Sero-epidemiology and molecular characterization of Rickettsiae infecting humans, selected animals and arthropod vectors in Asembo, western Kenya, 2007-2010

Alice Ngonyo Maina

A thesis submitted in partial fulfilmentfor the degree of Doctor of Philosophy in Molecular Medicine in the Jomo Kenyatta University of Agriculture and Technology.

2012

DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

Signed: _____Date: _____

Alice Ngonyo Maina

This thesis has been submitted for examination with our approval as university supervisors

Signed: _____Date: _____

Dr. M. Kariuki Njenga

CDC, Kenya

Signed: _____ Date: _____

Professor Zipporah Ng'ang'a

JKUAT, Kenya

Signed: _____ Date: _____

Dr. Solomon Mpoke

KEMRI, Kenya

DEDICATION

This work is dedicated to my parents, Maina Wachira and Gathoni Maina

ACKNOWLEDGEMENTS

I wish to sincerely thank my supervisors, Dr. Kariuki Njenga (Centers of Disease Control and Prevention, Kenya), Prof. Zipporah Ng'ang'a (Jomo Kenyatta University of Agriculture and Technology) and Dr. Solomon Mpoke (Kenya Medical Research Institute) for offering valuable guidance, encouragement and constructive criticisms. I most sincerely acknowledge the mentorship and guidance that I received from Dr. Darryn Knobel (University of Pretoria, South Africa), Dr. Sally Cutler (University of East London, UK) and Dr. Joanna Halliday (University of Glasgow, UK).

I am grateful to the Wellcome Trust UK (grant number081828/B/06/Z) for offering me a scholarship to pursue this study and Centers for Disease Control and Prevention through Global Disease Detection Division for their funding that made this study a success. I am indebted to the United States Department of Defence Global Emerging Infections Surveillance and Response system (DoD-GEIS) for the financial support in further characterization of rickettsial species (work unit number 188M.0931.001.A0074). Ju Jiang of the Naval Medical research Center is highly appreciated for training me on the Multi-locus sequence typing and her excellent provision of technical information and assistance. Dr. Anna Laudisoit (VAR, Brussels, Belgium) is highly appreciated for her extensive training on tick and flea identification.

The bulk of the work was carried out at the Naval Medical Research Center (NMRC), Silver spring, MD. USA. My sincere gratitude goes to Dr. Allen Richards

for inviting me to this institution, provision of laboratory space, reagents and accommodation. The warm and facilitating hospitality extended to me by the entire staff of the NMRC is highly appreciated.I acknowledge valuable assistance from the entire International Emerging Infections program (IEIP) staff for the role they played in the realization of the many objectives of this study.I appreciate the support of Miss Kabura Wamburu, Immaculate Amadi, Petty Ahenda, Brenda Rasowo, Leonard Nderitu, Sylvia Omulo, Terryson Yator, Cathleen Sonye and Beatrice Wanjiru for their helping hand when I wavered.

Finally, I feel indebted to my family for their genuine and constant support during the period of this study and to many other people who gave assistance in one way or another. Thank you.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

LIST OF ABBREVIATIONS AND ACRONYMS

ABI	Applied Biosystem
AG	Ancestral group
ATBF	African tick-bite fever
BLAST	Basic local alignment search tool
BP	base pair
CDC	Centers for disease Control and Prevention
C.I	Confidence interval
CT	Cycle Threshold
DEBONEL	Dermacentor-borne necrosis erythema and lymphadenopathy
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetraacetic acid
EID	Emerging Infectious Diseases
ELB	EL Labs
ELISA	Enzyme Linked Immunosorbent Assay
ERC	Ethical review committee
FAK	Focal adhesion kinase
FITC	Fluorescein isothiocyanate
GDDD	Global Disease Detection Division

HDSS	Human demographic surveillance study
IEIP	International Emerging Infections Program
IFA	Indirect fluorescence antibody
IgG	Immunoglobulin G (Gamma)
IgM	Immunoglobulin M (Mu)
ITROMID	Institute of Tropical Medicine and Infectious Diseases
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KEMRI	Kenya Medical Research Institute
LOCs	Livestock owning compounds
Mb	Megabyte
MSF	Mediterranean spotted fever
MLST	Multi-locus sequence typing
NCBI	National Center for Biotechnology Information
NMRC	Naval Medical Research Center
ompA	Outer membrane protein A
ompB	Outer membrane protein B
PBIDS	Population-based infectious disease surveillance
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
RMSF	Rocky Mountain spotted fever
ROC	Receiver operating characteristics
SARI	Severe acute respiratory illness
SSC	Scientific steering committee

Taq	*Thermus aquaticus*
TBE	Tris, borate EDTA
TG	Typhus group
TIBOLA	Tick-borne lymphadenopathy
TRG	Transitional group
SFG	Spotted fever group
UDG	Uracil DNAglycosylase
µl	Microlitre
USA	United States of America

ABSTRACT

Rickettsiae are gram negative obligate intracellular bacteria in the class α-*proteobacteria*. They are transmitted by arthropod vectors, including fleas, ticks, mites, andbody lice. The prevalence of rickettsial infections in humans, animals and arthropod vectors in Kenya is undefined.Thisstudy was conducted to investigate rickettsial infections in western Kenya. To assess previous exposure to *Rickettsia*, a randomly selected subset of sera from patients attending Lwak Hospital in western Kenya in 2007-8 were tested for IgG antibodies against *Rickettsia*(n=359) with indirect fluorescence antibody (IFA) test. To detect acute rickettsial infections, blood specimens from patients with acute febrile illness (n=699) and from asymptomatic individuals (n=236) were tested using four monoplex qPCR assays that target gene fragments of *gltA*,17-kDa and *ompB*. Buffy coats,spleen specimens collected from peri-domestic small mammals, ticks and fleas were tested for *Rickettsia* using *gltA*qPCR.Selected positive samples were further characterized by sequencing genes encoding the 17-kDa, *ompA*, *ompB* and *R. felis* plasmids.Further testing of the unique *Rickettsia* species was done using multi-locus sequence typing of *rrs*, *gltA*, *ompA*, *sca4* and *ompB* genes. Immunoglobulin G against all rickettsiae were detected in 57.4% of the patients (n = 357), including 56% positive for spotted fever group (SFG)*Rickettsiae* and and 14.5% positive for typhus group (TG)

rickettsiae.Seropositivity for SFG antibodies increased significantly with age (p <0.001). Febrile patients had 2.2 times higher odds of testing positive for rickettsial infection by PCR than asymptomatic individuals (7.15% vs 3.39%, odds ratio 2.20, CI; 1.03-4.70). From animals in the same region, *Rickettsia* DNA were detected by PCR in 3.68% (n=299) of the dogs and 7.69% (n=26) of the cats. No rickettsia was detected in cattle, sheep and goats. Partial sequences from 17-kDa gene showed 97% homology with specimens from cats, dogs and *R. felis* URRWXCal2. Of the ticks obtained from cattle and dogs, 96.9% (n=162 pools) and 20.34% (n=59) were positive for *Rickettsia*, respectively. Nucleotide sequences of *ompA* and *ompB* gene showed ≥ 98% similarity with tick specimens and *Rickettsia africae*. Of the flea specimens tested, 59.7% (n=134) were positive for *Rickettsiae*. Partial sequences of human isolates and one flea specimen were 100% homologous to *Rickettsia felis*, while11 other fleas were ≤93% homologous to *R. felis*. MLST of the 6/11 specimens determined the flea specimens to be unique and genetically similar to *Rickettsia* RF2125, a previously-described rickettsial agent of fleas and mites.This study provides evidence of *R. felis*-associated illness in febrile patients in western Kenya and demonstrates at least one novel rickettsial agent"*CandidatusRickettsia asemboensis*". Further work is needed to elucidate the biological characteristics and pathogenecity of the novel *Rickettsia* species.

CHAPTER ONE

1.0 INTRODUCTION

Human health is inextricably linked to animal health and production. In developing countries the link between humans, animals and the surrounding environment is very close and therefore, the environment plays a critical role in selection and emergence of zoonotic pathogens. According to definition given by Daszak *et al.* (2000), emerging diseases are medical conditions that have recently increased in incidence or geographical range, recently moved into new host populations, recently been discovered or are caused by newly evolved pathogens.

In the past two decades, the emergence of many infectious diseases has attracted increasing attention in humans, domestic animals and wildlife (Cleaveland *et al*., 2001). About 75 % of the new diseases that have affected humans have been caused by pathogens originating from animal or products of animal origin (Taylor *et al*., 2001). Many of these diseases have the potential to spread through various means over long distances to become global problems. Some factors may favour this phenomenon such as the increased interaction between humans and animals (Hechemy, 2006), increased production of food animals and wildlife, increased leisure activities, breakdown of host defences and deliberate efforts by human to cause harm like during the world wars (Kutz *et al*., 2004; Blancou *et al*., 2005). Catastrophic breakdown of social conditions such as impoverishment of some human populations amongst which all zoonoses can find suitable hosts due to poor

hygiene can result in re-emergence of diseases in epidemic form (Raoult *et al.*, 1997; Blancou *et al*., 2005). Emergence of these diseases is typically from a setting characterized by close association between humans and animals, both domestic and wild, highlighting the need for improved awareness, detection and co-ordination of both medical and veterinary health providers to combat new and emerging disease threats.

Microbial changes or adaptations such as mutations, genetic drifts, transformation, activation and silencing of genes are important in the epidemiology of zoonotic diseases. These adaptations may play a role in the natural selection and evolution of these pathogens(Kruse *et al*., 2004). New species or strains of *Rickettsia*, and the geographic spread of known *Rickettsiae* once thought to be geographically restricted are being discovered at an increasing rate (Hechemy *et al.,* 2003). Investigating and understanding emergence of rickettsial diseases in sub-Saharan Africa presents a number of challenges, the greatest of which is paucity of baseline data. The use of sentinel hospital-based studies built upon existing systems may provide useful clinical and public health information in countries that lack resources for long-term diagnostic testing (Archibald and Reller, 2001).

1.1 Problem statement

Emerging infectious diseases (EID) are increasing significantly over time. These EID events are dominated by zoonoses (60.3%) of which 54.3% are caused by bacteria or *Rickettsia* (Jones *et al*., 2008). While the burden of some infections (for

instance, malaria) in sub-Saharan Africa is believed to be substantial, the importance of others (for example rickettsial diseases) is undefined. In malaria endemic areas, most fevers are attributed to *Plasmodium falciparum* infections and presumptively treated with expensive combination therapies (Snow *et al.*, 2003). In addition, clinical manifestations of rickettsial illnesses are similar to those of malaria. In western Kenya for instance, febrile illness and respiratory infections are the most common reasons for seeking medical attention (Bigogo *et al*., 2010), but there is limited information on the frequency of specific infections. For this reason, these neglected febrile illnesses need to be given appropriate consideration.

Furthermore, species of *Rickettsia* continue to be described in many parts of the world, yet little is known about the circulating species or strains in Kenya. In a situation where emerging and re-emerging zoonotic infections are on the rise, it is important that studies extend to embrace all aspects of the disease epidemiology in a more holistic manner. In the absence of baseline knowledge, there remains some level of ambiguity between 'new emergence" or only detected in a region or host population (apparent emergence).

1.2 Justification

Despite reports of substantial burden from infectious diseases in sub-Saharan Africa, information is lacking on the incidence and aetiologies of important infectious disease syndromes in most African countries, including Kenya. Emerging zoonotic diseases pose a growing threat to human health, but little is known about the

relationship between infection in animals and humans, or the specific risk factors for zoonotic disease outbreaks in Africa.

Diagnosis of some of these zoonotic diseases is challenging and may be obscured by lack of obvious clinical signs in humans and animals and also the overwhelming cases of malaria that present as a febrile illness in humans. *Rickettsia* spp. are known to be highly infective sometimes causing fatal illnesses or otherwise debilitating disease and hence have been regarded as possible agents for bioterrorism.

This study seeks to determine the role of *Rickettsia*species as a cause of febrile illnesses in western Kenya. It would also establish the need for routine laboratory screening for *Rickettsia*and thus hasten appropriate patient management with reduction of the severity and progression of the pathology.

1.3Research questions

1. What is the prevalence of IgG antibodies to spotted fever group and typhus group *Rickettsia* among patients attending Lwak Hospital?
2. What is the optimum volume of blood clot and optimum cycle threshold (CT) for *gltA* qPCR assay as a screening test?
3. Is there a difference in *Rickettsia* prevalence rates between febrile and asymptomatic individuals in Lwak Hospital of Rarieda district?
4. What is prevalence of rickettsial infections in domestic animals, peri-domestic small mammals and arthropods in Asembo, western Kenya?

5. Are the *Rickettsia* species that infect humans homologous to those that are harboredby animalsand arthropods?

1.4General objective

To investigate *Rickettsia* infections in humans, animals and arthropod vectors in Asembo, western Kenya

1.5 Specific objectives

1. To assess the prevalence of IgG antibodies to spotted fever and typhus group *Rickettsia* among patients attending Lwak Hospital in Rarieda District
2. To optimize blood clot volume for maximum efficiency and determine the optimum cycle threshold (CT) cut-off for screening of *Rickettsia*using blood clot samples with *gltA*qPCR assay.
3. To compare the prevalence of acute*Rickettsia* infections in febrile patients with that of asymptomaticindividuals among patients attending Lwak Hospital in Rarieda District.
4. To determine the prevalence of *Rickettsia* infections in domestic animals, peri-domestic small mammals and arthropods in Asembo
5. To determine the homology of *Rickettsia* species found in humans, animals and arthropods

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 RICKETTSIAE

The *Rickettsiae* are obligate intracellular short rods, measuring 0.3 to 0.5 by 0.8 to 2.0 µm that have intimate association with eukaryotic cells (Woese, 1987). The bacterium has a Gram-negative trilamellar structure made of a bilayer inner membrane, a peptidoglycan layer and a bilayer outer membrane. The *Rickettsia* genome is small ranging between 1-1.6 Mb and consists of a single circular chromosome (Raoult and Roux, 1997). Members of the genus *Rickettsiae* do not have plasmids except *R. felis* which is reported to carry one plasmid (Gillespie *et al*., 2007).

2.1.1 THE HISTORY OF RICKETTSIOSES

Rickettsioses are thought to be one of the oldest infectious diseases as exemplified by epidemic typhus suspected to have been responsible for the Athens plague described by Thucydides during 5^{th} century BC (Raoult and Roux, 1997). Howard Taylor Ricketts discovered the "bacillus of the Rocky Mountain spotted fever" in 1906. He however died in 1910 of a laboratory-acquired typhus fever. The agent of the Rocky Mountain spotted fever was named *R. rickettsii* in honour of Ricketts (Hechemy, 2006).

In 1909, Stanislaus Von Prowazek discovered *R. prowazekii*, the causative agent of epidemic typhus fever. This was later followed by the discovery by Charlse Nicolle that epidemic typhus fever was louse-borne (Raoult *et al*., 2005a). Wolbach further studied the causative agent of epidemic typhus fever and named it *R. prowazekii*, after the discoverer (Hechemy, 2006). *Rickettsia conorii*, the causative agent for Mediterranean spotted fever (MSF)was first described by Conor and Bruch in Tunisia in 1910. This disease was more associated with dogs and more prevalent in people living in urban areas. In 1930, Pijper described a mild disease that was associated with cattle ticks in the bush. This disease was African tick-bite fever (ATBF) caused by *R. africae* (Raoult and Roux, 1997; Jensenius *et al.*, 2003a).

2.1.2 TAXONOMIC CLASSIFICATION OF RICKETTSIA

The genus *Rickettsia* refer to arthropod-borne bacteria which have over 25 validated species and several other uncharacterized species or arthropod amplificons (Fournier and Raoult, 2009).The genus *Rickettsia* belongs to the family *Rickettsiaceae* and order *Rickettsiales*. Historically, the order *Rickettsiales* was divided into three families namely; *Rickettsiaceae*, *Bartonellaceae*, and *Anaplasmataceae*. The family *Rickettsiaceae* was made up of three tribes *Rickettsieae*, *Ehrlichieae*, and *Wolbachieae* (Weisburg *et al*., 1989).The genera of *Coxiella*, *Rickettsia* and *Rochalimaea* belonged to the tribe *Rickettsieae*. Following recent taxonomic studies based on 16S rRNA, the two genera, *Coxiella*, and *Rochalimaea* were removed from the tribe *Rickettsieae* leaving the genus *Rickettsia* alone. *Rickettsia tsutsugamushi* an

only member of scrub typhus group was transferred into the genus, *Orentia* (Fournier *et al.*, 2003).

Traditionally rickettsial taxonomy was based on phenotypic characteristics. This had major limitations because *Rickettsia* being intracellular in nature, expresses few phenotypic characteristics (Raoult and Roux, 1997). These include their intracellular location which was thought to be determined by the ability to polymerize actin in the cytoplasm, growth temperature and cross-reaction with somatic antigens of three strains of *Proteus*, OX19, OX2 and OXK. Members of the genus *Rickettsia* were divided into spotted fever group (SFG), typhus group (TG), and the scrub typhus group (STG)on the basis ofphenotypic characteristics (Raoult and Roux, 1997).

Technological advancement in molecular genetics has led to extensive reorganization of genus *Rickettsia*. Comparison of different gene sequences allowed significant phylogenetic inferences to be made at different levels. Phylogenetic studies based on the 16 rRNA (*rrs*) genes have showed evolutionary unity in the genus*Rickettsia* (Figure 1.1). Inferences on the intragenus phylogeny were not possible because of the nucleotide similarity within the genus (Raoult and Roux, 1997). The major difficulties encountered in the nomenclature of *Rickettsia* is the determination of cut-off in the percent divergence among gene sequences that define a species, a subspecies or a strain within the *Rickettsia* genus (Parola *et al.*, 2005). Polyphasic taxonomy which integrates phenotypic and phylogenetic data has proved to be useful in rickettsiology. Taxonomic classification of *Rickettsiae* has been

controversial. It has been suggested that strains with more than 3% divergence belong to different species (Raoult *et al*., 2005).

Recent phylogenetic work shows that the genus is subdivided into four biotypes/ lineages. The Typhus group (TG) comprising *R. typhi* and *R. prowazekii*, the spotted fever group (SFG) comprises over 20 species with *R. conorii, R. africae* and *R. rickettsii,* the transitional group (TRG) which consist of *R. felis* and *R. akari* and the ancestral group (AG) which has *R. bellii* and *R.canadensis* (Gillespie *et al*., 2007). The number of genes varies within the genus *Rickettsia* with the typhus group having the fewest genes (*R. prowazekii*, 872 and *R. typhi*, 877). *Rickettsia felis* has the highest number of genes followed by *R. bellii* (Gillespie *et al*., 2007). Genome size of the typhus group is smaller and has a poorer G + C content as compared to *R. bellii* and *R. felis* (Blanc *et al*., 2007). The differences in the gene assemblages across the rickettsial genome may be due to differential gene losses from the ancestors.

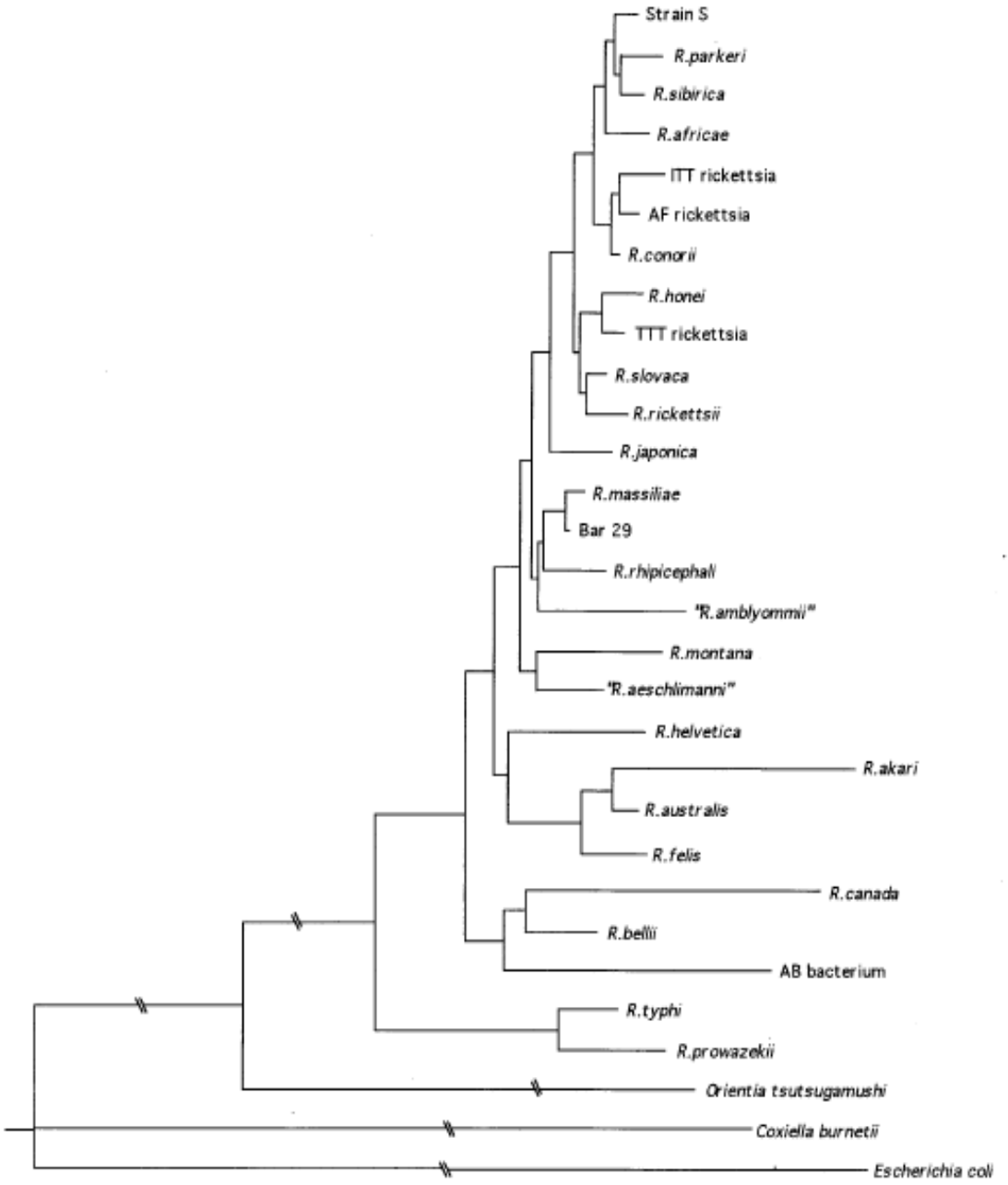


Figure 1.1 Phylogenetic tree derived from 16S rRNA gene of the bacteria belonging to *Rickettsia* genus (Source: Raoult and Roux, 1997)

Rickettsia genus is an excellent paradigm for understanding the process of reductive evolution. It is speculated that genome diversification is as a result of gene loss (Blanc *et al*., 2007). Genome reduction has been recognized in many bacteria living within the cells of higher eukaryotes and especially in obligate intracellular parasitism such as *Rickettsiae* and *Chlamydia* (Zientz *et al*., 2001). Bacteria living intracellulary adapt to such niches and may end up losing some of their functional genes required by free living bacteria. Most of these small genome pathogens show reduction in gene numbers in various functional categories such as basic cellular processes and biosynthesis of metabolic intermediates (Moran and Mira, 2001). Other intracellular organisms like the *Buchnera aphidicola* that have small genomes as compared to their ancestral counterparts are documented to have undergone large gene deletions soon after they acquired their endosymbiotic lifestyle (Moran and Mira, 2001).

2.1.2.1 Typhus fever group

This group cause typhus in humans and comprises of *R. typhi* and *R prowazekii*. They have an optimal growth temperature of 35ºC and G+C content of 29 %. They are found in the cytoplasm because of their inability to polymerize actin (Fournier and Raoult, 2009).

Rickettsia typhi causes murine typhus and is transmitted by rat fleas (*Xenopsylla cheopis*) and has worldwide distribution (Azad and Bearourd, 1998). Murine typhus is also known by other names like endemic typhus or urban typhus (Blanco and

Oteo, 2006). The vertebrate reservoirs are *Rattus norvegicus* and *Rattus rattus*. Transmission to human occurs through contaminated skin, conjunctiva or via respiratory route by aerosols of dust contaminated by infected flea faeces (Raoult and Roux, 1997).

*Rickettsia prowazekii*causes epidemic typhus and is known by various names in Europe; *fleckfieber* (German), *typhus exanthematique* (French) and *tabardillo* (Spain) (Blanco and Oteo, 2006). It is transmitted from one person to another by the human body louse (*Pediculus hominis corporis*). Other than humans, the flying squirrels are also documented as reservoir hosts for *R. prowazekii*. Transmission is through contact particularly when bite wounds get contaminated by faeces or crushed body lice (Gillespie *et al*., 2009). Outbreaks have occurred in refugee camps created by such upheavals as civil wars or volcanic eruptions due to conditions of overcrowding (Hechemy *et al*., 2006).

2.1.2.2 Spotted fever group

The spotted fever group is associated with spotted fever in humans. This group comprises of over 20 different species (Raoult and Roux, 1997). This group has an optimal growth temperature of 32º C, a G+C content of 32-33 %, and can polymerize actin and therefore can be found in the nuclei of the host cells (Fournier and Raoult, 2009). *Rickettsia conorii* the cause of Mediterranean spotted fever also known as "*botonneuse fever,*" "*Marseilles fever*" or "*Escaro-nodular fever,*" (Blanco

and Oteo, 2006) belongs to this group. It is transmitted by ticks in the genus *Rhipicephalus* (mainly the brown dog tick, *R. sanguineus*). Several serotypes of *R. conorii* have been described (Rovery *et al*., 2008).

Rickettsia africae causes African tick-bite fever and is transmitted by ticks in the genus *Amblyomma* mainly *A.habraeum* in Southern Africa and *A. variegatum* in the West, Central and East Africa. Ticks in this genus act as reservoirs in which the infection is maintained through transovarial and transtadial transmission (Jensenius *et al*., 2003).

Rickettsia rickettsii causes Rocky Mountain spotted fever and is transmitted by Ixodes ticks. Ticks serve both as vectors and reservoir for *R. rickettsii*. *Dermacentor variabilis* and *D. andersoni* are the main vectors in North America, while *Amblyomma canajensis* is the main vector in South America (OIE, 2005). Other reservoirs include rabbits, rodents, dogs and birds (Azad and Radulovic, 2003).

Other recently discovered *Rickettsiae*have been reported in Africa; *R. aeschlimanii*, and *R. massiliae* from ticks in Algeria (Bitam *et al*., 2006a), *R. sibirica subsp. Monglotimona* (Raoult and Roux, 1997), *R. heilongjiangensis* isolated from humans and ticks (Mediannikov *et al*., 2009) and *R. raoultii* in Europe and Russia (Mediannikov *et al*., 2008).

2.1.2.3 Transitional group (TRG) *Rickettsia*

This group is phylogenetically positioned between the spotted fever group (SFG) and the typhus group (TG) (Gillespie *et al*., 2007). *Rickettsia felis* the cause of flea-borne spotted fever (Zavala-Velezquez *et al*., 2000) and *Rickettsia akari* the cause of rickettsialpox belong to this group.

The gene encoding outer membrane protein A (*ompA*) is common in both the transitional group *Rickettsia* and the spotted fever group (SFG). This gene (ompA) is involved in *Rickettsia*-host cell attachment process (Zavala-Castro *et al*., 2008). Members of TRG *Rickettsia* were previously grouped together in the spotted fever group yet none of these has a tick vector. *Rickettsia felis* has been isolated in *Ctenocephalides felis* flea (Bauer *et al*., 2006; Marquez *et al*., 2006; Venzal *et al*., 2006) and *R. akari* in mites.

Rickettsia felis (previously known as ELB agent) received its first name, ELB agent from EL-labs (El Labs, Soquel, CA, USA), a company that maintained a commercial cat flea colony (Adams *et al*., 1990). It was first observed in 1990 within the midgut cells of cat fleas from a commercial colony manufactured by EL Labs. With reports of *Rickettsia felis* illnesses from all over the world, this newly described pathogen is emerging as a global threat to humans (Perez-Osorio *et al*., 2008). The cat fleas (*Ctenocephalides felis*) are documented to be the primary vector and reservoirs of *R. felis* (Reif and Macaluso, 2009). *Rickettsia felis* displays both genotypic and phenotyphic characteristics of the spotted fever and typhus fever groups. These

attributes are serological cross reactivity, transovarial maintenance in vectors, actin-based motility, hemolytic activity and association with insects (Gillespie *et al.*, 2007). *Rickettsia felis* is the only member of the genus *Rickettsia* so far documented to harbor plasmids known as *Rickettsia felis* plasmids (pRF) and a conjugative pili. It harbors two plasmids (pRF and pRFδ) that are 62,829 and 39,263 bp long, respectively (Ogata *et al.*, 2005). Other intracellular bacteria documented to possess plasmids are *Coxiella burnetii* (Maurin and Raoult, 1999), *Chlamydia* and *Chlamydophila*species (Pickett *et al.*, 2005). It is hypothesized that all members of ancestral group (AG) *Rickettsia* once contained functional plasmids that might have been lost in the process of evolutionary decay or the plasmidless *Rickettsia* may have had their plasmid genes incorporated in their chromosomes (Gillespie *et al.*, 2007). The polyphyletic nature of the pRF and chromosomal genes in *R. felis* points to the fact that the pRF genes may not have been vertically passed over time in the lineage but rather were inherited horizontally from other bacterial plasmids and non bacterial DNA (Gillespie *et al.*, 2007). This can be supported by the fact that majority of conjugative transfer genes and other structural proteins involved are present in the *R. felis* genome. The pRF plasmid in *R. felis* has proteins with high homology to bacterial conjugation proteins (*tra* genes). The pRF plasmid could therefore function as an (F) plasmid exchanging genetic material with other bacteria of the same kind through a pilus (Gillespie *et al.*, 2007). The pRF plasmid may have functions that benefit *R. felis* in the survival and expression of virulence. It is documented that the pRF contains a patatin like phospholipase (pRF11) which together with

hyaluronidase gene (pRF56) are implicated to be virulent factors (Gillespie *et al*., 2007).

2.1.2.4 Ancestral group (AG) *Rickettsia*

This group comprises the *Rickettsia bellii* and *R. canadensis*. This group is speculated to have diverged prior to the separation of typhus group and spotted fever group. It is basal in location to other pathogenic *Rickettsiae* in the phylogenetic tree (Gillespie *et al*., 2007). *Rickettsia bellii* is found in both soft and hard ticks and exhibits the largest arthropod host range among known members of the genus *Rickettsia* (Ogata *et al*., 2006). Its large size (1,522,076 bp) is comparable to that of *R. felis* (1,587,240 bp) (Gillespie *et al*., 2007) and the early branching in phylogenic position may suggest that it might have retained ancestral features lost in other *Rickettsiae* (Ogata *et al*., 2005). A conjugative sex pili-like appendage has been documented to occur in *R. bellii* that could allow conjugative DNA transfer.

2.1.3 EPIDEMIOLOGY OF RICKETTSIA

2.1.3.1 The vectors and reservoirs of the *Rickettsiae*

Arthropods are important vectors of various pathogenic bacteria, protozoa and viruses that cause disease in humans and animals worldwide. The main arthropods implicated in the life cycle of rickettsial diseases are ticks, fleasand mites. The *Rickettsiae* can multiply in all the organs of the invertebrate host. When the salivary

glands are infected, such *Rickettsiae* can be transmitted to vertebrate hosts during feeding. Transmission in ticks is both transovarial and transtadial (Raoult and Roux, 1997).

The important role that arthropods play in the transmission of medically significant zoonoses is becoming increasingly apparent. Most of these arthropods act as vectors and reservoirs of disease pathogens (Azad and Beard, 1998; Parola *et al*., 2005). The vectorial competence of an arthropod which is the ability to acquire, maintain and transmit microbial agents is determined by various intrinsic and extrinsic factors (Kilpatrick *et al*., 2007).

Intrinsic factors include ability of an infected vector to transmit the microbe to its offspring transovarially or transtadially. Although some arthropods manifest a symbiotic relationship with some agents, some act as true parasites where they can alter reproduction and manipulate cellular processes in the arthropod host to an extent of killing the vector. This is the case with *Rickettsia prowazekii*, and its vector, the body louse (Badiaga *et al*., 2005).

Extrinsic factors are mainly conducive climatic conditions and vector interaction with the host. The competence of reservoir vertebrate host is determined by the ability to acquire a pathogen from an infected vector (susceptibility), the ability of the host to infect uninfected vectors (infectivity), persistence of the infection which is determined by host immunity, life span which is majorly the population turnover

rate, dispersal mechanism and interaction with other agents which is either synergy or antagonism.

2.1.3.2 Ticks as vectors and reservoir of rickettsial diseases

Ticks are responsible for transmitting a variety of pathogens including *Rickettsia*, protozoa, bacteria and viruses to both humans and animals. They have been known to be vectors of disease since the 17th century. The "hard" ticks in the family *Ixodidae* act as vectors, reservoirs and amplifiers of spotted fever group rickettisae. Epidemiological and clinical aspects of tick-borne diseases are influenced by ecological characteristics of the tick vectors. This gives rise to seasonal patterns observed with many rickettsial diseases (Parola *et al*., 2005).

Ticks may acquire *Rickettsiae* vertically through transovarial passage (from mother to eggs and progeny), transtadially from one stage to another or transexually between adult sexes during mating. Ticks may also acquire infection through horizontal transmission which occurs through coxal fluid, systemic pathogenemia in saliva-activated transmission and during co-feeding where infected and non-infected ticks co-feed in space and time in absence of an infection in the host (Parola *et al*., 2005).

2.1.3.3 Fleas as vectors and reservoirs of rickettsial diseases

Fleas are distributed throughout the world in endemic foci and serve as important vectors for *Rickettsia typhi, R. felis* (Stevenson *et al*., 2005), *Yersinia pestis* and *Bartonella hensellae*(Stevenson *et al*., 2003). Fleas have also been documented to

transmit some of the most important and most prevalent human rickettsioses. The rat flea (*Xenopsylla cheopis*) and the cat flea (*Ctenocephalodes felis*) are the vectors for murine/endemic typhus caused by *R. typhi* and murine typhus-like fever caused by *R. felis* respectively (Blanco and Oteo, 2006; Sackal *et al*., 2008). Both *R. felis* and *R. typhi* are maintained transovarially in fleas. With advancement of molecular technology, *Rickettsiae* of unknown pathogenecity continue to be discovered in other flea's species. For instance, a *Rickettsia*spp. DQ166937 was detected in Egyptian chicken flea (*Echidnophaga gallinacea*) (Loftis *et al*., 2006). Changes in vector-host ecology due to environmental and human modification may result in re-emergence of vector-borne diseases in epidemic form (Daszak *et al*., 2000).

2.1.3.4 Rickettsioses in Animals

Evidence of antibodies against spotted fever group *Rickettsiae* was reported in cattle (90%) in Zimbabwe (Kelly *et al*., 1991), in dogs (range of 42.4-66.7%) and horses (80%) in Brazil (Sangioni *et al*., 2005; Saito *et al*., 2008). Previous studies in rodents have documented a substantial level of exposure to rickettsial agents,where up to 39.1% was recorded in Indonesia (Nurisa *et al*., 1999) and 21.1% in Spain (Lledo *et al*., 2003).In Kenya,there is very scanty information available on seroprevalence of *Rickettsiae* in rodents (Heisch, 1969; Heisch *et al*., 1969). There are numerous reports of molecular detection of *Rickettsiae* in animals, including detection of a *Rickettsia* spp. in sick dogs in Italy (Solano-Gallego *et al*., 2008) and *Rickettsia prowazekii* in Northern Carolina (Kidd *et al*., 2006). The isolates of *R. rickettsii* infecting dogs were shown to display high homology (≥99.7%) with those of humans

(Kidd *et al*., 2006).A spotted fever group *Rickettsia*, *R. parkeri* was detected in dogs' blood in Bolivia (Tomassone *et al*., 2010).

2.1.3.5 Rickettsioses around the world

Many species of *Rickettsia* have been identified all over the world both in humans and animals. Reports of rickettsioses in humans include case studies such as *R. honei* in Thailand (Jiang *et al*., 2005). Rocky Mountain spotted fever (RMSF) and *R. parkeri* are prevalent in the Americas (Thorner *et al*., 1998; Masters *et al*., 2003; Pachebo *et al*., 2006; Whitman *et al*., 2007) while *R. sibirica* is prevalent in the USSR and *R. australis* in Australia (Parola *et al*., 2005).Flea-borne rickettsioses caused by *R. typhi* and *R. felis* are widely distributed, especially in warm areas in the tropics and subtropics and in port cities and coastal regions where rodents are present. The murine typhus is prevalent in Spain (Hernandez-Cabrera *et al*., 2004) and Greece (Tselentis *et al*., 1996) where it has been identified in both rat and fleas. *Rickettsia felis* was detected in new world flea species *Anomiopsyllus nudata* in New Mexico (Stevenson *et al*., 2005).In a study conducted in children aged 1 to 17 years in South Texas, IgG antibodies against *R. typhi* were found to be prevalent (14 %) (Purcell *et al*., 2007).

Outbreaks of epidemic typhus have often been associated with periods of war, poverty, unhygienic conditions, hunger, and natural disasters (Blanco and Oteo, 2006; Raoult *et al*., 2006), and is more prevalent in regions with cold climatic conditions or during the colder months when infested clothings are not

laundered.Unhygienic conditions associated with war, hunger and natural catastrophies may provide conducive conditions for lice breeding or induce people to retreat in saturated atmospheres such as camps.Resurgences of epidemic typhus were most recently reported in a jail in Burundi (Raoult *et al*., 1998). Other newly recognised tick borne rickettsioses in Europe include *Rickettsia helvetica* (Fournier *et al*., 2000a), *R. sibirica monglotimonae* (Fournier *et al*., 2000b), *R. slovaca* and *R. raoultii* (Parola *et al*., 2009).

In Africa, morbidities and mortalities due to rickettsial diseases may be largely underestimated. The non specific pattern of rickettsial illnesses (Renvoise *et al*., 2009), difficulty in seeing a rash on dark skinned patients (Raoult *et al*., 1998) and rampant use of tetracycline for empirical treatment for most febrile illnesses may interfere with the recognition of these rickettsial illnesses (Kamalanathan and Rohani, 2003).

