DETECTION OF SPOTTED FEVER GROUP RICKETTSIOSES, *COXIELLA BURNETII* AND *THEILERIA ORIENTALIS* IN HUMAN BLOOD AND TICK SAMPLES FROM PASTORAL COMMUNITIES IN KENYA

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Detection of Spotted Fever Group Rickettsioses, *Coxiella Burnetii* and *Theileria Orientalis* in human blood and tick samples from pastoral communities in Kenya

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A thesis submitted in partial fulfillment of the degree of Master of Science in Medical Parasitology and Entomology in the Jomo Kenyatta University of Agriculture and Technology

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DECLARATION

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

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DEDICATION

This work is dedicated to my husband Alex T. Mavugano Lumadede and my children Joshua Mboya, Joy Kasandi and Jude Mafunu and to my late mum Celestina Koka and uncle Napoleon Wasyombwii who believed in the value of education.

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LIST OF ABBREVIATIONS AND ACRONYMS

ATBF	African tick bite fever
AVID	Arbovirus Incidence and Disease
BLAST	Basic Local Alignment Search Tool
bp	base pair
CCHFV	Crimean Congo Haemorrhagic Fever Virus
CDC	Center for Disease Control
CF	Complement Fixation
DNA	Deoxyribonucleic acid
HIV	Human Immunodeficiency Virus
ID	Identification
IgM	Immunoglobulin M
IF	Immunofluorescence
IRB	Institutional Review Board
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KEMRI	Kenya Medical Research Institute
LPS	Lipopolysaccharide
MSF	Mediterranean spotted fever

N/A	Not Applicable
OmpA	Outer membrane protein A
OmpB	Outer membrane protein B
PCR	Polymerase Chain Reaction
RMSF	Rocky Mountain spotted fever
SFG	Spotted Fever Group
SSC	Scientific Steering Committee
TG	Typhus Group
µl	Microlitre
USAMRD-K	US Army Medical Research Directorate-Kenya
VHF	Viral Haemorrhagic Fever
WRAIR	Walter Reed Army Institute of Research

ABSTRACT

The tick-borne human diseases caused by *Rickettsia* spp., *Coxiella burnetii* and *Babesia* spp. are rarely reported in Kenya and yet these infections are likely contributors to undiagnosed febrile disease especially among pastoral communities. The objective of the present study was to assess the prevalence of Spotted fever group *Rickettsia* spp., *Coxiella burnetii* and *Babesia* spp. in human blood and tick samples from pastoral communities in Kenya and to determine the tick species involved in their maintenance and transmission. Archived human blood samples (278) and ticks (380 pools) collected from several geographically dispersed pastoral communities in Kenya were tested for *Rickettsia* spp., *Coxiella burnetii* and *Babesia* spp. by PCR. For *Rickettsia* spp., three primers sets were used which target the citrate synthase gene (*gltA*) primer and the outer membrane protein gene (*ompA* and *ompB).* A primer targeting the transposon-like IS1111 region was used to detect *Coxiella burnetii.* The *Babesia* spp. primer targeted the conserved β-tubulin gene that is able to detect piroplasms of *Babesia* and *Theileria* species. A subset of all PCR positive samples were sequenced and data compared with reference sequences in the GenBank. *Rickettsia* spp. were detected in 14% (39/278) of human blood samples tested using the *gltA* primer set. On the other hand, 25% of all tick pools screened were positive for *Rickettsia* spp. using the *gltA* primer set. Subsequently, all *gltA* positive tick samples were tested using *OmpA* and *OmpB* primers, 21.1 % were positive for the *ompA* gene and 28.4 % were positive for the *ompB* gene. Ticks collected from camels (60%) were significantly more infected with *Rickettsia* spp. *C. burnetii* was

