A single representative sample sequence is included for each genotype identified in this study, with the exception of genotype 3g to illustrate the identical sequences obtained from *R. rattus* and *M. natalensis*. Sequences from this study are labelled with unique identifiers, with prefix "R" followed by sample identifier numbers, Genbank accession number, the rodent or flea host species, the genotype code and the number of samples yielding each genotype (in parentheses). Reference *Bartonella* sequences from rodents trapped elsewhere in East Africa obtained from GenBank are indicated by GenBank accession numbers in parentheses, rodent species and country code (Kenya (KE) [22], Uganda (UG) [26] Tanzania (TZ) [25], Democratic Republic of Congo (DRC) [25]). The sequence obtained for the known *Bartonella* positive control sample provided by a colleague from a previous study is included and indicated with a unique identification number (COB936) [24].

https://doi.org/10.1371/journal.pone.0223667.g003

Table 1. Summary of rodent species and their *Bartonella* detection status defined by *ssrA* qPCR and sequence confirmed by *gltA* PCR testing of spleen samples.

Rodent species	Number of spleen samples tested	*Bartonella ssrA* qPCR Positive n (%)	*Bartonella ssrA* qPCR Ct values of Positives	*Bartonella gltA* Product Sequence Positive n (%)
Acomys wilsonii	6	0 (0)	NA	0 (0)
Mastomys natalensis	8	2 (25.0)	33.23 & 33.54	1 (12.5)
Mus minutoides	3	0 (0)	NA	0 (0)
Mus musculus	44	0 (0)	NA	0 (0)
Paraxerus flavovittis	3	1 (33.3)	36.49	0 (0)
Rattus rattus	317	54 (17.0)	median value of 33.07, range 24.25–39.56	16 (5.0)
Total	**381**	**57 (15.0)**		**17 (4.4)**

https://doi.org/10.1371/journal.pone.0223667.t001

Logistic regression analysis identified a relationship between flea *ssrA* qPCR test status and the *ssrA* qPCR test status of the rodent that each flea was collected from (LRT: $\chi_2 = 20.73$, df = 1, $p < 0.001$). *X. cheopis* fleas collected from *ssrA* qPCR positive rodents were more likely to themselves be *ssrA* qPCR positive (OR 7.23, 95% CI 2.90–17.97, $p < 0.001$).

Table 2. Summary of rodent trapping data and the *Bartonella ssrA* and *gltA* genotypes detected in trapped rodents by village around Moshi, Tanzania.

Village code	District	Total number of rodents tested for *Bartonella*	Adjusted trap success [29]	*Bartonella* genotypes identified, with data on the number of individuals and rodent species each genotype was detected in	
				ssrA genotypes	*gltA* genotypes
A	Rural	12	9.79	1s–2 * *Rattus rattus*	3g–2 * *Rattus rattus*
B	Rural	13	4.28	7s–1 * *Rattus rattus* 10s–1 * *Rattus rattus*	-
C	Municipal	31	4.77	10s–1 * *Rattus rattus*	1g–1 * *Rattus rattus*
D	Municipal	25	2.68	10s–1 * *Rattus rattus* 2s–1 * *Paraxerus flavovittis*	1g–1 * *Rattus rattus*
E	Rural	39	5.28	4s–1 * *Rattus rattus* 10s–2 * *Rattus rattus*	1g–1 * *Rattus rattus* 3g–1 * *Rattus rattus*
F	Municipal	76	10.8	1s–5 * *Rattus rattus* 9s–1 * *Rattus rattus* 10s–1 * *Rattus rattus* 11s–5 * *Rattus rattus*	1g–1 * *Rattus rattus* 3g–3 * *Rattus rattus*
F (visit 2)	Municipal	33	4.42	1s–1 * *Rattus rattus* 11s–3 * *Rattus rattus*	
G	Rural	15	1.94	6s–1 * *M. natalensis*	3g–1 * *M. natalensis*
H	Rural	35	4.69	1s–1 * *Rattus rattus* 5s–5 * *Rattus rattus* 8s–1 * *Rattus rattus* 10s–3 * *Rattus rattus*	1g–2 * *Rattus rattus* 3g–1 * *Rattus rattus*
J	Rural	19	2.70	10s–4 * *Rattus rattus*	2g–1 * *Rattus rattus*
K	Municipal	23	3.19	10s–1 * *Rattus rattus*	-
L	Rural	22	2.93	1s–2 * *Rattus rattus* 10s–3 * *Rattus rattus*	1g–2 * *Rattus rattus*
M	Municipal	38	5.06	3s–1 * *Rattus rattus*	

Village codes correspond to village locations indicated in Fig 1. Distinct *ssrA* and *gltA* genotypes are identified by a sequential number and "s" or "g" respectively. Data on trap success are as reported in [29]

https://doi.org/10.1371/journal.pone.0223667.t002

Table 3. Summary of *Bartonella ssrA* genotypes identified in rodent spleens and fleas from Moshi, Tanzania.

ssrA genotypes	*ssrA* Group	Rodent species and number of positive samples	Flea species and number of positive samples	Closest *Bartonella* species (GenBank ID)[a]	% similarity to closest *Bartonella* spp. (number of base pair identities/ total base pair length)
1s	As	11 * *R. rattus* (MN25672)	11* *X. cheopis* (MN25673)	*B.tribocorum* (MF765681)	99 (240/244)
2s	As	-	1* *X. cheopis* (MN25674)	*B.tribocorum* (MF765681)	99 (217/222)
3s	As	1 * *R. rattus* (MN25675)	1* *X. cheopis* (MN25676)	*B.tribocorum* (MF765681)	99 (228/233)
4s	As	1 * *R. rattus* (MN25677)	-	*B.tribocorum* (MF765681)	99 (239/244)
5s	As	1 * *R. rattus* (MN25678)	1* *X. cheopis* (MN25679)	*B.tribocorum* (MF765681)	98 (237/244)
6s	Bs	1* *M. natalensis* (MN25680)	-	*B.elizabethae* (MF765617)	99 (222/224)
7s	Cs	1 * *R. rattus* (MN25681)	1* *X. cheopis* (MN25682)	*B.tribocorum* (MF765675)	99 (242/244)
8s	Cs	1 * *R. rattus* (MN25683)	-	*B.tribocorum* (MF765675)	99 (239/244)
9s	Cs	1 * *R. rattus* (MN25684)	-	*B.tribocorum* (MF765675)	99 (236/237)
10s	Cs	18 * *R. rattus* (MN25685)	9* *X. cheopis* (MN25686)	*B.tribocorum* (MF765675)	100 (244/244)
11s	Ds	8 * *R. rattus* (MN25687)	15* *X. cheopis* (MN25688)	*B.rochalimae* (MF765651)	100 (246/246)
12s	Es	1* *P. flavovottis* (MN25689)	-	*B.quintana* (HG518998)	98 (233/239)

The number of individuals of each rodents (n = 45) and fleas (n = 39) species from which each genotype was obtained are shown, as well as data on % similarity to reference *Bartonella* species sequences, with the number of base pair identities indicated in parentheses. The Genbank accession numbers for each genotype are also indicated in parentheses in columns 2 and 3.

[a]The closest reference sequences to the study sequences were selected from fully characterized sequences in Genbank obtained from cultures.

https://doi.org/10.1371/journal.pone.0223667.t003

Characterisation of *Bartonella* in Tanzanian rodents and fleas by *ssrA* sequencing

Full length sequences of the *ssrA* gene target were obtained from 45 of 57 rodents spleen samples (*Rattus rattus* n = 43, *Mastomys natalensis* n = 1 and *Paraxerus flavovottis* n = 1) and 39 of 53 *ssrA* positive *X. cheopis* fleas. From the 237bp *ssrA* sequences amplified, 12 unique genotypes (1s to 12s) were identified in the sequences from rodent and flea populations combined (Table 3; Fig 2). The 12 genotypes were grouped into monophyletic groups with ≥97% similarity within the group. The following groupings were identified: 1) group As: Genotypes 1s to 5s clustering with a *B. tribocorum* reference sequence from strain GDHL73 (GenBank accession number MF765681); 2) group Bs: genotype 6s was unique but clustered with a *B. elizabethae* reference sequence (GenBank accession number MF765617); 3) group Cs: genotypes 7s to 10s clustering with a sequence from *B. tribocorum* strain GDHL25 (GenBank accession number MF765675); 4) group Ds: genotype 11s clustering with a *B. rochalimae* reference sequence (GenBank accession number MF7651); and 5) group Es: genotype 12s from *P. flavovittis*, which was mostly closely related to a *B. quintana* reference sequence (GenBank accession number HG518998). The distribution of *ssrA* genotypes between trapping villages (Table 2) show that more frequently detected *ssrA* genotypes (1s, 10s and 11s) were present in rodents

Table 4. Summary of *gltA* genotypes identified in rodent spleens (n = 17) from Moshi, Tanzania and the correspondence with *ssrA* genotypes identified in the same species and individuals. Data on the % similarity to reference *Bartonella* species sequences, and the number of base pair identities are indicated. The Genbank accession numbers for each genotype identified in the study are indicated.* The closest reference sequences to the study sequences were selected from fully characterised sequences in Genbank obtained from cultures.

gltA Genotype	GenBank accession number	Rodent species and number of positive samples	Closest *Bartonella* species (GenBank ID)*.	% similarity to closest *Bartonella* spp. (number of base pair identities/ total base pair length)	*ssrA* group and genotype
1g	MK906044	8 * *R. rattus*	*B.elizabethae*(GQ225710)	99.65 (282/283)	Group C:10s
2g	MK906046	1 * *R. rattus*	*B.elizabethae* (GQ225710)	97 (278/284)	Group C:10s
3g	MK906043	5 * *R. rattus*	*B.elizabethae*(GU056192)	100 (283/283)	Group A: 1s
3g	MK906043	1 * *R. rattus*	*B.elizabethae*(GU056192)	100 (283/283)	Group A: 4s
3g	MK906045	1 * *M. natalensis*	*B.elizabethae*(GU056192)	100 (283/283)	Group B:6s
3g	MK906043	1 * *R. rattus*	*B.elizabethae*(GU056192)	100 (283/283)	No *ssrA* typing obtained

https://doi.org/10.1371/journal.pone.0223667.t004

trapped at multiple villages and provides no evidence of spatial segregation of the genotypes in this rodent population. Data on *ssrA* genotypes were available for fourteen pairs of *X. cheopis* fleas and *R. rattus* hosts (n = 11 *R. rattus* including three from which two *X. cheopis* were collected and tested). For seven pairs the *ssrA* genotype detected in fleas and rodent hosts were identical, but in the other seven pairs the genotypes differed. At the group level, 11 flea and host pairs had sequences from the same *ssrA* group and three pairs differed.