About six species of pathogenic Rickettsia (mainly tick borne spotted fever group) have been documented to occur in sub Saharan Africa. Among them are *R. conorii* causing Mediterranean spotted fever, *R. conorii caspia*, the agent for Astrakhan fever, *R. africae* the agent of African tick-bite fever, *R. aeschlimanii, R. sibirica mongolitimonae* and *R. massiliae* (Parola, 2006). *Rickettsia conorii* and *R. typhi* were detected among Somali refugees (Gray *et al*., 1995). *Rickettsia conorii* and *R. africae* were detected in ticks collected in Uganda and Djibouti (Socolovschi *et al*., 2007). Outbreaks of African tick-bite fever as a result of *R. africae* infection have

been reported in military personnel visiting Botswana (Smoak *et al.*, 1996). African tick-bite fever has plagued tourists visiting Africa (Raoult *et al.*, 2001; Jensenius *et al.*, 2003b; Oostvogel *et al.*, 2007). It has been suggested that sub-Saharan Africa is probably the region where rickettsioses is most prevalent (Raoult and Roux, 1997).

A serosurvey conducted in Tanzania using an immunoflourescent assay (IFA) revealed presence of previous exposure to *Rickettsia typhi* in pregnant women (Anstey *et al.*, 1997). *Rickettsia typhi, R. felis* and *R. prowazekii* were reported in a jail in Burundi (Raoult *et al.*, 1997). Other more recent reports include that of *R. conorii* and *R. africae* in Cameroon (Ndip *et al.*, 2004a; Ndip *et al.*, 2004b), Uganda and Djibouti (Socolovschi *et al.*, 2007). A newly recognized species, *R. felis* was detected in fleas collected in Democratic Republic of Congo (Sackal *et al.*, 2008). Rickettsioses have continually struck many thousands of persons living in sub Saharan Africa with civil war, famine and poor conditions (Raoult *et al.*, 1998; Jensenius, 2003; Parola, 2006).

A fatal infection by *Rickettsia conorii* was reported in a woman from the United States who had visited Kenya (Rutherford *et al.*, 2004). Another case report is that of a 52 years male Japanese traveler who had visited Kenya (Yoshikawa *et al.*, 2005). *Rickettsia africae* has been isolated from *Amblyomma variegatum* ticks collected in Maasai Mara in Kenya (Macaluso *et al.*, 2003). Another recent report is that of *R. felis* that was detected in febrile patients in Garissa Province (Richards *et al.*, 2010).In Kenya, the use of tetracylines for treatment of non-malarial fevers may

obscure the actual burden of rickettsioses (Kamalanathan and Rohani, 2003). Moreover, the situation may be worsened by inappropriate exposure to anti-malarial treatments: anecdotal reports and experimental studies suggest that sulfa-containing antimicrobial agents exacerbate the clinical severity of rickettsial infections (Topping, 1939; Beltrán and Herrero Herrero, 1992). A previous study has reported widespread use of anti-malarial drugs, especially sulphadoxine-pyrimethamine, for treatment of non-malaria fevers in Kenya (Yazoume *et al*., 2009).

2.1.4 Pathogenesis and clinical manifestation of Rickettsioses

2.1.4.1 Pathogenesis

Pathogenicity varies significantly between rickettsial species as does the extent of genomic decay found between these obligate intracellular pathogens. It has been hypothesised that those species associated with greater severity are those in which genetic decay is more advanced, potentially resulting from a loss of regulatory genes (Fournier *et al.*, 2009).

A few genes driving the pathogenic mechanisms of rickettsial diseases have been determined. Rickettsial genes involved in virulence and affecting pathogenesis include the adhesins (*omp*A and *omp*B), those effecting phagosomal escape (*pld*, *tlyC)*, actin-based mobility (*rickA*), other potential membranolytic activity (*tlyA*, *pat-1*), autotransporters (*omp*A, *omp*B and three *sca* proteins), type IV secretion system

(*virB*, *virD* genes and *invA* and others such as *sodB* (superoxide dismutase) *and* Lipopolysaccharide synthesis genes (Walker and Yu, 2005).

The best studied and most notorious members of this genus with regard to infective potential in vertebrate hosts are *R. prowazekii* (Badiaga *et al*., 2005) the cause of epidemic typhus; flea-borne murine typhus caused by *R. typhi* (Tselentis *et al*., 1996) and a clinically similar flea-borne infection by *R. felis*. Some of the best studied tick-borne spotted fever group *Rickettsiae* are *R. akari*, the cause of rickettsial pox; Rocky Mountain spotted fever by *R. rickettsii*; Boutonneuse fever by *R. conorii*; and Asian tick typhus following infection with *R. sibirica* (Parola et al., 2005).

Rickettsiae introduced through the skin spread via the lymphatics and blood vessels. *Rickettsiae* have a preference for the endothelial cells, lining small blood vessels (Thorner *et al*., 1998). The tropism for endothelial cells is poorly understood. They enter the cells by phagocytosis and then move from the phagosome to the cytoplasm, and sometimes to the nucleus where they reproduce by binary fusion. The main mechanism through which *Rickettsiae* asserts its pathogenic activity is through increased microvascular permeability. Generalized vascular injury follows, resulting in activation of clotting factors, extravasation of fluid, edema, hypovolemia, hypotension, and hypoalbuminemia. This leads to cerebral and non-cardiogenic pulmonary edema. Several mechanisms through which this process may occur are through accumulation of reactive oxygen species (ROS) and down regulation of enzymes involved in protection against oxidative injury (Olano, 2005; Woods *et al*., 2005).

Another way that *Rickettsiae* are thought to assert their pathogenic mechanism is through the role of phospholipase A_2. In an experiment, Phospholipase $A_2$of *R. prowazekii*and *R. conorii*was shown to cause a temperature cytotoxic effect on vero cells *in vitro.* Although the role of Phospholipase A_2 may not be fully determined, it is hypothesized that it mediates lysis of host cell membranes (Walker *et al*., 2001; Walker and Yu, 2005).

Entry of *Rickettsiae* into the host cell is an active process requiring active protein synthesis by the bacterium. The signalling pathways involved in internalization and adherence include recruitment of the actin nucleating complex Arp 2/3 to the site of attachment and interaction with other molecules like ATPase, cdc42, phosphoinositide 3 –kinase, protein tyrosine kinase (Src family) and focal adhesion kinase (FAK). Other adhesins studied include ompA and ompB. Once adhered to the host cell, the agent gains entry through induced phagocytosis, “zipper mechanism”. Focal re-arrangement of actin cytoskeleton occurs below the site of attachment. Rapid escape into the cytoplasm occurs to avoid phagolysosomal fusion. This process is mediated by up-regulation of genes encoding for membranolytic activity like the tlyC (hemolysin C) and pld (phospholipase D) (Olano, 2005). The infection foci enlarge as the *Rickettsiae* spread from cell to cell forming a continuous network of infected endothelial cells in the microvasculature of the dermis, brain, lungs, liver and other visceral organs and tissues (Walker, 1995).

2.1.4.2 Clinical manifestations of rickettsial infections

The spectrum of rickettsial infections differs from mild to severe fatal illness. The incubation period is 2-14 and 2-16 days for *R. rickettsii* and *R. conorii* respectively, after the tick bite (Thorner *et al*., 1998; Raoult *et al*., 1986). For some rickettsial diseases like the Rocky Mountain spotted fever (RMSF), the case fatality rate for untreated patients can be as high as 20% (Dalton *et al*., 1995).

Generally, the initial signs and symptoms of rickettsial illnesses in humans include; fever, headache, myalgia, chills, sweats and cutaneous eruptions (eschars) (Walker, 1995; Raoult *et al*., 1997; Raoult and Roux, 1997; Zavala-Velazquez *et al*., 2000; Rutherford *et al*., 2004; Mahara, 2006; Whitman, 2007; Rovery *et al*., 2008). Other symptoms reported include; lymphadenopathy (Parola, 2005), neurological signs (Raoult *et al*., 1997), nausea, vomiting (Thorner *et al*., 1998) and epistaxis (Buchau *et al*., 2006). Neurologic signs noted in an epidemic typhus outbreak in Burundi included; mental confusion, seizures, delirium and coma (Raoult *et al*., 1997).

A similar clinical picture has been observed in the newly recognized tick-borne lymphadenopathy (TIBOLA), also known as *Dermacentor*-borne necrosis erythema and lymphadenopathy (DEBONEL). TIBOLA/DEBONEL is an emerging tick-borne rickettsiosis caused by *R. slovaca* and *R. raoultii.* An eschar, alopecia and cervical lymphadenopathy are the peculiar triad of clinical signs associated with the latter (Parola *et al*., 2009). *Rickettsia sibirica mongolotimonae* on the other hand, has been

associated with a specific lymphagitis that extends from the inoculation eschar to the draining lymph node (Parola, 2006).

Rash, though considered the classic distinguishing characteristic of some rickettsial diseases like the RMSF, may not appear until 3 days or more into the illness. The rash may not appear at all in a significantly great proportion of RMSF cases, or some patients may have a few cutaneous lesions that are only detectable after a meticulous examination (Walker, 1995). About 50% of the patients suffering from murine typhus may develop a non specific rash (Raoult and Roux, 1997).

After inoculation of the pathogen, attachment, spread and growth, the rash develops in several different stages. The damage in the tissue begins as erythematous macules measuring 1-5 mm that frequently start on the ankles and wrists. It hence spreads to trunk, palms and soles. The macules consist of areas of vasodilation that blanch on pressure and later become maculopapular because of extravasations of fluid from the damaged vessels. Severe illness leads to more pronounced endothelial destruction resulting in pinpoint areas of haemorrhage (Petechiae) at the centre of the maculopapule (Walker, 1995). The events described herein may occur in all affected tissues including the brain and lungs which may be catastrophic.

Increased permeability of vascular endothelium due to damage of the vascular lining may lead to changes in endothelial function. Pathophysiologic events include altered

blood chemistry and haematological profile. Serum biochemistry may reveal hyponatremia, thrombocytopenia, increased alkaline phosphatase, lactate dehydrogenase and serum glutaminoxalo acetic transaminase (Raoult *et al.*, 1986).

Involvement of pulmonary microvascular endothelium may result in non cardiogenic edema and interstitial pneumonia which are associated with adult respiratory distress. Congestive heart failure may develop particularly in older patients and is manifested as arrhythmias. Neurological signs occurring as a result of encephalitis may be manifested as lethargy, confusion, seizures; other neurological signs may vary from cranial nerve palsies, hemiphlegia and paraplegia to complete paralysis. These neurological signs together with adult respiratory distress are major determinants of a fatal outcome (Walker, 1995). Neurological signs have also been documented with *Rickettsia felis* infection (Zavala-Velezquez *et al*., 2000).

Increased vascular permeability may lead to acute renal failure as a result of reduced glomerular filtration rate and hypovolemia. Acute tubular necrosis occurs as sequelae to prerenal azotemia and severe hypotensive shock. Focal hepatic necrosis has been documented which leads to elevated hepatic enzyme concentration. There is involvement of skeletal muscles manifested as myalgia as a result of skeletal muscle cell necrosis. This is associated with elevated serum creatinine kinase concentration (Walker, 1995). Dogs suffering from Rocky Mountain spotted fever manifest clinical signs that are strikingly very similar to those portrayed by people. These include fever, cutaneous lesions characterized by hyperaemia, edema,

petechiae, eechymosis and necrosis and nervous signs (ataxia, hyperesthesia, vestibular disease, and seizures) (Gasser *et al.*, 2001; Paddock *et al.*, 2002; Mikszewski and Vite, 2005).

2.1.5 Diagnosis of Rickettsial Infections

Clinical diagnosis of rickettsial illnesses is difficult in the absence of an epidemic and/or a simple laboratory procedure to establish the aetiology of the infection. Lack of a simple laboratory test can significantly contribute to the lack of recognition of clinical disease in endemic areas during interepidemic seasons. In addition, some diagnostic tests may not yield results in the acute stage or may have low sensitivity (Walker, 1995).

The need for an effective and versatile test to identify rickettsial illness early in the course of illness cannot be overemphasized. Until tests that are useful in early diagnosis are available, clinicians must be aware of clinical and epidemiological diagnosis that should trigger initial empirical treatment with appropriate antibiotic to curb potential fatal cases.

2.1.5.1 Epidemiological diagnosis of rickettsial illnesses

In epidemiological diagnosis, the factors to consider include seasonal, geographic distribution and human activities associated with exposition to the vectors. Activities such as game hunting and travelling to endemic areas have been associated with increased risk of infection. In a study involving 940 persons, travelling to southern

Africa in the month of November through April, was associated with increased risk of infection with African tick-bite fever (ATBF) (Jensenius *et al*., 2003).While these activities are associated with increased risk of infection with *Rickettsiae*, infection can occur in all ages. In another study involving 280 international travellers, 87.6% SFG rickettsiosis, were acquired in sub-Saharan Africa and were associated with higher age, male gender, travel to southern Africa and travel for tourism (Jensenius *et al*., 2009). Rickettsial illnesses therefore, should be highly suspected in age brackets likely to be at risk, especially the age between 5-9 years and those over 60 years. These two age categories may be at risk of getting exposed to arthropod vectors while playing outdoors or when indulging in outdoor leisure activities.

Rickettsial infections have been reported in all continents, although some *Rickettsiae* are confined in some geographical regions. Rickettsial illnesses seem to follow a spatial and temporal trend dictated by arthropod vector activity (Parola *et al*., 2005; Letaief, 2006). Rocky Mountain spotted fever (RMSF), Mediterranean spotted fever (MSF) and African tick-bite fever (ATBF) are associated with *Dermacentor*, *Rhipicephalus* and *Amblyomma* tick species, respectively. In Africa, a geographical confinement of some rickettsial pathogens is evident, where Mediterranean spotted fever is endemic in northern Africa in Egypt, Tunisia, Libya, Morocco and Algeria (Letaief, 2006). Isolated cases of *R.conorii* infections have been reported in Somalia and in Kenya where it is referred to as Kenya tick typhus(Raoult *et al*., 1997). *Rickettsia africae* causes African tick-bite fever (ABTF) and is the main cause of fever and illness in travellers returning from sub-Saharan Africa. Two

*Amblyomma*species, *A. hebraeum* in southern Africa and *A. variegatum* elsewhere in sub-Saharan Africa, are recognized as the principal reservoir hosts and vectors for *R. africae*. Several foci of epidemic typhus have been detected in Africa where it is endemic in Ethiopia, and sporadic cases in Burundi, Rwanda and Uganda (WHO, 1993; Raoult *et al.,* 1998).

2.1.5.2 Clinical diagnosis of rickettsial illnesses

An early diagnosis of rickettsial illness remains a clinical dilemma and represents an often difficult challenge, even to physicians who are acquainted with the disease. Rickettsioses have nonspecific clinical manifestations, making them difficult to diagnose in a clinical setting (McDade, 1990). There are several challenges that may hinder early clinical diagnosis of rickettsial diseases, good examples of such are discounting a diagnosis when there is no history of a tick bite. Some rickettsial diseases like the Rocky Mountain spotted fever (RMSF) and Mediterranean spotted fever (MSF) are endemic in some regions of the world and have been associated with seasonality (Raoult *et al.*, 1986; Thorner *et al.*, 1998), a fact that has led some clinicians to use inappropriate geographical and seasonal exclusion (Masters *et al.*, 2003). It is speculated that if clinicians recognize the possibility of rickettsial infection when febrile patients seek medical attention after outdoor activities, fatalities associated with the diseases could be substantially reduced. This is applicable to those rickettsial diseases with seasonal and geographical trends (Walker, 1995).

Whilst skin rash together with other clinical signs (fever and history of tick bite) in the triad may be pathognomonic for spotted fevers, this (rash) may not appear until 3 days into the illness in RMSF (Thorner *et al*., 1998) or may be absent altogether in 10% of the cases reported. This may complicate diagnosis further as it would increase the time it takes to reach a conclusive diagnosis. Physicians must remain on high alert for spotted fevers in patients with febrile illnesses with or without history of tick bites and/or rash. Laboratory testing may be needed to support the clinical diagnosis, although antibodies may not be detectable until convalescence. In addition, immunohistologic methods and other advanced molecular techniques for detection of *Rickettsiae* may not be available in local clinics (Walker, 1995).

2.1.5.3 Laboratory diagnosis of rickettsial diseases

In order to achieve acute phase diagnosis of rickettsioses, tests that would yield results in a timely manner to enhance adequate patient management must be developed. Immunohistologic detection of *Rickettsiae* in biopsy specimens of the rash lesions and the centrifugation-shell vial assay may offer such advantages. These tests are sensitive and specific as long as they are done before antimicrobial therapy is initiated. The major shortcomings of these tests are that they are time consuming and require expertise. It is also difficult to apply them under routine conditions. Circulating endothelial cells (CEC) may also be applicable in early diagnosis of rickettsioses. Isolation of *Rickettsiae* using centrifugation-shell vial technique and detection of *Rickettsiae* in CEC can yield results in 2-3 days and 3 hours of receipt of specimen, respectively (la Scola and Raoult, 1996).

Although serological tests have been used for diagnosis of rickettsioses, they suffer from a number of shortcomings such as; patients presenting early into the illness may have antibody levels below detection threshold of any test. These tests should not be used to confirm diagnosis of acutely ill patients, because they only provide retrospective diagnosis.Serology cannot be reliably used for specific diagnosis due to cross-reactivity of antigens resulting in group-specific rather than species-specific antibody production. Serological diagnosis requires a rising antibody titre preferably a 4-fold or greater increase in antibodies between 2 serum specimens at least 1-2 weeks apart and tested in parallel is presumptive of recent or current infection. However, early treatment may abrogate antibody production (Kaplan *et al*., 1986).

2.1.5.3.1 Cultivation of *Rickettsiae*

Several isolation methods have been used including inoculation in guinea pigs (Pinter and Labruna, 2006) voles, rats, rat fleas (Raoult and Roux, 1997) and cell cultures. The cell lines used for cultures are mainly the Vero cells, HEL, L929, XTC-2, or MRC5 cells (Parola *et al*., 2005).

In vitro cultivation of *Rickettsiae* has been carried out successfully in shell vials containing Vero cells (Emereeva *et al*., 2001; Pinter and Labruna, 2006) and human embryonic lung fibroblasts (HEL) (La Scola and Raoult, 1996).Confluent shell vials are inoculated with heparinised or EDTA-anticoagulated whole blood, skin biopsy specimens or arthropod homogenate (Parola *et al*., 2005) and incubated for a couple of days. Monitoring of positive cultures is by direct immunofluorescence technique

(La Scola and Raoult 1996), or by Giminez staining (Pinter and Labruna, 2006). Cytopathic effects can be observed in the cell cultures when incubated at a certain temperature (Pinter and Labruna, 2006).

Cultures may be negative if the samples were collected from patients treated with antibiotics. Positive cultures have been reported in patients suffering from Mediterranean spotted fever in France (La Scola and Raoult, 1996). Although this technique is considered to be the most definitive method of diagnosis, a large proportion of the rickettsial isolates are lost during passaging (approximately one-third may be lost) (Parola *et al.*, 2005), this may lead to under diagnosis.

2.1.5.3.2 Serological diagnosis of rickettsial diseases

Historically, serological diagnosis of rickettsial illness was performed using the Weil-Felix test which is based on rickettsial cross reactivity with *Proteus* OX-2 and OX-19 antigen (Parola *et al.*, 2005). There are several serological assays that have been used in recent years which include, indirect fluorescence antibody (IFA) test, enzyme linked immunosorbent assays (ELISA), indirect hemaglutination test, complement fixation test and latex agglutination test (Kaplan *et al*., 1986; Walker 1995). In a study, sensitivities of various serological tests were evaluated from onset of clinical signs (7 days) in 1,779 laboratory confirmed cases of Rocky Mountain spotted fever. Among the serological tests evaluated, IFA was the most sensitive (94%),while Weil Felix test was least sensitive (47%) (Kaplan *et al*., 1986). Indirect fluorescence antibody test is the reference method for diagnosis of rickettsioses (La

Scola and Raoult, 1997) and isalso the most commonly used (Walker, 1995; Thorner *et al.*, 1998, Hechemy *et al.*, 1979). The Weil-Felix test is incapable of detecting patients with Brill-Zinsser disease and *R. akari* infections (La Scola and Raoult, 1997).

Antibody tests may fail to identify rickettsioses early enough to affect the management of individual patients. Available serologic techniques vary considerably in their sensitivity and specificity. ELISAs are very sensitive (90%) and specific (100%) (Do *et al.*, 2009), but the general unavailability of specific diagnostic antigens reduces their specificity of this and other serologic techniques (McDade, 1991).

2.1.5.3.3 Histochemical and immunohistochemical techniques of detecting Rickettsial pathogens

Tissue specimens can be stained using Giemsa or Giminez to visualize *Rickettsiae*. Immunohistologic diagnosis based on demonstration of *Rickettsiae* in biopsy specimens of lesions is among the tests that can provide diagnostic results in a timely manner. Rickettsial organisms can be detected in the vascular endothelium using direct immunofluorescence or immunoperoxidase staining of skin biopsy specimens. This technique can also be applied on post mortem formalin fixed tissues (Rutherford *et al.*, 2004). Although this test has a high sensitivity (70-90 %), the antemortem applicability of this test is limited to patients who develop a skin rash (Thorner *et al.*, 1998). Direct immunofluorescence of skin biopsy specimens have

been proposed for diagnosis of RMSF and MSF. Availability of this test is limited to few hospitals and diagnostic laboratories (Walker, 1995). Similarly just like other serological tests, interpretation of results of immunohistochemical methods must be done with caution because they are most often group specific but not species specific (Parola *et al*., 2005).

2.1.5.3.4Detection of *Rickettsiae* in circulating endothelial cells (CECs)

Circulating endothelial cells (CECs) technique offers a solution in the acute stage diagnosis of rickettsioses but the sensitivity of this test is limited by the amount of circulating endothelial cells. Interestingly, this test is very useful even in situations where specific antibiotic therapy has been initiated or with concurrent serum antibodies (La Scola and Raoult, 1996). Circulating endothelial cells are isolated from blood by use of immunomagnetic beads detected using indirect immunofluorescence technique. This test has been used in the diagnosis of Mediterranean spotted fever with a sensitivity of 50 % being recorded (La Scola and Raoult, 1996). The low sensitivity may lead to under diagnosis of acute cases.

2.1.5.3.5 Molecular methods for detection of *Rickettsiae*

Polymerase chain reaction (PCR) relies on the detection of rickettsial DNA through amplification of specific sequences of conserved genes. Genes that have been used successfully to diagnose acute cases include the outer membrane proteins A and B

(*ompA, ompB*), Citrate Synthase (*gltA*), gene D (Parola *et al*., 2005) and genes encoding 16S rRNA and 17-kDaproteins.

The conventional PCR may suffer from low sensitivity (Walker, 1995) but ongoing research has enabled improvement of this technique. Suicide PCR which is a nested PCR (Raoult *et al*., 2000) is considered to be more specific and sensitive than culture and conventional PCR. The sample arrays used include blood collected in EDTA, skin biopsy samples and arthropod tissues (Ndip *et al*., 2004a, Whitman *et al*., 2007). The leukocytic buffy coat can also be utilized (Raoult *et al*., 1997).

The development of pathogen-specific fluorescent probes that can be used with quantitative PCR (qPCR) has improved diagnosis of rickettsial illnesses. Real time PCR has greater sensitivity (when compared to conventional PCR), it is faster, can be automated for high throughput, can allow for multiplexing and quantification of nucleic acids in the sample (Jiang *et al*., 2004).

Various DNA-based methods have been developed for species identification of *Rickettsiae*. Restriction fragment length polymorphism (RFLP) which is based on polymerase chain reaction and digestion of DNA with specific restriction endonucleases and agarose gel electrophoresis has been used in identification of *Rickettsia* species (Regnery *et al*., 1985; Roux *et al*., 1996).

Multi-locus sequence typing involves PCR amplification followed by DNA sequencing. The method characterizes isolates of bacterial species using DNA sequences of internal fragments of multiple housekeeping genes; a minimum of 5 genes is recommended (Fournier and Raoult, 2009). It directly measures the DNA sequence variation in a set of genes and characterizes strains by their unique allelic profiles (Vitorino *et al*., 2007; Fournier and Raoult, 2009).

Other high-resolution genotyping methods available that allow identification of rickettsial isolates at the species level are for example the multi-spacer typing (MST) (Fournier and Raoult, 2007). This method is capable of further differentiation of strains within some species. It is based on existence of short sequence repeats (SSRs) and variable number tandem repeats (VNTRs). In eukaryotic genomes the repetitive DNA is primarily located in extragenic regions. Short tandemly repeated sequences occur in several to thousands of copies dispersed through the genome of many eukaryotes. These sequences elements shows hyper variability among individual persons and genetic mapping can be used to prepare DNA fingerprints that are specific for an individual. These sequences were initially defined as mini and microsatellite DNA consisting of short sequence repeats (SSRs) or short tandem repeats (STRs). They have now been given a new term; variable number tandem repeats (VNTRs) (Van Belkum *et al*., 1998). Variable number tandem repeats are repeated elements present at a single genomic locus and show inter individual length variability. Short sequence repeats have been reported in prokaryotes (Goffeau *et al*., 1996).

2.1.6 Treatment and Prevention of rickettsial illnesses

Doxycycline has been recommended as a drug of choice for rickettsial infection except if the patient is pregnant or is allergic to the drug. Doxycycline is also contraindicated in the treatment of rickettsial illnesses in patients less than 9 years because of its potential to stain teeth permanently. Although tetracycline has been shown to cause a dose dependent staining of teeth in young children (Grossman *et al.*, 1971), doxycycline may not have this permanent discolouration as it binds less strongly to calcium as compared to tetracycline (Abramson and Givner, 1990). Tetracyclines may endanger the life of developing foetus.

Although chloramphenical is not as effective as the doxycycline, it has been recommended for children under 9 years and in pregnant women. It may be worthwhile to use Doxycycline for potentially fatal RMSF illness in children less than 9 years (Walker, 1995).Fluoroquinolones have been shown to have anti-rickettsial activity against the spotted fever group *Rickettsiae* in cell cultures, in dogs experimentally infected with *R. rickettsii* and in human patients with *R. conorii.* There are however no reports on their use in treatment of RMSF (Walker, 1995)

The recommended treatment regimens include; oral doxycycline at 200 mg/d or intravenously in two divided doses for adults. Oral tetracycline may be used at 25-50 mg/kg/d in four divided doses for adults and Chloramphenical at 50-75 mg/kg/d in four divided doses (Raoult and Drancourt, 1991; Walker, 1995; Kumar, 2010).

Other supportive treatment that may be required in severely ill patients include management at the intensive care unit with administration of intravenous fluids, intubation and mechanical ventilation, hemodialysis, antisiezure medication, packed erythrocyte and platelet transfusions.

Prevention of tick borne spotted fever is mainly by avoidance of the inoculation of *Rickettsiae* by ticks. This can be achieved by use of tick repellants, routine careful inspection of the body and keeping of tick free pets. Immediate and careful tick removal by use of forceps to avoid contamination of cutaneous lesion, mucous membrane and conjunctivae by tick fluid is highly recommended. There is no vaccine available for prevention of rickettsial diseases (Walker, 1995; Kumar, 2010). Improvement of hygienic conditions and practices, use of repellents, rodent control, and field sanitation has controlled rickettsial outbreaks and other vector-borne disease (Blanco and Oteo, 2006).

CHAPTER THREE

3.0 MATERIAL AND METHODS

3.1 STUDY AREA

The study was conducted in Asembo area, located in Rarieda (formerly Bondo) district of western Kenya (Figure 3.1). The rural site bordering Lake Victoria is part of a human demographic surveillance system (HDSS), run by the Kenya Medical Research Institute (KEMRI) and the Centers for Disease Control and Prevention (CDC) since 2001. Asembo area is approximately 225 square kilometres and is culturally homogenous, with 95% of people being ethnically Luo. It has a bimodal rainfall pattern with long rains occurring from March to May and short rains in September to November. The primary economic livelihood is subsistence farming and fishing. The community live in dispersed settlements. Houses are made of mud, cement or brick, with roof of iron sheet or thatch (Bigogo *et al.*, 2010).

Within the Asembo HDSS area, CDC in collaboration with KEMRI initiated population-based infectious disease surveillance (PBIDS) which has been in operation since 2005. The PBIDS focuses on four infectious disease syndromes, namely acute respiratory infections, diarrhoeal diseases, febrile illnesses and jaundice. The PBIDS catchment population includes 33 villages, in which 50% or more of each village's population must be within a five kilometre radius of Lwak hospital, the designated referral health facility (Bigogo *et al.*, 2010).

The syndromic study involves biweekly visits to all recruited households during which information is collected on symptoms and signs of recent or current illnesses for all household members. Participants showing recent or current signs and symptoms of pneumonia, diarrhoea, jaundice and/or fever are referred to the designated clinic (Lwak Mission hospital) for clinical evaluation, sample collection and treatment.

3.1.1 Human Population

The human population used for this study were specimens collected using an on-going study protocol, SSC No. 932 on, "Active population-based study of major infectious disease syndromes in Western Kenya and Nairobi". On June 1, 2007, the surveillance population included 25,489 persons of whom 3, 576 were <5 years of age living in approximately 6,000 households. All study participants must have resided permanently for 4 calendar months and have been registered into KEMRI/CDC human demographic surveillance study.The population undertake agricultural production under a traditional mixed crop-livestock system and fishing, and 95% belong to the Luo ethnic community (Feikin *et al.*, 2011).

3.1.2 Animal population

Animal population comprised of domestic ruminants, dogs, cats and peri-domestic small mammals (rodents and insectivores) living within the 33 villages of the

PBIDS. The study specimens were collected by an ongoing study, SSC No. 1191, "Integrated epidemiological study of zoonotic pathogens in linked human and animal population in rural and urban Kenya". Three hundred livestock owning compounds (LOC) were randomly selected and animalssampled. A LOC was defined as ownership of one or more of the following species; cattle, sheep, goats and chicken. According to the HDSS data (2008), there were 48.4% of PBIDS compounds that owned at least one goat, 48.9% (cattle), 17.5% (sheep) and 86.3% poultry. Within the LOCs, the mean herd size was 5.1 goats, 5.4 cattle, 4.7 sheep, 12.7 poultry and 149 compounds owning at least one dog (KEMRI/CDC, Unpublished data). There was no data on cat ownership.

3.1.3 Arthropod population

Arthropods used for this study included ticks and fleas that had been collected by an on-going study on,"Integrated epidemiological study of zoonotic pathogens in linked human and animal population in rural and urban Kenya,SSC No. 1191". All *Amblyomma variegatum* ticks from cattle and all ticks collected from the dogs were surveyed for *Rickettsia*. The inclusion criterion of ticks was based on the fact that *A. variegatum* is the natural reservoir and host for *R africae* and its preferred host is the cattle. While *Rhipicephalus sanguineus,*found predominantly in dogs, is the natural host for *Rickettsia conorii,* other tick species found in dogs are documented to contain *Rickettsiae.*Flea specimens used for this study included all fleas obtained from dogs, cats, peri-domestic small mammals andfleas trapped within the human

dwellings. Although mites and lice are documented vectors of *Rickettsiae*, these were not collected during the two on-going studies (SSC # 932 and 1191).

3.1.3 Ethical Approval

The study was approved by the scientific steering committee (SSC, Appendix 1), National Ethical Review Board (ERC approval, Appendix 2)and the Animal care and review committee (ACUC, Appendix 3).

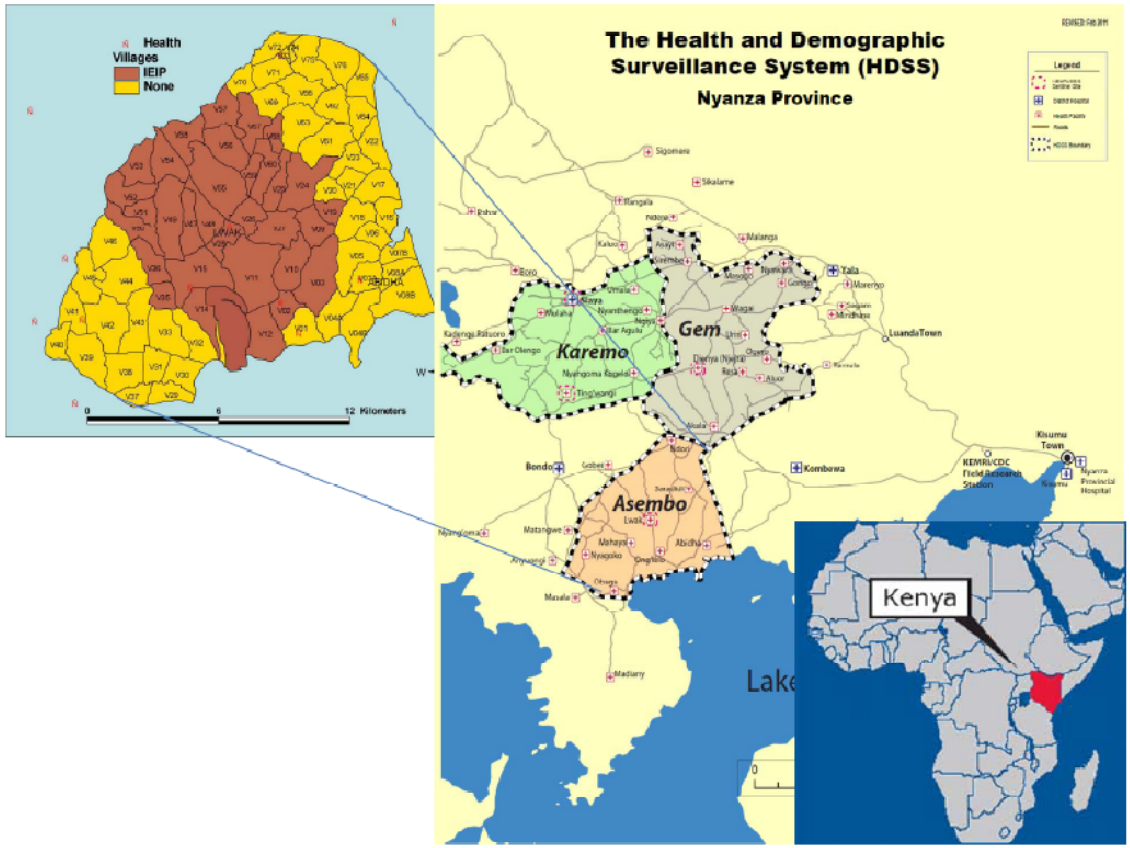


Figure 3.1 Locations of villages in Asembo area of western Kenya where the study was conducted (Source: KEMRI/CDC, Unpublished data)

3.2. Assessment of previous exposure to *Rickettsia* in humans

To determine whether the population in Asembo was exposed to *Rickettsiae,* a serological study was conducted. Specimens used for the sero-surveillance were collected between 30th November 2008 to 1st January 2007. Samples for testing were randomly selected using MS excel software from an existing biobank of 1318 stored sera. The required number of samples to elucidate previous exposure in the study area was estimated by assuming a sero-prevalence of 50% and a desired precision of 5% with 95% confidence intervals. The sample size was determined using the method described by Martin *et al*. (1987).

$$N = \frac{Z\alpha_{/2}{}^2 PQ}{L^2}$$

Where: N is the calculated sample size

L is the precision =5%

Z (z-score) standard normal deviate at 95% C.I is 1.96

P estimated prevalence =50%

Q is (1-P)

A total of 385 serum samples were selected for testing for IgG antibodies against *Rickettsiae*.

3.2.1 Indirect fluorescenceantibody (IFA) test for detection of IgG antibodies in human sera

Stored sera from an ongoing syndromic surveillance study were randomly selected. Using sample number as an identifier, Microsoft Excel function RAND () was used to generate random numbers between 0 and 1. The random numbers were then sorted by ascending order from 0 to 1; the first 385 serum samples were selected. Sera from patients who presented to Lwak Mission Hospital in 2007/2008 were tested by indirect fluorescence antibody (IFA) test kit (Fuller Laboratories, CA, USA) for IgG antibodies against SFG and TG *Rickettsiae* at a dilution of 1:128. Procedures were carried out as described by the manufacturer (Appendix 4). Sera were diluted with phosphate buffered saline (PBS). Ten microlitres of diluted sera were applied onto each well of 12-well antigen slides, and incubated in a humid chamber at 37 ºC for 30 minutes. The slides were rinsed three times with PBS and washed in a jar containing PBS for 10 minutes. The slides were allowed to air dry. Ten microlitres of fluorescein isothiocyanate (FITC) conjugated goat anti-human IgG were applied onto each well and incubated in a humid chamber at 37 ºC for 30 minutes. After incubation the slides were washed as before. The slides were mounted with 90% glycerin in PBS (mounting media). A cover slip was applied and examined under a fluorescence microscope (Nikon, Inc., Garden City, NY) at 400X magnification.

3.3 Optimization of blood clot volume for extraction of DNAfor qPCR assays

Approximately 500µl of human blood clot from a *Rickettsia* negative individualwas used in this experiment. To help ensure uniform replicates, blood from a single individual was resuspended in an equal volume of normal saline (500µl) and mixed thoroughly using a Pasteur pipette. The mixture was then aliquoted into different volumes which varied from 100µl, 200µl, 300µl and 400µl; adopted from the works of Tan *et al*. (2008). A known/ quantified plasmid DNA (100 ng/µl) of the tick borne spotted fever group (TBSFG) outer membrane protein B (*ompB*) donated by Naval Medical Research Center (NMRC), USA was used for optimization experiment. Five microlitres of the plasmid DNA was spiked into each volume and mixed by pulse vortexing. DNA was extracted from each volume using QIAamp®DNA Mini Kit (Qiagen, Valencia, CA) (Appendix 5) with the volumes of proteinase K, AL buffer and ethanol being adjusted depending on the volume of clot used. The ratio of proteinase K to sample was maintained at 1:10 while that of AL buffer and ethanol to sample at 1:2.Elution was done using a 100µl of buffer AE.Using 5µl as the template volume, a TBSFG *ompB* real time PCR assay was conducted on a 7500 Fast Real Time PCR System (Applied Biosystems) as described by Blair *et al*. (2006).