detected in 5.53% of the tick tested. On the contrary, all the human blood samples were negative for *Coxiella burnetii* DNA. All the human blood and tick samples tested were negative for *Babesia* spp. DNA. However, *Theileria orientalis,* a related piroplasm was detected in a tick pool collected from a goat in Marigat. In conclusion, the findings in this study suggest that *Rickettsia* spp. may contribute to a significant proportion of febrile illness in Kenya with multiple rickettsial species circulating among tick and human populations in pastoral communities. In addition, *R. aeschlimannii* and *R. raoultii,* which have never been reported before in human samples in Kenya, was detected in the blood samples we tested. Moreover, positive ticks from camels, implicated camels in the maintenance of SFG *rickettsia* in Kenya. Finally, the study underscores the need for increased diagnostic capacity for rickettsiosis and the monitoring of *Coxiella* and *Theileria* in livestock population. It also highlights the need for livestock to be sprayed with acaricides to control ticks and prevent transmission.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Vector-borne diseases transmitted by ticks are caused by a variety of pathogens. These include bacterial (e.g. Erhlichiosis, Borreliosis, Relapsing fevers, Q fever, Lyme disease, Tularemia and the Spotted fever group rickettsiosis), protozoan (e.g. Babesiosis and Theileria) and viral (e.g. Crimean Congo Haemorrhagic Fever Virus, Kyasanur Forest disease, Tick-borne encephalitis) pathogens. The risk of human infection differs largely and depends on the prevalence of pathogens within the reservoir host and the ticks in a particular location. One tick may transmit more than one infection (Swanson *et al.*, 2006). Tick-borne infections in Kenya are commonly reported in livestock (Latif *et al.*, 1995; Okuthe and Buyu, 2006). Several reports also show that tick borne infections in humans occur in Kenya (Jensenius *et al.*, 2003; Rutherford *et al.*, 2004; Wanzala and Ondiaka, 2013).

The seroprevalence of babesiosis in cattle is reported to range between 25-53% in a district in the Eastern province of Kenya (Wesonga, 2010). Studies on the prevalence of *Babesia* spp. in free-ranging non-human primates in Kenya have indicated that 22% of wild caught baboons and African green monkeys are infected with *B. microti* (Maamun *et al.*, 2010). No human sero-surveys on babesiosis have been reported so far.

A 1976 study on *C. burnetii* infections causing Q fever reported a 12.1% prevalence rate (Vanek, 1976) in both livestock and human populations in six of the seven provinces in Kenya. The North Eastern province which has large pastoral communities was not

included in the serosurvey (Vanek, 1976). Travelers returning to Europe from Kenya were found infected with Q fever. It was postulated that the infection was acquired through inhalation of fumes in a shack that was housing two goats (Potasman *et al.*, 2000). Q fever has also been recently reported in a rural village in western Kenya in domestic animals (Knobel *et al.*, 2013). Since infected animals also shed the bacteria in urine, milk, faeces and placenta, it is possible that infection rates are higher in pastoralist communities in Kenya.

In Africa, two rickettsial species *R. conorii* and *R. africae* have been reported to cause spotted fever rickettsiosis (Raoult *et al.*, 2001). These two rickettsial species, and the newly emerged *R. aeschlimannii* and *R. mongolotimonae* have also been reported in domestic animals and ticks from various parts of Kenya (Mutai *et al.*, 2013). Molecular studies have revealed the presence of *R. africae* variants in ticks collected from rural Western Kenya (Maina *et al.*, 2014). A new strain “moyalensis” has also been identified (Kimita *et al.*, 2016). *Rickettsia felis* was also detected in human samples from Garissa district, North Eastern province and Rarieda district in Western provinces (Richards *et al.*, 2010; Maina *et al.*, 2012). Recently, a sero-prevalence study in six sites (Garissa, Alupe**,** Kisumu, Malindi, Kisii and Marigat) in Kenya reported spotted fever group *rickettsia* at 10%, typhus group *rickettsia* at < 1% and scrub typhus *rickettsia* at 5% in febrile patients (Thiga *et al.*, 2015). Another national sero-prevalence study in febrile patients reported spotted fever group *rickettsia* sero-prevalence at 23% and typhus group *rickettsia* at 0.6% in Kenya (Omballa *et al.*, 2016).