Characterisation of *Bartonella* in Tanzanian rodents by *gltA* sequencing

Full length sequences of the *gltA* gene were obtained from 17 rodent spleen DNA extracts (*R. rattus* n = 16 and *M. natalensis* n = 1). To be conservative, the alignment was pruned to the length of the shortest sequence (283 bp) and three unique genotypes were identified in this fragment (Fig 3). The correspondence between *gltA* and *ssrA* genotypes and groups is shown in Table 4. Only one *R.rattus* individual had a *gltA* sequence without a corresponding *ssrA* sequence. For the 16 remaining *gltA* genotypes, the same individuals all yielded *ssrA* sequences falling into groups As to Cs (Table 4). Eight sequences (genotype 1g, GenBank accession number MK906044) collected from *R. rattus* were identical to a *B. elizabethae* sequence obtained from a *R. rattus* sampled previously at a rural site in Kenya (strain B29297 [22], GenBank accession number KM233489). A second genotype (2g, GenBank accession number MK906046) was identified in one *R. rattus* and showed and 97% similarity (278 of 284 base pair matches) to a *B.elizabethae* sequence (GQ225710). Eight sequences (genotype 3g) collected from *M. natalensis* (GenBank accession number MK906045) and from *R. rattus* (GenBank accession number MK906043) were identical to a sample identified in a *R. rattus* from an urban site in Kenya (strain B28391 [22], GenBank accession number: KC763968) and to a cultured reference strain of *B. elizabethae* (strain BR02, GenBank accession number: GU056192).

Discussion

This study reveals substantial variation in *Bartonella* genotypes among rodents and their fleas in a previously uncharacterised region of northern Tanzania (the Moshi Municipal and Moshi Rural Districts). *Rattus rattus* was the most common rodent species trapped and showed a high *Bartonella* prevalence defined by *ssrA* qPCR, which is consistent with the global distribution of *Bartonella* species in *Rattus* [42]. Within *R. rattus*, the probability of qPCR positivity was higher in reproductively mature individuals as compared to immature individuals, consistent with other studies (performed in the USA) that have found an association with age

[43,44]. Sequencing of *ssr*A and *glt*A gene fragments revealed a variety of genotypes and the majority of sequences obtained showed greatest similarity to *B. tribocorum* and *B. elizabethae* reference sequences, both of which have been isolated in humans with febrile illness [6]. Sequences similar to *B. rochalimae* and *B. quintana* were also identified based on *ssr*A sequencing. These species were not detected by sequencing of the *glt*A, indicating reduced sensitivity of the *glt*A conventional PCR for detection of *Bartonella* species as compared to the *ssr*A qPCR. This is consistent with the findings of a previous study [20].

The *ssr*A qPCR was used to estimate prevalence in rodents and fleas, and the sequencing of *ssr*A and *glt*A PCR products to assess genetic variation and characterise the *Bartonella* detected. The overall prevalence of *Bartonella* (15%) detected in rodents using the *ssr*A qPCR was lower than has been detected in many comparable studies of global rodent populations [21,45], including studies that have used a less sensitive *glt*A assay for prevalence determination [46,47]. In a previous Tanzanian study of indigenous rodent species an overall *glt*A prevalence of 41% was detected [25]. A Ugandan survey using *glt*A to test invasive and indigenous rodent populations found variable prevalence across species, with higher prevalence in indigenous species (60% in *Arvicanthus niloticus* and 61% in *Cricetomys gambianus*), but low prevalence (1.4%) was recorded in invasive *R. rattus* [26]. Similarly, in Kenya the *Bartonella* prevalence determined by culture varied by rodent species [22]. Considering the data from *R. rattus* only, the prevalence seen in this study and previous African studies reveals consistently lower prevalence in comparison to *R. rattus* sampled in Asia and tested using *ssr*A qPCR methods (e.g. 32.5% [48]). The prevalence of *Bartonella* detection in *X. cheopis* fleas in this study using the *ssr*A qPCR was also lower than has been recorded in this species in the USA, where 190 of 200 (95%) *X. cheopis* tested were positive for Bartonella DNA [32]. The low prevalence of *Bartonella* in *R. rattus* and *X. cheopis* observed in this study are consistent with several other studies conducted in Africa. It has been argued that this pattern of lower *Bartonella* prevalence in African *R. rattus* populations could be attributed to host escape during colonization [49]. Further investigation of native and invasive rodent populations across Africa would be needed to investigate this further, and also evaluate the possible implications for human disease risk on the continent.

Phylogenetic analysis of sequences from rodents and their fleas revealed high concordance of sequences between hosts and ectoparasites. Overall, 10 distinct *ssr*A genotypes were identified that were most similar to reference sequences of *B. tribocorum* and *B. elizabethae* (Groups As to Cs), with only one genotype (10s) showing an identical match to a published *B. tribocorum* sequence in GenBank. However, since all of the reference sequences that were most similar were from a single study in China [50] it is important to recognise the limited reference data available currently and need for future comparison to datasets from other geographic areas to further evaluate these data on the diversity and types of *Bartonella* found in Tanzania, Moreover, *B. elizabethae* and *B. tribocorum* share identical published *ssr*A sequences in Genbank and our results show clustering in the phylogenetic tree (Fig 2), so the two species cannot be distinguished by this *ssr*A fragment. The other two *Bartonella* species identified were: 1) a single sequence (11s) with greatest similarity (98%) to *B. quintana* obtained from a sample from a *P. flavovittis* host; and 2) a sequence obtained from multiple samples of *R. rattus* and *X. cheopis* that showed an identical match to *B. rochalimae*, emphasising the diversity of *Bartonella* present in rodents in Tanzania. To the best of our knowledge, this is the first report of molecular detection and characterization of *Bartonella* species in rodents and their associated ectoparasites in Africa using the *ssr*A gene target. The scope for comparison with other sequences is thus limited, as there is currently little reference material on *ssr*A sequences from *Bartonella* sampled elsewhere, particularly in Africa.

In contrast, the *glt*A gene has been widely used to study *Bartonella* globally. Phylogenetic analysis of sequences from 17 *glt*A PCR products from this Tanzanian rodent population (16 *R. rattus* and 1 *M. natalensis*) showed the highest similarity to reference sequences of *B. elizabethae*, which has multiple published sequences in Genbank, including many from east Africa. The association of sequences similar to *B. elizabethae* with *Rattus* spp. in this study is consistent with similar findings from Malaysia [51] and Thailand [48]. *B. elizabethae* has also been identified in different rodent species in Africa [25,26]. Identical *glt*A sequences were amplified from *R. rattus* and *M. natalensis* in our study, suggesting possible transmission between different rodent species in the Tanzania site or a shared common source of infection. Identical sequences were also identified previously in *R. rattus* sampled at both rural (strain B28297, accession number KM233489, from Asembo, Kenya) and urban (strain B28391, accession number KC763968, from Kibera, Nairobi Kenya) sites in Kenya [22]. This suggests that similar *Bartonella* could be found in rodents in Kenya and Tanzania. However, these comparisons are based on short sequences of a single gene target only. Longer sequences from multiple genes and greater sampling effort across the region would be required to robustly confirm sharing of genotypes to trace source populations or determine patterns of host connectivity

Several studies of zoonotic disease have shown that a variety of pathogens account for high proportions of febrile illness in northern Tanzania but that considerable proportions remain unexplained [16,52]. *Bartonella* species have been identified as important causes of human febrile illness in several global settings but there has been little investigation of the impact of bartonellosis upon human health, in Africa particularly. The finding of *Bartonella* genotypes that are most similar to *B. elizabethae*, *B. rochalimae* and *B. quintana* reference sequences in rodents trapped in and around households in Moshi, Tanzania, and the fleas collected from these rodents, indicates the possibility that *Bartonella* infection may be responsible for an as yet unknown proportion of febrile illnesses in this region. Efforts are needed to determine the clinical impact of bartonellosis in this region and increase awareness about *Bartonella* and other zoonotic pathogens among physicians and health care workers, especially where the cause of large proportions of febrile illness remains unknown. Our results also demonstrate that molecular detection tools can be effectively used for surveillance and diagnostic of zoonotic pathogens in resource limited settings.

Acknowledgments

The authors would like to thank Professor Gibson Kibiki and Professor Blandina Mmbaga, the former and sitting directors of Kilimanjaro Clinical Research Institute (KCRI) for their support in establishing zoonotic research programmes at KCRI including this study. Special thanks go to laboratory staff at the KCRI biotechnology laboratory, especially Dr. Ireen Kiwelu, Happiness Kumburu, Davis Kuchaka and the late Bora Almasy for assisting in laboratory work. We are grateful to Dassa Nkini and Elizabeth Kussaga for administrative support and to Anne Laudisoit (formerly at University of Antwerp and FRIA, Belgium) for providing positive control materials obtained during a previous study [24].

Author Contributions

Conceptualization: Ndyetabura O. Theonest, Julius D. Keyyu, Gabriel M. Shirima, Kate M. Thomas, Daniel T. Haydon, Joram J. Buza, Kathryn J. Allan.

Data curation: Kathryn J. Allan, Jo E. B. Halliday.

Formal analysis: Ndyetabura O. Theonest, Ryan W. Carter, Nelson Amani, Kate M. Thomas, Daniel T. Haydon, Kathryn J. Allan, Jo E. B. Halliday.

Funding acquisition: Daniel T. Haydon, Joram J. Buza, Kathryn J. Allan, Jo E. B. Halliday.

Investigation: Ndyetabura O. Theonest, Nelson Amani, Siân L. Doherty, Ephrasia Hugho, Julius D. Keyyu, Barbara K. Mable, Gabriel M. Shirima, Rigobert Tarimo, Kate M. Thomas, Daniel T. Haydon, Joram J. Buza, Kathryn J. Allan, Jo E. B. Halliday.

Methodology: Ryan W. Carter, Ephrasia Hugho, Rigobert Tarimo, Kathryn J. Allan, Jo E. B. Halliday.

Resources: Daniel T. Haydon, Kathryn J. Allan.