3.4 Assessment of acute *Rickettsia* infections in humans

To compare the occurrence of acute *Rickettsia* infections in febrile patients and asymptomatic individuals in the study area, blood samples collected from febrile patients and asymptomatic individuals from 1st December 2008, through to 28th of February 2010 were used. This date, 1st of Dec 2008 concided with the period when

PBIDS study (SSC No. 932) started preserving blood clots for future studies, and hence the blood clots were available for *Rickettsia* testing. Approximately 3ml of blood was collected from each febrile patient and asymptomatic individual by venipuncture using sterile technique. Serum sample were separated and the remaining blood clot was preserved and later used for testing for *Rickettsia* by PCR.

Febrile patients were defined as all patients presenting to Lwak Mission Hospital with documented fever (≥ 38 ºC) and from whom a blood sample was taken. Blood samples were collected from the first two people <5 years old and the first two people ≥5 years old with documented fever (≥38°C) presenting as outpatients to the Lwak Mission Hospital each day.

Asymptomatic individuals were defined as (patients or accompanying individuals) with no history (in the preceding two weeks) and no current episode of fever, respiratory illness, runny nose (greater than normal), cough, sore throat, earache, diarrhoea, vomiting, or jaundice. Samples from convalescent patients were excluded.

3.4.1 Extraction of DNA from human blood clots

Blood clots from febrile patients and asymptomatic individuals were extracted using QIAamp®DNA Mini Kit (Qiagen, Valencia, CA) (Appendix 5). Three hundred microlitres (300 µl) were first treated with 30 µl of proteinase K and lysed with 300 µl AL buffer. The mixture was incubated in a thermomixer at 56^{o} C for 10 minutes. An equal volume (300 µl) of absolute ethanol (Analytical grade) was added. This

was followed by 2 washes with 500 µl AW1 and AW2 wash buffers and a final elution with 100 buffer AE. The resultant DNA was stored at minus 80^{o}C.

3.4.2 Quantitative Polymerase reaction (qPCR) assays

A quantitative PCR (qPCR) targeting a 74 base pair (bp) fragment of *RickettsiagltA* gene of the citrate synthase (referred to as *gltA*) (Stenos *et al*., 2005) was used to screen human blood samples for *Rickettsia*. This pan-rickettsial gene was selected to detect members of the genus *Rickettsia*. A group specific assay was selected to test samples for presence of tick borne spotted fever group *Rickettsia* (TBSFG). This assay amplifies and detects a 128 bp fragment of the outer membrane protein B (*ompB*) (referred to as TBSFG *ompB*) and was used as described by Blair *et al.*(2004). Each PCR was conducted in a total volume of 25 µl of PCR reaction mix consisting of 5µl of DNA template, 12.5 µl of 2X Taqman Universal PCR master mix (Applied Biosystems, Roche, Branchburg, New Jersey USA), 0.5 µM of each primer and 0.25 µM probe of *Rickettsia* citrate synthase.The PCR was incubated at 94^{o}C for 10 minutes, followed by 50 cycles of amplification at 94^{o}C for 15 seconds and 60^{o}C for 1 minute on a 7500 Fast Real Time PCR System (Applied Biosystems).

To confirm findings and evaluate the performance of the *gltA* assay, a total of 100 samples previously tested with *gltA* and comprising of those with low cycle threshold (ct) values (less than 40), high ct (greater than 40) and negative ones (undetermined ct) were selected and tested at the Naval Medical Research Center (NMRC) with a second genus specific assay, Rick17b, that amplifies a 115 bp fragment of the 17kDa

surface protein (Jiang *et al*., 2004). An *R. felis* specific qPCR that detects a 129 bp fragment of the outer membrane protein B gene was also used as described by Henry *et al*. (2007). A total of 25 samples with lowest cycle threshold (ct≤37) were selected for sequencing.

Briefly, a final PCR reaction of 25 µl was prepared consisting of 2X PCR Supermix-UDG (Invitrogen, Life Technologies, California. USA) containing dNTP's, Uracil DNA glycosylase (UDG), Platinum® Taq DNA polymerase, Tris-HCl, KCl, $MgCl_2$ and stabilizers), 5mM of magnesium chloride ($MgCl_2$), 0.5µM of forward and reverse primers, 0.2µM of fluorescent labelled probe (Biosearch Technologies), sterile water, and 3µl of template DNA (or sterile water for negative controls). *Rickettsia* DNA was amplified on a Smart Cycler (Cepheid, Sunnyvale, CA USA) with the following cycling conditions: first hold at 50 °C for 2 minutes, second hold at 95 °C for 2 minutes, denaturation at 95 °C for 15 second and annealing and extension at 60 °C for 30 seconds.

An overview of the sample selection and testing is given in figure 3.2 below. The gene sequences for the assays are described in Table 3.1.

Table 3.1: Oligonucleotide primer sequences for quantitative PCR (Sigma Genosys, The Woodlands TX, USA)

Name used in text	Applied in samples	Gene targeted	Group detected	Primer & probe	Sequence (5`→3`)	Reference
Rickettsia gltA	Human Animal Tick Fleas	*gltA*	Genus	CS-1126F	TCG CAA ATG TTC ACG GTA CTT T	Stenos *et al*., 2005
				CS-1199R	TCG TGC ATT TCT TTC CAT TGT G	
				CS-1149P	FAM-TGC AAT AGC AAG AAC CGT AGG CTG GAT G-BHQ-1	
Rickettsia Rick17b	Human Animal Tick Fleas	*17kDa*	Genus	R17k128F	GGGCGGTATGAAYAAACAAG	Jiang *et al*., 2004
				R17k238R	CCTACACCTACTCCVACAAG	
				R17k202TaqP	FAM-CCGAATTGAGAACCAAGTAATGC-TAMRA	
TBSFG *ompB*	Human Animal Tick	*ompB*	TBSFG	Rr1595F	GCCGGAGTTGTCCAATTATCA	Blair *et al*., 2004
				Rr1722R	CCGCCGACAAGAGCAGTTT	
				Rr1654PB	FAM-CCGCGCCGGCATT"T"CCTAAACGTAACTCGGCAGCGCGG-BHQ-1	
R. felis ompB	Human Animal Flea	*ompB*	*R. felis*	Rf1396F	ACCCAGAACTCGAACTTTGGTG	Henry *et al*., 2007
				Rf1524R	CACACCCGCAGTATTACCGTT	
				Rf1448BP	FAM-CGCGACTTACAGTTCCTGATACTAAGGTTCTTACAGGTCGCG-BHQ-1	
R. africae	Tick	*ompB*	*R. africae*	Raf1797F	TTGGAGCTAATAATAAAACTCTTGGAC	Richards *et al*., Unpublished
				Raf1915R	GAATTGTACTGCACCGTTATTTCC	
				Raf1879P	FAM-CGCGATGTTAATAGCAACATCACCGCCACTATCGCG-BHQ-1	
R. typhi	Flea	*ompB*	*R. typhi*	Rt557F	TGGTATTACTGCTCAACAAGCT	Jiang *et al*., 2006
				Rt678R	CAGTAAAGTCTATTGATCCTACACC	
				Rt640BP	FAM-CGCGATCGTTAATAGCAGCACCAGCATTATCGCG-BHQ-1	

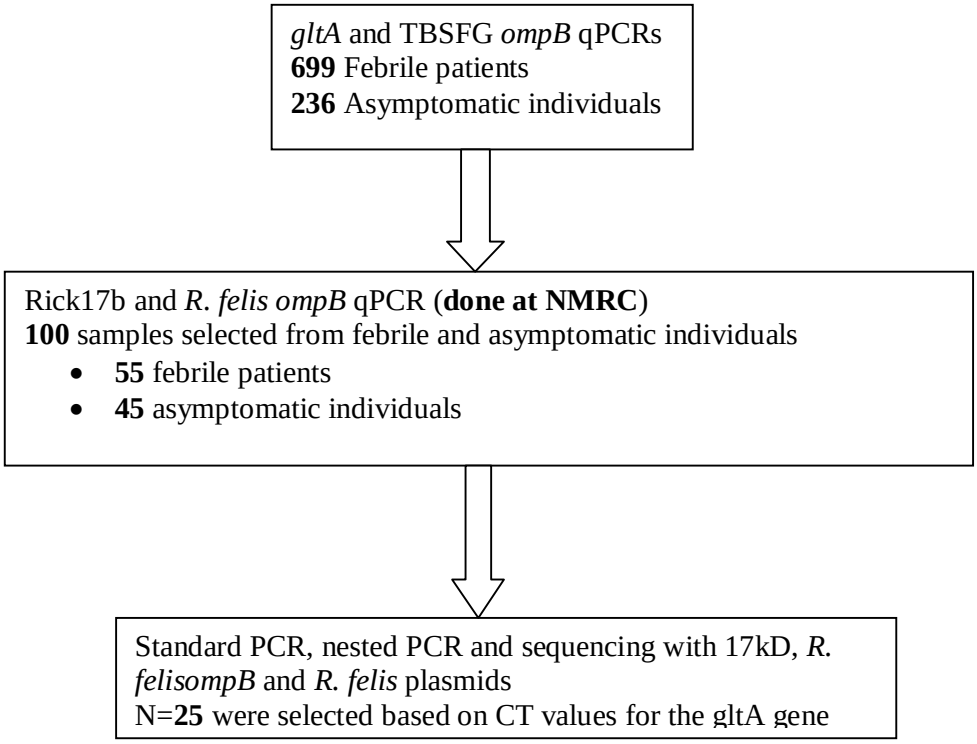


Figure 3.2. Sample selection and testing for acute rickettsial infection

3.4.3 Characterization of *Rickettsia* detected in human samples using 17kDa, *R. felis ompB* and plasmid genes

Further characterization of *Rickettsia* was done in a reference laboratory, Naval Medical Research Center (NMRC) (Silver Spring, USA). This involved standard PCR followed by a nested PCR. Three genes were used, namely; 17kDa (Richards and others NMRC, Unpublished), *R. felis* ompB (Roux and Raoult, 2000; Henry *et al.*, 2007) and *R. felis* plasmid targeting the short and the long fragment of the *R. felis* plasmids (Ogata *et al*., 2005).

Two microlitres of sample DNA preparation was used for standard PCR and 2 µl of PCR product were used in the nested PCR in a total of 25 µl total reaction mix containing 0.3 µM of each primer, 1X Platinum® PCR Supermix High Fidelity (Life Technologies, Grand Island, NY). Each PCR was performed in TGradient Thermocycler (Whatman Biometra Gottingen, Germany). It was incubated for 94 °C, for 1 minute followed by 40 (35 for nested PCR) cycles of denaturation at 94 °C for 30 seconds, annealing at 59 °C for 30 seconds and elongation at 68 °C for 1 minute. Final elongation was done at 72 °C for 7 minutes. No positive controls were used in these PCR and nested PCR procedures to decrease chances of contamination. A negative control was used with each reaction (molecular biology grade water, GIBCO). The PCR and nested PCR cycling conditions for 17kDa, *R. felis* ompB and *R. felis* plasmids are summarized in Table 3.2.

The PCR products were visualized using ethidium bromide (GIBCO BRL Life Technologies, Inc., Gaithersburg, MD, USA) on 1% agarose gel (Sigma, St. Louis)

in 1X TBE buffer (44.5 mM Tris-HCl, 44.5mM boric acid, 1mM EDTA; pH 8: (Appendix 4) for 45 minutes at 150 V after electrophoresis (Sambrook *et al*., 1989). The size of the amplified product was determined by comparison with Track It ^{TM} 1 kb molecular marker (Invitrogen).

Table 3.2. Primers for standard PCR, nested PCR and their thermo-cycling conditions (Sigma Genosys, The Woodlands TX, USA)

Gene target	Primer name	Product size	Step	Standard PCR			Nested PCR		
				Temp	Time	Cycles	Temp	Time	Cycles
17kDa Richards *et al*., Unpublished	R17k31F***^**	440bp*	1st hold	94 °C	1 minute		94 °C	1 minute	
	R17k469R*****		Denaturation	94 °C	30seconds	40	94 °C	30seconds	35
	R17k2608R^	434bp^	Annealing	57 °C	30seconds		59 °C	30seconds	
			Extension	68 °C	30seconds		68 C	30seconds	
			Final extension	72 °C	7 minutes	1	72 C	7 minutes	1
			Final hold	4 °C	∞		4 °C	∞	
ompB Raoult and Roux, 2000; Henry *et al*., 2007	RompB607F***^**	599bp*^	1st hold	94 °C	1 minute		94 °C	1 minute	
	Rf1524R***^**		Denaturation	94 °C	30seconds	40	94 °C	30seconds	35
			Annealing	57 °C	30seconds		59 °C	30seconds	
			Extension	68 °C	1 minute		68 C	30seconds	
			Final extension	72 °C	7 minutes	1	72 C	7 minutes	1
			Final hold	4 °C	∞		4 °C	∞	
R. felis plasmids (pRF & pRFδ) Ogata *et al*., 2005	pRFa***^**	1100bp	1st hold	94 °C	1 minute		94 °C	1 minute	
	pRFc***^**	1342bp	Denaturation	94 °C	30seconds	40	94 °C	30seconds	35
	pRFd*****	251bp	Annealing	52 °C	30seconds		52 °C	30seconds	
	pRFg^	429bp	Extension	68 °C	30seconds		68 C	30seconds	
			Final extension	72 °C	7 minutes	1	72 C	7 minutes	1
			Final hold	4 °C	∞		4 °C	∞	

Key:

* primer for PCR ^primers for nested PCR *^ for both PCR and nested PCR

3.4.4 DNA fragment recovery and purification

Nested PCR products which were the templates for downstream sequencing reactions were purified to get rid of leftover primers, unincorporated nucleotides and non-specific products. Nested PCR products from *17kDa, R. felis ompB, pRF* and *pRFδ* plasmid were purified using QIAquick PCR purification kit (Qiagen) (Appendix 7). A 100 µl of Buffer PBI was added to 20 µl of the nested PCR products and mixed by pulse vortexing. The resulting yellow mixture was applied into a spin column and centrifuged for one minute at 10,000 × *g* (or 13,000 revolutions per minute). The flow-through was discarded and a new collection tube was replaced. Seven hundred and fifty (750 µl) microlitre of buffer PE were added into the column and centrifuged for one minute at 10,000 × *g*. The flow-through was discarded and a new 2ml collection tube replaced. To ensure complete removal of buffer PE, the column was centrifuged for an additional one minute at 10,000 × *g*. The column was then placed in a clean 1.5ml centrifuge tube and 20 µl of buffer EB was added, followed by incubation for one minute at room temperature (25 °C). Purified PCR products were obtained by centrifuging for one minute at 10,000 × *g*.

3.4.5 Sequencing PCR

A Big Dye Terminator v 3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystem, Foster City, CA, USA) was used in subsequent sequencing reactions according to the manufacturer's instructions. One microlitre (1 µl) of purified PCR products was used for sequencing PCR in a total volume of 8 µl total reaction mix containing 0.3 µM of primer (either forward or reverse primer sequenced separately), one microlitre of Big dye terminator (Applied Biosystems), 1× big dye terminator

buffer. Each sequencing PCR was performed in TGradient Thermocycler (Whatman Biometra Gottingen, Germany) in 25 cycles of denaturation at 96 ℃ for 10 seconds, extension at 50 ℃ for five seconds, annealing at 60 ℃ for two minutes.

3.4.6 Purification of Big Dye terminator and sequencing

Purification of sequenced products was carried out to recover DNA free of excess nucleotides, excess dideoxynucleotides and buffer salts. Sequenced products were purified by using Dye Ex 2.0 spin kit (QIAGEN) (Appendix 8). Dye Ex spin columns were vortexed to re-suspend the resin. The cap was loosened, the bottom snapped off and the column was applied in a 2ml collection tube. This was spun for three minutes at 750 × *g*. The column were then transferred in a clean 1.5ml centrifuge tube and the sequence PCR products (8 µl) were applied in to the gel bed, spun for three minutes at 750 × *g*. The products were then dried using vacuum centrifuge, centrivap concentrator (Labconco) at 60 ℃ for 15 minutes. The dried products were resuspended using a 10 µl of formamide (Applied Biosystems) and loaded in to a 96-well PCR plate (Applied Biosystem) for sequencing. The 96-well PCR plate was heated at 95 ℃ for 10 minutes before loading to the sequencer.

Sequencing was done in ABI Prism 3130xl Genetic Analyzer (Applied Biosystem). Genomic sequencing was done using the dideoxy-synthesis termination method (Sanger*et al*., 1977). The primers used for sequencing were the same as the one for PCR. At least two reactions were done for each strand of DNA. Sequences from the 17kDa antigen gene, ompB and plasmid genes were assembled with Vector NTI advanced 11 software (Invitrogen), and BLAST searches were managed on the

National Center for Biotechnology Information website (http://blast.ncbi.nlm.hih.gov).

3.5 Surveillance of rickettsioses in domestic animals and peri-domestic small mammals

As part of a study on zoonotic diseases surveillance in Asembo site, 300 livestock owning compounds (LOCs) were selected from 4,528 LOCs in the 33 population-based infectious disease surveillance (PBIDS) villages. The sampling frame of LOCs was compiled from livestock census data collected by Human demographic surveillance system (HDSS). Domestic animals were sampled from January through May, 2009; this was the first cross sectional sampling for KEMRI/CDC surveillance of zoonotic diseases in Asembo (SSC No. 1191). Where present, up to a maximum of three animals per species were randomly selected from each LOC.Domestic animals from which samples were collected included cattle (*Bos taurus*), goat (*Capra hircus*), sheep (*Ovis aries*), dog (*Canis familiaris*) and cat (*Felis catus*). Samples collected from domestic animals included the buffy coats, sera and ticks. Fleas were collected from dogs, cats, rodents and from the households.

Additionally, the peri-domestic small mammal sampling was conducted in collaboration with the National Museums of Kenya using baited Sherman live traps over the period, July 30 to August 7, 2009. Fifty compounds were randomly selected from the 300 LOCs. Five traps were placed within dwellings, in outbuildings and outdoors in each compound for four consecutive nights. Trap sites were marked with flagging tape, and the GPS locations of key points were recorded. Captured animals were euthanized using an overdose of halothane inhalant anesthesia (Rhodia Limited, Avonmouth, Bristol, BS119YF, UK). Fleas were collected fromthese small animals

by combing. Spleen samples were collected from every individual using sterile technique.

3.5.1 DNA Extraction and qPCR testing of animal buffy coat, serumand rodent spleen samples

All samples collected from domestic ruminants, dogs, cats and peri-domestic animals were used for DNA extraction. Nucleic acid (DNA) was extracted using QIAamp®DNA Mini Kit (Qiagen, Valencia, CA) as described by the manufacturers. Briefly,a 200 µl sample was first treated with 20 µl of Proteinase K and lysed with 200 µl AL buffer. The mixture was incubated in a thermomixer at 56^{o} C for 10 minutes. An equal volume (200 µl) of absolute ethanol (Analytical grade) was added. This was followed by a two series of washes using wash buffers and a final elution with 100 buffer AE (Appendix 9).

To extract DNA from spleen samples, a 5mm steel bead was placed in a 2 ml microtube and pre-cooled for 15 minutes in dry ice. Approximately 25g of rodent spleen tissue was placed in the 2 ml microtube and incubated for another 15 minutes in dry ice. This was followed by 2 minutes incubation at room temperature and then mechanical disruption using a bead mill (Qiagen tissue lyser ^{LT}) at 30 Hz for 1 minute (Appendix 10). Genomic DNA was extracted from the mechanically disrupted samples using QIAamp DNA mini kit (QIAGEN, Valencia, CA, USA). The DNA purification involved lysing of spleen tissues using 20 µl of Proteinase K and 180 µl ATL buffer and incubating the mixture in a thermomixer at 56^{o} C for 3 hours. Buffer

200 µl AL was added. The mixture was incubated in a thermomixer at 70^{o} C for 10 minutes. The Resultant DNA was stored at -80 ^{o}C until use.

Deoxyribonucleic acid (DNA) extracted from buffy coat, serum and spleen samples was used for real time PCRusing *gltA* gene (Stenos *et al*., 2005) and Rick17b as described by Jiang *et al*. (2004). Standard and nested PCR of the 17kDa, *ompA* and *ompB* genes was done as described in table 3.2. Sequencing was done using the method described by Sangers *et al*. (1977). Sequences were assembled with Vector NTI advanced 11 software (Invitrogen), and BLAST searches were managed on the National Center for Biotechnology Information (NCBI) website (http://blast.ncbi.nlm.hih.gov).

3.6 Study of *Rickettsiae* in arthropods

To investigate the prevalence and species of *Rickettsia* circulating in arthropods vectors in the study area, selected vectors namely; ticks and fleas were tested.

3.6.1 Identification of ticks and fleas

Ticks collected from the domestic animals were preserved in 70% ethanol until delivered to the laboratory. Non-engorged adult ticks were pooled depending on the host, tick species, site and date of collection. All ticks from the genus *Amblyomma* from cattle (*Bos taurus*) and all ticks collected from dogs (*Canis familiaris)* were identified to species level using entomological keys(Walker *et al*., 2003).

Fleas were collected from dogs, cats, rodents and insectivores by combing and from the houses by light trapping (Fig. 3.3). Fleas were preserved in 70% ethanol before delivery to the laboratory. Fleas were pooled depending on the host of origin, flea species, sex, site and date of collection. All fleas obtained from dogs, cats, and rodents and in the houses were identified using entomological keys(Segerman, 1995).

3.6.2 DNA Extraction from ticks and fleas

After identification, tick and flea pools were processed separately to recover DNA. Each tick or flea pool was placed in a 15 ml falcon tube and washed in RNase free water by vigorous shaking. A 5mm steel bead was placed in a 2 ml microtube and pre-cooled for 15 minutes in dry ice. The tick or flea was placed in the 2 ml microtube and incubated for another 15 minutes in dry ice. This was followed by 2 minutes incubation at room temperature and then mechanical disruption using a bead mill (Qiagen tissue lyser ^{LT}) at 30 Hz for three(one for fleas) minutes (Appendix 10). Genomic DNA was extracted from the mechanically disrupted tick or flea samplesusing QIAamp DNA tissue kit (QIAGEN, Valencia, CA, USA). Following mechanical disruption, further lysis of ticks/ fleas was achieved using 20 µl of Proteinase K and 180 µl ATL buffer and incubating the mixture in a thermomixer at 56^{o}C for 3 hours. A total of 200 µl buffer AL was added. The mixture was incubated in a thermomixer at 70^{o} C for 10 minutes. An equal volume (200 µl) of absolute ethanol (Analytical grade) was added. This was followed by a series of washes using wash buffers and a final elution with 100 µl buffer AE according to the manufacturer's instructions.

3.6.3Real time PCR (qPCR) assays in ticks and fleas

Two genus specific real time PCR for *Rickettsia*, namely *gltA* (Stenos *et al*., 2005) and Rick17b (Jiang *et al*., 2004) were performed individually using DNA from ticks and fleas. An overview of the arthropod sample selection and testing is given in figure 3.3.

All DNA samples from ticks were also tested with a tick-borne spotted fever group target *ompB*(TBSFG ompB) (Blair *et al*., 2004) and a species specific assay for *R. africae* (Richards *et al*., Pers Comm) (Table 3.1). The cycling conditions for *R. africae* qPCR were first hold at 50 °C for 2 minutes, second hold at 95 °C for 2 minutes, denaturation at 95 °C for 15 second and annealing and extension at 62 °C for 1 minute.

DNA samples from fleas were tested with two species specific assays; a *R. felisompB* assay (Henry *et al*., 2007) and a *R. typhi* (Jiang *et al.*, 2006) (Table 3.1).

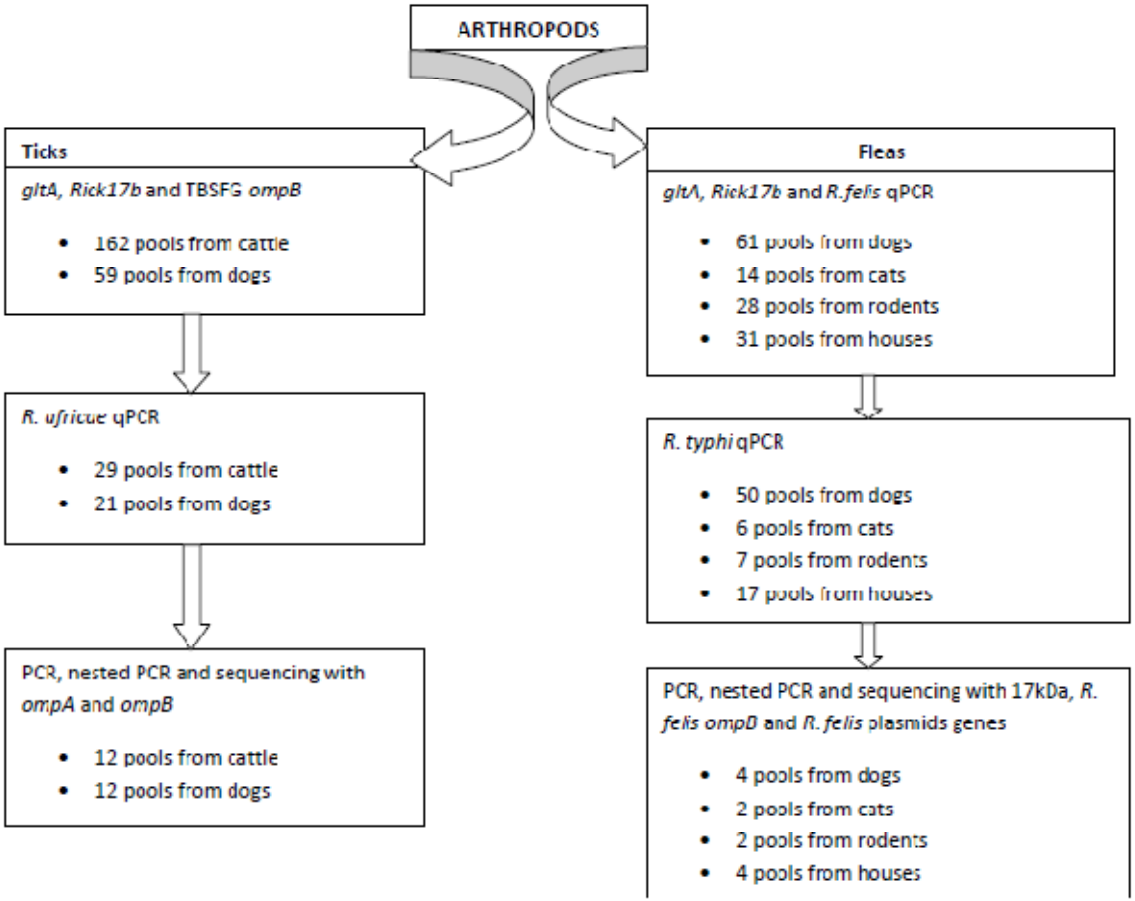


Figure 3.3 Arthropod sample selection for testing for *Rickettsia*

3.6.4 Characterization of *Rickettsia* detected in ticks samples using *ompA* and *ompB* gene

A 190 kDa outer membrane protein A (ompA3588F, ompA5238R) amplifying a 1651 bp amplicon was used for standard PCR. Nested PCR was done using primers RompA1F and ompA5044R which amplifies a 1437 bp product. A standard PCR targeting a 1902 bp fragment of the *ompB* gene utilizing primers RompB11F and RompB1902R was used for an initial PCR and was followed by a nested PCR amplifying a 1265 bp fragment with primers RompB607F and RompB1902R of the outer membrane protein B gene(*ompB*) (Raoult and Roux, 2000; Jiang *et al.*, 2005).

One (1 µl) microlitre of sample DNA preparation was used in a total of 25 µl total reaction mix containing 0.3 µM of each primer, 1X Platinum® PCR Supermix High Fidelity (Life Technologies, Grand Island, NY). One microlitre of PCR product was used in the nested PCR. Each PCR was performed in TGradient Thermocycler (Whatman BiometraGottingen, Germany) and incubated for 95 ℃, for 1 minute followed by 40 (35 for nested PCR) cycles of denaturation at 95 ℃ for 30 seconds, annealing at 50 ℃ for 1 minute and elongation at 68 ℃ for 2 (1.5 minutes for nested PCR). Final elongation was done at 72 ℃ for 7 minutes. No positive controls were used to decrease chances of contamination. RNase/ DNase free water was used as a negative control. The PCR products were visualized using ethidium bromide (GIBCO BRL Life Technologies, Inc., Gaithersburg, MD, USA) on 1% agarose gel after electrophoresis (Sambrook *et al*., 1989).

The nested PCR products from *ompA* were purified using QIAquick PCR purification kit according to manufacturer's instructions (Appendix 7). Nested PCR products from *ompB* were purified using gel extraction procedure as described by the manufacturer (QIAquick gel extraction kit, Qiagen) (Appendix 12).

A Big Dye Terminator v 3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) was used in subsequent sequencing reactions (Sanger*et al*., 1977). Sequenced products were purified by using Performa DTR Gel Filtration Cartridges(Edge BioSystems, Gaithersburg, MD, USA) (Appendix 13). Sequencing was done in ABI Prism 3130xlGenetic Analyzer (Applied Biosystems). Primers used for sequencing were the same as those for PCR. An additional forward primer RhoA4336F (Jiang *et al*., 2005) was included in the sequencing PCR for the *ompA* gene.Sequences were assembled with Vector NTI advanced 11 software (Invitrogen), and BLAST searches were managed on the National Center for Biotechnology Information website (http://blast.ncbi.nlm.hih.gov). Phylogenetic analyses of the tick sequences were performed using MEGA version 5 (Tamura *et al.*, 2011) based on the multi alignment of *ompA* and *ompB* sequences from the tick isolates and other rickettsial isolates from GenBank.

Table 3.3 Primers for standard PCR, nested PCR and their thermo-cycling conditions (Sigma Genosys, The Woodlands TX, USA)

Target	Name	Product size	Step	Standard PCR			Nested PCR		
				Temp	Time	Cycles	Temp	Time	Cycles
ompB Jiang *et al*., 2005; Raoult and Roux, 2000	RompB11F*	1902bp*	1st hold	94 °C	1 minute		94 °C	1 minute	
	120-607F^	1265bp^	Denaturation	94 °C	30seconds	40	94 °C	30seconds	35
	RompB1902R*^		Annealing	54 °C	30seconds		59 °C	30seconds	
			Extension	68 °C	2 minutes		68 C	90seconds	
			Final extension	72 °C	7 minutes	1	72 C	7 minutes	1
			Final hold	4 °C	∞		4 °C	∞	
ompA Jiang *et al.*, 2005;	190-3588F*	1651bp*	1st hold	94 °C	1 minute		94 °C	1 minute	
	190-5238R*	1437^	Denaturation	94 °C	30seconds	40	94 °C	30seconds	35
	RompA1F^		Annealing	50 °C	2 minutes		55 °C	1 minute	
	190-5044R^		Extension	68 °C	1 minute		68 C	30seconds	
	RhoA4336F#		Final extension	72 °C	7 minutes	1	72 C	7 minutes	1
			Final hold	4 °C	∞		4 °C	∞	

Key:
* primer for PCR
^primers for nested PCR
*^ for both PCR and nested PCR
# additional sequencing primer

3.6.5 Characterization of*Rickettsia* detected in fleas samples using 17kDa, *R. felisompB* and Plasmids genes

Three genes were used, namely; 17kDa, *R. felis* ompB (Richards *et al*., 2010) and *R. felis* plasmid targeting the short and the long fragment of the *R. felis* plasmids (Ogata *et al*., 2005).One microlitre of sample DNA preparation (Standard PCR) and 1 µl of PCR product (for nested PCR) was usedfor a PCR in a total of 25 µl total reaction mix containing 0.3 µM of each primer and 1X Platinum® PCR supermix High Fidelity(Life Technologies, Grand Island, NY). Each PCR was performed in TGradient Thermocycler (Whatman Biometra Gottingen, Germany). No positive controls were used in these PCR and nested PCR procedures to decrease chances of contamination. A negative control was used with each reaction (molecular biology grade water, GIBCO). The PCR and nested PCR cycling conditions for 17kDa, *R. felis* ompB and *R. felis* plasmids are summarized in Table 3.2.

3.6.6 Further identification of unique *Rickettsia* species in flea specimens

To identify novel *Rickettsiae*,selected positive (n=6) samples representing different locations and host species were further characterized by multi-locus sequence typing (MLST) targeting segments of five rickettsial gene; *rrs, gltA, ompB, ompA,* and *sca4* (Fournier *et al*., 2003). Standard and nested PCR were carried out using primers and procedures previously described (Roux and Roux, 2000; Jiang *et al* 2005).

PCR products from 17-kDa, *ompB, ompA, gltA, rrs, sca4* and two *R. felis* plasmid genes (pRF and pRFδ) were purified using QIAquick PCR purification kit according to the manufacturer's instructions (QIAGEN) (Appendix 7). Sequencing reactions were performed for both DNA strands using Big Dye Terminator v3.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions. After purification of sequenced products using Performa DTR Gel Filtration Cartridges(Edge BioSystems, Gaithersburg, MD) sequencing was run in an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems). Sequences were assembled with Vector NTI advanced 11 software (Invitrogen), and BLAST searches were managed on the National Center for Biotechnology Information website (http://blast.ncbi.nlm.hih.gov). Phylogenetic analyses of the unique flea sequences were performed using MEGA version 5 (Tamura *et al.*, 2011) based on the multi alignment of *rrs, gltA, ompA, ompB,* and *sca4* sequences from the flea isolates and other rickettsial isolates from GenBank. The unique sequences derived from the fleas were submitted to the GenBank.

3.6.7 Homology determination of*Rickettsia* isolates from human and fleas

Outer membrane protein (*ompB*) sequences derived from human specimens and those of fleas in this study were aligned against *Rickettsia*sequences selected from different geographical regions, sourced from the GenBank using MEGA version 5 (Tamura *et al.*, 2011).Phylogenetic tree was constructed using Neighbor-Joining method and the

Bootstrap analyses were performed with 1,000 replications using theMega 5 sequence analysis software.

3.7 Data analysis

The laboratory data collected from the above analyses was linked to existing HDSS data and analyzed for risk factors including gender and age. All statistical analyses were conducted in R version 2.10.0 (http://www.R-project.org).

A chi-square test for trend in proportions was used to test for linear trend in age-stratified seroprevalence of antibodies to TG and SFG *Rickettsiae*. Multivariate logistic regression analysis was conducted for the various age groups, gender, fever and acute respiratory illness. Variables with a p value <0.05 in the final multivariate logistic regression model were considered significant. For comparison of variables, χ^2 test and Fischer's test were used where applicable. Observed differences were considered significant at $p \leq 0.05$ for 2-tailed tests. Generalized linear models were used to examine the influence of individual status (febrile vs asymptomatic), age (<5 years vs >=5 yrs) and gender upon the *Rickettsia* PCR status (negative or positive in the *gltA* assay) of specimens. Disease episode was defined as the positive detection of the PCR target in the blood of a person presenting with acute febrile illness. Temporal patterns were examined by plotting monthly incidence data.

Performance of the test used for screening (*Rickettsia gltA*) of human samples was evaluated using receiver operating characteristics (ROC). The *gltA* qPCR was compared to Rick17bqPCR used at the reference laboratory in NMRC, USA. A hundred (100) samples previously tested at Centers for Disease Controls' International Emerging Infections Program (CDC-IEIP) laboratory Kenya using *gltA* gene of the citrate synthase were used. These samples were retested using Rick17b assay which was assumed to be a gold standard. Two by two tables were generated for each *gltA* gene cycle threshold results (a continuous variable) and the results of the Rick17b target (a categorical variable) positive or negative. Sensitivity and specificity values were calculated for each result of *gltA* assay (Appendix 14). A ROC graph was plotted using sensitivities and (1-specificity). A ROC graph was plotted using the sensitivity (true positive rate) on the y-axis and one minus specificity (false positive rate) on the x-axis (Fawcett, 2006). To minimize the rate of false positives and maximize on the sensitivity, the point where the sum of sensitivity and 1-specificity was largest, was marked and the CT corresponding with the largest sum of sensitivity and 1-specificity was used for calculation of the disease prevalence in humans.

CHAPTER FOUR

4.0 RESULTS

4.1 Assessment of previous exposure to *Rickettsia* in humans

A total of 385/1318stored patients sera collected in January 2007 through October 2008 were randomly selected from patients attending St Elizabeth Lwak Mission Hospital. Of these, 26 had insufficient volumes and hence they were not tested. Three hundred and fifty nine (359) sera were tested for presence of IgG against spotted fever group and typhus group *Rickettsiae*. One hundred and thirty six of these patients were males while 157 were females. There was no data ongender of 66of the 359 patients. Their mean age was 13.20 years (range 0-83 years). The sampled population comprised those from febrile patients 73.54% (264/359), acute respiratory illness patients 28.80% and 28.69% were in-patients. There were no diarrheic or pneumonic patients in this group and, only 7.8% (28/359) were positive for malaria. These signs and symptoms were not mutually exclusive.

4.1.1 Prevalence of immunoglobulin G against typhus group and spotted fever group *Rickettsia*

Of the 359 sera tested from clinical patients, two SFG and one TG test result could not be read. Two hundred of 357 serum samples tested (56.0%, 95% exact binomial confidence intervals [CI]: 50.7%-61.2%) were positive for IgG antibodies against *R.*

rickettsii antigen preparation (SFG-specific). Antibodies against *R. typhi* antigen preparation (TG-specific) were detected in 52 out of 358 sera tested (14.5%, 95% CI: 11.0%-18.6%). Forty seven out of those 52 samples positive for TG *Rickettsiae* were also positive for SFG, while only five (1.4%) reacted to TG *Rickettsiae* antigens alone.All negative control wells were non reactive while a positive reaction was defined as bright staining (at least 1+) of elementary bodies against a background of red/green counterstained material (plate 4.1). There was no association with gender for either TG (p=0.26) or SFG (p=0.41) (Table 4.1).