Acute febrile illness surveillance activities in tick populations from North Eastern Kenya, have shown that there is a high number of arboviruses in circulation (Lutomiah

et al., 2014). Recently, CCHFV was detected in ticks collected from various domestic animals in North Eastern Kenya (Sang *et al.*, 2011) and there is also serological evidence indicating exposure of humans to CCHFV in Ijara district in the North Eastern region (Lwande *et al.*, 2012). However, the aetiology of a proportion of these samples were not diagnosed.

This study therefore evaluated if the tick-borne non-viral pathogens, Babesiosis, Q fever and spotted fever group rickettsiosis, were contributors to the acute febrile illness in pastoral communities in Kenya by molecular analysis of human blood from febrile patients. In addition, a sample of ticks from domestic animals in these communities were identified and tested for these pathogens to establish the tick species carrying these infections and the host animals the ticks were feeding on.

1.2 Statement of the problem

The aetiology of blood samples from patients with febrile illness in Kenya remains undiagnosed after routine malarial and typhoid investigations. Babesiosis, Q fever and spotted fever group rickettsiosis are recognized tick-borne zoonotic diseases in Kenya and yet their prevalence in ticks and animal hosts and clinical impact as causes of human febrile disease are inadequately described.

1.3 Justification of the study

Nomadic pastoralist communities are likely to be exposed to tick-borne infections as a result of close interaction with a large number and diversity of domestic animals which harbor ticks. However, there is limited epidemiological surveillance in Kenya to

determine circulation of three key tick-borne pathogens namely Rickettsia, Q fever and Babesiosis in the human population. Therefore, more studies on the prevalence of these diseases especially in residents of tick-exposed communities, animal hosts and tick species that transmit them are needed. Awareness of occurrence and distribution of these infections would justify provision of diagnostic capacity to health care facilities. It would also facilitate appropriate treatment of acute phase clinical febrile illness cases to reduce disease morbidity and mortality.

1.4 Objectives

1.4.1 General Objective

To determine the prevalence of Spotted fever group rickettsiosis, Q fever and Babesiosis in undiagnosed febrile illness patient samples in tick-exposed communities in Kenya and identify the potential animal hosts and tick vectors involved in transmission.

1.4.2 Specific Objectives

1. To determine the prevalence of *Rickettsia* spp., *Coxiella burnetii* and *Babesia* spp. in archived acute febrile illness patient blood samples from the pastoralist communities in Kenya.
2. To determine the prevalence *Rickettsia* spp., *Coxiella burnetii* and *Babesia* spp. in archived tick samples from pastoralist communities in Kenya.
3. To determine the tick species that act as vectors and the animal hosts of these pathogens in the pastoralist communities in Kenya.

CHAPTER TWO

LITERATURE REVIEW

2.1 Ticks

2.1.1Taxonomy

Ticks are blood-feeding external parasites of mammals, birds and reptiles found throughout the world. Ticks are classified in the phylum *Arthropoda*, class *Arachnida* and sub-class *Acari*. *Acari* has four families *Argasidae, Ixodidae*, *Nuttalliellidae* and *Laelaptidae*. *Ixodidae* (Hard ticks) and *Argasidae* (Soft ticks) families are very well described. However, the last two families are relatively new classifications comprising of a single species each (Anderson and Magnarelli, 2008).

Approximately 900 tick species have been described worldwide (Estrada-Peña, 2015) The family *Argasidae* has 4 genera but only the genus *Ornithodoros* is of medical importance. The family *Ixodidae* is by far the largest and medically, most important family with 13 genera and approximately 650 species. Although Ixodid ticks have a worldwide distribution, they are more common in temperate regions (Swanson *et al.*, 2006).