Supervision: Barbara K. Mable, Daniel T. Haydon, Kathryn J. Allan, Jo E. B. Halliday.

Writing – original draft: Ndyetabura O. Theonest, Ryan W. Carter, Siân L. Doherty, Kathryn J. Allan, Jo E. B. Halliday.

Writing – review & editing: Ndyetabura O. Theonest, Ryan W. Carter, Nelson Amani, Barbara K. Mable, Daniel T. Haydon, Kathryn J. Allan, Jo E. B. Halliday.

References

1. Dehio C (2004) Molecular and cellular basis of *Bartonella* pathogenesis. Annu Rev Microbiol 58: 365–390. https://doi.org/10.1146/annurev.micro.58.030603.123700 PMID: 15487942
2. Castle KT, Kosoy M, Lerdthusnee K, Phalan L, Bai Y, Gage KL, et al. (2004) Prevalence and diversity of *Bartonella* in rodents of northern Thailand: a comparison with *Bartonella* in rodents from southern China. Am J Trop Med Hyg 70: 429–433. PMID: 15100459
3. Angelakis E, Khamphoukeo K, Grice D, Newton PN, Roux V, Aplin K, et al. (2009) Molecular detection of *Bartonella* species in rodents from the Lao PDR. Clin Microbiol Infect 15 Suppl 2: 95–97. https://doi.org/10.1111/j.1469-0691.2008.02177.x PMID: 19374642
4. Okaro U, Addisu A, Casanas B, Anderson B (2017) *Bartonella* Species, an Emerging Cause of Blood-Culture-Negative Endocarditis. Clin Microbiol Rev 30: 709–746. https://doi.org/10.1128/CMR.00013-17 PMID: 28490579
5. Saisongkorh W, Rolain JM, Suputtamongkol Y, Raoult D (2009) Emerging *Bartonella* in humans and animals in Asia and Australia. J Med Assoc Thai 92: 707–731. PMID: 19459936
6. Kosoy M, Bai Y, Sheff K, Morway C, Baggett H, Maloney SA, et al. (2010) Identification of *Bartonella* infections in febrile human patients from Thailand and their potential animal reservoirs. Am J Trop Med Hyg 82: 1140–1145. https://doi.org/10.4269/ajtmh.2010.09-0778 PMID: 20519614
7. Anderson BE, Neuman MA (1997) *Bartonella* spp. as emerging human pathogens. Clin Microbiol Rev 10: 203–219. PMID: 9105751
8. Iralu J, Bai Y, Crook L, Tempest B, Simpson G, McKenzie T, et al. (2006) Rodent-associated *Bartonella* febrile illness, Southwestern United States. Emerg Infect Dis 12: 1081–1086. https://doi.org/10.3201/eid1207.040397 PMID: 16836824
9. Bonilla DL, Cole-Porse C, Kjemtrup A, Osikowicz L, Kosoy M (2014) Risk factors for human lice and bartonellosis among the homeless, San Francisco, California, USA. Emerg Infect Dis 20: 1645–1651. https://doi.org/10.3201/eid2010.131655 PMID: 25280080
10. Breitschwerdt EB, Maggi RG, Chomel BB, Lappin MR (2010) Bartonellosis: an emerging infectious disease of zoonotic importance to animals and human beings. J Vet Emerg Crit Care (San Antonio) 20: 8–30. https://doi.org/10.1111/j.1476-4431.2009.00496.x PMID: 20230432
11. Regier Y, O'Rourke F, Kempf VAJ (2016) *Bartonella* spp.—a chance to establish One Health concepts in veterinary and human medicine. Parasite Vector 9. ARTN 330.
12. Kosoy M, Murray M, Gilmore RD, Bai Y, Gage KL (2003) *Bartonella* strains from ground squirrels are identical to *Bartonella washoensis* isolated from a human patient. J Clin Microbiol 41: 645–650. https://doi.org/10.1128/JCM.41.2.645-650.2003 PMID: 12574261
13. Welch DF, Carroll KC, Hofmeister EK, Persing DH, Robison DA, Steigerwalt AG, et al. (1999) Isolation of a new subspecies, *Bartonella vinsonii* subsp. *arupensis*, from a cattle rancher: identity with isolates found in conjunction with *Borrelia burgdorferi* and *Babesia microti* among naturally infected mice. J Clin Microbiol 37: 2598–2601. PMID: 10405408
14. Knap N, Duh D, Bidles R, Trilar T, Petrovec M, Avsic-Zupanc T (2007) Molecular detection of *Bartonella* species infecting rodents in Slovenia. Ferns Immunol Med Mic 50: 45–50. https://doi.org/10.1111/j.1574-695X.2007.00226.x PMID: 17374132

15. Probert W, Louie JK, Tucker JR, Longoria R, Hogue R, Moler S, et al. (2009) Meningitis Due to a "*Bartonella washoensis*"-Like Human Pathogen. J Clin Microbiol 47: 2332–2335. https://doi.org/10.1128/JCM.00511-09 PMID: 19439538

16. Crump JA, Morrissey AB, Nicholson WL, Massung RF, Stoddard RA, Galloway RL, et al. (2013) Etiology of Severe Non-malaria Febrile Illness in Northern Tanzania: A Prospective Cohort Study. PLoS Negl Trop Dis 7: e2324. https://doi.org/10.1371/journal.pntd.0002324 PMID: 23875063

17. Chinga-Alayo E, Huarcaya E, Nasarre C, del Aguila R, Llanos-Cuentas A (2004) The influence of climate on the epidemiology of bartonellosis in Ancash, Peru. Trans R Soc Trop Med Hyg 98: 116–124. https://doi.org/10.1016/s0035-9203(03)00017-8 PMID: 14964812

18. Breitschwerdt EB (2014) Bartonellosis: One Health Perspectives for an Emerging Infectious Disease. Ilar J 55: 46–58. https://doi.org/10.1093/ilar/ilu015 PMID: 24936029

19. Gutierrez R, Vayssier-Taussat M, Buffet JP, Harrus S (2017) Guidelines for the Isolation, Molecular Detection, and Characterization of *Bartonella* Species. Vector-Borne Zoonot 17: 42–50. https://doi.org/10.1089/vbz.2016.1956 PMID: 28055575

20. Diaz MH, Bai Y, Malania L, Winchell JM, Kosoy MY (2012) Development of a Novel Genus-Specific Real-Time PCR Assay for Detection and Differentiation of *Bartonella* Species and Genotypes. J Clin Microbiol 50: 1645–1649. https://doi.org/10.1128/JCM.06621-11 PMID: 22378904

21. Meheretu Y, Leirs H, Welegerima K, Breno M, Tomas Z, Kidane D, G et al. (2013) *Bartonella* prevalence and genetic diversity in small mammals from Ethiopia. Vector Borne Zoonotic Diseases 13: 164–175. https://doi.org/10.1089/vbz.2012.1004 PMID: 23421888

22. Halliday JE, Knobel DL, Agwanda B, Bai Y, Breiman RF, Cleaveland S, et al. (2015) Prevalence and diversity of small mammal-associated *Bartonella* species in rural and urban Kenya. PLoS Negl Trop Dis 9: e0003608. https://doi.org/10.1371/journal.pntd.0003608 PMID: 25781015

23. Pretorius AM, Beati L, Birtles RJ (2004) Diversity of bartonellae associated with small mammals inhabiting Free State province, South Africa. Int J Syst Evol Microbiol 54: 1959–1967. https://doi.org/10.1099/ijs.0.03033-0 PMID: 15545418

24. Laudisoit A, Falay D, Amundala N, Akaibe D, de Bellocq JG, Van Houtte N, et al. (2014) High prevalence of *Rickettsia typhi* and *Bartonella* species in rats and fleas, Kisangani, Democratic Republic of the Congo. Am J Trop Med Hyg 90: 463–468. https://doi.org/10.4269/ajtmh.13-0216 PMID: 24445202

25. Gundi VA, Kosoy MY, Makundi RH, Laudisoit A (2012) Identification of diverse *Bartonella* genotypes among small mammals from Democratic Republic of Congo and Tanzania. Am J Trop Med Hyg 87: 319–326. https://doi.org/10.4269/ajtmh.2012.11-0555 PMID: 22855765

26. Billeter SA, Borchert JN, Atiku LA, Mpanga JT, Gage KL, Kosoy MY (2014) *Bartonella* species in invasive rats and indigenous rodents from Uganda. Vector Borne Zoonotic Diseases 14: 182–188. https://doi.org/10.1089/vbz.2013.1375 PMID: 24575846

27. Home Office (2014) Guidance on the Operation of the Animals (Scientific Procedures) Act 1986.

28. Leary S, Underwood W, Anthony R, Cartner S, Corey D, Grandin T, et al. (2014) The AVMA Guidelines for the Euthanasia of Animals. Shaumburg, IL.: American Veterinary Medical Association.

29. Allan KJ, Halliday JEB, Moseley M, Carter RW, Ahmed A, Goris MGA, et al. (2018) Assessment of animal hosts of pathogenic *Leptospira* in northern Tanzania. PLoS Negl Trop Dis 12. ARTN e0006444. https://doi.org/10.1371/journal.pntd.0006444 PMID: 29879104

30. Cunningham D, Moors P. (1996) A guide to the identification and collection of New Zealand Rodents. Wellington: New Zealand: Wildlife Service Department.

31. Pratt HD, Wiseman JS (1962) Fleas of public health importance and their control. Communicable Disease Center, Atlanta, Georgia.