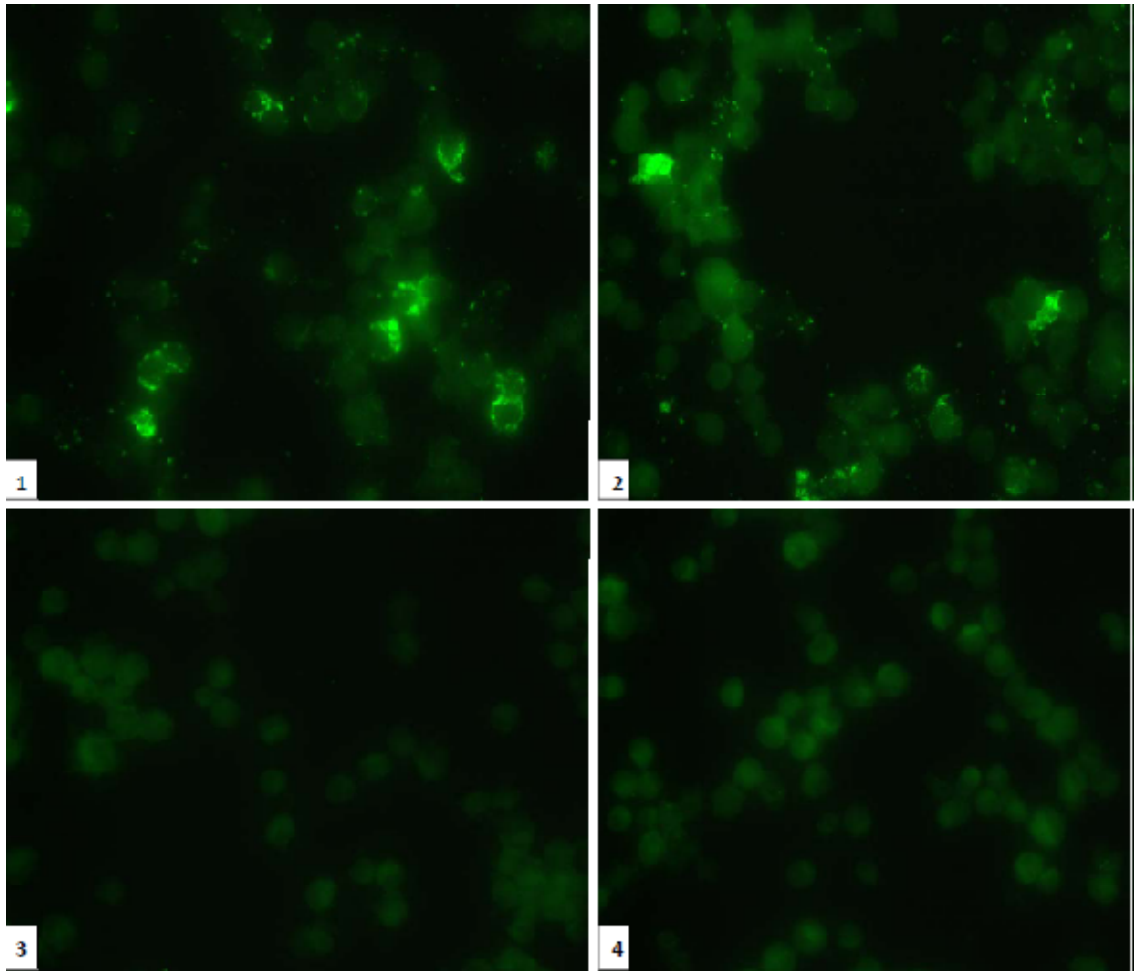


Plate 4.1 IFA picture showing a patient sample positive for IgG antibodies at 1:128 dilution (Panel 1), a positive control at 1:256 dilution (panel 2), a patient sample negative for IgG antibodies at 1:128 (panel 3) and negative control (panel 4)at ×400 magnification photograph taken using Olympus Microscope (Nikon, Inc., Garden City, NY) on 25th February 2009,

Table 4.1 Immunoglobulin G against typhus group (TG) and spotted fever group (SFG) *Rickettsia* by gender

	Gender	Positive (%)	Negative (%)	χ^2	Df	p-value
TG						
(*R.typhi*)	Male	16(37.21)	119(47.79)	1.25	1	0.26
	Female	27(62.79)	130(52.21)			
	Missing	66				
SFG						
(*R. rickettsii*)	Male	76 (43.93)	59(49.58)	0.69	1	0.41
	Female	97 (56.07)	60 (50.42)			
	Missing	65				

4.1.2 Age-stratified seroprevalence for IgG antibodies against spotted fever group and typhus group *Rickettsia*

A chi square test for trend conducted to establish whether there was any association between age and IgG seropositivity showed that there was no statistically significant trend for typhus group and age (χ^2 test for trend: $\chi = 3.41$, df = 1, p-value =0.065). An incremental linear association was demonstrated between age and IgG seropositivity to spotted fever group rikettsia (χ^2 test for trend: $\chi = 45.46$, df = 1, p-value <0.001) (Figure 4.1). In a multivariate logistic regression involving the various age groups, gender, fever and acute respiratory illness, age and fever were significantly associated with seropositivity (P<0.05).

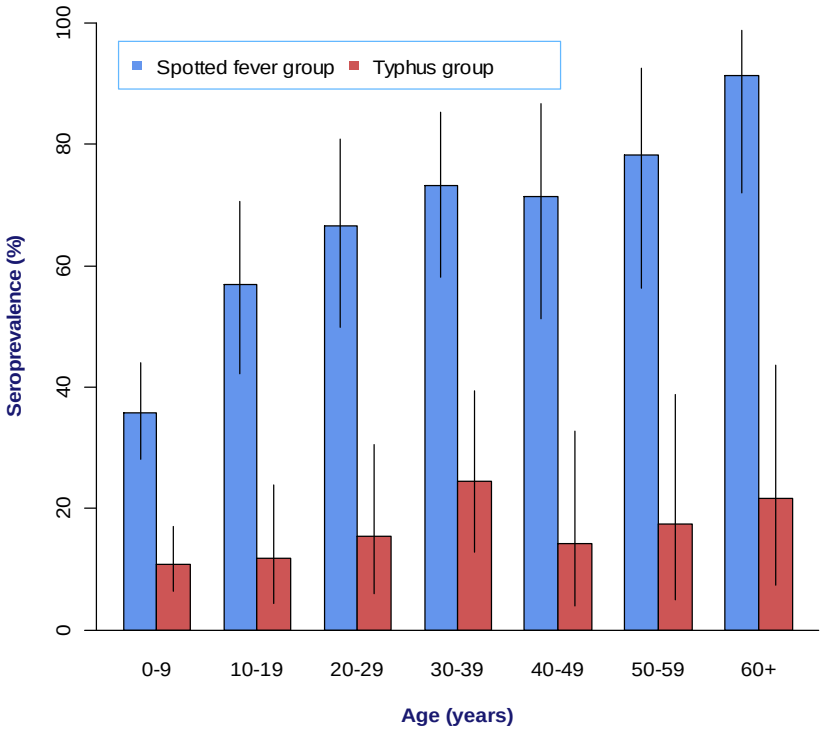


Figure 4.1 Age-stratified seroprevalence of IgG antibodies to *Rickettsia* among IEIP patients in 2007/2008

4.2 Blood clot volume optimization

The results of specimen volume optimization of the blood clots involving different volume are presented in Figure 4.2 below. Three hundred (300 µl) microlitre of diluted blood clot had reasonably lower cycle threshold (CT) (mean= 34.85) in comparison to other volumes used. On this basis, the volume was considered the most appropriate for extraction of deoxyribonucleic acid (DNA).

The 300 µl volume was selected as most optimum volume that gave the lowest average cycle threshold (CT) average of 34.85.

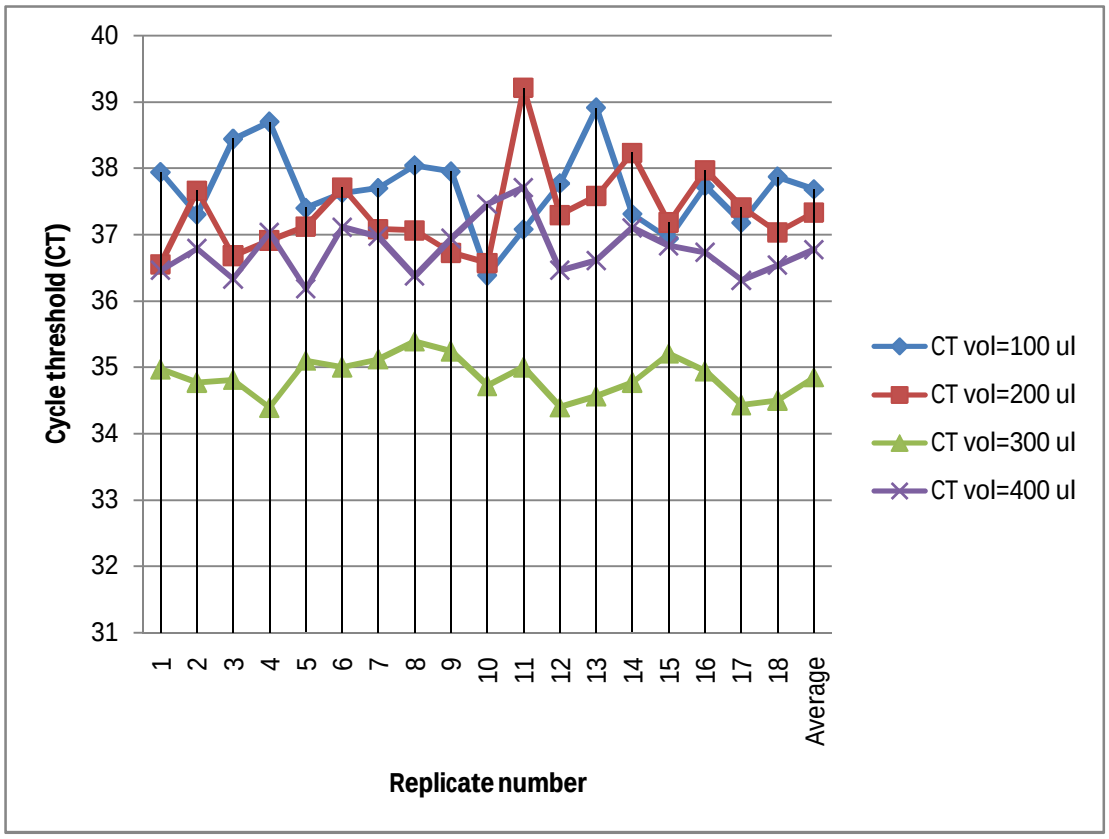


Figure 4.2 Cycle thresholds for different volumes of blood clots in 18 replicates

4.3 Assessment of acute *Rickettsia* infections in human

Overall, 699 out of 749 patients met the selection criteria for febrile patients while 236 out of 299 individuals met the criteria for asymptomatic individuals and were tested by PCR using the *gltA* gene of the citrate synthase. Blood clot specimens of 50 out of 749 febrile patientswere not available for testing, while 63/299 asymptomatic individuals

were dropped (without replacement) because they were convalescent patients on a return visit and had been sampled and tested during their acute phase testing. Febrile patients and asymptomatic individuals differed significantly by age (χ^2=147.65, df=6, p<0.001) and gender (χ^2=26.63, df=1, p<0.001; Table 4.2).

Table 4.2 Demographic characteristics of febrile patients and asymptomatic individuals enrolled in the hospital-based survey

	Febrile (%) n=699	Afebrile (%) n=236	P-value
Age group (y)			<0.001
0-9	540 (77.25)	86 (36.75)	
10-19	93 (13.30)	47 (20.09)	
20-29	30 (4.29)	27 (11.54)	
30-39	14 (2.00)	33(14.10)	
40-49	11 (1.57)	23 (9.83)	
50-59	6(0.86)	12(5.13)	
+60	5(0.72)	6(2.56)	
Gender			<0.001
Male	352 (50.57)	73 (30.93)	
Female	344 (49.43)	163(69.07)	

4.3.1 Receiver operating characteristics (ROC) test performance evaluation

A total of 699 febrile patients and 236 asymptomatic individuals visiting St Elizabeth Lwak hospital were tested for *Rickettsia* using the *gltA* gene qPCR. A hundred (100) samples selected for performance evaluation with the Rick17b assay comprised of 55 from febrile patients and 45 from asymptomatic individuals. The rationale of selecting the 100 samples was to ensure that the whole range of cycle thresholds is included in evaluation of *gltA* qPCR assay. The maximum sum of sensitivity and specificity was

recorded to be 1.61 corresponding with cycle threshold of 39.74 ~ Rounded to 40 (Appendix 15). A cycle threshold of 40 was selected to be the most optimum for analysis. Samples demonstrating a logarithmic increase in fluorescence beyond a minimum default threshold value were identified as positive and with a cycle threshold (CT) value equal to or less than forty. Figure 4.3shows the ROC curve (receiver operating characteristics) for various sensitivities and specificities.

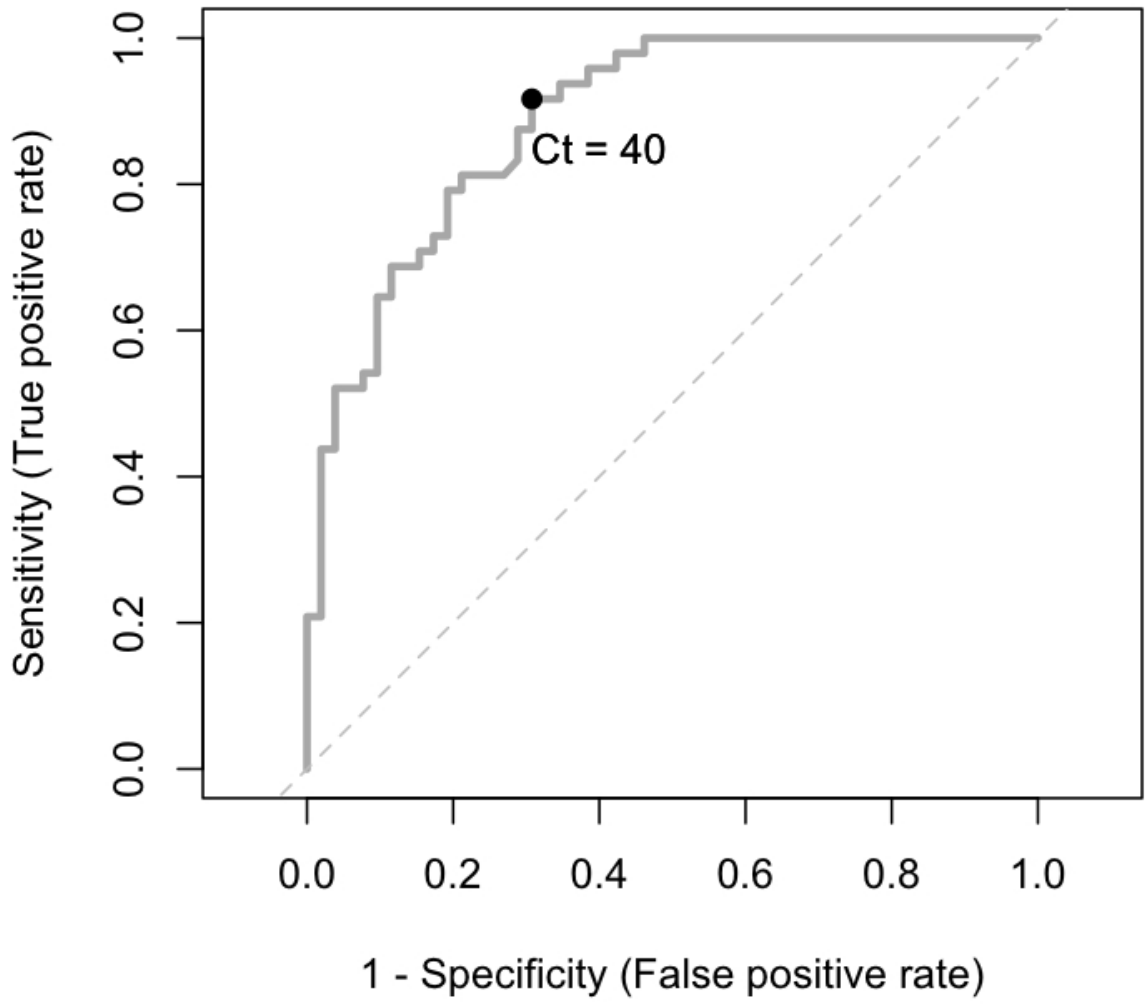


Figure 4.3ROC curve for the *gltA* and Rick17b assays for 100 samples tested. The black dot (ct=40) signifies the point at which the sum of sensitivity and specificity was greatest.

4.3.2 Quantitative PCR (qPCR) using *gltA* target

Of the 699 febrile patients tested, 50 (7.15%, 95% exact binomial CI: 5.36-9.32%) were positive for *Rickettsia* by PCR while eight out of 236 asymptomatic individuals (3.39%, 95% CI 1.47%-6.57%) were positive. The mean age of *Rickettsia* positive (by *gltA* gene) patients was 5.36 years with a range of 1 to 43 years. All febrile patients and asymptomatic individuals were negative for TBSFG *ompB* qPCR. *Rickettsia felis* qPCR on a subset of samples showed that 77.05% of the *gltA* positive samples were *R. felis.*The proportion of *Rickettsia* positive among the febrile patients differed marginally from that of asymptomatic individuals ($\chi 2=3.67$, df= 1, p=0.055). Univariate logistic regression of the influence of individual status (febrile versus afebrile) on PCR status showed that febrile patients had higher odds of being positive than afebrile individuals (odds ratio 2.20, 95% CI 0.98-5.09, p = 0.055) (Table 4.3). Multivariate analysis was also conducted to examine the influence of individual status in a model that included age (<5 years old vs. ≥5 years old) and gender variables. In this model, the odds ratio for febrile patients versus afebrile individuals was similar to the univariate model (odds ratio = 2.11, 95% CI 0.86-5.20) but the effect seen was no longer statistically significant.

Table 4.3 Univariate logistic regression of rickettsioses by *gltA* PCR in febrile patients and asymptomatic individuals

	Febrile patients (n=699) (%)	Asymptomatic individuals (n=236) (%)	Odds ratio	95% CI
Negative	649 (92.85)	228 (96.61)		
Positive	50 (7.15)	8(3.39)	2.20	0.98-5.09

4.3.3 Relationship between gender and age of febrile patients and *Rickettsia* status

Of the 50 *Rickettsia* positive febrile patients, 31 were males and 19 were females. There were marginally more males than females who tested positive for *Rickettsia* among the febrile cases. There was no association between genderand *Rickettsia* status ($\chi 2=2.34$, df=1, p =0.13). A total of 44/50 (88%) of *Rickettsia* positive febrile patients were 10 years and below. There was no linear association between age and *Rickettsia* status (P =0.07, Table 4.4).

Table 4.4 Percentage of *Rickettsia* PCR positive and negative febrile patients by age category

	Positive (%) n=50	Negative (%) n=649	P-value
Age group (years)			0.07
0-9	44(8.15)	496 (91.85)	
10-19	5 (5.38)	88 (94.62)	
20-29	0 (0.00)	30(100.00)	
30-39	0(0.00)	14(100.00)	
40-49	1(9.01)	10 (90.91)	
50-59	0(0.00)	6(100.00)	
+60	0(0.00)	5(100.00)	

4.3.4 Clinical signs and symptoms manifested by febrile patients

The common clinical manifestations among the *Rickettsia* positive patients were fever (100%), headache (100%), chills (93.8%), muscle aches (68.8) and joint pains (68.8%). Malaria was recorded in 79.2% and 73.4% of *Rickettsia* positive and *Rickettsia* negative patients using giemsa staining technique, respectively. Some symptoms such as headache, chills, muscle aches and joint pain were only recorded in patients 5 years and

above (≥60 months). There were 16 *Rickettsia* positive febrile patients who were ≥60 months, while the rest 277 were negative for *Rickettsia*. Of all the other clinical signs and symptoms recorded, there was no statistically significant association with rickettsiosis (Table 4.5). In alogistic regression, none of the predictors, namely age, gender, fever, rash was significantly associated with dependent variable (*Rickettsia* status by gltA) ($p>0.05$).

Table 4.5 Percentage of *Rickettsia* PCR positive and negative febrile patients presenting with specific clinical signs/symptoms

Symptom/sign	Positive (n=50)	Negative (n=649)	P-value*
Fever	100.0	100.0	
Headaches†	100.0	91.7	0.62‡
Chills†	93.8	76.8	0.21‡
Muscle aches†	68.8	46.6	0.14
Joint pain†	68.8	50.2	0.24
Rash	4.4	2.4	0.34‡
Malaria smear +ve	79.2	73.4	0.48

P-value of Chi-square test, unless otherwise stated

†Symptoms only recorded for patients ≥60 months (*Rickettsia* positive=16, *Rickettsia* negative=277)

‡P-value of Fisher's exact test

4.3.5 Temporal patterns for Rickettsiosis in febrile patients and asymptomatic individuals

A temporal trend for *Rickettsia* positives by PCR was observed over the study period with 4 peaks being observed in the months January 2009, May 2009, November 2009 and January 2010 (Figure 4.4).

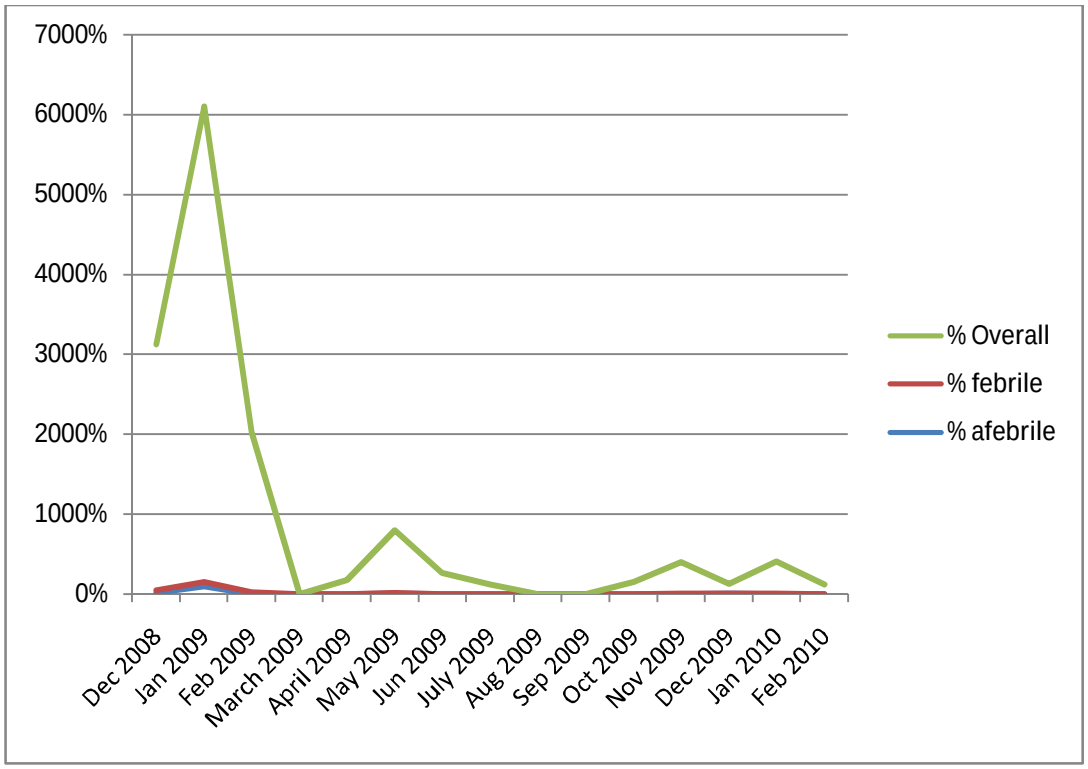


Figure 4.4 The number of *Rickettsia* positive among the febrile patients and asymptomatic individuals by month for febrile patients and asymptomatic individuals

4.3.6 Standard PCR, Nested PCR and sequencing for the *17kDa* gene, *ompB* gene and *R. felis* plasmids

To identify which *Rickettsiae* was detected in the human blood samples, standard and nested PCR of a 17 kDa, *R. felis ompB* and *R. felis* plasmid (pRF and pRFδ)were conducted. The negative control consistently produced no detectable product. A 434bp fragment was produced by the nested PCR in 22/25 samples tested. Sequences of up to 427-bp were obtained after assembling the sequences of the two fragments. One sample was not sequenced because it did not yield adequate product for sequencing. BLAST searches conducted showed the 21 samples to be 100% identical to the published sequences of *R. felis* strain URRWXCal2 (GenBank accession number CP000053.1).

Nested PCR using primers RompB607F and Rf1524R detected a 599-bp amplicons in 15/25 samples analyzed using this assay. A 548 to 594-bp fragment was obtained after assembling the sequences of the 2 fragments. Sequence of this product was found to have 100% identity with *R. felis* URRWXCal2complete sequence (GenBank accession number CP000053.1) (Table 4.7). A 1324-bp product was detected using primers pRFc-pRFd standard PCR in 2/25 samples. A nested PCR using primers pRFc-pRFg detected 429-bp fragment in 7/25 samples tested. Sequence of this product had a 100% identity with to *R. felis* URRWXCal2 pRF plasmid, complete sequence (GenBank accession number CP000054.1). None of the 25 samples produced a pRFδ product with standard and nested PCR (Table 4.6).

Table 4.6 Human samples sequence summary for 17kDa, *R. felis* ompB and plasmid genes from human samples Elizabeth Lwak Hospital

DNA No.	17kDa gene sequence	ompB sequence	pRF gene seq	pRFδ gene seq
1	+	+	-	-
2	+	+	+	-
3	+	+	-	-
4	+	+	-	-
5	+	+	-	-
6	+	+	-	-
7	+	+	-	-
8	+	+	-	-
9	+	-	-	-
10	+	+	+	-
11	+	-	-	-
12	+	-	-	-
13	+	-	-	-
14	+	+	-	-
15	+	+	+	-
16	+	+	-	-
17	+	-	-	-
18	+	+	+	-
19	+	+	+	-
20	+	-	+	-
21	+	+	+	-

4.4*Rickettsiae* in animals

Overall, 516 cattle were surveyed in the 300 LOCs; of these, 162 animals (31.4%) carried *Amblyomma variegatum* ticks and 471 had buffy coat samples collected. One or more rodents were trapped in 27 of the 50 compounds selected for rodent's survey. A total of 55 peri-domestic small mammals comprising of rodents and insectivores were trapped from a total of 1,142 trap placements.

4.4.1Quantitative PCR (qPCR) using *gltA* and Rick17b assays in domestic animals and rodents

Rickettsial DNA was detected by *gltA* gene qPCR in 11/299 (3.68%) dog buffy coats and 2/26 (7.69%) cat buffy coats tested. These amplification curves produced were very weak with cycle threshold (CT) above thirty nine. Only one out of the 471 cattle buffy coat tested produced a weak positive. No amplification was obtained in the 312 goat buffy coat, 334 sheep buffy coat and 54 rodent spleen samples using *gltA* qPCR assay. On retesting with Rick17b antigen gene, 10/11 dog and 2/2 cat buffy coat samples were positive. Retesting of the single positive cattle buffy coat sample with Rick17b qPCR was negative. The 12 *gltA* positive dog and cat samples were negative for TBSFG *ompB* qPCR.

4.4.2 Standard PCR, nested PCR and sequencing for the *17kDa* gene, *ompB* gene

A 434-basepair fragment was generated in a nested PCR using *17kDa* primer *R17k31F* and R17k2608R. Sequence of this fragment was found to have 97% identity to the *R. felis* complete genome URRWXCal2 (Accession number CP000053.1) in seven PCR products. Nucleotide deviation from reported examples was as a result of two strong signals occurring in 3-4 bases. Standard PCR using *ompB* gene did not produce any amplicons. Two 1265-bp amplicons were observed in the nested PCR using primers RompB120-607F and RompB1902R. Sequencing of these products was unsuccessful.

There was no amplification in both standard and nested PCR using *Rickettsia ompA* gene using primers RompA190-3588F/ RompA190-5238R (standard PCR) RompA1F/ RompA190-5044R (nested PCR).

4.5 Rickettsioses in ticks

A total of 258 adult *Amblyomma variegatum* ticks were collected off 162 cattle in the study area, pooled by individual host. One hundred and two ticks were collected off 36 dogs. Fifty nine pools were identified comprising of *Rhipicephalus sanguineus,Rhipicephalus appendiculatus*, and unidentified *Rhipicephalus* species,*Amblyomma variegatum, Boophilus decoloratus* and *Haemaphysalis leachi.*

4.5.1 Quantitative PCR (qPCR) assays in ticks

Of the 162 *Amblyomma variegatum* tick pools tested, 96.9% (95% CI; 92.9%-98.0%) were positive for *Rickettsia* by *gltA* gene qPCR. A total of 134/162 (82.72%) were positive for *Rickettsia* using the Rick17b qPCR. A tick-borne spotted fever group *Rickettsia* was identified in 124/162 (76.54%) of the ticks using TBSFG *ompB* qPCR. A total of 25/27 (93.1%) pools of *A. variegatum* selected for further typing were positive with a species specific *Rickettsia africae* qPCR.

Overall 12/59 (20.34) tick pools obtained from dogs were positive for *Rickettsia* by *gltA* qPCR. Majority of the positive tick pools (50%) were *Amblyomma variegatum.* None of the ticks in the species *R. sanguineus* was positive for *Rickettsia*. Of the 12 *gltA* positive ticks, 10 were positive with the Rick17b, TBSFG *ompB* and *R. africae* qPCRs. Percent prevalence of *Rickettsia* by *gltA* qPCR in ticks obtained from dogs is summarized in Table 4.7.

Table 4.7 Prevalence of *Rickettsia* in ticks obtained from dogs by *gltA* PCR

Tick species	Total pools	% abundance	No. positive	% per spp
Amblyomma variegatum	10	16.95	6	50
Boophilus decoloratus	5	8.47	1	8.3
Haemaphysalis leachi	10	16.95	1	8.3
Rhipicephalus appendiculatus	9	15.25	1	8.3
Rhipicephalus sanguineus	15	25.42	0	0
Rhipicephalus spp	10	16.95	3	25
Grand Total	**59**	**100**	**12**	**99.9**

4.5.2 Standard PCR, nested PCR and sequencing using *ompA*and *ompB* gene

Quantitative PCR methods were verified by sequencing products of the nested PCR of *ompA* and *ompB* gene. A 1437-basepair amplicon from the *Rickettsia ompA* gene was amplified by nested PCR of 22 of 24 *gltA* positive ticks. A 1364 bp sequence was obtained after assembling sequences of RompA1F, ompA190-5044R and RhoA4346F primers. Blast searches showed that this sequence was a 99% identical with that of *Rickettsia africae* ESF-2500 (Accession number U83436) and*Rickettsia africae* ESF-5 complete genome (Accession number CP001612.1). Twenty one 1265-bp amplicons were produced in *ompB* nested PCR. Sequencing of these PCR products was found to

have >98% homology to *Rickettsia africae* ESF-5 complete genome (Accession number CP001612.1).

4.5.3 Phylogenetic analysis of the *Rickettsia**ompA* and *ompB* genes in ticks

The *ompA* sequences from tick specimens from this study clustered together with *Rickettsia africae* ESF-2500(U83436)(Figure 4.5).Analysis carried out based on the nucleotide alignment of partial segment of the *ompB* gene resulted in >98% similarity with *Rickettsia africae* ESF-5 (CP001612) (Figure 4.6).*Rickettsia* detected in ticks from this study were not identical to each other, although they were >99% and >98% similar to the reference strain (*R. africae* ESF) for *ompA* and *ompB* gene, respectively,

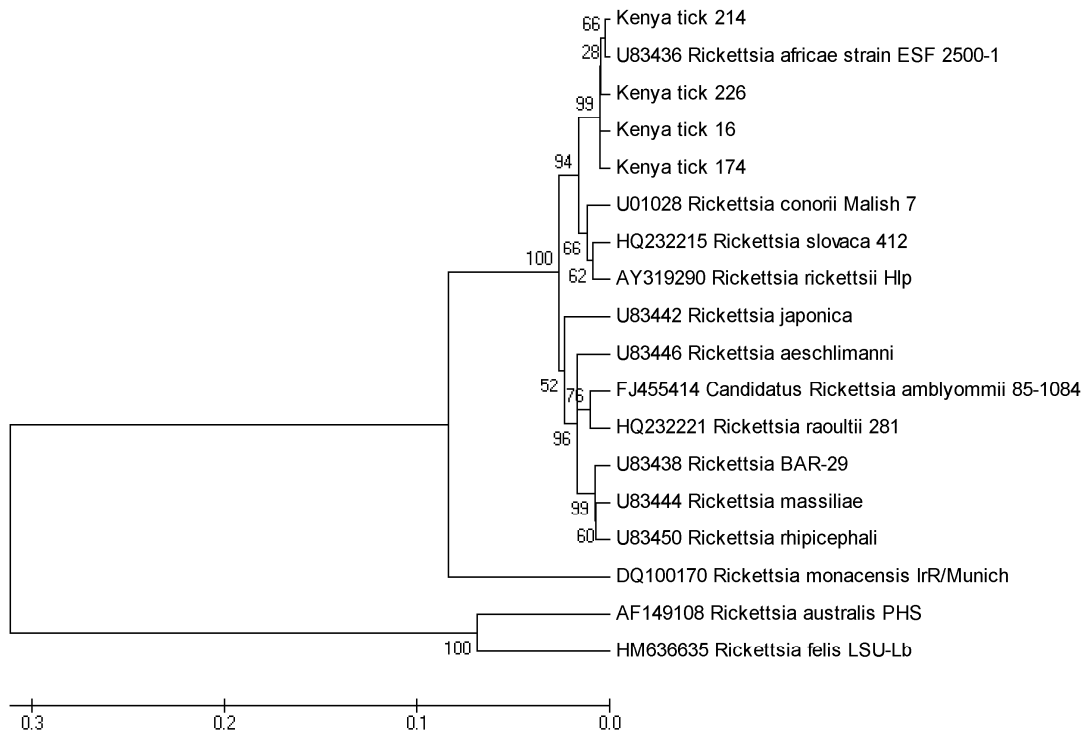


Figure 4.5 A Phylogenetic tree derived from *ompA* gene (partial genome) of 4*Rickettsia* isolates from ticks obtained from cattle and dogs in western Kenya.

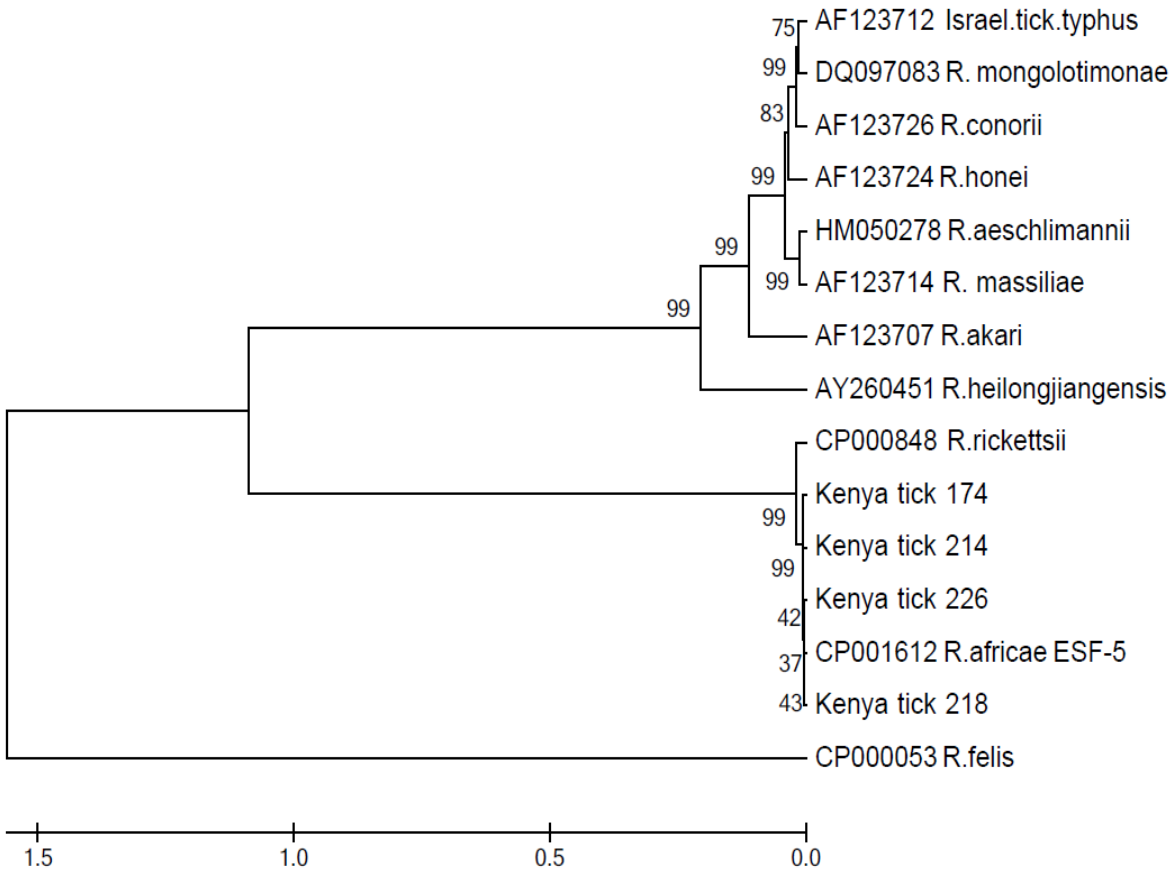


Figure 4.6 A Phylogenetic tree derived from *ompB* gene (partial genome) of 4*Rickettsia* isolates from ticks obtained from cattle and dogs in western Kenya.

4.6 Prevalence of *Rickettsia* in fleas obtained from Asembo

Fleas were collected from dogs, cats, and rodents, and by light traps from households, pooled by individual host and household. A total of 134 pools of fleas were collected from dogs, cats, rodents and in the houses. The pools comprised of *Ctenocephalides felis, C. canis, Echidnophaga gallinacea*, *Xenopsylla cheopis* and *Pulex irritans.* Overall *C. felis* was most abundant flea species (>50%) particularly found on dogs while *C. canis* was the least prevalent (~3%). *Echidnophaga gallinacea* was mostly identified on cats while *X. cheopis* was the most abundant flea species on rodents. Majority of fleas captured by the light traps in the house environment were *Ctenocephalisdes* spp. (48.4%) and*X. cheopis*(29%) (Table 4.8).