2.1.2 External morphology of Ixodid ticks

Hard ticks have a scutum on the dorsal surface. In the male hard tick; the scutum is large, completely covering the dorsal surface. The scutum covers only a part of the dorsal surface in females and is almost obscured when engorged. There is sometimes ornamentation on the scutum of some species. There may be festoons also present on the margin of the scutum. The capitulum of hard ticks extends forward from the anterior end

of the body, bearing some resemblance to a true head. Palps are swollen and club shaped. The cheliceral sheaths are covered with small denticles. A cement-like substance from the salivary gland glues the mouthparts to allow attachment for longer periods. Four pairs of legs each ending in a claw are present (Service, 2008). These morphological features are used in the identification of species.

2.1.3 Distribution and habits of Ixodid ticks

Hard ticks are found in habitats that support a large numbers of vertebrate hosts, such as mammals, ground-dwelling birds, and lizards. Ticks of domestic animals are especially common. Ticks flourish more in countries with warm, humid climates or during wet seasons, because they require a certain amount of moisture in the air to undergo metamorphosis and because low temperatures inhibit their development from egg to larva. When not attached to a host, the ticks shelter under leaves, grass, soil, stones or shrubs (Walker *et al.*, 2003).

2.1.4 Life cycle and feeding behaviour of Ixodid ticks

Hard ticks have three distinct life stages. Larvae which emerge from the egg have six legs. After obtaining a blood meal from a vertebrate host, they molt to the nymph stage with eight legs. Nymphs feed and molt into the adult stage which also has eight legs. Mating occurs on the host and after feeding, the adult female tick drops off and lays one batch of thousands of eggs and then dies. Both sexes are blood feeders but adult males feed for shorter periods than females. In each stage, the tick seeks out a host, attaches and feeds for several days. After engorgement, the tick detaches and drops off the host. It finds a resting place where it can digest its blood meal and then molts to the next

feeding stage. Their host seeking behavior known as 'questing' allows them to get onto a preferable host and feed again. Most species feed on a different host during each stage, but there are some one-host and two-host species. This characteristic of ticks that requires them to obtain a blood meal before moulting is essential for disease transmission. The tick life cycle may take from less than a year in tropical regions to over three years in cold climates. At certain stages ticks may enter diapause, a state of arrested development, until hosts are available (Parola and Raoult, 2001).

2.1.5 Tick control

In many countries acaricides have been used to control ticks. Acaricides are sprayed on the animal host or the environment (Rajput *et al.*, 2006). Introduction of natural predators like insectivorous birds, parasitoid wasps, nematodes and bacteria (*Bacillus thuringiensis*) or deuteromycete fungi in tick infested areas have also been used for tick control (Ostfeld *et al.*, 2006). Another strategy is to have livestock vaccinated against tick-borne diseases depending on the availability of vaccines (Jongejan and Uilenberg, 1994).

2.2 Rickettsia

Rickettsiae are small (0.3 -1.0 μm) obligate gram-negative intracellular bacteria found in the cytosol of human endothelial cells and various cells of their arthropod hosts. The order Rickettsiales is currently comprised of the genera *Rickettsia, Orientia, Ehrlichia, Neorickettsia, Neoehrlichia,* and *Anaplasma*, belonging to the class *Alphaproteobacteria* (Fournier and Raoult, 2009).