32. Billeter SA, Gundi VA, Rood MP, Kosoy MY (2011) Molecular detection and identification of *Bartonella* species in *Xenopsylla cheopis* fleas (Siphonaptera: Pulicidae) collected from *Rattus norvegicus* rats in Los Angeles, California. Appl Environ Microbiol 77: 7850–7852. https://doi.org/10.1128/AEM.06012-11 PMID: 21908631

33. Sanger F, Nicklen S, Coulson AR (1977) DNA Sequencing with Chain-Terminating Inhibitors. P Natl Acad Sci USA 74: 5463–5467. https://doi.org/10.1073/pnas.74.12.5463 PMID: 271968

34. Norman AF, Regnery R, Jameson P, Greene C, Krause DC (1995) Differentiation of *Bartonella*-like isolates at the species level by PCR-restriction fragment length polymorphism in the citrate synthase gene. J Clin Microbiol 33: 1797–1803. PMID: 7545181

35. Kumar S, Stecher G, Tamura K (2016) MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol Biol Evol 33: 187904.

36. Frank HK, Boyd SD, Hadly EA (2018) Global fingerprint of humans on the distribution of *Bartonella* bacteria in mammals. PLoS Negl Trop Dis 12: e0006865. https://doi.org/10.1371/journal.pntd.0006865 PMID: 30439961

37. Bai Y, Hayman DTS, Mckee CD, Kosoy MY (2015) Classification of *Bartonella* Strains Associated with Straw-Colored Fruit Bats (*Eidolon helvum*) across Africa Using a Multi-locus Sequence Typing Platform. PLoS Negl Trop Dis 9. ARTN e0003478. https://doi.org/10.1371/journal.pntd.0003478 PMID: 25635826

38. R Core Team (2018) R: A Language and Environment for Statistical Computing. Vienna, Austria.

39. Dorai-Raj S (2009) binom: Binomial Confidence Intervals For Several Parameterizations. R package version 1.0–5.

40. Bates D, Maechler M, Bolker B, Walker S (2015) Fitting Linear Mixed-Effects Models Using lme4. J Stat Softw 67: 1–48. https://doi.org/10.18637/jss.v067.i01

41. Tamura K (1992) Estimation of the Number of Nucleotide Substitutions When There Are Strong Transition-Transversion and G+C-Content Biases. Mol Biol Evol 9: 678–687. https://doi.org/10.1093/oxfordjournals.molbev.a040752 PMID: 1630306

42. Buffet JP, Kosoy M, Vayssier-Taussat M (2013) Natural history of *Bartonella*-infecting rodents in light of new knowledge on genomics, diversity and evolution. Future Microbiol 8: 1117–1128. https://doi.org/10.2217/fmb.13.77 PMID: 24020740

43. Firth C, Bhat M, Firth MA, Williams SH, Frye MJ, Simmonds P, et al. (2014) Detection of zoonotic pathogens and characterization of novel viruses carried by commensal *Rattus norvegicus* in New York City. MBio 5: e01933–01914. https://doi.org/10.1128/mBio.01933-14 PMID: 25316698

44. Peterson AC, Ghersi BM, Alda F, Firth C, Frye MJ, Bai Y, et al. (2017) Rodent-Borne *Bartonella* Infection Varies According to Host Species Within and Among Cities. Ecohealth 14: 771–782. https://doi.org/10.1007/s10393-017-1291-4 PMID: 29164472

45. Gundi VA, Kosoy MY, Myint KS, Shrestha SK, Shrestha MP, Pavlin JA, et al. (2010) Prevalence and genetic diversity of *Bartonella* species detected in different tissues of small mammals in Nepal. Appl Environ Microbiol 76: 8247–8254. https://doi.org/10.1128/AEM.01180-10 PMID: 21037303

46. Lipatova I, Paulauskas A, Puraite I, Radzijevskaja J, Balciauskas L, Gedminas V (2015) *Bartonella* infection in small mammals and their ectoparasites in Lithuania. Microbes Infect 17: 884–888. https://doi.org/10.1016/j.micinf.2015.08.013 PMID: 26344603

47. Kamani J, Morick D, Mumcuoglu KY, Harrus S (2013) Prevalence and diversity of *Bartonella* species in commensal rodents and ectoparasites from Nigeria, West Africa. PLoS Negl Trop Dis 7: e2246. https://doi.org/10.1371/journal.pntd.0002246 PMID: 23738028

48. Klangthong K, Promsthaporn S, Leepitakrat S, Schuster AL, McCardle PW, Kosoy M, et al. (2015) The Distribution and Diversity of *Bartonella* Species in Rodents and their ectoparasites across Thailand. Plos One 10. ARTN e0140856.

49. Torchin ME, Lafferty KD, Dobson AP, McKenzie VJ, Kuris AM (2003) Introduced species and their missing parasites. Nature 421: 628–630. https://doi.org/10.1038/nature01346 PMID: 12571595

50. Li X-Y, Chen J-D, Li C-R (2018) Genotype characteristics of *Bartonella* and the infection in rodents in Guangdong Province, China. Zhongguo Ren Shou Gong Huan Bing Za Zhi 34: 482–486.

51. Tay ST, Mokhtar AS, Zain SNM, Low KC (2014) Short Report: Isolation and Molecular Identification of *Bartonellae* from Wild Rats (*Rattus* Species) in Malaysia. Am J Trop Med Hyg 90: 1039–1042.

52. Chipwaza B, Mhamphi GG, Ngatunga SD, Selemani M, Amuri M, Mugasa JP, et al. (2015) Prevalence of bacterial febrile illnesses in children in Kilosa district, Tanzania. PLoS Negl Trop Dis 9: e0003750. https://doi.org/10.1371/journal.pntd.0003750 PMID: 25955522

DOI: 10.1002/vms3.401

ORIGINAL ARTICLE

WILEY

Molecular detection of *Coxiella burnetii* infection in small mammals from Moshi Rural and Urban Districts, northern Tanzania

Ndyetabura O. Theonest[1,2] | Ryan W. Carter[3] | Elizabeth Kasagama[2] | Julius D. Keyyu[4] | Gabriel M. Shirima[1] | Rigobert Tarimo[2] | Kate M. Thomas[2,5] | Nick Wheelhouse[6] | Venance P. Maro[7] | Daniel T. Haydon[3] | Joram J. Buza[1] | Kathryn J. Allan[3] | Jo E.B. Halliday[3]

[1]School of Life Sciences and Bioengineering, Nelson Mandela African Institution of Science and Technology, Arusha, Tanzania

[2]Kilimanjaro Clinical Research Institute, Moshi, Tanzania

[3]The Boyd Orr Centre for Population and Ecosystem Health, Institute of Biodiversity Animal Health and Comparative Medicine, College of Medical Veterinary and Life Sciences, University of Glasgow, Glasgow, UK

[4]Tanzania Wildlife Research Institute, Arusha, Tanzania

[5]Centre for International Health, Dunedin School of Medicine, University of Otago, Dunedin, New Zealand

[6]School of Applied Sciences, Edinburgh Napier University, Edinburgh, UK

[7]Kilimanjaro Christian Medical University College, Moshi, Tanzania

Correspondence
Ndyetabura O. Theonest, School of Life Sciences and Bioengineering, Nelson Mandela African Institution of Science and Technology, Arusha, Tanzania.
Email: t.ndyetabura@kcri.ac.tz

Funding information
This work was supported by a Leverhulme–Royal Society Africa Award (grant number AA130131; https://www.leverhulme.ac.uk. https://royalsociety.org). KJA was supported by the Wellcome Trust (grant number 096400/Z/11/Z; https://wellcome.ac.uk/). JEBH, GMS and DTH received support from the Research Councils UK, UK Department for International Development, and UK Biotechnology and Biological Sciences Research Council (BBSRC) (grant numbers BB/J010367/1, BB/L018845; http://www.bbsrc.ac.uk/). KMT received support from the Research Councils UK, UK Department for International Development, and UK Biotechnology and Biological Sciences Research Council (BBSRC) (grant number BB/L017679; http://www.bbsrc.ac.uk/). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abstract

Coxiella burnetii is an obligate intracellular bacterium that causes Q fever, a zoonotic disease of public health importance. In northern Tanzania, Q fever is a known cause of human febrile illness, but little is known about its distribution in animal hosts. We used a quantitative real-time PCR (qPCR) targeting the insertion element IS1111 to determine the presence and prevalence of *C. burnetii* infections in small mammals trapped in 12 villages around Moshi Rural and Moshi Urban Districts, northern Tanzania. A total of 382 trapped small mammals of seven species were included in the study; *Rattus rattus* ($n = 317$), *Mus musculus* ($n = 44$), *Mastomys natalensis* ($n = 8$), *Acomys wilson* ($n = 6$), *Mus minutoides* ($n = 3$), *Paraxerus flavovottis* ($n = 3$) and *Atelerix albiventris* ($n = 1$). Overall, 12 (3.1%) of 382 (95% CI: 1.6–5.4) small mammal spleens were positive for *C. burnetii* DNA. *Coxiella burnetii* DNA was detected in five of seven of the small mammal species trapped; *R. rattus* ($n = 7$), *M. musculus* ($n = 1$), *A. wilson* ($n = 2$), *P. flavovottis* ($n = 1$) and *A. albiventris* ($n = 1$). Eleven (91.7%) of twelve (95% CI: 61.5–99.8) *C. burnetii* DNA positive small mammals were trapped within Moshi Urban District. These findings demonstrate that small mammals in Moshi, northern Tanzania are hosts of *C. burnetii* and may act as a source of *C. burnetii* infection to humans and other animals. This detection of *C. burnetii* infections in small mammals

should motivate further studies into the contribution of small mammals to the transmission of *C. burnetii* to humans and animals in this region.

KEYWORDS
Coxiella burnetii, detection, prevalence, small mammal, Tanzania, zoonoses

1 | INTRODUCTION

Coxiella burnetii is an obligate intracellular bacterium, the causative agent of Q fever, a zoonotic disease of public health importance worldwide except in New Zealand (Marrie et al., 2015; Schimmer et al., 2014; Toman et al., 2009). *C. burnetii* can infect a wide range of vertebrate and invertebrate hosts. Domestic ruminants (sheep, goats and cattle) are considered the main reservoirs of *C. burnetii* (Duron et al., 2015; Van den Brom & Vellema, 2009).

In recent years, an increasing number of studies have reported the detection of *C. burnetii* in small mammals. Investigation of patients in the Netherlands indicated an association between small mammal sightings and Q fever case occurred during the 2007 outbreak (Karagiannis et al., 2009). Similarly, wild rodents, marsupials, bats and other wild mammals captured around the houses of Q fever case patients in French Guiana were more likely to test *C. burnetii* positive as compared to animals trapped at greater distance from residential houses, and sighting of these animals especially rodents was identified as a risk factor for human *C. burnetii* infection (Gardon et al., 2001).

Data on the presence and prevalence of *C. burnetii* in small mammals and their epidemiology in Tanzania are limited. Globally, studies on the presence and prevalence of *C. burnetii* in small mammals and other animals have demonstrated significant variation in the prevalence of *C. burnetii* depending on factors such as species, sex, age, season of sampling (wet or dry) and sampling location (Foronda et al., 2015; Gardon et al., 2001; Webster et al., 1995; Yadav et al., 2019). In many African countries, there are few studies on *C. burnetii* presence and data on prevalence in both animal and humans are scarce (Salifu et al., 2019). Based on conventional PCR detection methods targeting *C. burnetii* 16rRNA and IS1111 genes, the overall prevalence of *C. burnetii* in African small mammal populations has been found to range from 2.1% (4/194) in peridomestic rodents in Nigeria (Kamani et al., 2018) to 45% (9/20) in Zambia (Chitanga et al., 2018).