4.6.1Quantitative PCR assays using *gltA*, Rick17b, *R. felis ompB* and *R. typhi* in fleas

A 74 bp fragment was amplified using primers (CS-1126F and CS1199R) and a fluorescence labelled Taqman probe (CS-1149P).All four pools (100%) of *C. canis*, and 97.3% of 75 pools of *C. felis* collected off domestic carnivores and from the home environments tested positive for *Rickettsia*.Positive pools of *C. felis* came from 37 dogs, five cats, and nine households. Of all *Xenopsylla cheopis* collected off the rodents and in the houses, 8.57% of the 35 pools were positive for *Rickettsia*. Other fleas collected were *Echidnophaga gallinacea* and *Purex irritans* all of which were negative for *Rickettsia*.Flea samples were tested by two species specific qPCR assays. A 129-bp

fragment was amplified using an *R. felis* ompB gene qPCR in all fleas that had tested positive with *gltA* and 17kDa antigen gene. Out of the 80 pools of fleas tested with an *R. typhi* qPCR, only one sample was weakly positive (CT=42.61). This was negative on retesting for the second time. The results of the qPCR using *gltA* gene are presented in Table 4.8.

Table 4.8*Rickettsia* PCR results by flea species. For each species, n represents the number of animals or houses

	Number of pools	Number Positive	Percent positive
Dogs (n=39 dogs)			
Ctenocephalides felis	58	56	96.6
Echidnophaga gallinacea	3	0	0
Cats (n=9 cats)			
Ctenocephalides canis	1	1	100
Ctenocephalides felis	5	5	100
Echidnophaga gallinacea	8	0	0
Rodents (n=18 rodents)			
Echidnophaga gallinacea	2	0	0
Xenopsylla cheopis	26	2	7.7
Light trap (n=16 households)			
Ctenocephalides canis	3	3	100
Ctenocephalides felis	12	12	100
Echidnophaga gallinacea	2	0	0
Pulex irritans	5	0	0
Xenopsylla cheopis	9	1	11.1

4.6.2 PCR, nested PCR and sequencing using *Rickettsia felisompB* gene

Three PCRs were performed to further type the *Rickettsia* detected in fleas. All 12 *R. felis* positive fleas tested by a standard and nested PCR targeting a 599-bp fragment (RompB607F and Rf1524R) of the *R. felis* ompB gene yielded expected bands in the nested PCR. One (1/12) of the 12 flea sequences (from *X. cheopis* collected from *Rattus rattus*) produced 549-bp sequence that was 100% identical to those already reported for *R. felis* URRWXCal2 (CP000053.1). Sequence of the 11/12 products were found to be unique, the closest match in the GenBank was *R. felis* but only at 92-93% identity (GeneBank accession number CP000053.1).

4.6.3 PCR, nested PCR and sequencing using *17kDa* gene

A standard PCR targeting a 440-bp fragment of *Rickettsia* 17kDa gene (R17k31F and R17k469R) was negative. A nested PCR of the same gene targeting a 434-bp fragment (utilizing primers R17k31F and R17k2608R) yielded expected amplicons in ten of the 12 flea samples tested with 17kDa gene.One of the ten sequences from a flea (*X. cheopis*) collected from *Rattus rattus* identified a 396-bp sequences that had 100% similarity to *R. felis* URRWXCal2 (Accession number CP000053.1). The remaining nine showed 96% homology when compared with the published *R. felis* URRWXCal2 above. BLAST searches of the nine flea sequences identified a 371-bp R17K sequence (excluding the primer site) that was 100% identical to *Rickettsia* spp. SE313 detected in *E. gallinacea* in Egypt (GenBank DQ166937) and *Rickettsia* spp.Cf1 and5 detected in *C.*

felis in South Carolina (GenBank AY953286). The nine unique amplicons were from *Ctenocephalides felis*(4) *C. Canis*(4) and *Xenopsylla cheopis* (1).

4.6.3 PCR, nested PCR and sequencing using *Rickettsia felis* plasmid gene

Standard (pRFa-pRFd) and nested (pRFa-pRFg) PCR targeting the short fragment of the *R. felis* plasmid were negative for all the 12 samples tested. Standard PCR targeting the larger fragment (1342-bp) of *R. felis* plasmid (pRFc-pRFd) did not yield any band. However a 429-bp fragment was amplified using pRFc-pRFg nested PCR in one out of the 12 flea samples tested. *Xenopsylla cheopis,* a flea species collected from *Rattus rattus* yielded the expected amplicon.A 427-bp sequence of this fragment was found to have 100% identity to the *R. felis* complete genome URRWXCal2 (Accession number CP000054.1). Sequence summaries are presented in table 4.9.

Table 4.9 Flea samples sequence summary for 17kDa, *R. felis* ompB and plasmid genes

DNA number	Flea species	17kDa	ompB gene	pRF	pRFδ
2	*X. cheopis*	100% R.felis URRWXCal2	100% R.felis URRWXCal2	100% *R. felis URRWXCal2*	-
9	*X. cheopis*	-	92% *R.felis* URRWXCal2	-	-
30	*C. canis*	100% *Rickettsia* spp. SE313 and 100% *Rickettsia* spp. cf1and5 96% *R.felis* URRWXCal2	93% *R.felis* URRWXCal2	-	-
36	*C. felis*	100% *Rickettsia* spp. SE313 and 100% *Rickettsia* spp. cf1and5 96% *R.felis* URRWXCal2	92% *R.felis* URRWXCal2	-	-
51	*C. canis*	100% *Rickettsia* spp. SE313 and 100% *Rickettsia* spp. cf1and5 96% *R.felis* URRWXCal2	92% *R.felis* URRWXCal2	-	-
58	*C. canis*	100% *Rickettsia* spp. SE313 and 100% *Rickettsia* spp. cf1and5 96% *R.felis* URRWXCal2	93% *R.felis* URRWXCal2	-	-
60	*C. canis*	100% *Rickettsia* spp. SE313 and 100% *Rickettsia* spp. cf1and5 96% *R.felis* URRWXCal2	93% *R.felis* URRWXCal2	-	-
63	*C. felis*	-	92% *R.felis Cal2*	-	-
77	*C. felis*	100% *Rickettsia* spp. SE313 and 100% *Rickettsia* spp. cf1and5 96% *R.felis* URRWXCal2	93% *R.felis* URRWXCal2	-	-
82	*C. felis*	100% *Rickettsia* spp. SE313 and 100% *Rickettsia* spp. cf1and5 96% *R.felis* URRWXCal2	92% *R.felis* URRWXCal2	-	-
89	*C. felis*	100% *Rickettsia* spp. SE313 and 100% *Rickettsia* spp. cf1and5 96% *R.felis* URRWXCal2	93% *R.felis* URRWXCal2	-	-
117	*C. felis*	100% *Rickettsia* spp. SE313 and 100% *Rickettsia* spp. cf1and5 96% *R.felis* URRWXCal2	92% *R.felis* URRWXCal2	-	-

4.6.4 Further identification of unique*Rickettsia* species in flea specimens

The sequences of the 1395bp fragment of *rrs* and 1130bp of *gltA* from the new isolate were closely related to *R. felis* URRWXCal2 (Figure 4.7) (99.5% and 98.0%, respectively) and *Rickettsia* spp. RF2125 (99.6% for *gltA* only) (Figure 4.8), indicating that this new isolate belongs within the genus *Rickettsia*. A 1517 bp fragment of *ompA* (3'end) was amplified providing evidence that the new isolate is a member of SFG of rickettsiae, but the fragment of the 5' end of *ompA* was not amplified by a SFG universal primer set, which suggests that the gene might be truncated or the sequence was not recognized by one or both of the primers. The sequence of the 1517 bp fragment of *ompA* was most similar to *R. felis* URRWXCal2 (92.3%) (Figure 4.9). The 1034 bp fragment of *sca4*from the new agent was most similar to *R. felis* URRWXCal2 (95.5%) (Figure 4.10), while the 4,311 bp fragment of *ompB* sequencefrom the novel *Rickettsia* was most similar*R. felis* URRWXCal2 (94.8%) (Figure 4.11). The percent cut-off point for the minimum 5 genes (Fournier *et al*., 2003) used to determine whether a new *Rickettsia* isolate fits to be classified as a new species are illustrated in Table 4.10.

Table 4.10 Percent identity reference cut-off for selected genesand percent identity of the new *Rickettsia*isolate from fleas

Gene	Cut-off (Reference)	% identity to *R.felis*	Amplicon length
rrs	>99.8	99.5%	1395bp
gltA	>99.9%	97.96%	1130bp
ompA	>99.8%	92.34%	1517bp
ompB	>99.2%	94.36%	1481bp
sca4	>99.3%	95.45%	1034bp

The sequences generated by various genes used for further characterization of the new *Rickettsia* isolates were deposited in GenBank. The accession numbers for selected flea specimens; isolate flea 30 (F30) from *Ctenocephalides Canis* and isolate flea 82 (F82) from *C. felis* are shown in Table 4.11.

Table 4.11 Accession numbers for sequences of the new *Rickettsia* isolates from fleas deposited in the GenBank

Gene	Accession numbers for flea 30	Accession numbers for flea 82
Rrs	JN315967	JN315973
gltA	JN315968	JN315974
17 kDa	JN315969	JN315975
sca4	JN315970	JN315976
ompA	JN315971	JN315977
ompB	JN315972	

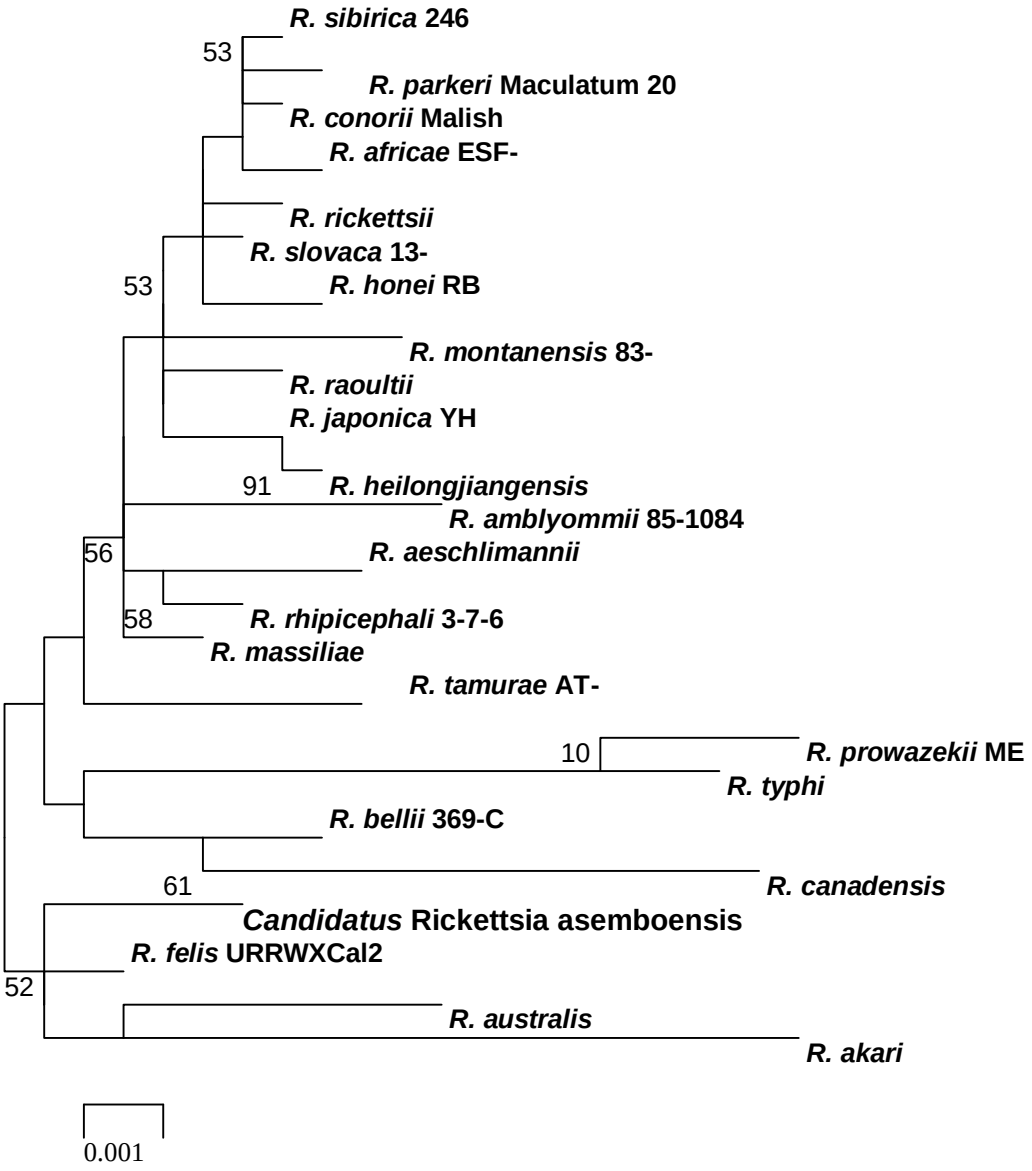


Figure 4.7 Phylogenetic relationships: *Candidatus Rickettsia asemboensis* with other *Rickettsiae*, tree was constructed for *rrs*(1395 bp) using Neighbor-joining method. (MEGA software v4).

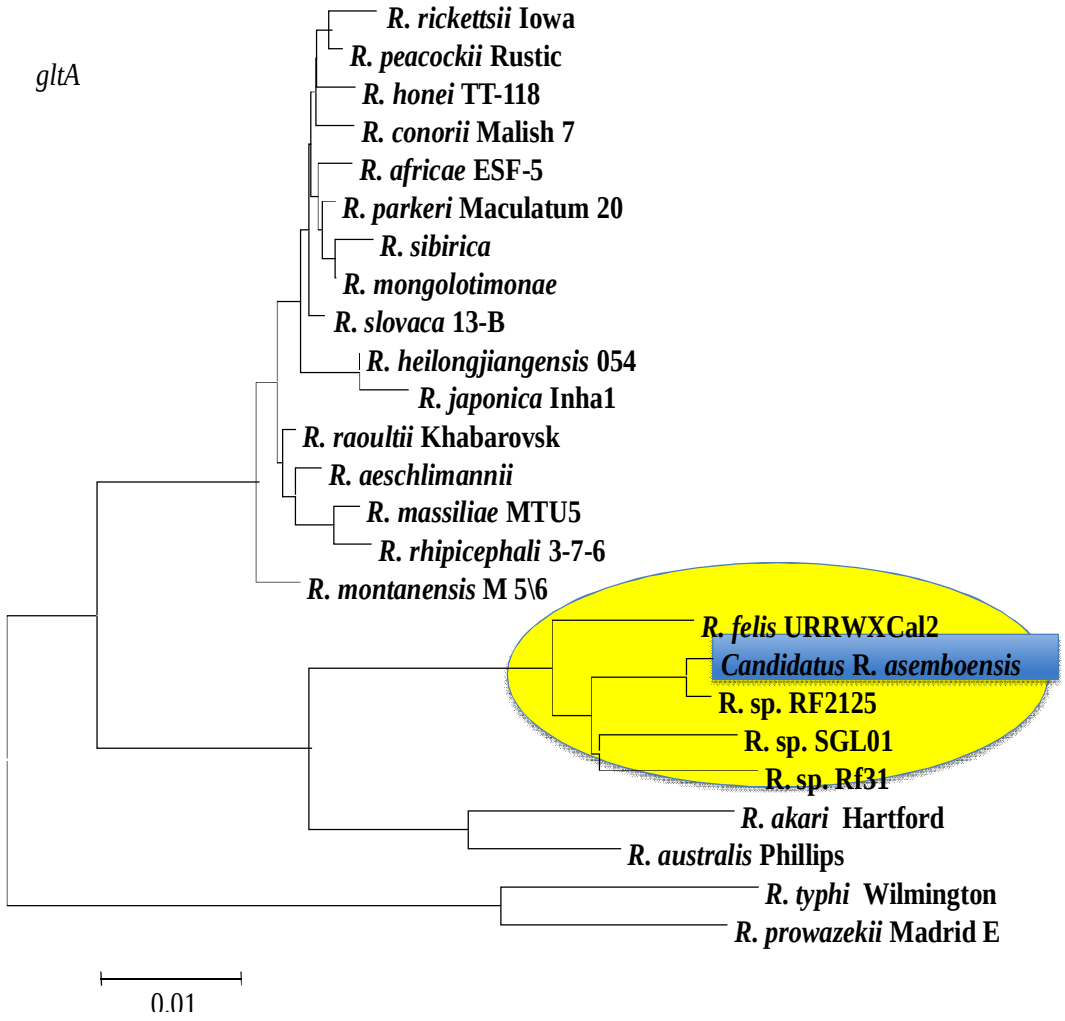


Figure 4.8 Phylogenetic relationships: *CandidatusRickettsia* asemboensis with other *Rickettsiae*, tree constructed for *gltA*(1130 bp) using Maximum composite likelihood model, Neighbor-joining method. (MEGA software v5)

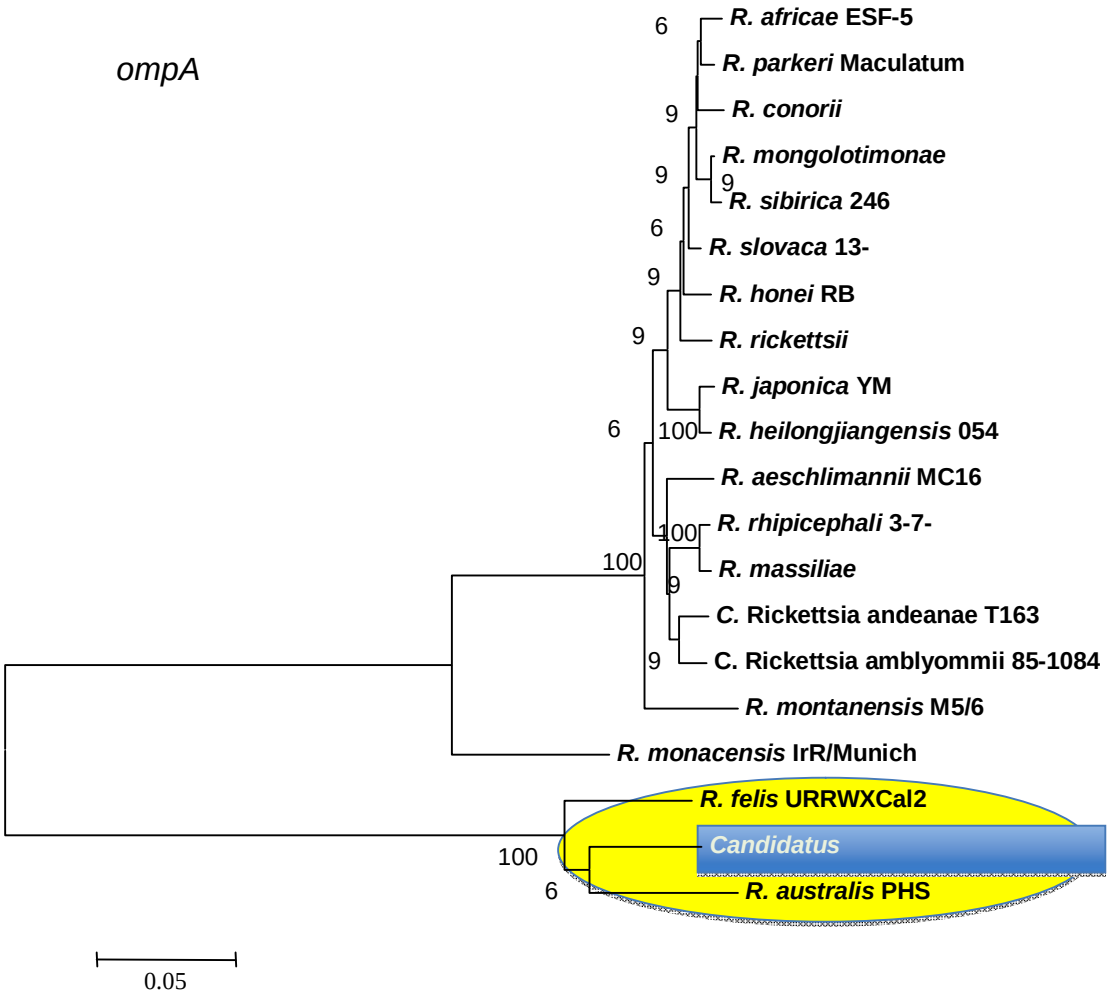


Figure 4.9 Phylogenetic relationships: *CandidatusRickettsiaasemboensis* with other *Rickettsiae*, tree constructed for *ompA* (1517 bp) using Neighbor-joining method. (MEGA software v5).

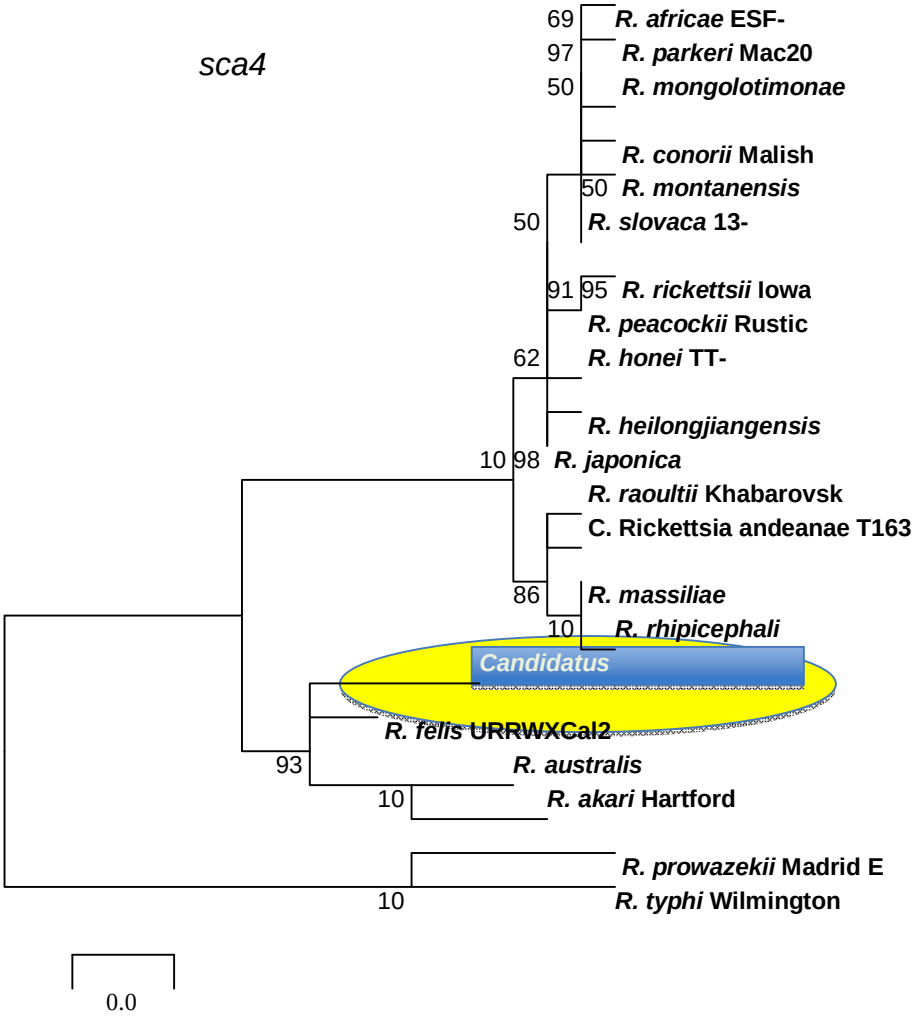


Figure 4.10 Phylogenetic relationships: *CandidatusRickettsiaasemboensis* with other *Rickettsiae*, tree constructed for *sca4* (1034 bp) using Neighbor-joining method. (MEGA software v5).

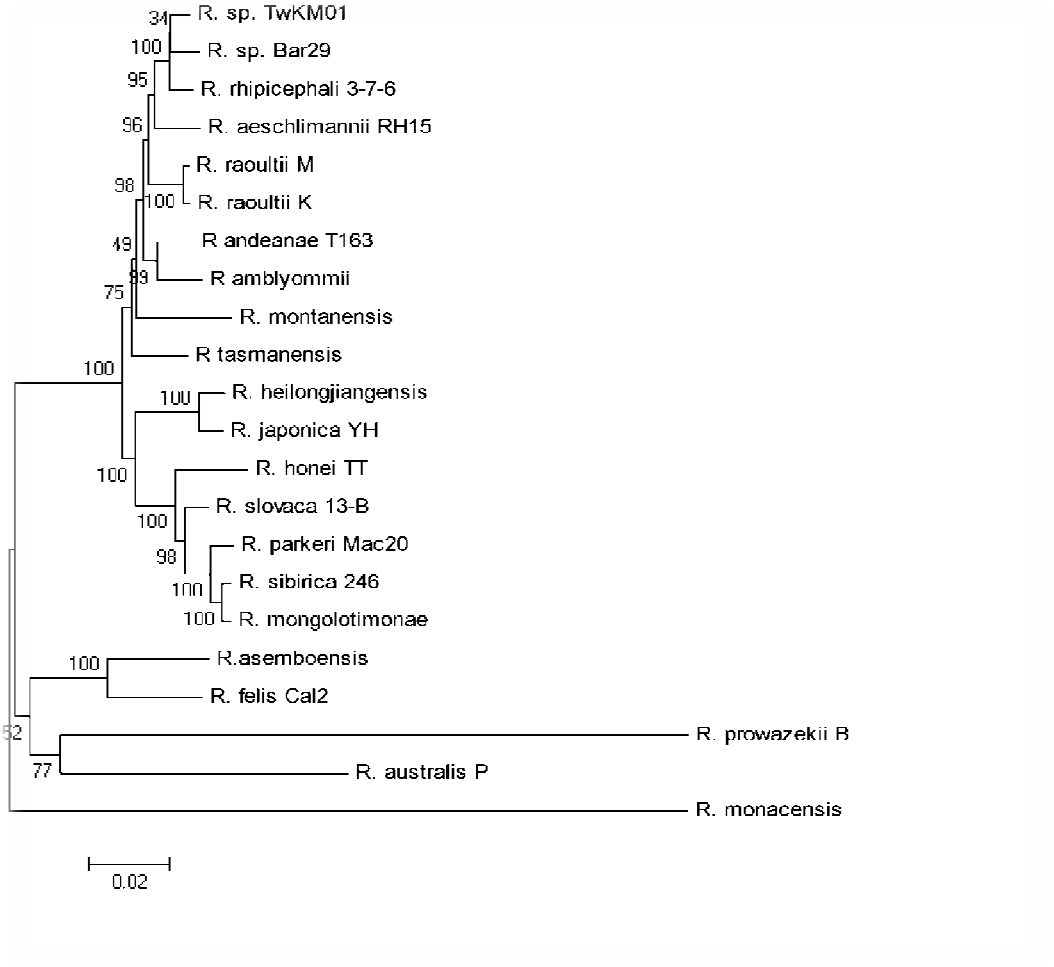


Figure 4.11 Phylogenetic relationships: *CandidatusRickettsiaasemboensis* with other *Rickettsiae*, tree constructed for *ompB*(1481bp) using Maximum composite likelihood model, Neighbor-joining method. (MEGA software v5)

4.7 Homology determination and phylogenetic analysis of *R. felis ompB* in human and flea samples

Phylogenetic analysis of *Rickettsia* species inferred from comparison of *ompB* gene sequences showed that highest percentage similarity (100%) was obtained forthe already published*R. felis* URRWXCal2 strain, human samples and *Rickettsia* detected in one of the *X. cheopis* (Oriental flea_2). Human samples (Human 596, 598, 599 and Human 639) fell into one clade with Oriental flea 2 and *Rickettsia felis* URRWXCal2 with bootstrap values of 100. Another separate clade was formed by the unique *Rickettsia* isolate from the cat flea, dog fleaand another oriental flea 9 (Figure 4.12)

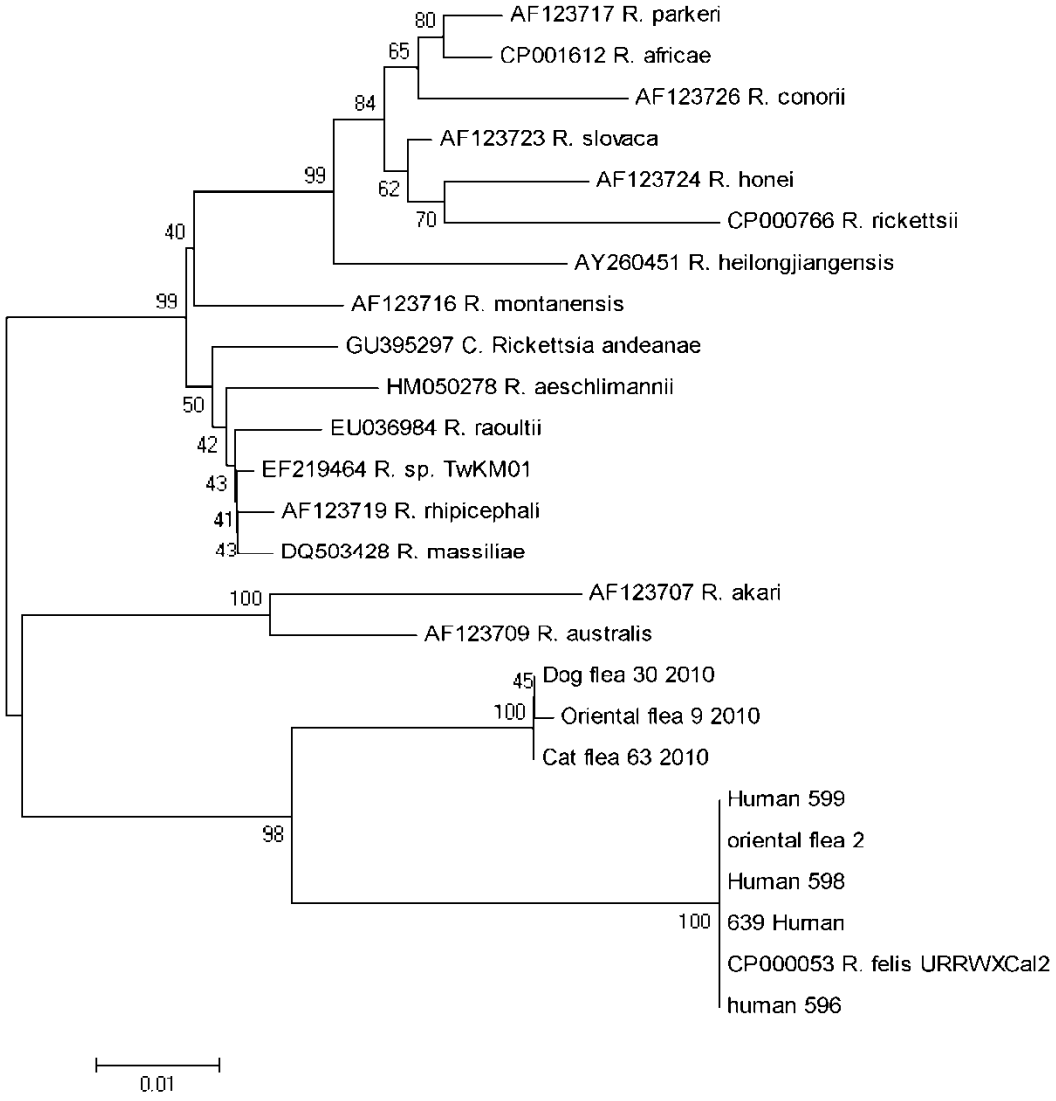


Figure 4.12 Dendrogram representing phylogenetic relationship between *Rickettsia* species inferred using neighbor joining method (1000 bootstrap replications) *ompB* partial genome

CHAPTER FIVE

5.0 DISCUSSION

Rickettsial infections are rarely considered when evaluating patients with acute febrile illnesses in Kenya. To elucidate presence of previous exposure to spotted fever group and/or typhus group *Rickettsia* in the study area, an indirect fluorescence antibody test (IFA) was used. The serosurvey indicates 56% and 14.5% of patients attending Lwak hospital had serological evidence of previous exposure to SFG and TG *Rickettsia*, respectively. Previous studies conducted in other African countries have reported high seroprevalence (range between 28-58%) to *Rickettsiae* (Kelly *et al*., 1991; Corwin *et al*., 1993; Anstey *et al*., 1997; Kaabia *et al*., 2006). Out of 52 (14.5%) patients seropositive for *R. typhi* antigen preparation, 47 (90.4%) were positive for both *R. rickettsii* and *R. typhi* antigen preparations. Because IFA cannot distinguish between species, within the same *Rickettsia* biogroup, diseases caused by *Rickettsia* are dichotomized as either SFG (sera react with *R. rickettsii* antigen preparation) or TG rickettsiosis (sera react with *R. typhi* antigen preparation (Jensenius *et al*., 2009). This high number of patients seropositive for both *R. rickettsii* and *R. typhi* antigen preparations may be attributed to cross-reactivity to both SFG and TG rickettsial antigens (Ormsbee *et al*., 1978). Results from studies in animal models however, show that cross-reactivity between SFG and TG *Rickettsiae* is not a consistent feature (Mediannikov *et al*., 2010a). Znazen *et al.* (2006) speculate that antibodies to *R.*

felis may be the major cause of cross-reactions to TG-specific and SFG-specific antigens. These authors found cross-reactivity to TG and SFG antigens in serum specimens from five of seven patients with confirmed *R. felis* infection. Due to unavailability of sufficient sera to test by PCR, none of the serosurvey specimens were tested by molecular methods and hence this study was unable to evaluate specifically which *Rickettsia* spp. was responsible for the observed serological reactions.

Previously, high seroprevalence (90%) to SFG in the sub-Saharan Africa (Kelly *et al.*, 1991) was attributed to *R. africae* infections as explained by the high prevalence of *Amblyomma* ticks in this region (Raoult and Roux, 1997; Niang *et al.*, 1998). Today, it is recognized that infections with *R. felis* are likely to be contributing to the high seroprevalence, as it has been found to be prevalent in this region together with its many biological vectors. Although serologically grouped in the spotted fever group using monoclonal and polyclonal antibodies (Fang and Raoult, 2003), *Rickettsia felis* had been shown to react with monoclonal and polyclonal antibodies, previously thought to be specific for *R. typhi* (Azad *et al.*, 1992). Recent studies grouped *R. felis* in the transitional group which is phylogenetically positioned between SFG and TG group *Rickettsia* (Gillespie *et al.*, 2007).

Seropositivity for SFG antibodies was associated with increasing age, with >90% of patients aged 60 years and above being seropositive (P<0.001). This increase with age of the prevalence of IgG antibodies to SFG *Rickettsia* can in part be accounted for by cumulative exposure to the pathogen and lifelong persistence of IgG antibodies. Mediannikov *et al.* (2010a) observed a similar association in Senegal where patients, 40 years and above, showed higher seroprevalence. Persistence of IgG antibodies against SFG has been described previously (Sarov *et al.*, 1990). Gender was not found to be an independent risk factor for exposure to SFG or TG *Rickettsia*. This finding is consistent with a study done by Yagupsky *et al.* (1990)in children of up to 17 years of age. Conversely, a study conducted on 47,915 international travellersby Jensenius *et al.* (2009), revealed that men were more commonly infected with rickettsial diseases.

The QIAamp DNA Mini Kit (QIAGEN, Valencia, CA) instructs one to use 200µl of blood for DNA extraction. This study used blood clot which was semi solid and had to be resuspended in equal volume of normal saline to dissolve the clot. To get the optimum volume of resuspended clot for DNA extraction, different volumes (100, 200, 300, 400µl) of resultant clot were analyzed. The volume of 300µl of resuspended blood clot yielded the lowest mean cycle threshold and was selected as the most optimum for extraction of DNA from blood clot. This method was adopted from Tan *et al.* (2009) who observed that the purification efficacy decreased drastically above 100µl of fresh blood input volume. The volume of

sample used for extraction of DNA should maximize on high quality DNA yield while minimizing the presence of extraneous materials such as proteins and chemicals in the final eluted product, which may in turn inhibit PCR and downstream applications.

Using the Rick17b qPCR assay used in the reference laboratory (NMRC) as a gold standard, the performance of the *gltA* assay was evaluated by calculating the sensitivity and specificity at different ct cutoff points of *gltA* assay, and constructing a receiver operating characteristic curve. A cycle threshold (ct) cutoff value of 40 was determined to be the most optimal cutoff for *gltA* qPCR assay as a screening test for *Rickettsia*. The end product resulting from a qPCR assay is a continuous variable, compared to conventional PCR which is a binary outcome.Evaluation of an optimal ct value cutoff for real time PCR assays is therefore necessary in order to minimize overall misclassification of disease positives and negatives. Evidence based selection of ct cutoff value for qPCR assays is essential in analytical and epidemiological approaches. In real time PCR, ct values are interpreted as amplification, cross-contamination or fluorescence artifacts due to breakdown of probes, spurious products and primer dimers (Caraguel *et al.*, 2011). Receiver operating characteristic curve has been used by other studies to evaluate performance of diagnostic tests and to determine limit of detection (Villella *et al.*, 2004; Rantala *et al.*, 2010; Caraguel *et al.*, 2011).

The prevalence of *Rickettsia* by PCR was 7.15% and 3.39% in the febrile patients and asymptomatic individuals, respectively. The febrile patients were 2.2× more likely to have rickettsial infection than the asymptomatic individuals. The excess rate (3.76%) of rickettsiosis in febrile patients suggests that rickettisoses may be contributing to the burden of febrile illness in western Kenya.This is similar to findings in northern Tanzania, where acute rickettsiosis was serologically confirmed in 8% of febrile hospital inpatients (Prabhu *et al*., 2010), and from rural Senegal where 6% of febrile patients without malaria tested positive for *Rickettsia* (Socolovschi *et al*., 2010). Identification of DNA of *Rickettsia felis* in both febrile patients and asymptomatic individuals visiting St. Elizabeth Lwak hospital of western Kenya establishes the occurrence of this pathogen in the indigenous population, and corroborates previous reports of the occurrence of a similar strain in humans in Kenya (Richards *et al.*, 2010) and in Senegal (Socolovoschi *et al*., 2010). The detection of rickettsial DNA in the asymptomatic individuals suggests a possibility of a mild or asymptomatic form of disease. It also highlights a possibility of exposure to the same vectors. Consequently, it is not surprising that both febrile patients and asymptomatic individuals yielded positive PCR results for *Rickettsia felis*. Furthermore, clinical manifestations of most rickettsial illness range from mild to severe illness (Raoult and Roux, 1997: Jensenius *et al.*, 2003b).