Members of the genus *Rickettsia* have been classically divided into two genetically similar groups: - typhus group (TG) and spotted fever group (SFG). This is based on host specificity, intracellular location, *in vitro* growth conditions, and antigenic characteristics; the molecular sequences of conserved genes, clinical features, and epidemiology. However, the genus has been subdivided further based on phylogenetic analysis to include a transitional group (TGR) and an ancestral group (AG) (Gillespie *et al.*, 2008). Seventeen species of the genus *Rickettsia* are categorized within the SFG rickettsiae. With the exception of *Rickettsia akari* (mite-borne) and *R. felis* (flea-borne), the remaining SFG *rickettsia* species are recognized as tick-borne rickettsiae. The SFG *rickettsia* include *Rickettsia aeschlimannii*, *Rickettsia africae*, *Rickettsia australis*, *Rickettsia conorii*, *Rickettsia heilongjiangensis*, *Rickettsia helvetica*, *Rickettsia honei*, *Rickettsia japonica*, *Rickettsia marmionii* subspecies, *Rickettsia massiliae*, *Rickettsia rickettsii*, *Rickettsia sibirica*, *Rickettsia sibirica mongolotimonae* and *Rickettsia slovaca* (Blair *et al.*, 2004).

Several tick-borne rickettsiae have been identified as human pathogens and are responsible for a significant portion of acute febrile illness in man (Vitorino *et al.*, 2007). These diseases include Rocky Mountain spotted fever (RMSF)-*Rickettsia rickettsii*, Mediterranean spotted fever or boutonneuse fever- *Rickettsia conorii* subsp. *conorii*, African tick bite fever (i.e., Kenya tick-bite fever, African tick typhus)-*Rickettsia africae*, Siberian tick typhus or North Asian tick typhus-*Rickettsia sibirica* subsp. s*ibirica*, Queensland tick typhus-*Rickettsia australis*, Astrakhan fever-*Rickettsia conorii* subsp. *caspia*, Flinders Island spotted fever -*Rickettsia honei*, Japanese or Oriental spotted fever-*Rickettsia japonica*, Israeli spotted fever-*Rickettsia conorii* subsp.

israelensis among others. It is also important to note that several other rickettsia have been identified in ticks but there is no evidence that they infect humans (Parola *et al.*, 2005).

2.2.1 Epidemiology of rickettsioses

Rickettsial infections occur throughout the world. The incidence of spotted fever group is much higher in the northern hemisphere but is thought to be underreported in Africa, Central and South America (Buelow, 2011). In the United States, the RMSF is the most prevalent SFG rickettsiae (Mediannikov Oleg *et al.*, 2010). In Africa, many countries such as Sudan, Burundi, Mali and South Africa (Pretorius and Birtles, 2004) have reported the presence of rickettsios. *Rickettsia africae* is the predominant species found in these countries followed by *R. conorii* and *R. aeschlimannii.* In Kenya, recent studies have documented that human infections with *R. felis* (Maina *et al.*, 2012) occurs and that all age groups were susceptible with prevalence levels increasing with age.

Rickettsial pathogens are mainly transmitted by ticks, fleas, lice or mites through bites or cutaneous abrasions contaminated by flea or louse feces. The spotted fever group ricketsiosses are transmitted predominantly by ixodid ticks. Another factor associated with transmission is seasonality. In summer and spring months, ticks are abundant and quest for hosts actively (Huntzinger, 2007). This is also the time that people tend to be involved in more activities outdoors increasing the risk of tick contact and transmission of infection. International travelers are at risk when they travel to endemic areas (Raoult *et al.*, 2001) as some rickettsial diseases such as the Mediterranean spotted fever infections occur over a large geographical region and can be quite severe.