Zoonotic infections are of great importance to public health in many parts of the world but their clinical importance is typically under-appreciated (Angelakis et al., 2014; Crump et al., 2013). *C. burnetii* infection was diagnosed in 5.0% of 482 febrile patients tested in a retrospective study performed in Moshi, northern Tanzania (the same area as this study) (Crump et al., 2013). This finding together with several recent outbreaks of Q fever highlights the importance of *C. burnetii* as a public health problem and need for continued efforts to identify reservoirs of *C. burnetii* to achieve better control and prevention.

Several PCR-based diagnostic methods have been successfully applied for the direct detection of *C. burnetii* (Herrin et al., 2011; Kersh et al., 2010; Klee et al., 2006; Piñero et al., 2014; Schneeberger et al., 2010). The use of quantitative real-time PCR (qPCR), targeting the IS1111 insertion element which is present in multiple copies, has been reported to be highly sensitive for the detection of *C. burnetii* DNA (Bruin et al., 2011). In this study, we aimed to use a qPCR assay targeting the transposase gene of insertion element IS1111 to determine the presence and prevalence of *C. burnetii* DNA in spleen tissue samples of small mammals from Moshi Rural and Urban Districts, northern Tanzania.

2 | MATERIALS AND METHODS

2.1 | Study site

The study was conducted in the Kilimanjaro Region of northern Tanzania. Trapping of the small mammals was conducted in two of seven districts of Kilimanjaro Region in a previous study (Allan et al., 2018). The two districts, Moshi Municipal (Urban) and Moshi Rural (Figure 1), were chosen as the study site due to the previous finding of a high prevalence of Q fever in febrile patients from this area (Crump et al., 2013). The climate in the study area is tropical with an average temperature for the year of 74.2°F (23. 4°C) and two patterns of rains; long rains from March to May and short rains from October to December. The coolest months coinciding with the long dry season from June to September. The warmest month, on average, is February with an average temperature of 77.9°F (25.5°C). The coolest month on average is July, with an average temperature of 69.3°F (20.7°C) (Climate-data.org, 2020). Subsistence farming is common. Agriculture, which is mainly mixed cropping and livestock farming, is the main economic activity in the study area.

2.2 | Small mammal sampling and data collection

Small mammal spleen samples for this study were obtained from a previous cross-sectional study (Allan et al., 2018) for which small mammal sampling was conducted within Moshi Rural and Urban Districts. Small mammals were trapped from a total of seven villages within Moshi Rural District and five villages within Moshi Urban District (Table 1 and Figure 1). The villages were randomly selected from a list of villages that were home to people that had sought care, and had been enrolled in previous febrile illness surveillance studies at local hospitals (Crump et al., 2013). As in the previous study,

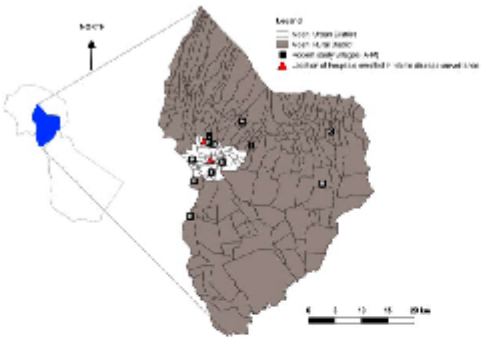


FIGURE 1 Map of Moshi Urban and Moshi Rural Districts, showing representative locations of small mammal study villages. Letters indicate the different villages in which small mammal trapping was conducted. Polygons in the main image show local administrative boundaries. Insert map on left shows outline of the Kilimanjaro region of Tanzania and the location of study districts within the region. This figure is adapted from a version published previously (Allan et al., 2018)

the target sample size was 50 small mammals per sub-village to give power ($\alpha = 0.95$, $\beta = 0.8$) to estimate *Leptospira* (Allan et al., 2018) and *C. burnetii* infection prevalence of 10%.

Small mammal trapping, identification and sampling are as previously described in another study (Allan et al., 2018). Data gathered for every trapped small mammal included: species (determined by observation of phenotypic characteristics and measurement of morphometric features), sex and reproductive maturity status (mature or immature determined based on external sexual characteristics) (Allan et al., 2018). Spleen tissues previously stored at −80°C were retrieved for the extraction of DNA used in this study.

2.3 | DNA extraction

DNA was extracted from approximately 10 milligrams (mg) of spleen tissue (previously heat treated in PBS at 67°C for 1 hr) using the DNeasy Blood and Tissue Kit spin-column protocol for DNA purification from tissues (Qiagen) performed in a biological safety cabinet (NuAire) at Kilimanjaro Clinical Research Institute Biotechnology Laboratory in Moshi, Tanzania. DNA was eluted in 100 µl of AE buffer and quantified using a Nano-Drop spectrophotometer (Thermo Scientific). A no-template extraction control (PCR-grade water) was included for every 20 samples. DNA extracts were stored at −20°C prior to testing. To minimize the potential for qPCR inhibition due to the high concentration of host DNA, extracts were diluted in 20 µl of AE buffer to a standard DNA concentration of 10–50 ng/µl for qPCR testing.

TABLE 1 Categorical variable summaries and *C. burnetii* qPCR IS1111 for small mammals trapped from Moshi, Tanzania ($n = 382$)

Variable		Number of small mammals tested for *C. burnetii*	*C. burnetii* positive *n* (%)	95% CI
Village Code	A	12	0 (0.0)	0.0–26.5
	B	13	1 (7.7)	0.2–36
	C	31	0 (0.0)	0.0–11.2
	D	26	7 (26.9)	11.6–47.8
	E	39	0 (0.0)	0.0–9.0
	F	109	2 (1.8)	0.2–6.5
	G	15	0 (0.0)	0.0–21.8
	H	35	0 (0.0)	0.0–10.0
	J	19	0 (0.0)	0.0–17.6
	K	23	0 (0.0)	0.0–14.8
	L	22	0 (0.0)	0.0–15.4
	M	38	2 (5.3)	0.6–17.7
District	Rural	155	1 (0.6)	0.02–3.5
	Urban	227	11 (4.8)	2.4–8.5
Sex	Male	163	7 (4.3)	1.7–8.6
	Female	219	5 (2.3)	0.7–5.2
Age	Mature	225	10 (4.4)	2.2–8.0
	Immature	157	2 (1.3)	0.2–4.5
Season	Wet	266	9 (3.4)	1.6–6.3
	Dry	116	3 (2.6)	0.5–7.4
Overall	NA	382	12 (3.1)	1.6–5.4

2.4 | Determination of sensitivity and limit of detection of the IS1111 qPCR assay

Initial set-up and verification of the assay was performed on the Rotor-Gene Q/6000 System (Qiagen). The approximate sensitivity and limit of detection (LoD) of the IS1111 qPCR assay for this study (295 bp target) was determined using a 10-fold dilution series of DNA from *C. burnetii* Nine Mile RSA493 reference strain. The primers and probe were as follows: Forward primer (5′-CATCACATTGCCGCGTTTAC-3′), Reverse primer (5′-GGTTGGTCCCTCGACAACAT-3′), and 6-carboxyfluorescein FAM-labelled probe (5′-AATCCCCAACAACACCTCCTTATTCCCAC-BHQ1-3′) as described in previous study (Roest et al., 2011).

2.5 | IS1111 qPCR for detection of *C. burnetii* DNA in small mammal spleens

DNA extracts from small mammal spleens were screened for the presence of *C. burnetii* by qPCR using the same primers and probe as described above. The qPCR reactions were carried out in total volumes of 20 µl comprising of 10 µl QuantiNova qPCR mix (Qiagen), 0.8 µl of each primer (10 µM) and probe (5 µM), 2.6 µl nuclease-free water and 5 µl DNA template. Positive controls (Nine Mile RSA493 strain), extraction controls (AE buffer) and no template controls (PCR-grade water) were included in each qPCR run. Assays were performed on a Rotor-Gene Q/6000 with thermocycling conditions as follows: 1 cycle of 95°C for 2 min followed by 45 cycles of 95°C for 5 s then 60°C for 5 s. Fluorescence readings were acquired via the green (510 nm) detection channel at the end of each annealing/extension phase. A qPCR run was considered valid when the negative controls showed no amplification and the positive controls amplified with Ct value < 40. Samples were tested in duplicate initially and then in an additional three wells if amplification (Ct < 40) was seen in one of two initial duplicate wells. A sample was considered positive for *C. burnetii* if at least two test wells, out of the maximum five, produced amplification with Ct < 40 and all other assay conditions were fulfilled.

2.6 | Statistical analysis

Statistical analyses were performed in R (R Development Core Team, 2018). Binomial proportions and 95% confidence intervals for prevalence estimates were calculated using the package "binom" version 1.0-5 for selected variables (Dorai-Raj, 2014).

3 | RESULTS

3.1 | Sample characteristics

A total of 382 spleen samples from small mammals were available for testing. The majority (n = 317, 83. 0%) were from indigenous black rats (*Rattus rattus*). Other small mammal species tested included: house mice (*Mus musculus*, n = 44, 11.5%); multimammate mice (*Mastomys natalensis*, n = 8, 2.1%); spiny mice (*Acomys wilsonii*, n = 6, 1.6%); African pygmy mice (*Mus minutoides*, n = 3, 0.8%); striped bush squirrels (*Paraxerus flavovittis*, n = 3, 0.8%) and the four-toed hedgehog (*Atelerix albiventris*, n = 1, 0.2%). Of the tested small mammal population, 219 individuals (57.3%) were female. Based on examination of external sexual characteristics, 224 (58.6%) were classified as sexually mature. The majority of small mammals (n = 266, 69.6%) were trapped during the wet season. Of the tested small mammal population, 227 (59.4%) were trapped from Moshi Urban District.