Although gender was not independently associated with rickettsiosis status, there were marginally more males positive for *Rickettsia* by PCR than females. This

finding is in support of other previous studies on rickettsial diseases (Raoult *et al.*, 1986; Dalton *et al.*, 1995). Jensenius *et al*. (2009) found that old age was associated with *R. africae* infections, nevertheless, this study observed a larger proportion of *Rickettsia* positive patients (88%) was found among the younger age group (i.e. children less than 10 years). The mean age of febrile patients positive for *Rickettsia* by PCR was 5.36 years, which is comparable to the findings by Socolovschi *et al.* (2010), who found that the attack rate was higher in children less than 10 years old and Dalton *et al*. (1995) in children 5-9 years of age. This may suggest that the infection occurs at an early age; and later when one is older, both humoral and cell mediated immune response mechanisms develop to rapidly clear the rickettsiae from the body. These findings therefore, suggest that rickettsial infections should be considered in the differential diagnosis of febrile illnesses in western Kenya.

The clinical picture observed in this study (fever, headache, chills, muscle aches and joint pains) is comparable to what has been described for rickettsial diseases elsewhere (Jelinek and Loscher, 2001). In addition malaria was detected in 79.2% of febrile patients tested. Occurrence of multiple agent infection among acute febrile patients has been reported in Egypt (Parker *et al.*, 2007). Other co-infections reported are malaria and leptospirosis (Wonsrichanalai *et al*., 2003), malaria and HIV (Skinner-Adams *et al*., 2008) and malaria and Q fever (Brouqui *et al*., 2005). There is paucity of literature addressing possible co-infections of *Rickettsia* and *Plasmodium* spp. among febrile patients in Kenya. This study

provides a compelling evidence for rickettsiosis-malaria co-infection in western Kenya, and raises dual issues concerning diagnosis and treatment. First, rickettsial illnesses manifest with unspecific clinical signs and can easily be confused with malaria. Secondly, it is a common practice in a malaria-endemic area that when acutely febrile patient is found to be malaria-positive, malaria is assumed as the sole cause of the fever (Wonsrichanalai *et al*., 2003). Arthropod vectors such as ticks and fleas are abundant in the tropics, a factor that may encourage exposition of humans to one or more vector-borne disease. Patients included in this study resided in the rural area of western Kenya where houses are made of earth/ mud, which may be conducive for flea breeding. There is also close interaction between people and animals where the two may share the houses especially with cats, dogs and chickens. This close proximity with farm animals puts people at a greater risk of exposure to rickettsial agents.

Spotted fever group *Rickettsia* cases were found to peak in the months of March, April and May in a study conducted in a span of 12 years in international travellers (Jensenius *et al*., 2009). In this study however, four peaks were observed in the months of January, May and November spanning from 1st of December 2008 to 28th of February 2010. The peaks observed in this study may be due to seasonal dynamics of fleas as determined by humidity and temperature. This hypothesis may be supported by a December and January peak in rodent fleas that was observed in Lushoto District of Tanzania, where seasonal dynamics of rodent fleas were studied (Makundi and Kilonzo, 1994). Similarly, drastic increase in the

number of ectoparasites in the months of April to July was observed in another study conducted in Iran between January 2006 and 2007 (Bahrami, 2010). The reason for the significantly higher infection rate in the month of January 2009 remains unclear. The data collected in this study cannot explain the high peak of *R. felis* infections observed in the month of January 2009, and can only speculate to have been caused by increased precipitation that may in turn cause the rats to seek shelter in human dwellings, bringing the vector in close to humans. This study was done in a period of 15 months which may not be adequate to elucidate proper temporal trends.

Rickettsia felis was detected in blood obtained from humans in western Kenya, after sequencing three genes namely; 17kDa, *R. felis ompB* and plasmid genes.This is the second time that *R. felis* was detected and reported in humans in Kenya, after a report in North Eastern Province (Richards *et al*., 2010). It also confirms previous reports on the occurrence of *R. felis* in in Senegal in West Africa (Socolovschi *et al*., 2010) and North Africa in Tunisia (Znazen *et al*., 2006). These findings demonstrate the importance of flea borne spotted fever in the indigenous population in Africa.*Rickettsia felis* is characterized by nonspecific clinical signs like fever, headache, joint pains and nausea (Perez-Arellano *et al*., 2005; Richards *et al*., 2010). As in other previous studies reported, cutaneous rash and/or inoculation eschar(s) were uncommon (Raoult *et al*., 2001b; Perez-Arellano *et al*., 2005; Socolovschi *et al*., 2010). Unusual signs reported elsewhere with *R.*

*felis*infection but not present in this study include neurological signs (Zavala-Velazquez *et al*., 2000). More studies have provided evidence of the presence of *R. felis* organism in febrile patients in Spain (Oteo *et al*., 2006; Perez-Arellano *et al*., 2005; Bernabeu-Wittel *et al*., 2006) and Germany (Richter *et al*., 2002).

In the present study, a *Rickettsia* plasmid was detected (pRF plasmid, strain URRWXCal2) in seven human samples and one flea sample (*Xenopsylla cheopis*) collected from *Rattus rattus*. The data corroborate results from previous reports by Pornwiroon *et al*. (2006) who detected a single plasmid (pRF) in cat fleas (*Ctenocephalides felis*) and Richards *et al*. (2010) in human samples. Other workers are of the opinion that the plasmid content may vary and that both plasmids may be present in some strains of *R. felis* (Fournier *et al*., 2008).

Domestic animals are important hosts for ticks and fleas and may act as useful sentinels for surveillance of rickettsial diseases. In this study, none of the ruminant (Cattle, goat and sheep) buffy coat and rodent spleen samples were positive for *Rickettsia* by *gltA* gene real-time PCR. However, a small proportion of cat (7.69%) and dog (3.68%) samples tested were positive using the *gltA* qPCR. Sequences from 17kDa antigen gene obtained from dog's and cat's specimens were 97% similar to *Rickettsia felis* URRWXCal2. *Rickettsia felis* has not previously been detected in mammals by PCR other than in experimental infection studies (Wedincamp and Foil, 2000). Cats exposed to fleas naturally infected with *R.*

*felis*were shown to seroconvert (Tsai *et al*., 2009). The role of animals in the epidemiology of flea-borne spotted fever has not been elucidated. Some workers speculate that animals may act as amplifier hosts (Capelli *et al*., 2009). Previously, only *Rickettsia parkeri* and *R. rickettsii* which cause a disease in dogs with similar clinical manifestation with RMSF in humans, that has been cultured and detected by molecular methods successfully (Kidd *et al*., 2006). *Rickettsia parkeri* was also detected in dogs' blood in Bolivia (Tomassone *et al*., 2010). Although *Rattus rattus* was documented to be the main reservoir of murine typhus in Kenya (Heisch, 1969; Heisch *et al*., 1962) and Indonesia (Okabayashi *et al*., 1999), spleen samples from small mammals tested in this study were negative for *Rickettsia* by PCR.

Tick borne spotted fever is the second most frequently identified cause of systemic febrile illness after malaria, among travellers to sub-Saharan Africa, (Freedman *et al*., 2006). This study demonstrates a high prevalence (96.9%) of infection with *R. africae* in adult *Amblyomma variegatum* collected from cattle and dogs in rural western Kenya. Other tick species from dogs in which *R. africae* was detected include *Haemaphysalis leachi*, *Boophilus decoloratus* and unidentified *Rhipicephalus* spp. These findings suggest endemicity of this pathogen in western Kenya and underscore the risks for zoonotic transmission to humans. This report corroborates similar findings from other studies where up to 100% infection rates of *A. variegatum* with *R. africae* were documented (Socolovschi *et al*., 2009).While very high infection rates in adult *A. variegatum* ticks were observed

in this study, significantly lower infection rates (15.5%) were reported in the same species of ticks collected in Maasai Mara in the Rift Valley province of Kenya (Macaluso *et al*., 2003) and elsewhere in Africa (Parola *et al*., 2001; Ndip *et al*., 2004b). In Africa, *Rickettsia africae* was also detected in other *Amblyomma*ticks species;*Amblyommalepidum* collected in Sudan (Parola *et al*., 2001) and in Djibouti (Socolovschi *et al*., 2007).

Amblyomma species are the main tick vectors of *R. africae*. They are primarily parasites of cattle and wild ungulates; they have been shown to readily feed on humans and hence are not host specific. Their aggressive nature has been documented where as high as 54% of patients had multiple inoculation eschars (Raoult *et al*., 2001a). In some parts of Africa, 100% attack rate with *R. africae* was observed in travellers to Swaziland emphasizing the risk of African tick-bite fever in Sub-Saharan Africa (Oostvogel *et al*., 2007).

For the first time in Kenya, *R. africae* was detected in *Haemaphysalis leachi* and *Boophilus decoloratus* ticks collected from dogs. Detection of *R. africae* in *Rhipicephalus (Boophilus) decoloratus* was not unexpected because it had been reported in *Boophilus decoloratus* in Botswana (Portillo *et al*., 2007) and in other closely related species, *Boophilus microplus* in the Caribbean (Robinson *et al*., 2009) and in *Boophilus annulatus* in Senegal (Mediannikov *et al*., 2010a). The finding of *Rickettsia africae* in *Haemaphysalis leachi* contrasts findings by Socolovschi *et al*. (2007) who found a different rickettsia (*Rickettsia conorii*) in

Haemaphysalis punctaleachi ticks collected in Uganda. *Rickettsia rickettsii* has previously been detected in a rabbit tick *Haemaphysalis leporispalustris* in Costa Rica (Hun *et al*., 2008). *Rhipicephalus sanguineus* is the principal vector for *Rickettsia conorii*. Although *R. sanguineus* was the most frequently encountered tick species in dogs (25.42%) in the study area, *Rickettsiae* were not detected in this tick species. This is in agreement with the report by Socolovschi *et al*. (2007) who failed to detect any *Rickettsiae* in *R. sanguineus*. Demma *et al*. (2005) however, found *Rickettsia rickettsii* in *R. sanguineus* ticks in Arizona, United States. These results clearly show that *Amblyomma variegatum* parasitizing cattle and dogs are highly infected with *Rickettsia africae* which may be a potential source of infection to humans. Although *R. africae* causes a milder form of diseases, physicians need to be aware of this disease so that they do not confuse it with other potentially more devastating illnesses requiring more aggressive therapy, and that appropriate treatment is provided.

The results of this study showed that indeed the dogs, cats, rodents and homes in the study area contained arthropod vectors known to transmit flea-borne *Rickettsiae*. It emerged that the most prevalent flea in dogs and house environment was *Ctenocephalides felis,* while in cats it was *Echidnophaga gallinacea*. In the rodents however, *Xenopsylla cheopis* was the most abundant flea species. This is consistent with a previous study conducted in Egypt on flea collection in rodents (Loftis *et al*., 2006) and Brazil (Milagres *et al*., 2010). Themajority of houses

within the study area have earth floors (Bigogo *et al*., 2010), a condition that makes excellent breeding ground for fleas. The earth floors and the presence of free-roaming chickens, dogs and cats may explain high prevalence of fleas in the study area. In a study conducted in Northern Iran, it was demonstrated that dogs kept in houses with floors covered by soil and grass were more likely to have ectoparasites compared to those in cemented floors (Bahrami, 2010).

High infection rates with *Rickettsia spp*. (96-100%) were observed in *Ctenocephalides spp* in this study. Overall infection rate of *X. cheopis* (8.57%) was lower than that of *Ctenocephalides* species. The implied prevalence rate is comparable to a previous study where 90% prevalence of *R. felis* was recorded in *C. felis*fleas from dogs in Brazil (Horta *et al*., 2005). In contrast to the findings of this study, significantly lower infection rates of *R. felis*were recorded in naturally infected *C. felis* collected from rats in Cyprus (5.6%) (Psarouki *et al*., 2006) and *C. felis* from cats and dogs in Israel (7.6%) (Bauer *et al*., 2006). Other studies reported >90% infection rates with the *R. felis* (ELB agent) in colonized cat fleas (Adams *et al.*, 1990; Reif *et al*., 2008). Horta *et al*., (2010) noted 100% prevalence of *R. felis* in a cat flea colony (*Ctenocephalides felis*). In a study conducted in Italy, *C. canis* collected from dogs and cats were negative for *Rickettsia*(Capelli *et al*., 2009).Nevertheless, this study reported 100% infection rate of *C. canis* with *Rickettsia spp*.In the present study, rickettsial pathogens were not detected in *Echidnophaga gallinacea, Pulex irritans* and *Tunga Penetrans*. On the

contrary,previous studies observed that rickettsiae were prevalent in these flea species (*P. irritans, E. gallinacea* and *T. penetrans*) (Sackal *et al*., 2008; Mourad *et al*., 2009). *Purex* species and *Tunga penetrans* (Muehlen et al., 2003) readily feed on humans and may represent a source of human exposure to *Rickettsia*. In New Mexico (USA), *R. felis* was detected in wild rodent (*Neotoma albigula*) flea species (*Amomiopsyllus nudata*) (Stevenson *et al.*, 2005).

The identity of the *Rickettsiae* was confirmed by sequencing of 17kDa and *R. felisompB* genes, which determined that of 12 samples assessed, only one was *R. felis*, the other 11 samples contained DNA of unique rickettsial agent. While pRF plasmid was detected in one of 12 fleas samples assayed, no plasmids were amplified in the 11/12 flea DNA samples. The flea specimen (*Xenopsylla cheopis*)from which *R. felis* was detected was collected from a rodent (*Rattus rattus*). This is the first report of *R. felis* naturally infecting the Oriental flea (*X. cheopis*) in Kenya. *Rickettsia felis* was previously detected in *X. cheopis* in Indonesia (Jiang *et al*, 2006) and the Democratic Republic of Congo (Sackal *et al*., 2008). *Xenopsylla cheopis* is the vector of *Yersinia pestis*, the cause of plague and *R. typhi*, the cause of murine typhus. Jiang *et al*. (2006) also detected *R. typhi* from *X. cheopis* fleas (12.82%) in Indonesia. In contrast, *R. typhi* was not detected in this study. *Xenopsylla cheopis* has previously been shown to be efficient in transmitting *Rickettsia* and was suggested to be a possible vector for *Rickettsia* species (Heisch, 1969). The finding of unique sequences using 17kDa antigen

gene and *ompB* gene necessitated further work using Multi-locus sequence typing (MLST) as described by Fournier *et al.*, (2003). Further characterization of this agent by MLST determined that it was most closely related to the one validated species, *R. felis*, but did not have enough nucleotide homology to *R. felis* to be considered as the same species. The new rickettsial agent was found to have close nucleotide similarity (>99%) to other unvalidated *Rickettsiae*; *Rickettsia* spp. RF2125, *R*ickettsia spp. RF31, SGL01 and *Candidatus R. dielmo*, *R. felis*-like genotypes found in fleas, mites and tsetse flies (Parola *et al* 2003, Reeves *et al* 2005, Bitam *et al*., 2006, Loftis *et al* 2006, Reeves *et al* 2007, Nelder *et al* 2009, Hornok *et al* 2010).

It was noted that the *R. felis* qPCR assay (Henry *et al* 2007) previously thought to be *R. felis* specific should no longer be considered species-specific as the target sequence is found both in *R. felis* and in *CandidatusR. asemboensis*. Sequencing of 17kDa and *ompB* gene showed that 11/12 flea specimen contained the new agent. Although testing of the flea specimens showed that the new agent is very prevalent (>90%) among fleas known to bite humans, it is intriguing to note that none of the human patients tested from the same study area had this new *Rickettsia* agent. Instead, *Rickettsia felis* was detected in 50 of 699 febrile patients tested. Similarly, in a study of non-malaria fever patients in North Eastern Province of Kenya, 6 of 163 patients had evidence of FBSF, 6 of 6 samples were verified by sequencing to

be *R. felis* (Richards *et al* 2010). Thus, it appears that *Candidatus* R. asemboensis may not be highly pathogenic for humans.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

- High prevalence (56%) of IgG antibodies against spotted fever group *Rickettsia* was detected among the patients attending Lwak hospital in western Kenya.
- Three hundred microlitres of blood clot resuspended in an equal volume of normal saline was the most optimum for DNA extraction. A cycle threshold of ≤40 was determined to be the most optimum cut-off for screening of patients for *Rickettsia* using the *gltA* assay.
- Febrile patients were 2.2 times more likely to have *R. felis* compared to asymptomatic individuals; therefore *R. felis* infection may be contributing to the burden of febrile illness in western Kenya.
- At least two human pathogenic *Rickettsia*e;*Rickettsia africae*,*R. felis*and one novel species, "*Candidatus Rickettsia asemboensis*" was described in this study in ticks and fleas.*Rickettsiae* are not prevalent in domestic ruminants and peri-domestic small mammals (0%) and hence they may only be involved indirectly in the maintainance of the vectors (*Amblyomma* spp). *Rickettsiae* were detected in cat and dog blood, suggesting that they may play an important role in the life cycle of *Rickettsiae*.

- Despite the multiplicity of circulating strains, this study detected similar *Rickettsia* spp. in fleas obtained from peri-domestic small mammals and humans in the same locality. A high degree of homology (100%) among the human isolates and one isolates from *Xenopsylla cheopis* was recorded.

6.2 Recommendations and scope for further work

- Testing of clinical serum with specific antigens of *R. africae* and *R. felis* (the two *Rickettsiae* found to be prevalent in the study area) and *R. typhi* would explain the *Rickettsia* that is responsible for high IgG seroprevalence in the study area and clarify the issue of cross-reactivity.
- Whether detection of *R. felis* in humans with fever was directly related or incidental remains to be assessed through future epidemiologic investigations.
- Further surveillance for *Rickettsiae* may detect additional vectors and/or hosts that may be involved in transmission of pathogenic strains to humans.
- Exploration of temporal and spatial patterns of disease occurrence, and relation to vector activity and environmental variables may allow for prediction of disease risk and identification of potential control measures.
- There is need to do isolation of *Candidatus Rickettsia asemboensis*, perform pathogenecity testing and interference studies to determine the biological characteristic of this agent. *Rickettsia felis* qPCR assay (Henry et

al., 2007) is no longer species specific hence, there is need to develop species specific qPCR assays to detect *R. felis* and *Candidatus Rickettsia asemboensis*, respectively.

REFERENCES

Abramson J.S. and Givner L.B. (1990). Should tetracyclines be contraindicated for therapy of presumed Rocky Mountain Spotted fever in children less than 9 years of age? *Pediatrics,* **86**: 123-124.

Adams J.R., Schmidtmann E.T. and Azad A.F. (1990). Infection of Colonized Cat Fleas, *Ctenocephalides felis* (Bouche') with a *Rickettsia*-like Microorganism. *American Journal of Tropical Medicine and Hygiene*, **43**(2): 400-409.

Anstey N.M., Dupont H.T., Hahn C.G., Mwaikambo E.D., McDonald M.I., Raoult D. and Sexton D.J.(1997).Seroepidemiology of *Rickettsia typhi*, Spotted Fever Group *Rickettsiae*, and *Coxiella burnetii* Infection in Pregnant Women from Urban Tanzania. *American Journal of Tropical Medicine and Hygiene*, **57**(2):187-189.

Archibald L.K. and Reller L.B. (2001). Clinical Microbiology in Developing Countries. *Emerging Infectious Diseases,* **7**:302-305

Azad A.F. and Beard C.B. (1998). Rickettsial Pathogens and their Arthropod Vectors. *Emerging Infectious Diseases*, **4(**2): 179-186**.**

Azad A.F. and Radulovic S. (2003). Pathogenic *Rickettsiae* as Bioterrorism Agents. *Annals of New York Academy of Sciences*, **990**: 734-738.

Azad A.F., Sacci J.B., Nelson W.M., Dasch G.A., Schmidtmanns E.T. and Carl M. (1992). Genetic Characterization and Transovarial Transmission of a Typhus-like *Rickettsia* found in Cat Fleas. *Proceedings of National Academy of Sciences.* USA, **89**: 43-46

Badiaga S., Brouqui P. and Raoult D. (2005). Autochthonous Epidemic typhus associated with Bartonella Quintana Bacteraemia in a Homeless person. *American Journal of Tropical Medicine and Hygiene*. **72**(5): 638-639.

Bahrami A.M. (2010). Common Ectoparasites Species of Domestic dogs in Western Iran. *World Applied Sceinces Journal*, **8**(10):1277-1281.

Barandika J.F., Hurtado A., Garcia-Esteban C., Gil H., Escudero R., Barral M., Jado I., Juste R.A., Anda P. and **Garcia-Perez A.L.** (2007). Tick Borne Zoonotic Bacteria in Wild and Domestic small mammals in Northern Spain. *Applied and Environment Microbiology*, **73**(19): 6166-6171.

Bauer O., Baneth G., Eshkol T., Shaw S.E. and Harrus S. (2006). Polygenic Detection of *Rickettsia felis* in Cat Fleas (*Ctenocephalides felis*) from Israel. *American Journal of Tropical Medicine and Hygiene*, **74**(3): 444-448

Beltrán, R.R. and Herrero Herrero J.I. (1992). Deleterious effect of trimethoprimsulfamethoxazole in Mediterranean spotted fever. *Antimicrobial Agents and Chemotherapy*. **36**:1342-3

Bernabeu-Wittel M., Del Toro M.D., Nogueras M.M., Muniain M.A., Cardenosa N., Márquez F.J., Segura F. and Pachón J. (2006). Seroepidemiological study of *Rickettsia felis, Rickettsia typhi,* and *Rickettsia*

conorii infection among the population of southern Spain. *European Journal of Clinical Microbiology and Infectious Diseases*, **25**(6): 375-381.

Bigogo G., Audi A., Aura B., Aol G., Breiman R.F. and Feikin D.R. (2010). Health seeking patterns among Participants of Population-based Morbidity Surveillance in Rural western Kenya: Implications for calculating Disease rates. *International journal of Infectious diseases*. **14**:967-973

Bitam I., Parola P., Dittmar De La Cruz K., Matsumoto K., Baziz B., Rolain J.M., Belkaid M. and Raoult D. (2006). First Molecular Detection of *Rickettsia felis* in Fleas from Nigeria. *American Journal of Tropical Medicine and Hygiene*, **74**(4): 532-535.

Bitam I., Parola P., Matsumoto K., Rolain J.M., Baziz B., Boubidi S.C., Harrat Z., Belkaid M. and Raoult D. (2006a). First Molecular Detection of *Rickettsia conorii, R. aeschlimannii* and *R. massiliae* in Ticks from Algeria. *Annals of New York Academy of Sciences*, **1078**: 368-372.

Blair P.J., Schoeler G.B., Moron C., Anaya E., Cespedes M., Cruz C., Felices V., Guevara C., Mendoza L., Villaseca P., Sumner J.W., Richards A.L. and Olson J.G. (2004). *Journal of Clinical Microbiology*. **42**(11): 4961-4967.

Blanc G., Ogata H., Robert C., Audic S., Suhre K., Vestris G., Claverie J. and Raoult D. (2007). Reductive Genome Evolution from the Mother of *Rickettsia*. *PLoS Genetics*, **3**(1): 0103-0114

Blanco J.R. and Oteo J.A. (2006). Rickettsiosis in Europe. *Annals New York Academy of Sci*ence, **1078**: 26-33.

Blancou, J., Chomel, B.B., Belotto, A. and **Meslin, F.X.** (2005). Emerging or re-emerging bacterial zoonoses: factors of emergence, surveillance and control.*Veterinary Research*,**36**: 507–522

Brouqui P., Rolain J.M., Foucault C. and Raoult D. (2005). Q fever and plasmodium falciparum malaria co-infection in a Patient returning from the Comoros Archipelago. *American Journal of Tropical Medicine and Hygiene*, **73**(6): 1028-1030.

Buchau, A.S., Wurthner J.U., Reifenberger, J. And Ruzicka T. (2006). Fever, Episcleritis, Epistaxis, And Rash After Safari Holiday In Swaziland Archives *of Dermatolology*, 142:1365-1366.

Capelli G., Montarsi F., Porcellato E., Maioli G., Furnari C., Rinaldi L., Oliva G. and Otranto D. (2009). Occurrence of *Rickettsia felis* in dog and Cat Fleas (*Ctenocephalides felis*) from Italy. *Parasites and Vectors*, **2**(Supplement 1).

Caraguel C.G.B., Stryhn H., Gagne N., Dohoo I.R. and **Hammell K.L.** (2011). Selection of a cutoff point for a real-time polymerase chain reaction results to fit a diagnostic purpose: analytical diagnostic purpose. *Journal of Veterinary Diagnostic Investigation*. **23**:2-15

Cleaveland S., Laurenson M.K. and Taylor L.H. (2001). Diseases of Humans and their Domestic Mammals: Pathogen Characteristics, Host Range and the Risk of Emergence. *Philosophical Transaction of the Royal Society B*, **356**: 991-999

Corwin, A., M. Habib M., Olson J.G., Scott D., Ksiazek T.G. and Watts D.M. (1993). The prevalence of arboviral, rickettsial, and Hantaan-like viral antibody among school children in the Nile river delta of Egypt. *Transaction of Royal Society of Tropical Medicine Hygiene*, **86:** 677–679.

Dalton.M.J., Clarke M.J., Holman R.C., Krebs J.W., fishbein D.B., Olson J.G., and Childs J.E. (1995). National Surveillance for Rocky Mountain spotted Fever, 1981-1992: Epidemiologic Summary and Evaluation of Risk Factors for Fatal Outcome. *American Journal for Tropical Medicine and Hygiene*, **52**(50):405-413.

Daszak P., Cunningham A.A. and Hyatt A. (2000). Emerging Infectious diseases of Wildlife- Threats to Biodiversity and Human Health. *Science*, **287**: 443-449.

Demma L.J., Traeger M.S., Nicholson W.L., Paddock C.D., Blau D.M., Eremeeva M.E., Dasch G.A., Levin M.L., Sigleton J., Zaki S.R., Cheek J.E., Swerdlow D.L. and Mcquiston J.H. (2005). Rocky Mountain Spotted Fever from Unexpected Tick Vector in Arizona. *The New England Journal of Medicine*, **353**(6): 587-594.

Do E.J., Kim J.E., Park J.M., Lee K.M., Jung M.Y., Lee H.J., Cho H.W., Choi Y.J., Lee S.H., Park K.H. and Jang W.J. (2009). Development of Recombinant ompA and ompB Proteins as Diagnostic Antigens for Rickettsial Disease. *Microbiology and Immunology*. **53**(7): 368-374

Eremeeva. M.E., Dasch G.A. and Silverman D.J. (2001). Quantitative Analyses Of Variations In The Injury Of Endothelial. *Clinical and Diagnostic Laboratory Immunology*, **8**(4):788–796.

Fang R. and Raoult D. (2003). Antigenic Classification of *Rickettsia felis* by Using Monoclonal and polyclonal Antibodies. *Clinical and Diagnostic Laboratory Immunology*, **10**(2): 221-228.

Fawcett T. (2006). An introduction to ROC analysis. *Patterns Recognition Letters*, **27**:861-874

Feikin D.R., Olack B., Bigogo G.M., Audi A., Cosmas L., Aura B., Burke H., Njenga M.K., Williamson J. and Breiman R.F. (2011). The Burden of Common Infectious Disease Syndromes at the Clinic and Household Level from a Population-based surveillance in rural and Urban Kenya. *Plos One*. **6**:e16085.

Fournier P.E., Belghazi L., Robert C., Elkarkouri k., Richards A.L., Greub G., Collyn F., Ogawa M., Portillos A., Oteo J.A., Psaroulaki A., Bitam I. and Raoult D. (2008). Variations of Plasmid Content in *Rickettsia felis.Plos ONE*, **3**(5): e2289.

Fournier P. and Raoult D. (2009) Current Knowledge on Phylogenyand Taxonomy of *Rickettsia* spp**.** *Annals New York Academy of Science*, **1166**: 1–11.

Fournier P.E., Dumler S., Greub G., Zhang J., Wu Y. and Raoult D. (2003). Gene Sequence-based Criteria for Identification of New *Rickettsia* Isolates

and Description of *Rickettsia heilongjiangensis* sp. Nov. *Journal of Clinical Microbiology*. **41**(12): 5456-5465.

Fournier P., Tissot-Dupont H., Gallais H., Raoult D. (2000b). *Rickettsia mongolotimonae*: A Rare Pathogen in France. *Emerging Infectious Diseases*, **6** (3): 290-292

Fournier P.E. and Raoult D. (2007). Identification of rickettsial Isolates at the species Level using multi-spacer typing. *BMC Microbiology*, **7**:72-77

Fournier, P.E., Grunnenberger F., Jaulhac B., Gstinger G. **and Raoult D.** (2000a). Evidence Of *Rickettsia Helvetica* Infection In Humans, Eastern France. *Emerging Infectious Diseases,* **6:** 389–392

Fournier, P.E., Karkouri E.L., Leroy K., Robert Q., Giumelli C., Renesto B., Socolovschi P., Parola C., Audic P. and Raoult, D. (2009). Analysis of the *Rickettsia africae* genome reveals that virulence acquisition in *Rickettsia*species may be explained by genome reduction. *BMC Genomics*, **10**:166

Freedman D.O., Weld L.H., Kozarsky P.E., Fisk T., Robins R., Sonnenburg F.V., Keystone J.S., Pandey P. and Cetron M.S. (2006). Spectrum of Disease and Relation to Place of Exposure among Ill Returned Travellers. *New England Journal of Medicine*, **354**(2): 119-130.

Gasser A.M., Birkenheuer A.J. and Breitschwerdt E.B. (2001). CanineRockyMountain Spotted fever: a retrospective study of 30 cases. *Journal of the American Animal Hospital Association*, **37**(1): 41-48

Gillespie J.J., Ammerman N.C., Dreher-Lesnick S.M., Rahman M.S., Worley M.J., Sebtubal J.C., Sobral B.S. and Azad A.F. (2009a). An Anomalous Type IV Secretion System in *Rickettsia* is Evolutionarily Conserved. *PLoS one*, **4**(3): 1-24

Gillespie J.J., Ammerman N.C., Beier-Sexton M., Sobral B.S. and Azad A.F. (2009b). Louse-and Flea-borne rickettsioses: biological and genomic analyses. *Veterinary Research*, **40**(2):12

Gillespie, J.J., Beier, M.S., Rahman, M.S., Ammerman, N.C., Shallom, J.M., J.M., Purkayastha, A., Sobral, B.S. and **Azad A.F.** (2007). Plasmids and Rickettsial Evolution: Insight from *Rickettsia felis*. *PLoS ONE*, **2**(3): e266. pp 1-17.

Goffeau A., Barell B.G., Bussey H., Davis R.W., Dujon B., Feldmann H., Galibert F.Hohessel J.D., Jacq C. and Others. (1996). Life with 6000 Genes. *Science*. **274** (5287): 546

Gray G.C., Rodier G.R., Matra-Maslin V.C., Honein M.A., Ismail E.A., Botros B.A. Soliman A.K., Merrell B.R., Wang S.P. and Grayston T.J. (1995). Serologic evidence of Respiratory and Rickettsial Infections among Somali Refugees. *American Journal of Tropical Medicine and Hygiene*, **52**(4): 349-353.

Grossman E.R., Walchek A. and Freedman H. (1971). tetracyclines and permanent teeth: the relation between dose and tooth colour. *Pediatrics*, **47**:567-570.

Hechemy K.E., Avsic-Zupanc T., Childs J.E. and **Raoult D.A.** (2003). Rickettsiology: Present and future directions. *Annals of New York Academy of Sciences,***990**(1): 264-266.

Hechemy K., Oteo J.A., Raoult D., Silverman D.J. and Blanco J.R. (2006). A century of Rickettsiology: Emerging, Re-emerging Rickettsioses, Clinical epidemiologic and Molecular Diagnostic Aspects and Emerging Veterinary Rickettsioses. *Annals of New York Academy of Sciences,* **1078**:1-14.

Hechemy K.E., Stevens R.W., Sasowski S., Michaelson E.E., Casper E.A. and Phillip R.N. (1979). Discrepancies in Weil-Felix and Micro-immunofluorescence Test Results for Rocky Mountain Spotted Fever. *Journal of Clinical Microbiology***. 9:**409-415.

Heisch R.B., Grainger W.E., Harvey E.C. and Lister G. (1962). Feral Aspects of Rickettsial Infections in Kenya. *Transaction of the Royal Society of Tropical Medicine and Hygiene,* **56**: 272-286

Heisch R.B., McPhee R. and Rickman L.R. (1957). The Epidemiology of Tick-Typhus in Nairobi. *East African Medical Journal*, **34**(9): 459-477

Heisch RB. (1969). Urban *Rattus* as the main Reservoir of Murine Typhus in Kenya. *Journal of Tropical Medicine and Hygiene*. **72**:195-196

Henry K.M., Jiang J., Rozmajzl P.J., Azad A.F., Macaluso K.R. and Richards A.L. (2007). Development of Quantitative Real-time PCR assays to detect *Rickettsia typhi* and *Rickettsia felis*, the causative agents of Murine typhus and Flea-borne Spotted Fever. *Molecular and Cellular probes,* **21**:17-23.

Hernandez-Cabrera, M., Angel-Moreno A., Sanatana E., Bolanos M., Frances A., Martin-Sanchez A and Perez-Arellano J.L. (2004). Murine Typhus with Renal Involvement in Canary Islands, Spain. *Emerging Infectious Diseases*, **10:** 740–743

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

Horta M.C., Scott F.B., Correia T.R., Fernandes J.I., Richtzanhain L.J. and Labruna M.B. (2010). *Rickettsia felis* Infections in Cat Fleas *Ctenocephalides felis felis. Brazilian Journal of Microbiology*, **41**: 813-818.

Horta M.C., Pinter A., Cortez A., Soares R.M., Gennari S.M., Schumaker T.T.S. and Labruna M.B. (2005). *Rickettsia felis* (*Rickettsiales: Rickettsiaceae*) in *Ctenocephalides felis felis* (Siphonaptera: Pulicidae) in the State of Sao Paulo, Brazil. *Brazilian Journal of Veterinary Medicine*, 57(3):321-325

http://blast.ncbi.nlm.hih.gov

http://www.R.project.org

Hun L., Cortes X. and Taylor L. (2008). Molecular Characterization of *Rickettsia rickettsii* Isolated from Human Clinical Samples and from the Rabbit Tick Haemaphysalis leporispalustris collected at Different Geographical Zones in Costa Rica. *American Journal of Tropical Medicine and Hygiene*, **79**(6): 899-902.

Jado I., Escudero R., Gil H., Jime´nez-Alonso M.I., Sousa R., Garcı´a-Pe´rez, A.L., Rodrı´guez-Vargas M., Lobo B. and **Anda P.** (2006). Molecular method for identification of *Rickettsia* spp. In clinical and environmental samples. *Journal of Clinical Microbiology*, **44(**12): 4572-4576.

Jelinek T. and loscher T. (2001). Clinical Features and Epidemiology of Tick Typhus in Travellers. *Journal of Travel Medicine*, **8**:57-59.

Jensenius M., Davis X., Sonnenburg F.V., Schwartz E., Keystone J.S., Leder K., Lopez-Velez R., Caumes E., Cramer J.P., Chen L. and Parola P. (2009). Muilticenter Geosentinel Analysis of Rickettsial Diseases in International Travellers, 1996-2008. *Emerging Infectious Diseases*, **15**(11): 1791-1798.

Jensenius M., Fournier, P.E., Kelly, P., Myrvang, B., and Raoult, D. (2003) African Tick-Bite Fever. *Lancet Infectious Diseases*, **3**:557-64.

Jensenius M., **Fournier P.E., Vene S., Hoel T., Hasle G., Henriksen A.Z., Hellum K.B., Raoult D. and Myrvang B**. (2003b). African Tick-Bite Fever in Travellers to Rural Sub-equatorial Africa. *Clinical Infection Diseases*, 36: 1411-1417.

Jiang J., Chan T., Temenek J.J., Dasch G.A., Ching W. and **Richards A.L.** (2004). Development of a Quantitative Real time Polymerase Chain Reaction Assay Specific for Orentia Tsutsugamushi. *American Journal of Tropical Medicine and Hygiene*, **70(4):** 351-356

Jiang J., Sangkasuwan, V., Lerdthusnee.K. Sukwit, S., Chuenchitra, T., Rozmajzl, P.J., Chirapa Eamsila., Jones, J.W. and Richards, A.L. (2005**).**

Human Infection with *Rickettsia honei*, Thailand. *Emerging Infectious Diseases*, 11(9): September 2005.

Jiang J., Soeatmadji D.W., Henry K., Ratiwayanto S., Bangs M.J., and **Richards A.L.** (2006). *Rickettsia felis* in *Xenopsylla cheopis*. Java. Indonesia, *Emerging Infectious Diseases*, **12**:1281-1283.

Jones K.E., Patel N.G., Levy M.A., Storeygard A., Balk D., Gittleman J.L. and Daszak P. (2008). Global Trends in Emerging Infectious Diseases. *Nature*, **451**:99-993.

Kamalanathan T. and Rohani M.Y. (2003). Antibody Prevalence of *Orentia Tsutsugamushi, Rickettsia typhi* and TT118 spotted Fever Group *Rickettsiae* among Malaysian Blood Donors and Febrile Patients in the Urban Areas. *Southeast Asian Journal of tropical medicine and Public health*, **34**(1): 165-170

Kaplan J.E. and Schonberger L. B (1986). The Sensitivity of Various Serologic Tests in the Diagnosis of Rocky Mountain Spotted Fever. *Journal of Tropical Medicine and Hygiene*,**35**(4): 840-844.

Kelly P.J., Mason P.R., Mathewman L.A. and Raoult D. (1991). Seroepidemiology of Spotted Fever Group Rickettsial Infections in Humans in Zimbabwe. *Journal of Tropical Medicine and Hygiene*, **94**: 304-309.

Kidd L., Hegarty B., Sexton D. and Breitschwerd E. (2006).Molecular Characterization of *Rickettsia Rickettsii* Infecting Dogs And People In North Carolina. *Annals of New York Academy of Sciences*, **1078**: 400–409.