2.2.2 Clinical presentation of rickettsioses

Clinical manifestations may vary depending on the virulence of the rickettsial agent and host factors, such as age, gender and immunity. Common symptoms include fever, headache, malaise, nausea and vomiting. Most symptoms associated with acute rickettsial infections are non-specific and mimic many viral illnesses (Huntzinger, 2007) therefore making diagnosis difficult. Other symptoms that are more distinct include a rash with macule and papules, blisters or reddish spots or an eschar at the site of the tick bite. Swollen lymph nodes and inflamed blood vessels have been documented in severe cases (La Scola and Raoult, 1997; Sexton and Walker, 2011). For most SFG rickettsioses, an eschar (a swelling at the site of the tick bite) is common. Certain key factors enable an early diagnosis. For instance, a history of tick bite or exposure, recent travel to endemic areas, and similar illness in family members and coworkers. Most symptomatic rickettsial diseases cause moderate illness with case fatalities ranging between 0-30% (Oberoi and Singh, 2010). However, some of the SFG rickettsioses are virulent. *Rickettsia prowazekii* is one such species and kills a significant proportion of infected persons, unless the diseases are treated sufficiently early in the course of infection. Despite the variability in clinical presentations, many pathogenic *Rickettsia* spp. cause debilitating diseases making them potential biological weapons (Oberoi and Singh, 2010).

2.2.3 Diagnosis of rickettsioses

Rickettsioses have a non-specific clinical manifestation that makes diagnosis difficult in a clinical setting. A laboratory test is usually needed to confirm diagnosis (McDade, 1991). Most clinical laboratories are not able to isolate rickettsial agents by culture because some of them are virulent and require higher levels of biological containment. Rickettsiae can be isolated by the classic animal or embryonated egg inoculation (Raoult, 2010). Shell vial cell culture has also been used recently to isolate *R. prowazekii* (Birg *et al.*, 1999). Rickettsiae can also be detected by immunofluorescent assays but these techniques lack sensitivity. Fortunately, rickettsial deoxyribonucleic acid (DNA) can be detected in the acute phase (10-15 days) using blood specimens by polymerase chain reaction (PCR) and this technique offers the prospect of prompt diagnosis and treatment (Sexton and Walker, 2011). Serologic testing remains the most frequently used and inexpensive diagnostic method. However, antibody tests usually fail to identify rickettsioses early enough and are thus not effective in prompt patient management. In addition, available serologic techniques vary considerably in their sensitivity and specificity. The general unavailability of species-specific diagnostic antigens reduces specificity leading to increased cross-reactivity between recognized pathogenic and non-pathogenic species (Regnery *et al.*, 1991).

2.2.4 Treatment and management of rickettsioses

Two drugs are recommended for the treatment of rickettsioses. Tetracycline is a first line drug while chloramphenicol is better suited for pregnant or patients with severe illness that may be co-infected with a meningococcal infection. Treatment should be started

early and should not wait for confirmatory testing. Other drugs that can be used include azithromycin and fluoroquinolones (Huntzinger, 2007). No vaccine is available for preventing rickettsial infections. Antibiotics are not recommended for prophylaxis of rickettsial diseases due to antibiotic resistance. Since environmental tick control is not feasible as many of the insecticide are unsafe, travelers should be instructed to minimize exposure to infectious arthropods. Self-examination after visits to tick-infested areas helps to reduce the risk. For the public and military personnel deployed in endemics area, use of repellents on skin, wearing protective clothing or use of insecticide impregnated clothing is recommended (Kelly *et al.*, 2002).

2.3 Q fever

Q fever is a zoonotic disease caused by *Coxiella burnetii,* a small (0.2–0.4 mm wide, 0.4–1.0 mm long) obligate gram-negative intracellular bacterium. The genus *Coxiella* is morphologically similar to *Rickettsia*. As a result, *Coxiella* had been classified in the order Rickettsiales. This classification is clearly seen in older literature and review articles. Advances in molecular technology in the 1990s indicated that several bacteria originally classified in the order Rickettsiales did not belong to the α-subclass of the *Proteobacteria* phylum. As a consequence, *C. burnetii* was reclassified within the order Legionellaceae and family *Coxiellaceae* (Arricau-Bouvery and Rodolakis, 2005; Fournier and Raoult, 2009). The name "Q fever" was first used to describe a febrile illness in abattoir workers in Brisbane, Queensland, Australia in 1937. It was proposed by Edward Holbrook Derrick after his investigation of the outbreak. The name was applied at the time because the causative agent was unknown (Maurin and Raoult,