3.2 | IS1111 limit of detection and prevalence of *C. burnetii* in spleen samples from small mammals

The assay limit of detection, with 100% reproducibility was estimated at approximately 10 genome copies per µl. *C. burnetii* DNA was detected by IS1111 qPCR in a total of 12 (3.1%) of 382 (95% CI: 1.6–5.4) spleen samples from small mammals. This included positive individuals from five of seven of the species tested; *Rattus rattus* (n = 7), *Mus musculus* (n = 1), *Acomys wilson* (n = 2), *Paraxerus flavovottis* (n = 1) and *Atelerix albiventris* (n = 1). Eleven (91.7%) of twelve (95% CI: 61.5–99.8) *C. burnetii* positive small mammals were trapped within Moshi Urban District. Five (2.3%) of 219 females (95% CI: 0.7–5.4) and seven (4.3%) of 163 males (95% CI: 1.7–8.6) were positive for *C. burnetii*. Nine (3.4%) of 266 (95% CI: 1.6– 6.3) small mammals sampled during the wet season were *C. burnetii* positive and three (2.6%) of 116 (95% CI: 0.5–7.4) small mammals sampled during the dry season were *C. burnetii* positive (Table 1).

4 | DISCUSSION

C. burnetii DNA was detected in 3.1% of small mammals trapped from Moshi Rural and Moshi Urban Districts in northern Tanzania between May 2013 and September 2014. To the best of our knowledge, this is the first study to demonstrate the presence and prevalence of *C. burnetii* in small mammals from Tanzania. Infected small mammals may act as a source of *C. burnetii* infection for both humans and other animals in the study area. The findings of this study provide evidence to inform Q fever control programs.

There is a significant variation in the prevalence of *C. burnetii* reported in different small mammal populations and at different locations within Africa (Abdel-Moein & Hamza, 2018; Chitanga et al., 2018; Kamani et al., 2018). Knowledge of the prevalence of *C. burnetii* in different small mammal populations and an improved understanding of the factors that drive this variation will be important to inform the design of *C. burnetii* control programs.

In this study, there are indications of variation in the proportion of small mammals that are *C. burnetii* positive across different small mammal species, season of sampling, age, species, sex and location of sampling (rural vs. urban districts and villages) (Table 1 and 2). The small

TABLE 2 *Coxiella burnetii* IS1111 qPCR-positive samples and the characteristics of positive small mammal trapped from Moshi Urban and Rural districts, Tanzania ($n = 12$)

Small mammal sample ID	Sex	Species	Locations	Season	Age	Ct Values		
						Ct1	Ct2	Av.Ct
R0024	Male	*R. rattus*	Rural	Wet	Mature	32.22	31.78	32.00
R0062	Male	*A. wilson*	Urban	Wet	Mature	30.56	30.87	30.72
R0063	Female	*A. wilson*	Urban	Wet	Mature	27.16	32.35	29.76
R0065	Female	*R. rattus*	Urban	Wet	Mature	32.59	32.84	32.72
R0067	Male	*R. rattus*	Urban	Wet	Immature	33.65	34.86	34.26
R0078	Male	*A. albiventris*	Urban	Wet	Mature	36.99	34.27	35.63
R0083	Male	*R. rattus*	Urban	Wet	Mature	31.32	32.11	31.72
R0084	Female	*R. rattus*	Urban	Wet	Mature	35.17	35.09	35.13
R0168	Male	*R. rattus*	Urban	Wet	Mature	27.90	27.76	27.83
R0330	Female	*R. rattus*	Urban	Dry	Mature	20.01	20.06	20.04
R0339	Female	*R. rattus*	Urban	Dry	Mature	27.40	28.54	27.97
R0393	Male	*R. rattus*	Urban	Dry	Immature	27.40	27.54	27.47

number of positive individuals identified in this study limits the scope for statistical analyses of these patterns, but the factors that determine prevalence in these populations should be investigated further. In this study, the majority of *C. burnetii* positive small mammals were trapped from Moshi Urban District. Previous studies have suggested that emerging and re-emerging zoonotic diseases and pathogens are linked with increasing globalization and urbanization (Amitai et al., 2010; Buzan et al., 2017; Comer et al., 2001) and there is a clear rationale for further investigation of the links between urbanization and *C. burnetii* prevalence. In this study, the small mammals sampled were trapped in or around households, indicating a potential risk of *C. burnetii* transmission to humans, pets and livestock.

Observations from previous studies and the raw data from this one indicate that specific small mammal species appear more likely to carry and maintain *C. burnetii* bacteria than others in a given geographical area (Burgdorfer et al., 1963; Reusken et al., 2011; Rozental et al., 2017). A study to assess susceptibility of rodent species to *C. burnetii* and other rickettsiae species indicated that variation in host genetic factors that determine macrophage responses, the infecting strain of *C. burnetii* and the route of infection may explain variation in *C. burnetii* infection prevalence in small mammal (Rehácek et al., 1992). Similarly, more *C. burnetii* positive individuals were classified as mature small mammals as compared to immature, consistent with previous findings of increased *C. burnetii* infection in mature mice (Leone et al., 2007). Q fever and a number of other bacterial infections are typically considered as diseases of mature adults due to age-associated physiological and anatomical changes, and dysfunction of the immune system (Gavazzi & Krause, 2002). More male small mammals trapped in this study were *C. burnetii* positive as compared to females, also consistent with previous findings (Thompson et al., 2012). Male small mammals have been demonstrated to exhibit frequent and long-distance movements in search of female mates or defence of their territory. This behaviour may increase their risk of acquiring *C. burnetii* infection from the environment or from their multiple mates (Adler, 2011; Kozakiewicz et al., 2007; Nelson, 1995).

Studies conducted in the Netherlands, suggest a role for rodents in maintaining the cycle of *C. burnetii* infection between wildlife and domestic animals, and consequently transmission to humans (Reusken et al., 2011). Similar *C. burnetii* transmission scenarios may be happening in Tanzania, where the main source for human *C. burnetii* infection is poorly understood. In the USA and Canada *C. burnetii* has been detected in small mammal species trapped in the forest and pristine environments, where human activities such as livestock keeping do not occur, suggesting that small mammals in these livestock-free areas could be acting as a reservoir of *C. burnetii* (Burgdorfer et al., 1963; Thompson et al., 2012).

5 | CONCLUSIONS

In Tanzania febrile illnesses caused by zoonotic pathogens, including *C. burnetii*, are of public health importance but are often underappreciated or misdiagnosed. In this study, we demonstrate the detection of *C. burnetii* in small mammals trapped in and around household premises from the same area where a previous study has reported high prevalence of Q fever in humans. These data provide a clear rationale for further investigation of the epidemiology of *C. burnetti* in this setting and the role that small mammals play in this multi-host epidemiology. Additional work is needed to understand the role of small mammals in the maintenance and transmission of *C. burnetii* infection in this region of Tanzania and to examine linkages between human, livestock and small mammal infections. *C. burnetii* strains circulating in small mammals should be typed and compared with isolates from human, other animals and environmental sources. This will provide information on the role of small mammals in *C. burnetii* transmission.

ETHICS STATEMENT

Approval for the study was granted by the Tanzania Commission for Science and Technology (COSTECH 2012-471-ER-2005-141 & 2015-71-NA-2011-199); Kilimanjaro Christian Medical Centre (KCMC) Ethics Committee (535 & 537); National Institute of Medical Research (NIMR), Tanzania (NIMR/HQ/R.8a/Vol.IX/1499 & NIMR/HQ/R.8a/Vol.IX/1522); Tanzania Wildlife Research Institute (TAWIRI); University of Glasgow College of Medical, Veterinary and Life Sciences Ethics Committee (200,120,020), and University of Glasgow Faculty of Veterinary Medicine Ethics and Welfare Committee (01a/13 & 02a/13). Written consent for study participation was obtained for each participating household, using forms translated into Swahili (Allan et al., 2018). Small mammal sampling was performed in accordance with the UK Guidance on the Operation of the Animals (Scientific Procedures) Act 1986 and American Veterinary Medical Association Guidelines for the Euthanasia of Animals (Home Office, 2014; AVMA Panel on Euthanasia, 2013).

ACKNOWLEDGEMENTS

The authors thank Professor Gibson Kibiki and Professor Blandina Mmbaga, the former and sitting directors of Kilimanjaro Clinical Research Institute (KCRI) for their support in establishing zoonotic research programmes at KCRI including this study. The authors also thank laboratory staff at the KCRI biotechnology laboratory, especially those working in zoonoses laboratory section. We are grateful to Dassa Nkini and Elizabeth Kussaga for administrative support.

CONFLICT OF INTEREST

The authors have no conflict of interests concerning the work reported in this manuscript.

AUTHOR CONTRIBUTION

NDYETABURA O THEONEST: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Writing-original draft; Writing-review & editing. **Ryan W Carter:** Data curation; Investigation; Methodology; Writing-review & editing. **Elizabeth Kasagama:** Investigation; Methodology; Writing-review & editing. **Julius D Keyyu:** Conceptualization; Funding acquisition; Project administration; Supervision; Writing-review & editing. **Gabriel Mkilema Shirima:** Conceptualization; Funding acquisition; Investigation; Supervision; Writing-review & editing. **Rigobert Tarimo:** Investigation; Methodology; Writing-review & editing. **Kate M Thomas:** Data curation; Investigation; Methodology; Validation; Writing-review & editing. **Nick Wheelhouse:** Data curation; Supervision; Validation; Writing-review & editing. **Venance P Maro:** Conceptualization; Funding acquisition; Investigation; Project administration; Supervision; Writing-review & editing. **Daniel T Haydon:** Conceptualization; Funding acquisition; Investigation; Project administration; Supervision; Writing-review & editing. **Joram J Buza:** Conceptualization; Funding acquisition; Investigation; Project administration; Supervision; Writing-review & editing. **Kathryn J Allan:** Conceptualization; Data curation; Funding acquisition; Investigation; Methodology; Project administration; Resources; Supervision; Validation; Visualization; Writing-review & editing. **Jo E. B. Halliday:** Conceptualization; Data curation; Funding acquisition; Investigation; Methodology; Project administration; Resources; Supervision; Validation; Visualization; Writing-review & editing.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1002/vms3.401.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study will be openly available after publication through: http://dx.doi. org/10.5525/gla.researchdata.948.