Kilpatrick A.M., Kramer L.D., Jones M.J., Marra P.P. Daszak P. and Foneseca D.M. (2007). Genetic Influence on Mosquito Feeding Behavior and the Emergence of Zoonotic Pathogens. *Journal of Tropical Medicine and Hygiene*, **77** (4) 667-671.

Kruse H. Kirkemo, A.M. and **Handeland K.** (2004). Wildlife as a Source of Zoonotic Infections. *Emerging Infectious Diseases*, **10**:2067-2072

Kumar S. (2010). Travel Acquired Scrub Typhus. *JKScience*. **12**(2): 65-66.

Kutz S.J., Hoberg E.P., Nagy J. Polley L. and **Elkins B**. (2004). "Emerging" Parasitic infections in Artic Ungulates. *Integrative and Comparative Bioliology*, **44**: 109-118.

La Scola B. and Raoult D. (1996). Diagnosis of Mediterranean Spotted Fever by Cultivation of *Rickettsia conorii* from Blood and Skin Samples Using the Centrifugation-Shell Vial Technique and by Detection of *R. conorii* in Circulating Endothelial Cells: a Six-Year Follow-Up. *Journal of Clinical Microbiology*, **34**(11): 2722-2727

La Scola B. and Raoult D. (1997). Laboratory Diagnosis of Rickettsioses: Current Approaches to Diagnosis of Old and New Rickettsial Diseases. *Journal of Clinical Microbiology*, 35(11): 2715-2727.

Letaief A. (2006). Epidemiology of Rickettsioses in North Africa. *Annals of New York Academy of Sciences*, **1078**: 34-41.

Lledo L., Gequndez I., Ruiz E., Rodriguez L., Bacellar F. and Saz J.V. (2003). *Rickettsia typhi* Infection in Wild Rodents. *Annals of Tropical Medicine and Parasitology***97**(4) 411-414.

Loftis A.D., Reeves W.K., Szumkas D.E., Abbassy M., Helmy I.M., Moriarity J.R. and **Dasch G.A.** (2006). Surveillance of Egyptian Fleas for Agents of Public Health Significance: *Anaplasma, Bartonella, Coxiella, Ehrlichia, Rickettsia* and *Yersinia pestis. American Journal for Tropical medicine and Hygiene*, **75**(1): 41-48.

Macaluso, J.D., Davis, J., Alam, U., Korman, A., Rutherford, J.S., Resenberg, R., and Azad, A.F. (2003) Spotted Fever Group *Rickettsiae* in Ticks from the Maasai Mara Region of Kenya. *American Journal of Tropical Medicine and Hygiene*, **68**(5): 551-3.

Mahara F. (2006). Rickettsioses in Japan and Far East. *Annals of New York Academy of Sciences*, 1078: 60-73.

Makundi R.H. and Kilonzo B.S. (1994). Seasonal Dynamics of Rodent Fleas and its Implication on Control Strategies in Lushoto District, North-Eastern Tanzania. *Journal of Applied Entomology*, **118**:165-171.

Marquez F.J., Muniain M.A., Rodriguez-Liebana J.J., Del Toro M.D., Bernabeu Wittel M and Pacho A.J. (2006). Incidence and Distribution Pattern of *Rickettsia Felis* In Peri-domestic Fleas From Andalusia, Southeast Spain. *Annals of New York Academy of Sciences*, **1078**: 344–346.

Martin, S.W., Meek, A.H. and Willeberg, P. (1987). Veterinary Epidemiology, Principles and Methods. Iowa State University Press, Ames, Iowa.

Masters E.J., Olson G.S. Scott J. Weiner S.J. and Paddock C.D. (2003). Rocky Mountain Spotted Fever: A Clinician's Dilemma. *Archives Internal Medicine,* **163**:769-774

McDADE, J.E. (1990). Presented at the 4th International Symposium on *Rickettsiae* and Rickettsial Diseases. Piestany, C.S.F.R., 1–6 October, 1990.

Mediannikov O., Diatta G., Fenollar F., Sokhna C., Trape J.F. and Raoult D. (2010a). Tick-borne Rickettsioses, Neglected Emerging Diseases in Rural Senegal. *PloS Neglected Tropical Diseases,* 4(9):e821.

Mediannikov O., Makarova V., Tarasevich I., Sidelnikov Y. and Raoult D. (2009). Isolation of *Rickettsia heilongjiangensis* Strains from Humans and Ticks and its Multispacer Typing. *European society of Clinical Microbiology and Infectious Diseases*. CMI, **15**(supplement 2): 288-289.

Mediannikov O., Matsumoto K., Samoylenko I., Drancourt M., Roux V., Rydkina E., Davoust B., Tarasevich I., Brouqui P. and Fournier P.E. (2008). *Rickettsia raoultii sp*. Nov., a Spotted Fever Group *Rickettsia* Associated with *Dermacentor* Ticks in Europe and Russia. *International Journal of Systematic and Evolutionary Microbiology,* **58**:1635-1639.

Mikszewski J.S. and Vite C.H. (2005). Central Nervous system Dysfunction Associated with Rocky Mountain Spotted fever Infection in Five Dogs. *Journal of the American Animal Hospital Association*. **41**: 259-266

Milagres B.S., padilha A.F., Barcelos R.F., Gomes G.G., Montandon C.E., Pena D.C.H., Nieri Bastos F.A., Silveira I., Pacheco R., Labruna M.B., Bouyer D.H., Freitas R.N., Walker D.H., Mafra C.L. and Galvao M.A.M. (2010). *Rickettsia* in Synaanthropic and Domestic Animals and their Hosts from Two Areas of Low Endemicity for Brazilian Spotted Fever in the Eastern Region of Minas Gerais, Brazil. *American Journal of Tropical Medicine and Hygiene*. **83**(6): 1305-1307.

Moran N.A. and Mira A. (2001).The process of genome shrinkage in the obligate symbiont *Buchnera aphidicola Genome Biology*,**2**(12): RESEARCH 0054

Mourad W.G., Henn J., Foley P., Chomel B.B. (2009). Zoonotic Bartonella Species in Fleas Collected on Gray Foxes (*Urocyon cinereoarggenteus*). *Vector-Borne and Zoonotic Diseases*,**9**(6): 597-602

Muehlen M., Heukelbach J., Wilcke T., Winter B., Mehlhorn H. and Feldmeier H. (2003). Investigation on the Biology, Epidemiology, Pathology and Control of *Tunga penetrans* in Brazil. *Parasitology Research*, **90**:449-455

Ndip, L.M., Bouyer, D.H., Amelia, P.A., Travassos, D.A., Titanji, V.P.K., Tesh, R.B., and Walker, D.H. (2004a). Acute Spotted Fever Rickettsiosis among Febrile Patients, Cameroon. *Emerging Infectious Diseases*, **10**(3): 432-437.

Ndip, L.M., Fokam, E.B. Bouyer, D.H., Ndip, R.N., Titanji, P.K., Walker., D.H., and McBRIDE, J.W. (2004b). Detection of *Rickettsia africae* in

patients and ticks along the coastal region of Cameroon. *Journal of Tropical Medicine and Hygiene*, 71 (3): 363-366.

Nelder MP, Reeves WK, Adler PH, Wozniak A, Wills W. (2009). Ectoparasites and associated pathogens of free-roaming and captive animals in zoos of South Carolina. Vector Borne Zoonotic Diseases. 9:469-77.

Niang M., Parola P., Tissot-Dupont H., Baidi Lo., Brouqui P. and Raoult D. (1998). Prevalence of Antibodies to *Rickettsia conorii*, *Rickettsia africae*, *Rickettsia typhi* and *Coxiella burnetii* in Mauritania. *European Journal of Epidemiology*, **14**: 817-818.

Nurisa I., Okabayashi T., Ristiyantfvo, Lestari E.W., Yanase T., Maramatsu Y., Ueno H. and Morito C. (1999). Serosurvey of Wild rodents for Rickettsioses (spotted fever, murine typhus and Q fever) in Java Island, Indonesia. *European Journal of Epidemiology*. **15**(1): 89-93

Ogata H., La Scola B., Audic S., Renesto P., Blanc G., Robert C., Fournier P.E., Claverie J.M. and Raoult D. (2006). Genome Sequence of *Rickettsia bellii* Illuminates the Role of *Amoebae* in Gene exchanges between Intracellular Pathogens. *PloS Genetics*. **2**(5): 733-744.

Ogata H., Renesto P., Audic S., Robert C., Blanc G., Fournier P., Parinello H., Claverie J. and Raoult D. (2005). The Genome Sequence of *Rickettsia felis* identifies the First Putative Conjugative Plasmid in an Obligate Intracellular Parasite. *PLoS Biology*, **3**(8): 1391-1402.

O.I.E., (2005). Rocky Mountain spotted fever. The Center for Food Security and Public Health. IOWA State University.

Olano J.P. (2005). Rickettsial Infections. *Annals of New York Academy of Sciences*, **1063**: 187–196 (2005).

Oostvogel P.M., Van Doornum G.J., Ferreira R., Vink J., Fenollar F. and Raoult D. (2007). African Tick-bite Fever in Travellers, Swaziland. *Emerging Infectious Diseases*, **13(**2): 353-355.

Ormsbee R., PeaCock M., Philip R., Casper E., Plorde J., Gabre-Kidan T. and Wright L. (1978). Antigenic Relationships between the Typhus and spotted fever Groups *Rickettsiae. American Journal of Epidemiology*, **108**(1): 53-59

Oteo J.A., Portillo A., Santibanez S., Blanco J.R., Perez-Martinez L. and Ibarra V. (2006). Cluster of cases of Human *Rickettsia felis* Infection from Southern Europe (Spain) Diagnosed by PCR. *Journal of Clinical Microbiology*, **44**(7): 2669-2671.

Pachebo R.C., Venzal J.M., Richtzenhain L.J. and Labruna M.B. (2006). *Rickettsia parkeri* in Uruguay. *Emerging Infectious Diseases*, *12*(11): 1804-1805.

Paddock C.D., Brenner O., Vaid C., Boyd B.D., Berg J.M., Joseph R.J., Zaki S.R. and Childs J.E. (2002). Short Report: Concurrent Rocky Mountain Spotted Fever In A Dog And Its Owner.*Journal of Tropical Medicine and Hygiene*,**66**(2): 197–199

Parker T.M., Murray C.K., Richards A.L., Samir A., Ismail F., Fadeel M.A., Jiang J., Wasfy M.O. and Pimentel G. (2007). Concurrent Infections in acute Febrile Illness Patients in Egypt. *American Journal of Tropical Medicine and Hygiene*, **77**(2): 390-392.

Parola P., Inokuma H., Camica J.L., Brouqui P. and Raoult D. (2001). Detection and Identification of Spotted Fever Group *Rickettsiae* and *Ehlichiae i*n African Ticks. *Emerging Infectious Diseases*, **7**(6): 1014-1017

Parola P., Paddock C.D., and Raoult D. (2005). Tick-Borne Rickettsioses Around The World: Emerging Diseases Challenging Old Concepts. *Clinical Microbiology Reviews*, **18**(4): 719–756

Parola P., Rovery C., Rolain J.M., Brouqui P., Davoust B. and Raoult D. (2009). *Rickettsia slovaca* and *R. raoultii* in Tick-borne Rickettsioses. *Emerging Infectious Diseases*, **15**(7):1105-1108

Parola, P. (2006). Rickettsioses in Sub-Saharan Africa. *Annals of New York Academy of Sciences*, **1078**:42-47.

Parola P, Sanogo OY, Lerdthusnee K, Zeaiter Z, Chauvancy G, Gonzalez JP, Miller RS, Teleford SRIII, Wongsrichanalai C, Raoult D. (2003).Identification of *Rickettsia* spp. and *Bartonella* spp. in fleas from the Thai-Myanmar border. *Annals of New York Academy of Sciences.* 990:173-81.

Perez-Arellano J., Fenollar F., Angel-Moreno A., Balanos M., Hernandez M., Santana E., Hemmersbach-Miller M., Martin A.M. and

Raoult D. (2005). Human *Rickettsia felis* Infection, Canary Islands, Spain. *Emerging Infectious Diseases*, **11**(12): 1961-1964.

Perez-Osorio C.E., Zavala-Velazquez J.E., Leon J.J.A. and Zavala-Castro J.E. (2008). *Rickettsia felis* as Emergent Global Threat for Humans. *Emerging Infectious Diseases*, **14**(7): 1019-1023.

Pickett M.A., Eversons J.S., Pead P.J. and Clarke I.N. (2005). The Plasmid of *Chlamydia trachomatis* and *Chlamydiphila pneumoniae* (N16): Accurate determination of Copy Number and the paradoxical effect of Plasmid-curing Agents. *Microbiology*, **151**:893-903

Pinter A. and Labruna M.B. (2006). *Rickettsia bellii* in Cell Culture from theTick *Amblyomma aureolatum* in Brazil. *Annals of New York Academy of Sciences*, **1078**: 523–530

Pornwiroon W., Pourciau S.S., Foil L.D. and Macaluso K.R. (2006). *Rickettsia felis* from Cat Fleas: isolation and Culture in a Tick-Derived Cell Line. *Applied and Environmental Microbiology*, **72**(8):5589-5595.

Portillo A., Perez-Martinez L., Santibanez S., Blanco J.R., Ibarra V et al., (2007). Detection of *R. africae* in *Rhipicephalus* (*Boophilus*) *decoloratus* ticks from the Republic of Botswana, South Africa. *American Journal of Medicine and Hygiene*, **77**:376-377

Prabhu M., Nicholson W., Roche A., Kersh G., Fitzpatrick K., Oliver L., Massung R.F., Morrisey A.B., Bartlett J.A., Onyango J.J., Maro V.P., Kinabo G.D., Saganda W. and Crump J.A. (2010). Spotted fever group and

typhus group rickettsioses among hospitalized febrile patients in northern Tanzania. In: Abstracts of the 59th Annual Meeting of the American Society for Tropical Medicine and Hygiene; Atlanta; 2010 Nov 3-7; Abstract 1036.

Psarouki A., Antoniou M., Papreustathiou A., Toumazos P., Loukaides F. and Tselentis Y. (2006). Short Report: First Detection of *Rickettsia felis* in *Ctenocephalides felis* Fleas Parasitizing Rats in Cypress. *American Journal of Tropical Medicine and Hygiene*, **74**(1): 120-122.

Purcell K., **Fergie J., Richman K. and Rocha L.** (2007). Murine Typhus in Children, South Texas. *Emerging Infectious Diseases*, 13(6): 926-927.

Rantala A.M., Taylor S.M., Trottman P.A., Luntamo M., Mbewe B., Maleta K., Kulmala T., Ashorn P. and **Meshnick S.R.**. (2010). Comparison of real time PCR and microscopy for malaria parasite detection in Malawian pregnant women. *Malaria Journal*. **9**:269

Raoult D., Aboudharam G., Crubezy E., Larrouy G., Ludes B. and Drancourt M. (2000). Molecular identification by ‘‘suicide PCR’’ of Yersinia pestis as the agent of Medieval Black Death. *Proceedings of the National Academy of Sciences. USA*, **97**: 12800–12803.

Raoult D. and Drancourt M. (1991). Antimicrobial Therapy of Rickettsial Diseases: *Antimicrobial Agents and Chemotherapy*, **35**(12): 2457-2462.

Raoult D., Dutour O., Houhamdi L., Jankauskas R., Fournier P.E., Ardagna Y., Drancourt M., Signoli M., La Dang V., Macia Y. and

Aboudharam G. (2006). Evidence for Louse-transmitted Diseases in Soldiers of Napoleon's Grand Army in Vilnius. Journal of Infectious Diseases. **193:** 112-120

Raoult D., and Roux, V. (1997). Rickettsioses as Paradigms of New of Emerging Infectious Diseases. *Clinical Microbiology Reviews*, **10** (4): 694-719.

Raoult D., Fournier P., Eremeeva M., Graves S., Kelly P.J., Oteo J.A., Sekeyova Z., Tamura A., Tarasevich I. and Zhang L. (2005a).NamingOf *Rickettsiae* and Rickettsial Diseases**.** *Annals of New York Academy of Sciences*, **1063:** 1–12.

Raoult D., Fournier P.E., Fenollar F., Jensenius M., Prioe T., De Pina J.J., Caruso G., Jones N., Laferl H., Rosenblatt J.E. and Marrie T.J. (2001a). *Rickettsia africae*, a Tick-Borne Pathogen in travellers to Sub-Saharan Africa. *New England Journal of Medicine*, **344**(20): 1504-1510.

Raoult D., La Scola B., Enea M., Fournier P.E. Roux V., Fenollar F., Galvao M. and Lamballerie X. (2001b). A Flea-Associated Rickettsia Pathogenic for Human. *Emerging Infectious Diseases*, **7**(1): 73-81.

Raoult D., Ndihokubwayo J.B.,Tissot- Dupont H., Roux V., Faugere B., Abegbinni R., and Birtles R.J (1998). Outbreak of Epidemic typhus associated with trench fever in Burundi. *Lancet*, 352: 353-358.

Raoult D., Roux V., Ndihokubwayo J.B., Bise G., Baudon D., Martet, G. and Birtles R. (1997). Jail fever (Epidemic Typhus) Outbreak in Burundi. *Emerging Infectious Diseases*, **3**:357-359**.**

Raoult D., Weiller P.J., Chagnon A., Chaudet H., Gallais H. and Casanova P. (1986). Mediterranean spotted Fever: Clinical, Laboratory and Epidemiological Features of 199 Cases. *American Journal of Tropical Medicine and Hygiene*, **35**(4): 845-850.

Regnery R.L., Fu Z.Y. and Spruill C.L. (1985). Flying squirrel-Associated *Rickettsia prowazekii* (Epidemic Typhus *Rickettsiae*) Characterized by specific DNA fragment produced by restriction Endonuclease Digestion. *Journal of Clinical Microbiology*, **23**(1): 89-191

Reeves WK, Loftis AD, Szumlas DE, Abbassy MM, Helmy IM, Hanafi HA, Dasch GA. (2007). Rickettsial pathogens in the tropical rat mite *Ornithonyssus bacoti* (Acari: Macronyssidae) from Egyptian rats (*Rattus* spp.). *Experimental and Applied Acarology*. 41:101-7.

Reeves W.K., Nelder M.P. and Korecki J.A.(2005). Bartonella and *Rickettsia* in Fleas and Lice from Mammals in South Carolina, USA. *Journal of Vector Ecology*, **30**(2): 310-315

Reif K.E. and Macaluso K.R. (2009). Ecology of *Rickettsia felis*: A Review. *Journal of Medical Entomology*, **46**(4): 723-736

Reif K.E., Stout R.W., Henry G.C., Foil L.D. and Macaluso K.R. (2008). Prevalence and Infection Load Dynamics of *Rickettsia felis* in Actively Feeding Cat Fleas. *PLoS ONE*, **3**(7): e2805

Renvoise A., Joliot A.Y. and Raoult D. (2009). *Rickettsia felis* Infection in Man, France. *Emerging Infectious Diseases*, **15**(7):1126-1127.

Richards A.L.,Jiang J., Omulo S., Dare R., Abdirahman K., Ali A., Sharif S.K., Feikin D.R., Breiman R.F. and Njenga M.K.(2010). Human infection with *Rickettsia felis*, Kenya. *Emerging Infectious Diseases*, **16**: 1081-1086.

Richter J., Fournier P.E., Petridou J., Haussinger D. and Raoult D. (2002). *Rickettsia felis* Infection Acquired in Europe and Documented by Polymerase Chain Reaction. *Emerging Infectious Diseases*, **8**(2): 207-208.

Robinson S.A., Emereeva M.E., Olson P.E., Thornton S.A., Medina M.J., et al. (2009). New Approaches to Detection and Identification of *Rickettsia africae* and *Ehrlichia ruminantium* in *Amblyomma variegatum* (*Acari*: *Ixodidae*) Ticks from the Caribbean. *Journal of Medical Entomology*, **46**: 942-951.

Rousset E., Durand B., Berri M., Dufour P., Prigent M., Russo P., Delcroix T., Touratier A., Rodolakis A and Aubert M. (2007). Comparative diagnostic potential of three serologicaltests for abortive Q fever in goat herds. *Veterinary Microbiology*, **124:** 286–297

Roux, V., Fournier, P.E. and Raoult, D. (1996). Differentiation of Spotted Fever Group *Rickettsiae* by Sequencing and Analysis of Restriction Fragment Length Polymorphism of PCR Amplified DNA of the Gene Encoding the Protein rOmpA. *Journal of Clinical Microbiology*, **34**(9): 2058-65.

Roux V. and Raoult D. (2000). Phylogenetic Analysis of Members of the Genus *Rickettsia* using the Gene Encoding the Outer Membrane Protein rompB (ompB). *International Journal of Systematic and Evolutionary Microbiology*, **50**:1449-1155

Rovery C., Brouqui P. and Raoult D. (2008). Questions on Mediterranean spotted Fever a Century after its Discovery. *Emerging Infectious Diseases*, **14**(9): 1360-1367.

Rutherford, S.J., Macaluso, K.R., Smith, N., Zaki, S.R., Paddock, S.D., Davis, J., Peterson, N., Azad, A.F., and Rosenberg, R. (2004). Fatal Spotted Fever Rickettsiosis, Kenya. *Emerging Infectious Diseases*, **10**(5): 910-913

Sackal C., Laudisoit A., Kosoy M., Massung R., Eremeeva M.E., Karpathy S.E., Wyk K.V., Gabitzsch E. and Zeidner N.S. (2008). *Bartonella spp*. and *Rickettsia felis* in fleas, Democratic Republic of Congo. *Emerging Infectious Diseases*, **14**(12): 1971-1974.

Saito T.B., Cunha-Filha N.A., Pachebo R.C., Ferreira F., Pappen F.G., Fariah N.A.R., Larssen C.E. and Labruna M.B. (2008). Canine Infection by *Rickettsiae* and *Ehrlichiae* in Southern Brazil. *American Journal of Tropical Medicine and Hygiene*, **79**(1): 102-108.

Sambrook J., Fritsch E.F. and Maniatis T. (1989). Gel Electrophoresis of DNA. In Sambrook J., Fritsch E.F. and Maniatis T. (Eds). Molecular Cloning: A Laboratory Manual. New York: Cold Spring Harbor Laboratory Press, Cold spring Harbor, NY, USA. Chapter 6

Sanger F., Nicklen S. and Coulson A.R. (1977). DNA sequencing with Chain-terminating inhibitors. *Proceedings of the national Academy of Sciences*. **74**(12): 5463-5467.

Sangioni L.A., Horta M.C., Vianna M.C.B., Gennari S.M., Soares R.M., Galvao M.A.M., Schumaker T.T.S., Ferreira F., Vidotto O. and Labruna M.B. (2005). Rickettsial Infections in Animals and Brazilian Spotted Fever Endemicity. *Emerging Infectious Diseases*, **11**(2): 265-270.

Sarov B., Galil A., Sikuler E., Yagupsky P., Saah A., Gilad E., Naggan L. and Sarov I. (1990). Prospective Study on Symptomatic versus Asymptomatic Infections and Serological Response to Spotted Fever Group *Rickettsiae* in Two Rural Sites in the Negev (Southern Israel). *Annals of New York Academy of Sciences*, 243-245

Segerman J. (1995). Siphonaptera of southern Africa. Handbook for Identification of Fleas. Publication of the South African Institute Medical Research. No 57. South African Institute for Medical Research, Johannesburg, South Africa.

Skinner Adams T.S., McCarthy J.S., Gardiner D.L. and Andrews K.T. (2008). HIV and Malaria Co-infections: Interactions and Consequences of Chemotherapy. *Trends in Parasitology*. 24(6): 264-271.

Smoak B.L., McClain J.B., Brundage J.F., Broadhurst L., Kelly D.J. Dasch G.A. and Miller R.N. (1996). An Outbreak of Spotted Fever Rickettsioses

in US Army Troops Deployed to Botswana. *Emerging Infectious Diseases*, **2**(3): 217-221.

Socolovschi C., Matsumoto K., Marie J., Davoust B., Raoult D., and Parola P. (2007). Identification of *Rickettsiae*, Uganda and Djibouti. *Emerging Infectious Diseases*, **13** (10): 1508-1509.

Socolovschi C., Mediannikov O., Raoult., D. and Parola P. (2009). The Relationship between Spotted Fever Group *Rickettsiae* and Ixodid Ticks. *Veterinary Research*, **40**:34-53.

Socolovschi C., Mediannikov., Sokhna C., Tall A., Diatta G., Bassene H., Trape J. and Raoult D. (2010). *Rickettsia felis*- associated Uneruptive Fever, Senegal. *Emerging Infectious Diseases*,2010 Jul [24/01/2010]. http://www.cdc.gov/EID/content/16/7/1140.htm

Sollano-Gellago L., Trotta M., Caldin M. and **Furlanello T.** (2008) Molecular Survey of *Rickettsia* spp. In Sick Dogs in Italy. *Zoonosis and Public Health*, in press.

Snow RW, Eckert E, Teklehaimanot A. (2003). Estimating the Needs for Artesunate-based Combination Therapy for Malaria Case Management in Africa. *Trends in Parasitology*, **19**:363–9.

Stenos J., Graves S.R. and **Unsworth N.B.** (2005). A highly sensitive and specific real- time PCR assay for the detection of spotted fever and typhus group *Rickettsia*. *American Journal of Tropical Medicine and Hygiene*, **73**(6): 1083-1085.

Stevenson. H.L., Bai Y., Kosoy M.Y., Montenieri J.A., Loweli J.L., Chu M.C. and Gage K.L. (2003). Detection of Novel Bartonella Strains and Yersinia pestis in Prairie Dogs and their Fleas (Siphonaptera: Ceratophyllidae and Pulicidae) Using Multiplex Polymerase Chain Reaction. *Journal of Medical Entomology*, **40** (3) 329-337.

Stevenson H.L., Labruna M.B., Montenieri J.A., Kosoy M.Y., Gage K.L. and Walker D.H. (2005). Detection of *Rickettsia felis* in a New World Flea species *Anomiopsllus nudata* (*Siphonaptera*: *Ctenophthalmidae*). *Journal of Medical Entomology*, **42** (2): 163-167.

Tamura K., Peterson D., Peterson N., Stecher G., Nei M. and Kumar S (2011) MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Molecular Biology and Evolution

Tan E., Giese H. And Hartmann D.M. (2008). Microfluidic DNA extraction and purification from Forensic Samples; Towards Rapid, Fully Integrated STR analysis. *Final Report*. Network Biosystems, IB Gill Street, Wobourn, MA 01801

Taylor L.H., Latham S.M. and Woolhouse M.E. (2001). Risk Factors for Human Disease Emergence. *Philosophical Transactions of Royal Society of London. Series B Biological Sciences*, **356**(1411): 983-989

Thorner A.R., Walker D.H. and Petri W.A. Jr. (1998). Rocky Mountain Spotted Fever. *Clinical Infectious Diseases*, **27:**1353–1360

Tomassone L., Conte V., Parrilla G. and Meneghi D. (2010). *Rickettsia* Infection in dogs and *Rickettsia parkeri* in *Amblyomma tigrinum* Ticks, Cochabamba Department, Bolivia. *Vector-borne and Zoonotic Diseases*, **10**(10): 953.

Topping N.H. (1939). Experimental Rocky Mountain Spotted fever and epidemic typhus treated with prontosil or Sulfapyridine. *Public Health Report*.**54**: 1143-7

Tsai KH., Lu H.Y., Huang J.H., Wang P.J., Wang H.C., Huang C.J., Wu W.J. and Shu P.Y. (2009). *Rickettsia felis* in Cat fleas in Taiwan. *Vector Borne Zoonotic Diseases*, **9**(5): 561-563

Tselentis, Y., A. Psaroulaki, J. Maniatis, Spyridaki I. and Babalis T. (1996). Genotypic identification of murine typhus *Rickettsia* In rats and their fleas in an endemic area of Greece by the polymerase chain reaction and restriction fragment length polymorphism. *American Journal of Tropical Medicine and Hygiene*, **54:** 413–417.

Van Belkum A., Scherer S., Van Alphen L. and Verbrugh H. (1998). Short-Sequence DNA Repeats in Prokaryotic genomes. *Microbiology and Molecular Biology Reviews*, **62(**2): 275-293

Venzal J.M.,Perez-Martinez, L., Felix M.L., Portillo A., Blanco J.R. and Oteo J.A. (2006) Prevalence of *Rickettsia felis*in *Ctenocephalides felis*and *Ctenocephalides canis*from Uruguay. *Annals of New York Academy of Sciences*, **1078:** 305–308

Villella V.D., Yao J., Getty R.R., Juliar B.E., Yiannoutsos C., Hartwell J.R., Cai S., Sadat M., A., Cornetta K., Williams D.A. andPollok K.E. (2004). Real-time PCR: A tool for measuring transduction efficiency in Haematopoietic progenitor cells. *Molecular Therapy*. **11**(3): 483-491

Vitrino L., Chelo I.M., Bacellar F. and Zeze L. (2007). *Rickettsia* phylogeny: a multigenic approach. *Microbiology*, **153**:160-168.

Walker A.R., Bouattour A., Camicas J.L., Estrada-Pena A., Horak I.G., Latif A.A., Pegram R.G. and Preston P.M. (2003). Ticks of Domestic Animals in Africa: A Guide to Identification of Species. Bioscience Reports. Edinburgh, Scotland, U.K.

Walker D.H. (1995). Rocky mountain Spotted Fever: A seasonal Alert. *Clinical Infectious Diseases*, **20**: 1111-1117.

Walker D.H. and Yu X. (2005). Progress in Rickettsial gene Analysis from Pioneering of *Rickettsia prowazekii* to the recent *Rickettsia typhi*. *Annals of New York Academy of Sciences*, 1**063**: 13-25

Walker, D.H., H.M. Feng and V.L. Popov. (2001). Rickettsial phospholipase A2 as a pathogenic mechanism in a model of cell injury by typhus and spotted fever group *Rickettsiae*. *American Journal of Tropical Medicine and Hygiene*, **65:** 936–942.

Wedincamp J.J. and Foil L.D. (2000). Infection and Seroconversion of Cats exposed to Cats Fleas (*Ctenocephalides felis* Bouche) Infected with *Rickettsia felis*. *Journal of Vector Ecology*, 25(1): 123-126.

Weisburg, W. G., M. E. Dobson, J. E. Samuel, G. A. Dasch, L. P. Mallavia, O. Baca, L. Mandelco, J. E. Sechrest, E. Weiss, and C. R. Woese. C. (1989). Phylogenetic diversity of the *Rickettsiae*. *Journal of Bacteriology*, **171:**4202–4206.

Whitman T.M., Richards, A.L., Paddock, C.D., Tamminga, C.L., Sniezek, P.J., Jiang, J., Byers, D.K., and Sanders, J.W. (2007). *Rickettsia parkeri* Infection after Tick Bite , Virginia. *Emerging Infectious Diseases*, **13(**2).

Woese, C. R. (1987). Bacterial evolution. *Microbiology Reviews*, **51:**222–270

Wongsrichanalai C., Murray C.K., Gray M., Miller R.S., McDaniel P., Liao W.J., Pickard A.L. and Magill A.J. (2003). Co-infection with Malaria and Leptospirosis. *American Journal of Tropical Medicine and Hygiene*, **68(**5): 583-585.

Woods M.E.,Wen G.And Juan P. Olano J.P. (2005). Nitric Oxide As A Mediator Of Increased Microvascular Permeability During Acute Rickettsioses. *Annals of New York Academy of Sciences*, **1063**: 239–245.

World Health Organization. (1993). Global surveillance of rickettsial diseases. Memorandum from WHO meeting. *Bulletin of World Health Organization*. **71**: 293-296

Yagupsky P., Sarov B., Naggan L., Sarov I., Avy K. andGoldwasser R. (1990). The prevalence of IgG antibodies to spotted fever *Rickettsiae* among Urban and Rural Dwelling Children in Southern Israel. *Scandinavian Journal of Infectious Diseases*, **22**(1): 19-23.

Yazoume, Y, Madise, N, Ndugwa, R, Ochola, S. et al. (2009). Fever treatment in the absence of malaria transmission in an urban informal settlement in Nairobi, Kenya. *Malaria Journal*. **8**:160-166

Yoshikawa H., Kimura M., Ogawa M., Rolain J. and Raoult D. (2005) Laboratory-Confirmed Mediterranean Spotted Fever in a Japanese Traveler to Kenya. *American Journal of Tropical Medicine and Hygiene*, **73**(6):1086-9.

Zavala-Castro J.E., Dzul-Rosado K.R., León J.J.L., Walker D.H and Zavala-Velázquez J.E. (2008).Short Report: *Rickettsia felis* Outer Membrane Protein A: A Potential Tool forDiagnosis of Patients with Flea-Borne Spotted Fever. *American Journal of Tropical Medicine and Hygiene*,**79**(6): 903–906.

Zavala-Velazquez J.E., Ruiz-Sosa J.A., Sanschez-Elias R.A., Becerra-Carmona G. And Walker D.H. (2000). *Rickettsia felis* Rickettsiosis in Yucatan. *The Lancet*, **356**: 1079-1080.

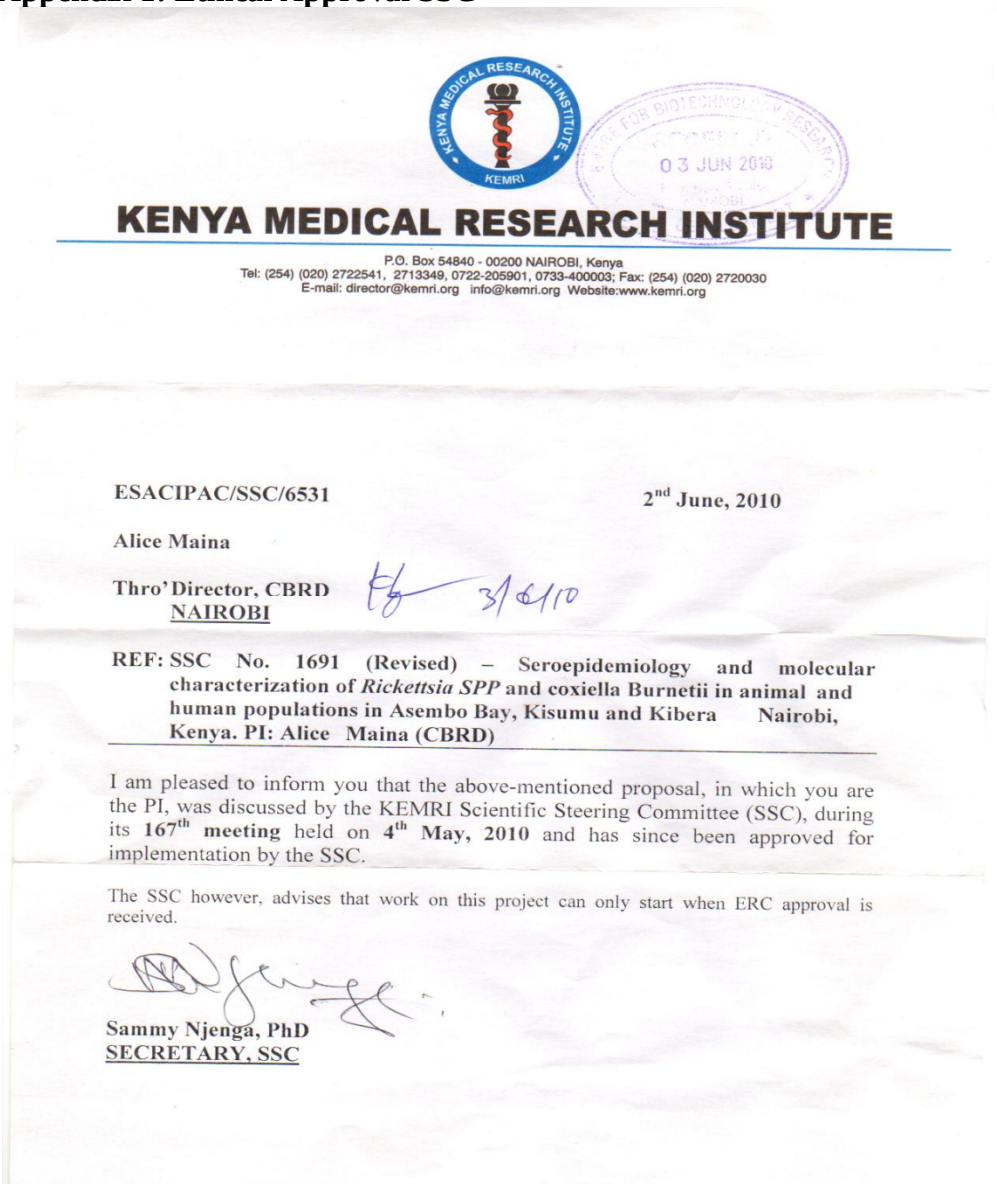
Zhu Y., Fournier P.E., Emereeva M. and Raoult D. (2005). Proposal to create subspecies of *Rickettsia conorii* based on Multi-locus sequence typing and an amended description of *Rickettsia conorii*. *BMC Microbiology*, **5:**11

Zientz E., Silvia F.J. and Gross R. (2001). Genome Interdependence in Insect-Bacterium Symbioses. *Genome Biology*, **2**(12):1032

Znazen A., Rolain J.M., Hammami N., Hammami A., Jemaa M.B. and Raoult D. (2006). *Rickettsia felis* Infection, Tunisia. *Emerging Infectious Disease*, **12**(1): 138-140.