1999). Q fever has emerged as an important human and veterinary public health problem worldwide (Sprong *et al.*, 2012). It causes an acute febrile disease of low mortality but significant morbidity and it has been described as a possible biological weapon by CDC due to its highly infectious nature (Madariaga *et al.*, 2003; Cutler *et al.*, 2007). Although the disease is under-reported, outbreaks have been reported in many European countries (Angelakis and Raoult, 2010; Sprong *et al.*, 2012; Vilibic-Cavlek *et al.*, 2012). New Zealand was the only country where this disease had not been found (Norlander, 2000) but recently cases have been reported (Woldehiwet, 2004).

2.3.1 Epidemiology and transmission risk factors for *Coxiella burnetii*

The primary reservoirs of *C. burnetii* are cattle, sheep and goats. Q fever is therefore often an occupational disease affecting farmers, veterinarians and abattoir workers (Maurin and Raoult, 1999).

Transmission to humans and susceptible animals occurs primarily through inhalation of *C. burnetii* spores. Humans may also get infected through contact with milk, urine, faeces, vaginal mucus, or semen of infected animals. The infected animal also sheds the bacterium through the placenta**.** Animal infections are mainly asymptomatic but still births, late abortion, delivery of weak offspring and infertility are reported to occur (Norlander, 2000). Contamination of the environment leads to airborne dissemination of the bacterium and infection of persons in close contact with livestock (Hart, 1973; Woldehiwet, 2004; Cutler *et al.*, 2007). *C. burnetii* is highly resistant to environmental changes and can survive standard disinfectants. This phenomena of resistance is

attributed to one of its pleomorphic forms and as a result, it is hard to eliminate from the environment (Arricau-Bouvery and Rodolakis, 2005). Direct tick transmission of Q fever appears to be rare in humans and livestock as well. Nevertheless, Ixodid ticks are considered the natural primary vectors of this disease (Maurin and Raoult, 1999). The incidence of human disease is seasonal and is associated with spring and summer months when lambing and shearing leads to environmental contamination (Honarmand, 2012).

2.3.2 Clinical manifestation of *Coxiella burnetii*

C. burnetii bacteria have two antigenic variations in their lipopolysaccharide (LPS) which play an important role in the pathogenesis of Q fever in man and animals (Woldehiwet, 2004; Angelakis and Raoult, 2010). In humans, the diseases may be asymptomatic or manifest as a mild disease that is self-limiting. The incubation period is between 9 – 40 days after exposure. Two forms of Q fever are known to occur in humans. The first form is acute Q fever which is characterized by fever, chills, myalgia and headache, and in some cases pneumonia. *C. burnetii* can establish a persistent, latent infection that may reactivate months or years after initial exposure (OIE, 2010).

The second form of the disease is chronic Q fever which is mainly associated with patients who are immunocompromised (Baca, 1991) or who have pre-existing heart valve defects and most commonly presents as an endocarditis (Maurin and Raoult, 1999; Fard and Khalili, 2011). Features of chronic infection include non-specific febrile illness, pneumonia, sub-acute endocarditis, hepatitis and less commonly, granulomatous lesions in bone, soft tissues or body organs.

2.3.3 Diagnosis and treatment of *Coxiella burnetii*

Cell culture is still used as a sensitive tool for routine detection of *C. burnetii* but this method is time-consuming and requires level 3 biosafety containment (Maurin and Raoult, 1999). Serological tests are commonly used in the detection of *C. burnetii* (Stein and Raoult, 1992). Acute Q fever can be diagnosed by a fourfold rise in specific complement fixation (CF) antibodies or by direct immunofluorescence (IF) antibody testing between acute and convalescent sera collected at least 14 days apart. Q fever IgM may persist for many months after infection, hence its presence does not necessarily confirm the diagnosis of acute disease. PCR is a highly sensitive and specific method for the detection of *C. burnetii* in clinical samples (Lorenz *et al.*, 1998). Doxycycline is the drug of choice for treating acute Q fever. In pregnant women and children less than eight years doxycycline is contraindicated. The alternative treatment is co-trimoxazole. Treatment is most effective when initiated within the first three days. Chronic Q fever is difficult to treat. However, treatment of chronic Q fever endocarditis can be improved by the use of the combination of doxycycline and chloroquine (Maurin and Raoult, 1999).