ORCID

Ndyetabura O. Theonest https://orcid.org/0000-0002-9030-140X

Jo E.B. Halliday https://orcid.org/0000-0002-1329-9035

REFERENCES

Abdel-Moein, K. A., & Hamza, D. A. (2018). Rat as an overlooked reservoir for *Coxiella burnetii*: A public health implication. *Comparative Immunology, Microbiology and Infectious Diseases*, *61*(November), 30–33. https://doi.org/10.1016/j.cimid.2018.11.002

Adler, G. H. (2011). Spacing patterns and social mating systems of echimyid rodents. *Journal of Mammalogy*, *92*(1), 31–38. https://doi.org/10.1644/09-MAMM-S-395.1

Allan, K. J., Halliday, J. E. B., Moseley, M., Carter, R. W., Ahmed, A., Goris, M. G. A., Hartskeerl, R. A., Keyyu, J., Kibona, T., Maro, V. P., Maze, M. J., Mmbaga, B. T., Tarimo, R., Crump, J. A., & Cleaveland, S. (2018). Assessment of animal hosts of pathogenic *Leptospira* in northern Tanzania. *PLoS Neglected Tropical Diseases*, *12*(6), e0006444. https://doi.org/10.1371/journal.pntd.0006444

Amitai, Z., Bromberg, M., Bernstein, M., Raveh, D., Keysary, A., David, D., Pitlik, S., Swerdlow, D., Massung, R., Rzotkiewicz, S., Halutz, O., & Shohat, T. (2010). A large Q fever outbreak in an urban school in central Israel. *Clinical Infectious Diseases*, *50*(11), 1433–1438. https://doi.org/10.1086/652442

Angelakis, E., Mediannikov, O., Socolovschi, C., Mouffok, N., Bassene, H., Tall, A., Niangaly, H., Doumbo, O., Znazen, A., Sarih, M., Sokhna, C., & Raoult, D. (2014). *Coxiella burnetii*-positive PCR in febrile patients in rural and urban Africa. *International Journal of Infectious Diseases*, *28*, 107–110. https://doi.org/10.1016/j.ijid.2014.05.029

AVMA Panel on Euthanasia. (2013). *AVMA guidelines for the euthanasia of animals* (2013 ed.). American Veterinary Medical Association.

Bruin, A. D., Groot, A. D., Heer, L. D., Bok, J., Wielinga, P. R., Hamans, M., Van Rotterdam, B. J., Janse, I., de Bruin, A., de Groot, A., de Heer, L., Bok, J., Wielinga, P. R., Hamans, M., van Rotterdam, B. J., & Janse, I. (2011). Detection of *Coxiella burnetii* in complex matrices by using multiplex quantitative PCR during a major Q fever outbreak in the Netherlands. *Applied and Environmental Microbiology*, *77*(18), 6516–6523. https://doi.org/10.1128/AEM.05097-11

Burgdorfer, W., Pickens, E. G., Newhouse, V. F., & Lackman, D. B. (1963). Isolation of *Coxiella burnetii* from rodents in western montana. *Journal of Infectious Diseases*, *112*(2), 181–186. https://doi.org/10.1093/infdis/112.2.181

Buzan, E., Zupan, S., & Jugovic, J. (2017). Changes in rodent communities as consequence of urbanization and inappropriate waste management. *Applied Ecology and Environmental Research*, *15*(1), 573–588. https://doi.org/10.15666/aeer/1501_573588

Chitanga, S., Simulundu, E., Simuunza, M. C., Changula, K., Qiu, Y., Kajihara, M., Nakao, R., Syakalima, M., Takada, A., Mweene, A. S., Mukaratirwa, S., & Hang'ombe, B. M. (2018). First molecular detection and genetic characterization of *Coxiella burnetii* in Zambian dogs and rodents. *Parasites & Vectors*, *11*(1), 40. https://doi.org/10.1186/s13071-018-2629-7

Climate-data.org. (2020). *OpenStreetMap project*. https://en.climate-data.org/africa/tanzania/kilimanjaro/moshi-3115/

Comer, J. A., Paddock, C. D., & Childs, J. E. (2001). Urban zoonoses caused by *Bartonella*, *Coxiella*, *Ehrlichia*, and *Rickettsia* species. *Vector Borne and Zoonotic Diseases (Larchmont, N.Y.)*, *1*(2), 91–118. https://doi.org/10.1089/153036601316977714

Crump, J. A., Morrissey, A. B., Nicholson, W. L., Massung, R. F., Stoddard, R. A., Galloway, R. L., Ooi, E. E., Maro, V. P., Saganda, W., Kinabo, G. D., Muiruri, C., & Bartlett, J. A. (2013). Etiology of severe non-malaria febrile illness in Northern Tanzania: A Prospective Cohort Study. *PLoS Neglected Tropical Diseases*, *7*(7), e2324. https://doi.org/10.1371/journal.pntd.0002324

Dorai-Raj, S. (2014). *binom: Binomial confidence intervals for several parameterizations*. R Package Version, 1, 1.

Duron, O., Sidi-Boumedine, K., Rousset, E., Moutailler, S., & Jourdain, E. (2015). The importance of ticks in Q fever transmission: What has (and Has Not) been demonstrated? *Trends in Parasitology*, *31*(11), 536–552. https://doi.org/10.1016/j.pt.2015.06.014

Foronda, P., Plata-Luis, J., del Castillo-Figueruelo, B., Fernández-Álvarez, Á., Martín-Alonso, A., Feliu, C., Cabral, M. D., & Valladares, B. (2015). Serological survey of antibodies to *Toxoplasma gondii* and *Coxiella burnetii* in rodents in north-Western African islands (Canary Islands and Cape Verde). *Onderstepoort Journal of Veterinary Research*, *82*(1), 4–7. https://doi.org/10.4102/ojvr.v82i1.899

Gardon, J., Héraud, J., Laventure, S., Ladam, A., Capot, P., Fouquet, E., Favre, J., Weber, S., Hommel, D., Hulin, A., Couratte, Y., & Talarmin, A. (2001). Suburban transmission of Q fever in French Guiana: evidence of a wild reservoir. *The Journal of Infectious Diseases*, *184*(3), 278–284. https://doi.org/10.1086/322034

Gavazzi, G., & Krause, K.-H. (2002). Ageing and infection. *The Lancet Infectious Diseases*, *2*(11), 659–666. https://doi.org/10.1016/S1473-3099(02)00437-1

Herrin, B., Mahapatra, S., Blouin, E. F., & Shaw, E. I. (2011). Growth of *Coxiella burnetii* in the Ixodes scapularis –derived IDE8 tick cell line. *Vector-Borne and Zoonotic Diseases*, *11*(7), 917–922. https://doi.org/10.1089/vbz.2010.0126

Home Office. (2014). *Guidance on the operation of the Animals (Scientific Procedures) Act 1986*. In Publications GOV.UK. https://doi.org/ISBN 1474100287- 9781474100281.

Kamani, J., Baneth, G., Gutiérrez, R., Nachum-Biala, Y., Mumcuoglu, K. Y., & Harrus, S. (2018). *Coxiella burnetii* and *Rickettsia conorii*: Two zoonotic pathogens in peridomestic rodents and their ectoparasites in Nigeria. *Ticks and Tick-Borne Diseases*, *9*(1), 86–92. https://doi.org/10.1016/j.ttbdis.2017.10.004

Karagiannis, I., Schimmer, B., Van Lier, A., Timen, A., Schneeberger, P., Van Rotterdam, B., De Bruin, A., Wijkmans, C., Rietveld, A., & Van Duynhoven, Y. (2009). Investigation of a Q fever outbreak in a rural area of The Netherlands. *Epidemiology and Infection*, *137*(9), 1283–1294. https://doi.org/10.1017/S0950268808001908

Kersh, G. J., Wolfe, T. M., Fitzpatrick, K. A., Candee, A. J., Oliver, L. D., Patterson, N. E., Self, J. S., Priestley, R. A., Loftis, A. D., & Massung, R. F. (2010). Presence of *Coxiella burnetii* DNA in the environment of the United States, 2006 to 2008. *Applied and Environmental Microbiology*, *76*(13), 4469–4475. https://doi.org/10.1128/AEM.00042-10

Klee, S. R., Tyczka, J., Ellerbrok, H., Franz, T., Linke, S., Baljer, G., & Appel, B. (2006). Highly sensitive real-time PCR for specific detection and quantification of *Coxiella burnetii*. *BMC Microbiology*, *6*, 1–8. https://doi.org/10.1186/1471-2180-6-2

Kozakiewicz, M., Choluj, A., & Kozakiewicz, A. (2007). Long-distance movements of individuals in a free-living bank vole population: An important element of male breeding strategy. *Acta Theriologica*, *52*(4), 339–348. https://doi.org/10.1007/BF03194231

Leone, M., Bechah, Y., Meghari, S., Lepidi, H., Capo, C., Raoult, D., & Mege, J. L. (2007). *Coxiella burnetii* infection in C57BL/6 mice aged 1 or 14 months. *FEMS Immunology and Medical Microbiology*, *50*(3), 396–400. https://doi.org/10.1111/j.1574-695X.2007.00272.x

Marrie, T. J., Minnick, M. F., Textoris, J., Capo, C., & Mege, J.-L. (2015). Coxiella. In Y.-W. Tang, D. Liu, J. Schwartzman, M. Sussman, & I. Poxton (Eds.), *Molecular medical microbiology* (Vol. 3, pp. 1941–1972). Elsevier. https://doi.org/10.1016/B978-0-12-397169-2.00106-2

Nelson, J. (1995). Determinants of male spacing behavior in microtines: An experimental manipulation of female spatial distribution and density. *Behavioral Ecology and Sociobiology*, *37*(4), 217–223. https://doi.org/10.1007/BF00177400

Piñero, A., Barandika, J. F., Hurtado, A., & García-Pérez, A. L. (2014). Evaluation of *Coxiella burnetii* status in dairy cattle herds with bulk-tank milk positive by ELISA and PCR. *Transboundary and Emerging Diseases*, *61*(2), 163–168. https://doi.org/10.1111/tbed.12013

R Development Core Team. (2018). *A language and environment for statistical computing*. In R Foundation for Statistical Computing (Vol. 2, p. https://www.R-project.org). https://doi.org/R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, http://www.R-project.org.

Rehácek, J., Urvölgyi, J., Kocianová, E., & Jedlicka, L. (1992). Susceptibility of some species of rodents to rickettsiae. *Folia Parasitologica*, *39*(3), 265–284.