APPENDICES

Appendix 1: Ethical Approval SSC



KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840 - 00200 NAIROBI, Kenya
Tel: (254) (020) 2722541, 2713349, 0722-205901, 0733-400003; Fax: (254) (020) 2720030
E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

03 JUN 2010

ESACIPAC/SSC/6531 2nd June, 2010

Alice Maina

Thro' Director, CBRD
NAIROBI

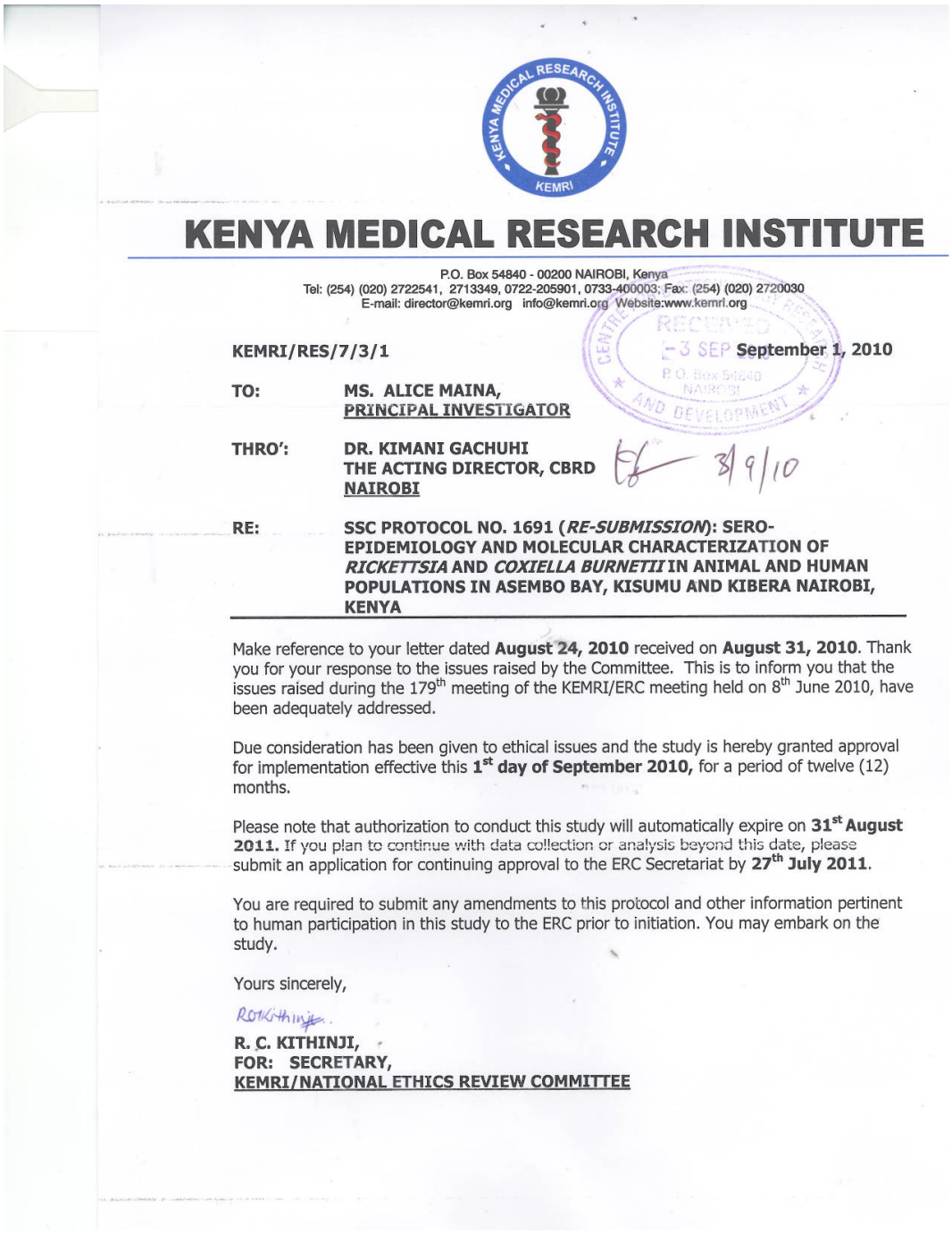
REF: SSC No. 1691 (Revised) – Seroepidemiology and molecular characterization of *Rickettsia SPP* and coxiella Burnetii in animal and human populations in Asembo Bay, Kisumu and Kibera Nairobi, Kenya. PI: Alice Maina (CBRD)

I am pleased to inform you that the above-mentioned proposal, in which you are the PI, was discussed by the KEMRI Scientific Steering Committee (SSC), during its 167th meeting held on 4th May, 2010 and has since been approved for implementation by the SSC.

The SSC however, advises that work on this project can only start when ERC approval is received.

Sammy Njenga, PhD
SECRETARY, SSC

Appendix 2: Approval ERC



KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840 - 00200 NAIROBI, Kenya
Tel: (254) (020) 2722541, 2713349, 0722-205901, 0733-400003; Fax: (254) (020) 2720030
E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

RECEIVED 3 SEP

KEMRI/RES/7/3/1 **September 1, 2010**

TO: **MS. ALICE MAINA,**
PRINCIPAL INVESTIGATOR

THRO': **DR. KIMANI GACHUHI**
THE ACTING DIRECTOR, CBRD
NAIROBI

3/9/10

RE: **SSC PROTOCOL NO. 1691 (*RE-SUBMISSION*): SERO-EPIDEMIOLOGY AND MOLECULAR CHARACTERIZATION OF *RICKETTSIA* AND *COXIELLA BURNETII* IN ANIMAL AND HUMAN POPULATIONS IN ASEMBO BAY, KISUMU AND KIBERA NAIROBI, KENYA**

Make reference to your letter dated **August 24, 2010** received on **August 31, 2010**. Thank you for your response to the issues raised by the Committee. This is to inform you that the issues raised during the 179th meeting of the KEMRI/ERC meeting held on 8th June 2010, have been adequately addressed.

Due consideration has been given to ethical issues and the study is hereby granted approval for implementation effective this **1st day of September 2010,** for a period of twelve (12) months.

Please note that authorization to conduct this study will automatically expire on **31st August 2011.** If you plan to continue with data collection or analysis beyond this date, please submit an application for continuing approval to the ERC Secretariat by **27th July 2011**.

You are required to submit any amendments to this protocol and other information pertinent to human participation in this study to the ERC prior to initiation. You may embark on the study.

Yours sincerely,

R.C. Kithinji

R. C. KITHINJI,
FOR: SECRETARY,
KEMRI/NATIONAL ETHICS REVIEW COMMITTEE

Appendix 3: Ethical Approval ACUC

KENYA MEDICAL RESEARCH INSTITUTE

Centre for Virus Research P.O. Box 54628 - 00200, NAIROBI - Kenya
Tel: (254) (020) 2722541, 2713349: 0722-205901, 0733-400003; Fax: (254) (020) 2726115
E-mail: cvr@kemri.org

1ST March 2010

KEMRI/ACUC/ 01.03.10

Dr. Alice Maina,
KEMRI/CDC

Dr. Alice,

RE: SSC No.1691 – Seroepidemiology and molecular characterization of Rickettsia species and Coxiella burnetii in animal and human populations in Asembo bay,Kisumu and kibera Nairobi,Kenya.

The KEMRI animal care and use committee acknowledges the receipt of the above mentioned proposal for review.

The committee has established that the samples to be used in this study have been and continue to be collected under the SSC Protocol 1191 **"An integrated epidemiological study of zoonotic pathogens in linked human and animal populations in rural and urban Kenya".** The latter protocol has KEMRI ACUC approval to collect samples from various domestic animals in Kisumu and Kibera.

The committee grants you the approval to proceed with your work after obtaining all the other necessary approvals that may be required and wishes you all the best in your study.
Yours sincerely,

Dr. Konongoi Limbaso,
Chairperson, KEMRI ACUC

Appendix 4: Indirect immunofluorescence antibody (IFA) test

Specimen Preparation

Prepare 1:128 screening dilutions of patient sera by mixing 1 part patient serum with 127 parts of phosphate buffered saline

IFA against IgG for Rickettsia rickettsii Fuller Laboratories

1. To determine endpoint titers, use reconstituted PBS to serially dilute the screening dilution.
2. Remove slides from +4ºC and allow them to attain room temperature to avoid condensation.
3. Apply 10 µl of spotted fever or typhus group positive controls to appropriate slide wells.
4. Apply 10 µl of negative control to appropriate slide well (do not dilute).
5. Apply 10 µl of each patient samples to be tested to appropriate slide wells (Diluted appropriately) and record the position for later reference.
6. Incubate slides in a humid chamber for 30 minutes at 35-37 ºC.
7. Remove slides from humidity chamber and rinse wells with gentle stream of PBS from wash bottle (3) times. Then allow beads of PBS to remaining the wells for at least 5 minutes. Shake or tap excess PBS from slides and go directly to the next step without allowing the slides to dry.
8. Add 10 µl (1 drop from dropper tip) IgG conjugate to each slide well.
9. Incubate slides in a dark humid chamber for 30 minutes at 35-37 ºC.
10. Repeat the washing step **7** above.
11. Place a few (2-3) drops of mounting medium on the slide and cover with 24 x 50 mm cover slip.

12. Read the slides at a final magnification of 400X on a proper fluorescein microscope. Slides should be read the same day the assay is performed, and if this is not possible, store the slides at +4 ºC up to 24 hours

.**Quality control**

- The negative control serum and dilutions of positive control serum should be assayed with each daily run.
- The **negative contro**l well is an example of a non reactive serum, with either **uniform red** counterstain or slight, but **uniform greenish** staining
- The **positive control** wells should give end point titer from 1:256 to 1:1024. The fluorescence intensity at 1:512 may be used as the cutoff level required for a patient reaction to be called positive.
- If the controls do not react as specified, the assay run should be considered void, reagent components and procedural steps rechecked and the assay should be repeated from the beginning.
- If a negative control well show bright green staining similar to that seen in positive control wells, there has been a breakdown in technique and assay must be repeated.

Appendix 5: Extraction of blood clots

1. **Pipette 30 µl Qiagen Protease** (Proteinase K) into the bottom of a 1.5 ml micro centrifuge tube.
2. **Add 300 µl samples** to the micro centrifuge tube (blood clot).
3. **Add 300 µl Buffer AL** to the sample. Mix by vortexing for 15 seconds.Incubate at 56°C for 10 minutes.Briefly **centrifuge** the 1.5 ml Micro centrifuge tube to remove drops from the inside of the lid.
4. **Add 300 µl ethanol** (96-100 %) to the sample, and mix again by pulse-vortexing for 15 seconds. After mixing, briefly centrifuge the 1.5 ml micro centrifuge tube to remove drops from the inside of the lid.
5. **Apply the mixture** from step **4** to the **QIAmp Mini spin column** (in a 2ml collection tube) without wetting the rim. ***The total volume in step 4 cannot fit the spin column and has to be applied in two successful steps of 650 µl.*** Close the cap, **centrifuge at 8000 rpmfor 1 minute**. Place the QIAmp Mini spin column in a clean 2 ml collection tube and discard the tube containing the filtrate.
6. Carefully open the QIAmp Mini spin column and add **500 µl buffer AW1** without wetting the rim. Close the cap and **centrifuge at 8000 rpm for 1 minute.** Place the QIAmp Mini spin column in a clean 2 ml collection tube and discard the tube containing the filtrate.
7. Carefully open the QIAmp Mini spin column and add **500µl buffer AW2** without wetting the rim. Close the cap and **centrifuge at full speed** (14,000 rpm) **for 3 minute**. Place the QIAmp Mini spin column in a clean 2 ml collection tube and discard the tube containing the filtrate.
8. Place the QIAmp Mini spin column in a new 2 ml collection tube, centrifuge at **14000 rpm for 1 minute. (To eliminate the chance of possible Buffer AW2 carryover.**
9. **Place the QIAmp mini spin column in a clean 1.5 micro centrifuge tube and discard the collection tube containing the filtrate. Carefully open the QIAmp Mini spin column and add 100 µl** buffer AE (Elution buffer) or distilled water. Incubate at room temperature (15-25 °C) for 5 minute, and centrifuge at 8000 rpm for 1 minute.
10. DNA was stored at -80^{0}C. DNA is stable for up to one year when stored at -20^{0}C or -80^{0}C.

Appendix 6: Preparation of Tris borate-EDTA (TBE) buffer and 1% agarose

1. To prepare 500ml of 5X buffer TBE

Weigh Tris, Boric acid and EDTA (ethylenediamine tetraacetic acid) as listed below and mix with 500ml of double distilled water

Reagent	Weight in grams	5X stock concentration
Tris Base	13.5	222.5 mM
Boric acid	6.8	222.5mM
EDTA	0.93	5mM

2. To prepare 200ml 1X buffer TBE

Add 160ml of double distilled water to 40ml of 5X buffer TBE, mix by inverting the bottle. The final 1X concentration will be 44.5 mM Tris-HCl, 44.5mM boric acid, 1mM EDTA; pH 8

3. To prepare 1% agarose

Add 1gm of agarose to 100ml of 1X buffer TBE; bring to the mixture to boil to dissolve the agarose completely

Wait for the agarose to cool at room temperature and add 3µl of Ethidium bromide (0.001% Et br)

Appendix 7: QIAquick PCR purification kit

Protocol

QIAquick PCR Purification Kit Protocol

using a microcentrifuge

This protocol is designed to purify single- or double-stranded DNA fragments from PCR and other enzymatic reactions (see page 8). For cleanup of other enzymatic reactions, follow the protocol as described for PCR samples or use the new MinElute Reaction Cleanup Kit. Fragments ranging from 100 bp to 10 kb are purified from primers, nucleotides, polymerases, and salts using QIAquick spin columns in a microcentrifuge.

Notes:
- Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
- All centrifuge steps are at ≥10,000 x *g* (~13,000 rpm) in a conventional tabletop microcentrifuge.

1. **Add 5 volumes of Buffer PB to 1 volume of the PCR sample and mix. It is not necessary to remove mineral oil or kerosene.**

   For example, add 500 µl of Buffer PB to 100 µl PCR sample (not including oil).
2. **Place a QIAquick spin column in a provided 2 ml collection tube.**
3. **To bind DNA, apply the sample to the QIAquick column and centrifuge for 30–60 s.**
4. **Discard flow-through. Place the QIAquick column back into the same tube.**

   Collection tubes are re-used to reduce plastic waste.
5. **To wash, add 0.75 ml Buffer PE to the QIAquick column and centrifuge for 30–60 s.**
6. **Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the column for an additional 1 min at maximum speed.**

   IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.
7. **Place QIAquick column in a clean 1.5 ml microcentrifuge tube.**
8. **To elute DNA, add 50 µl Buffer EB (10 mM Tris-Cl, pH 8.5) or H_2O to the center of the QIAquick membrane and centrifuge the column for 1 min. Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.**

   IMPORTANT: Ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA. The average eluate volume is 43 µl from 50 µl elution buffer volume, and 28 µl from 30 µl elution buffer.

   Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, make sure that the pH value is within this range, and store DNA at –20°C as DNA may degrade in the absence of a buffering agent. The purified DNA can also be eluted in TE (10 mM Tris-Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.

Appendix 8: Dye Ex 2.0 spin kit (QIAGEN)

1. **Gently vortex the spin column to resuspend the resin.**
2. **Loosen the cap of the column a quarter turn.**

   This is necessary to avoid a vacuum inside the spin column.
3. **Snap off the bottom closure of the spin column (Figure 1), and place the spin column in a 2 ml collection tube (provided).**
4. **Centrifuge for 3 min at the calculated speed.**
5. **Carefully transfer the spin column to a clean centrifuge tube. Slowly apply the sequencing reaction (10–20 µl) to the gel bed (Figure 2).**

   Notes:
   - Pipet the sequencing reaction directly onto the center of the slanted gel-bed surface (Figure 2). Do not allow the reaction mixture or the pipet tip to touch the sides of the column. The sample should be pipetted slowly so that the drops are absorbed into the gel and do not flow down the sides of the gel bed. Avoid touching the gel-bed surface with the pipet tip.
   - This protocol is suitable for sequencing reactions with volumes of 10–20 µl. For easier handling, more reproducible pipetting, and reduced error with sample volumes <10 µl, we recommend adjusting the volume to 20 µl using distilled water, before application to the gel-bed.
   - It is not necessary to remove mineral oil or kerosene prior to cleanup of dye-terminator sequencing reactions.
   - It is not necessary to replace the lid on the column.
6. **Centrifuge for 3 min at the calculated speed.**
7. **Remove the spin column from the microcentrifuge tube.**

   The eluate contains the purified DNA.

   Optional: If using the ABI PRISM 3700 with a water loading protocol, it is possible to load the eluate directly onto the sequencer without drying down the sample.
8. **Dry the sample in a vacuum centrifuge and proceed according to the instructions provided with the DNA sequencer.**

Appendix 9: Procedure for extraction of DNA from serum, whole blood or Buffy coat

1. **Pipette 20 µl Qiagen Protease** (Proteinase K) into the bottom of a 1.5 ml micro centrifuge tube.
2. **Add 200 µl samples** to the micro centrifuge tube (Serum, whole blood or Buffy coat).
3. **Add 200 µl Buffer AL** to the sample. Mix by vortexing for 15 seconds.
4. Incubate at 56°C for 10 minutes.
5. Briefly **centrifuge** the 1.5 ml Micro centrifuge tube to remove drops from the inside of the lid.
6. **Add 200 µl ethanol** (96-100 %) to the sample, and mix again by pulse-vortexing for 15 seconds. After mixing, briefly centrifuge the 1.5 ml micro centrifuge tube to remove drops from the inside of the lid.
7. **Apply the mixture** from step 6 to the **QIAmp Mini spin column** (in a 2ml collection tube) without wetting the rim. Close the cap, **centrifuge at 8000 rpmfor 1 minute**. Place the QIAmp Mini spin column in a clean 2 ml collection tube and discard the tube containing the filtrate. (**Note:** when preparing DNA from Buffy coat, centrifugation at full speed is recommended to avoid clogging).
8. Carefully open the QIAmp Mini spin column and add **500 ul buffer AW1** without wetting the rim. Close the cap and **centrifuge at 8000 rpm for 1 minute.** Place the QIAmp Mini spin column in a clean 2 ml collection tube and discard the tube containing the filtrate.
9. Carefully open the QIAmp Mini spin column and add **500 µl buffer AW2** without wetting the rim. Close the cap and **centrifuge at full speed** (14,000 rpm) **for 3 minute**. Place the QIAmp Mini spin column in a clean 2 ml collection tube and discard the tube containing the filtrate.
10. Place the QIAmp Mini spin column in a new 2 ml collection tube, centrifuge at **14000 rpm for 1 minute. (To eliminate the chance of possible Buffer AW2 carryover.**
11. **Place the QIAmp mini spin column in a clean 1.5 micro centrifuge tube and discard the collection tube containing the filtrate. Carefully open the QIAmp Mini spin column and add 50 µl** buffer AE (Elution buffer) or distilled water. Incubate at room temperature (15-25 °C) for 5 minute, and centrifuge at 8000 rpm for 1 minute.
12. Store the DNA at – 20°C

Appendix 10: Disruption of tissues for purification of nucleic acids

Procedure

1. Place 2 ml microcentrifuge tubes containing 1 stainless steel bead (5 mm mean diameter) on dry ice for at least 15 min. Keep the insert of the TissueLyser LT Adapter at room temperature (15–25°C).
2. Transfer up to 25 mg fresh or frozen tissue to the precooled tubes and incubate for another 15 min on dry ice.
3. Place the tubes into the insert of the TissueLyser LT Adapter, and incubate at room temperature for 2 min to avoid freezing of lysis buffer in step 4.

   Do not incubate for longer than 2 min, otherwise the tissue will thaw, resulting in potential DNA degradation.
4. Immediately add the appropriate volume of lysis buffer (e.g., Buffer ATL) to each tube.
5. Place the insert with sample tubes into the base of the TissueLyser LT Adapter, which is attached to the TissueLyser LT. Place the lid of the TissueLyser LT Adapter over the insert, and screw the knob until the lid is securely fastened.
6. Operate the TissueLyser LT for 40 s at 30 Hz.

   Note: Depending on the type of tissue, exceeding this homogenization time and intensity may lead to significant fragmentation of genomic DNA. However, for tough samples, it may be necessary to exceed this homogenization time and/or intensity to improve disruption efficiency.

   If working with fibrous tissues, cutting the tissue into smaller pieces before starting disruption will improve disruption efficiency.
7. Proceed with DNA purification.

   Do not reuse the stainless steel beads.

Appendix 11: QIAamp tissues kit extraction procedure

Protocol: DNA Purification from Tissues (QIAamp DNA Mini Kit)

This protocol is for purification of total (genomic, mitochondrial, and viral) DNA from tissues using the QIAamp DNA Mini Kit.

Important points before starting

- All centrifugation steps are carried out at room temperature (15–25°C).
- Use carrier DNA if the sample contains <10,000 genome equivalents (see page 18).
- Avoid repeated freezing and thawing of stored samples, since this leads to reduced DNA size.
- Transcriptionally active tissues, such as liver and kidney, contain high levels of RNA which will copurify with genomic DNA. RNA may inhibit some downstream enzymatic reactions, but will not inhibit PCR. If RNA-free genomic DNA is required, include the RNase A digest, as described in step 5a of the protocol.

Things to do before starting

- Equilibrate the sample to room temperature (15–25°C).
- Heat 2 water baths or heating blocks: one to 56°C for use in step 3, and one to 70°C for use in step 5.
- Equilibrate Buffer AE or distilled water to room temperature for elution in step 11.
- Ensure that Buffers AW1 and AW2 have been prepared according to the instructions on page 17.
- If a precipitate has formed in Buffer ATL or Buffer AL, dissolve by incubating at 56°C.

Procedure

1. **Excise the tissue sample or remove it from storage. Determine the amount of tissue. Do not use more than 25 mg (10 mg spleen).**

   Weighing tissue is the most accurate way to determine the amount.

   If DNA is prepared from spleen tissue, no more than 10 mg should be used.

   The yield of DNA will depend on both the amount and the type of tissue processed.

   1 mg of tissue will yield approximately 0.2–1.2 µg of DNA.

2. **Cut up (step 2a), grind (step 2b), or mechanically disrupt (step 2c) the tissue sample.**

   The QIAamp procedure requires no mechanical disruption of the tissue sample, but lysis time will be reduced if the sample is ground in liquid nitrogen (step 2b) or mechanically homogenized (step 2c) in advance.

2a. **Cut up to 25 mg of tissue (up to 10 mg spleen) into small pieces. Place in a 1.5 ml microcentrifuge tube, and add 180 µl of Buffer ATL. Proceed with step 3.**

   It is important to cut the tissue into small pieces to decrease lysis time.

   2 ml microcentrifuge tubes may be better suited for lysis.

2b. **Place up to 25 mg of tissue (10 mg spleen) in liquid nitrogen, and grind thoroughly with a mortar and pestle. Decant tissue powder and liquid nitrogen into 1.5 ml microcentrifuge tube. Allow the liquid nitrogen to evaporate, but do not allow the tissue to thaw, and add 180 µl of Buffer ATL. Proceed with step 3.**

2c. **Add up to 25 mg of tissue (10 mg spleen) to a 1.5 ml microcentrifuge tube containing no more than 80 µl PBS. Homogenize the sample using the TissueRuptor or equivalent rotor–stator homogenizer. Add 100 µl Buffer ATL, and proceed with step 3.**

   Some tissues require undiluted Buffer ATL for complete lysis. In this case, grinding in liquid nitrogen is recommended. Samples cannot be homogenized directly in Buffer ATL, which contains detergent.

3. **Add 20 µl proteinase K, mix by vortexing, and incubate at 56°C until the tissue is completely lysed. Vortex occasionally during incubation to disperse the sample, or place in a shaking water bath or on a rocking platform.**

   Note: Proteinase K must be used. QIAGEN Protease has reduced activity in the presence of Buffer ATL.

   Lysis time varies depending on the type of tissue processed. Lysis is usually complete in 1–3 h. Lysis overnight is possible and does not influence the preparation. In order to ensure efficient lysis, a shaking water bath or a rocking platform should be used. If not available, vortexing 2–3 times per hour during incubation is recommended.

4. **Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.**

5. **If RNA-free genomic DNA is required, follow step 5a. Otherwise, follow step 5b.**

   Transcriptionally active tissues, such as liver and kidney, contain high levels of RNA which will copurify with genomic DNA. RNA may inhibit some downstream enzymatic reactions, but will not inhibit PCR.

5a. **First add 4 µl RNase A (100 mg/ml), mix by pulse-vortexing for 15 s, and incubate for 2 min at room temperature. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid before adding 200 µl Buffer AL to the sample. Mix again by pulse-vortexing for 15 s, and incubate at 70°C for 10 min. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid.**

It is essential that the sample and Buffer AL are mixed thoroughly to yield a homogeneous solution.

A white precipitate may form on addition of Buffer AL. In most cases it will dissolve during incubation at 70°C. The precipitate does not interfere with the QIAamp procedure or with any subsequent application.

5b. **Add 200 µl Buffer AL to the sample, mix by pulse-vortexing for 15 s, and incubate at 70°C for 10 min. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid.**

It is essential that the sample and Buffer AL are mixed thoroughly to yield a homogeneous solution.

A white precipitate may form on addition of Buffer AL, which in most cases will dissolve during incubation at 70°C. The precipitate does not interfere with the QIAamp procedure or with any subsequent application.

6. **Add 200 µl ethanol (96–100%) to the sample, and mix by pulse-vortexing for 15 s. After mixing, briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid.**

It is essential that the sample, Buffer AL, and the ethanol are mixed thoroughly to yield a homogeneous solution.

A white precipitate may form on addition of ethanol. It is essential to apply all of the precipitate to the QIAamp Mini spin column. This precipitate does not interfere with the QIAamp procedure or with any subsequent application.

Do not use alcohols other than ethanol since this may result in reduced yields.

7. **Carefully apply the mixture from step 6 (including the precipitate) to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.***

Close each spin column to avoid aerosol formation during centrifugation.

It is essential to apply all of the precipitate to the QIAamp Mini spin column.

Centrifugation is performed at 6000 x *g* (8000 rpm) in order to reduce noise. Centrifugation at full speed will not affect the yield or purity of the DNA. If the solution has not completely passed through the membrane, centrifuge again at a higher speed until all the solution has passed through.

* Flow-through contains Buffer AL or Buffer AW1 and is therefore not compatible with bleach. See page 8 for safety information.

8. **Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW1 without wetting the rim. Close the cap, and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the collection tube containing the filtrate.***
9. **Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x *g*; 14,000 rpm) for 3 min.**
10. **Recommended: Place the QIAamp Mini spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.**

    This step helps to eliminate the chance of possible Buffer AW2 carryover.
11. **Place the QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube (not provided), and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 200 µl Buffer AE or distilled water. Incubate at room temperature for 1 min, and then centrifuge at 6000 x *g* (8000 rpm) for 1 min.**
12. **Repeat step 11.**

    A 5 min incubation of the QIAamp Mini spin column loaded with Buffer AE or water, before centrifugation, generally increases DNA yield.

Appendix 12: QIAquick gel extraction kit, Qiagen

QIAquick Gel Extraction Kit Protocol

using a microcentrifuge

This protocol is designed to extract and purify DNA of 70 bp to 10 kb from standard or low-melt agarose gels in TAE or TBE buffer. Up to 400 mg agarose can be processed per spin column. This kit can also be used for DNA cleanup from enzymatic reactions (see page 8). For DNA cleanup from enzymatic reactions using this protocol, add 3 volumes of Buffer QG and 1 volume of isopropanol to the reaction, mix, and proceed with step 6 of the protocol. Alternatively, use the new MinElute Reaction Cleanup Kit.

Notes:

- The yellow color of Buffer QG indicates a pH <7.5.
- Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
- Isopropanol (100%) and a heating block or water bath at 50°C are required.
- All centrifugation steps are carried out at ≥10,000 x *g* (~13,000 rpm) in a conventional table top microcentrifuge.
- 3 M sodium acetate, pH 5.0, may be necessary.

1. **Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.**

   Minimize the size of the gel slice by removing extra agarose.

2. **Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg ~ 100 µl).**

   For example, add 300 µl of Buffer QG to each 100 mg of gel. For >2% agarose gels, add 6 volumes of Buffer QG. The maximum amount of gel slice per QIAquick column is 400 mg; for gel slices >400 mg use more than one QIAquick column.

3. **Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). To help dissolve gel, mix by vortexing the tube every 2–3 min during the incubation.**

   IMPORTANT: Solubilize agarose completely. For >2% gels, increase incubation time.

4. **After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose).**

   If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow.

   The adsorption of DNA to the QIAquick membrane is efficient only at pH ≤7.5. Buffer QG contains a pH indicator which is yellow at pH <7.5 and orange or violet at higher pH, allowing easy determination of the optimal pH for DNA binding.

5. **Add 1 gel volume of isopropanol to the sample and mix.**

   For example, if the agarose gel slice is 100 mg, add 100 µl isopropanol. This step increases the yield of DNA fragments <500 bp and >4 kb. For DNA fragments between 500 bp and 4 kb, addition of isopropanol has no effect on yield. Do not centrifuge the sample at this stage.

6. **Place a QIAquick spin column in a provided 2 ml collection tube.**
7. **To bind DNA, apply the sample to the QIAquick column, and centrifuge for 1 min.**

   The maximum volume of the column reservoir is 800 µl. For sample volumes of more than 800 µl, simply load and spin again.
8. **Discard flow-through and place QIAquick column back in the same collection tube.**

   Collection tubes are re-used to reduce plastic waste.
9. **(Optional): Add 0.5 ml of Buffer QG to QIAquick column and centrifuge for 1 min.**

   This step will remove all traces of agarose. It is only required when the DNA will subsequently be used for direct sequencing, in vitro transcription or microinjection.
10. **To wash, add 0.75 ml of Buffer PE to QIAquick column and centrifuge for 1 min.**

    Note: If the DNA will be used for salt sensitive applications, such as blunt-end ligation and direct sequencing, let the column stand 2–5 min after addition of Buffer PE, before centrifuging.
11. **Discard the flow-through and centrifuge the QIAquick column for an additional 1 min at ≥10,000 x *g* (~13,000 rpm).**

    IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.
12. **Place QIAquick column into a clean 1.5 ml microcentrifuge tube.**
13. **To elute DNA, add 50 µl of Buffer EB (10 mM Tris·Cl, pH 8.5) or H_2O to the center of the QIAquick membrane and centrifuge the column for 1 min at maximum speed. Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge for 1 min.**

    IMPORTANT: Ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA. The average eluate volume is 48 µl from 50 µl elution buffer volume, and 28 µl from 30 µl.

    Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, make sure that the pH value is within this range, and store DNA at –20°C as DNA may degrade in the absence of a buffering agent. The purified DNA can also be eluted in TE (10 mM Tris·Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.

Appendix 13: Performa Gel Filtration Procedure

EdgeBio

PERFORMA® DTR Gel Filtration Cartridges

Product	Catalog #	Purifications
Performa DTR Gel Filtration Cartridges (36 cartridges)	98780	36
Performa DTR Gel Filtration Cartridges (108 cartridges)	42453	108

Description

Performa DTR (Dye Terminator Removal) Gel Filtration Cartridges are 0.8-ml spin columns packed with a gel matrix optimized to effectively remove dye terminators, dNTPs, and other low molecular weight materials from sequencing reactions. These columns also remove DNA primers and fragments up to 15 bases, buffers, and nucleotides labeled with biotin, isotopes and other assorted markers. The column is pre-packed with the matrix fully hydrated in water.

Components	98780	42453
Performa Gel Filtration Cartridges	36 carts. (PN 4050167)	108 carts. (3 x PN 4050167)
1.5-ml Microcentrifuge Tubes	36 tubes (PN 4050090)	108 tubes (PN 4050087)

Equipment and Materials Required

1. Variable speed centrifuge (benchtop or floor model) capable of 850 *x g*.
2. Carriers for microcentrifuge tubes.

Storage Condition

Store at +4°C. Do not freeze.

Quality Control

Tested for sequence quality, including signal strength, removal of fluorescent contaminants, and sequencing accuracy on a gel sequencer.

Recommended Protocol for use with BigDye™ v3.1

1. **Centrifuge the Performa Gel Filtration Cartridge for 3 minutes at 850 *x g*.**
   - The time and speed of centrifugation are important.
   - The drier the packing (longer centrifugation times and/or higher *g* forces), the longer it takes to recover product and the lower the overall recovery.
   - Conversely, shorter spin times and lower speeds result in elution volumes higher than the input sample volume.
   - See "Notes" for determination of RPM from RCF or visit our website at www.edgebio.com and click on Technical Support.
2. **Transfer the cartridge to the provided 1.5-ml microcentrifuge tube and add the sample to the packed column. Be sure the fluid runs into the gel.**
   - If using a microcentrifuge or other centrifuge which uses a fixed angle rotor, place the sample in the center of the slanted gel bed surface to obtain optimal performance.
3. **Close the cap and centrifuge for 3 minutes at 850 *x g*. Retain eluate.**
   - Up to 4 µl may be lost during sample processing.
   - If the volume loss is greater than 4 µl, this is an indication of an overly dry gel. To optimize recovery of sample, repeat centrifugation.

Recommended Protocol for all other Dye Terminators

1. **Centrifuge the Performa Gel Filtration Cartridge for 2 minutes at 750 x *g*.**
2. **Transfer the cartridge to the provided 1.5-ml microcentrifuge tube and add the sample to the packed column. Be sure the fluid runs into the gel.**
3. **Close the cap and centrifuge for 2 minutes at 750 x *g*. Retain eluate.**

Appendix 14. Receiver operating characteristics (ROC) raw data

	Cut-off	Ct	spec	1-sp	sens	Sp+Se
1	Inf	-	1.00	0.00	0.00	1
2	18.51993	31.48007	1.00	0.00	0.02	1.02083333
3	16.95361	33.04639	1.00	0.00	0.04	1.04166667
4	16.92904	33.07096	1.00	0.00	0.06	1.0625
5	16.24479	33.75521	1.00	0.00	0.08	1.08333333
6	16.19699	33.80301	1.00	0.00	0.10	1.10416667
7	15.83031	34.16969	1.00	0.00	0.13	1.125
8	15.2586	34.7414	1.00	0.00	0.15	1.14583333
9	15.20129	34.79871	1.00	0.00	0.17	1.16666667
10	14.82405	35.17595	1.00	0.00	0.19	1.1875
11	14.65639	35.34361	1.00	0.00	0.21	1.20833333
12	14.51189	35.48811	0.98	0.02	0.21	1.18910256
13	14.44541	35.55459	0.98	0.02	0.23	1.2099359
14	14.44367	35.55633	0.98	0.02	0.25	1.23076923
15	14.32	35.68	0.98	0.02	0.27	1.25160256
16	14.30386	35.69614	0.98	0.02	0.29	1.2724359
17	14.26683	35.73317	0.98	0.02	0.31	1.29326923
18	14.24502	35.75498	0.98	0.02	0.33	1.31410256
19	13.96344	36.03656	0.98	0.02	0.35	1.3349359
20	13.9535	36.0465	0.98	0.02	0.38	1.35576923
21	13.69069	36.30931	0.98	0.02	0.40	1.37660256
22	13.66372	36.33628	0.98	0.02	0.42	1.3974359
23	13.3061	36.6939	0.98	0.02	0.44	1.41826923
24	12.94998	37.05002	0.96	0.04	0.44	1.39903846
25	12.85	37.15	0.96	0.04	0.46	1.4198718
26	12.8	37.2	0.96	0.04	0.48	1.44070513
27	12.54825	37.45175	0.96	0.04	0.50	1.46153846
28	12.33	37.67	0.96	0.04	0.52	1.4823718
29	12.2001	37.7999	0.94	0.06	0.52	1.46314103
30	12.2	37.8	0.92	0.08	0.52	1.44391026
31	12.11848	37.88152	0.92	0.08	0.54	1.46474359
32	12.05	37.95	0.90	0.10	0.54	1.44551282
33	12.02	37.98	0.90	0.10	0.56	1.46634615
34	11.93642	38.06358	0.90	0.10	0.58	1.48717949
35	11.77611	38.22389	0.90	0.10	0.60	1.50801282
36	11.70005	38.29995	0.90	0.10	0.63	1.52884615
37	11.6968	38.3032	0.90	0.10	0.65	1.54967949

	cut-off	Ct	spec	1-sp	sens	Sp+Se
38	11.65279	38.34721	0.88	0.12	0.65	1.53044872
39	11.64487	38.35513	0.88	0.12	0.67	1.55128205
40	11.53	38.47	0.88	0.12	0.69	1.57211539
41	11.40016	38.59984	0.87	0.13	0.69	1.55288462
42	11.34	38.66	0.85	0.15	0.69	1.53365385
43	11.27193	38.72807	0.85	0.15	0.71	1.55448718
44	11.2	38.8	0.83	0.17	0.71	1.53525641
45	11.06232	38.93768	0.83	0.17	0.73	1.55608974
46	11.06	38.94	0.81	0.19	0.73	1.53685897
47	10.94684	39.05316	0.81	0.19	0.75	1.55769231
48	10.93851	39.06149	0.81	0.19	0.77	1.57852564
49	10.76259	39.23741	0.81	0.19	0.79	1.59935897
50	10.76	39.24	0.79	0.21	0.79	1.58012821
51	10.75506	39.24494	0.79	0.21	0.81	1.60096154
52	10.69	39.31	0.77	0.23	0.81	1.58173077
53	10.67501	39.32499	0.75	0.25	0.81	1.5625
54	10.52925	39.47075	0.73	0.27	0.81	1.54326923
55	10.48	39.52	0.71	0.29	0.83	1.5448718
56	10.44	39.56	0.71	0.29	0.85	1.56570513
57	10.39873	39.60127	0.71	0.29	0.88	1.58653846
58	10.30861	39.69139	0.69	0.31	0.88	1.56730769
59	10.27007	39.72993	0.69	0.31	0.90	1.58814103
60	10.26	39.74	0.69	0.31	0.92	1.60897436
61	10.17	39.83	0.67	0.33	0.92	1.58974359
62	9.62	40.38	0.65	0.35	0.92	1.57051282
63	8.35	41.65	0.65	0.35	0.94	1.59134615
64	8.07	41.93	0.63	0.37	0.94	1.57211539
65	7.87	42.13	0.62	0.38	0.94	1.55288462
66	6.18	43.82	0.62	0.38	0.96	1.57371795
67	6.01	43.99	0.60	0.40	0.96	1.55448718
68	4.53	45.47	0.58	0.42	0.96	1.53525641
69	4.1	45.9	0.58	0.42	0.98	1.55608974
70	2.99	47.01	0.56	0.44	0.98	1.53685897
71	1.3	48.7	0.54	0.46	0.98	1.51762821
72	1.12	48.88	0.54	0.46	1.00	1.53846154
73	0.72	49.28	0.52	0.48	1.00	1.51923077
74	0	50	0.00	1.00	1.00	1