Reusken, C., van der Plaats, R., Opsteegh, M., de Bruin, A., & Swart, A. (2011). *Coxiella burnetii* (Q fever) in *Rattus norvegicus* and *Rattus rattus* at livestock farms and urban locations in the Netherlands; could *Rattus* spp. represent reservoirs for (re)introduction? *Preventive Veterinary Medicine*, *101*(1–2), 124–130. https://doi.org/10.1016/j.prevetmed.2011.05.003

Roest, H. I. J., Ruuls, R. C., Tilburg, J. J. H. C., Nabuurs-Franssen, M. H., Klaassen, C. H. W., Vellema, P., Van den Brom, R., Dercksen, D., Wouda, W., Spierenburg, M. A. H., van der Spek, A. N., Buijs, R., de Boer, A. G., Willemsen, P. T. J., & van Zijderveld, F. G. (2011). Molecular epidemiology of *Coxiella burnetii* from ruminants in Q fever outbreak, The Netherlands. *Emerging Infectious Diseases*, *17*(4), 668–675. https://doi.org/10.3201/eid1704.101562

Rozental, T., Ferreira, M. S., Guterres, A., Mares-Guia, M. A., Teixeira, B. R., Gonçalves, J., Bonvicino, C. R., D'Andrea, P. S., & de Lemos, E. R. S. (2017). Zoonotic pathogens in Atlantic Forest wild rodents in Brazil: *Bartonella* and *Coxiella* infections. *Acta Tropica*, *168*, 64–73. https://doi.org/10.1016/j.actatropica.2017.01.003

Salifu, S. P., Bukari, A. R. A., Frangoulidis, D., & Wheelhouse, N. (2019). Current perspectives on the transmission of Q fever: Highlighting the need for a systematic molecular approach for a neglected disease in Africa. *Acta Tropica*, *193*, 99–105. https://doi.org/10.1016/j.actatropica.2019.02.032

Schimmer, B., Schotten, N., van Engelen, E., Hautvast, J. L. A., Schneeberger, P. M., & van Duijnhoven, Y. T. H. P. (2014). *Coxiella burnetii* seroprevalence and risk for humans on dairy cattle farms, the Netherlands, 2010–2011. *Emerging Infectious Diseases*, *20*(3), 417–425. https://doi.org/10.3201/eid2003.131111

Schneeberger, P. M., Hermans, M. H. A., van Hannen, E. J., Schellekens, J. J. A., Leenders, A. C. A. P., & Wever, P. C. (2010). Real-time PCR with serum samples is indispensable for early diagnosis of acute Q fever. *Clinical and Vaccine Immunology*, *17*(2), 286–290. https://doi.org/10.1128/CVI.00454-09

Thompson, M., Mykytczuk, N., Gooderham, K., & Schulte-Hostedde, A. (2012). Prevalence of the bacterium *Coxiella burnetii* in wild rodents from a Canadian natural environment park. *Zoonoses and Public Health*, *59*(8), 553–560. https://doi.org/10.1111/j.1863-2378.2012.01493.x

Toman, R., Skultety, L., & Ihnatko, R. (2009). *Coxiella burnetii* glycomics and proteomics-tools for linking structure to function. *Annals*

of the New York Academy of Sciences, *1166*(1), 67–78. https://doi.org/10.1111/j.1749-6632.2009.04512.x

Van den Brom, R., & Vellema, P. (2009). Q fever outbreaks in small ruminants and people in the Netherlands. *Small Ruminant Research*, *86*(1–3), 74–79. https://doi.org/10.1016/j.smallrumres.2009.09.022

Webster, J. P., Lloyd, G., & Macdonald, D. W. (1995). Q fever (*Coxiella burnetii*) reservoir in wild brown rat (*Rattus norvegicus*) populations in the UK. *Parasitology*, *110*(1), 31–35. https://doi.org/10.1017/S0031182000081014

Yadav, J. P., Malik, S. V. S., Dhaka, P., Kumar, M., Bhoomika, S., Gourkhede, D., Kumar, B., Ram Verma, M., Barbuddhe, S. B., & Rawool, D. B. (2019). Seasonal variation in occurrence of *Coxiella burnetii* infection in buffaloes slaughtered in India. *Biological Rhythm Research*, 1–7. https://doi.org/10.1080/09291016.2019.1627643

How to cite this article: Theonest NO, Carter RW, Kasagama E, et al. Molecular detection of *Coxiella burnetii* infection in small mammals from Moshi Rural and Urban Districts, northern Tanzania. *Vet Med Sci*. 2020;00:1–8. https://doi.org/10.1002/vms3.401

Poster presentation

Molecular detection of rodent borne zoonoses in the Northern zone of Tanzania: A one health approach towards understanding of zoonotic cause of febrile illness

Ndyetabura Theonest[1,2], Kathryn Allan[3], Julius Keyyu[4], Hugo[1] Ephrasia Hugo[1], Joram Buza[2], Gabriel Shirima[2], Dan Haydon[3], Jo Halliday[3]
1. School of Life Science, The Nelson Mandela African Institution of Science and Technology, Arusha, 2. Kilimanjaro Clinical Research Institute, Moshi 3. Institute of Biodiversity Animal health and Comparative Medicine, College of Medical Veterinary and Life Science, University of Glasgow, Scotland, 4. Tanzania Wildlife Research Institute, Arusha

Background Introduction

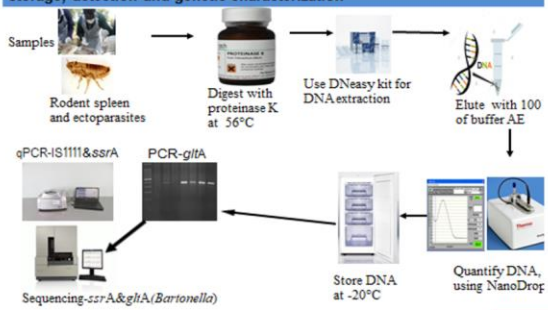
➢Zoonotic diseases poses greater public health problem in the world we live in today than ever before. For centuries rodents or their associated ectoparasites have been linked with emergence of several zoonotic diseases of public health importance. In the Northern Tanzania zoonotic p athogens have been reported to cause large proportional of people admit ted in hospital or seeking health care due to febrile illness. Little is known about animal source and hosts for these pathogens. Use of molecular meth ods for detection zoonotic pathogens could significant improve on informed patient care , management and policy plan or reforms

Problem statement

Febrile illness is the major clinical presentation among persons pres enting complaint seeking healthcare in Moshi, northern Tanzania . Large proportional are zoonotic however animal hosts for several zoonotic pathogens are poorly known in this area thus limiting intervention programs.

Material and methods

Methodology: Flowchart of various steps for DNA extraction ,quantification, storage, detection and genetic characterization



Objectives

Main Objective

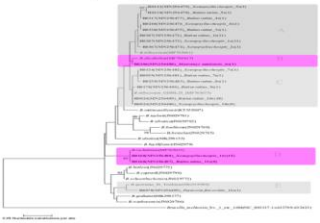
To determine the presence and the prevalence , genetic characteristics and risk factors for *C. burnetii* and *Bartonella* spp in smalls and their fleas from Moshi , Tanzania.

Specific Objectives

- To determine the presence and prevalence of *C. burnetii* and *Bartonella* spp infections in rodents from Moshi, Northern Tanzania .
- To determine the genetic characteristics of *Bartonella* spp in rodents and the ir ectoparasites in Moshi, Northern Tanzania.
- To analyse the risk factors for *C. burnetii* and *Bartonella* spp infections in rod ents from Moshi, Northern Tanzania.

Results

A. *Bartonella* genotypes, rodent spleen& fleas as detected by ssrA qPCR



-Five clusters-A,B,C, & E
-Seq.within cluster are ≥97.0% to Genbank ref.seq
-Seq.within a cluster are 100% to each other
-Outgroup-*B. meltensis*
-Seq.deposited in Genbank: MN256472-89

- Overall, 12 (3.1%) of 382 (95% CI: 1.6-5.4) spleens from small mammal tested were positive for *C. burnetii* DNA.
- *Coxiella burnetii* DNA was detected in five (71.4%) of seven (95% CI: 29.0-96.3) small mammal species; *Rattus rattus* (n=7), *Mus musculus* (n=1), *Acomys wilsoni* (n=2), *Paraxerus flavovottis* (n=1) and *Atelerix albiventris* (n=1).
- Eleven (91.7%) of twelve (95% CI: 61.5-99.8) *C. burnetii* DNA positive were trapped within Moshi Urban District.
- Overall, 57 (15.0%) of 381 (95% CI: 11.3-18.5) small mammal spleens tested positive for *Bartonella* DNA.
- *Bartonella* DNA was detected in three species (*R. rattus* n=54, *M. natalensis* n=2 and *P. flavovottis* n = 1) using qPCR targeting the *ssr*A gene.
- Analysis of *R. rattus* species only for risk of *Bartonella* infection indicated that *Bartonella* infection was more likely in reproductively mature as compared to immature small mammal (OR = 3.42, p<0.001)

CONCLUSION AND RECOMMENDATION

- These findings demonstrate that rodents in Moshi are hosts of *C. burnetii* and *Bartonella* species and may act as a source of these pathogens to humans and animas
- Further studies covering broad range of potential hosts should be considered in order to understand the distribution of these in other animals
- Efforts are needed to determine the clinical impact of *C. burnetii* and *Bartonella* infections in humans

Contact
Ndyetabura Theonest
The Nelson Mandela African Institution of Science and Technology
P.O.Box 447, Tengeru – Arusha
E-mail: teoctan@yahoo.com/theonestn@nm-aist.ac.tz
Tel: +255 767926634

References

1. Crump, J. A., Morrissey, A. B., Nicholson, W. L., Massung, R. F., Stoddard, R. A., Galloway, R. L., Ooi, E. E., Maro, V. P, Saganda, W., Kinabo, G. D., Muiruri, C., & Bartlett, J. A. (2013). Etiology of Severe Non-malaria Febrile Illness in Northern Tanzania: A Prospective Cohort Study. *PLoS Neglected Tropical Diseases*, 7(7), e2324. https://doi.org/10.1371/journal.pntd.0002324
2. Abbassy, M. M., Loftis, A. D., Dasch, G. A., Szumlas, D. E., Helmy, I. M., Moriarity, J. R., & Reeves, W. K. (2006). Surveillance of Egyptian fleas for agents of public health significance: *Anaplasma, Bartonella, Coxiella, Ehrlichia, Rickettsia* and *Yersinai Pestis*. *The American Journal of Tropical Medicine and Hygiene*, 75(1), 41–48. https://doi.org/10.4269/ajtmh.2006.75.41