2.3.4 Prevention and management of Coxiella burnetii

Control of the disease in animals will effectively reduce the level of disease in man. Effective vaccines exist for animals. Immunization of those in high risk occupational groups such as abattoir workers is the primary preventive measure against Q fever. However, human vaccines are currently not available in most countries (Angelakis and Raoult, 2010). Manure must be covered and composted or treated with 0.4% calcium

cyanamide before spreading it in the field. Sterilization of milk from infected flocks is recommended. Training is recommended for medical researchers intending to conduct Q fever screening and vaccination. Fetuses and placenta must be destroyed by incineration. Items contaminated with blood, sputum and excreta should be disinfected using standard precautions. However, it is important to note that *C. burnetii* spores survive for long periods in the environment and thus the best preventive measure is to vaccinate all uninfected animals (Arricau-Bouvery and Rodolakis, 2005; Honarmand, 2012).

2.4 Babesia

Babesiosis is an emerging infectious disease caused by an intra-erythrocytic protozoan parasite of the genus *Babesia*. It commonly infects mammalian hosts especially birds and rodents. Bovine, canine, and equine babesiosis are among the most economically relevant infections of domestic animals (Pérez de León *et al.*, 2010). The incidence of human babesiosis is increasing in many parts of the world. Babesiosis usually presents as a febrile syndrome with anaemia and haemoglobinuria (Tavassoli *et al.*, 2013). The genus *Babesia* belongs to the phylum *Apicomplexa*, class *Sporozoasida*, order *Eucoccidiorida*, suborder *Piroplasmorina* and family *Babesiidae*. At least, 100 known species of *Babesia* have been identified (Bock *et al.*, 2004).

Human babesiosis is caused by several species of *Babesia*. A significant number of infections in humans are caused by *Babesia divergens*, a parasite of cattle in Europe. In the United States, *Babesia microti,* a parasite of rodents has been implicated in the disease. Other *Babesia* species of unknown identity, currently designated WA1 type,

MO1, and CA1 to CA4 have been found to be pathogens of considerable concern (Persing *et al.*, 1995; Hunfeld *et al.*, 2002).

2.4.1 Epidemiology and transmission risk factors for babesiosis

The protozoan parasite is transmitted to mammals by ixodid ticks. Although most infections are acquired by tick bites, cases of infection by blood transfusion have been reported (Gelfand and Callahan, 2003). Cases of congenital transmission have been reported but are rare (Vannier and Krause, 2009). Increased outdoor activities, especially in the spring and summer months when ticks are abundant, predispose humans to infection (Blevins *et al.*, 2008).

2.4.2 Clinical manifestation of babesiosis

The clinical spectrum of human babesiosis ranges from an asymptomatic infection to a severe malaria-like disease resulting in severe haemolysis and occasionally in death (Krause *et al.*, 1998; Vannier and Krause, 2009). Symptomatic infection is common in asplenic patients, older patients, and patients with underlying medical conditions, including HIV. Symptoms begin seven days after the tick bite. Common symptoms include fever, malaise, fatigue, anorexia, shaking chills, headache, nausea, vomiting, abdominal pain and dark urine or haemoglobinuria (Tavassoli *et al.*, 2013).

2.4.3 Diagnosis of babesiosis

The laboratory diagnosis of babesiosis is dependent on demonstration of the characteristic parasites on Giemsa-stained thin blood films. In Giemsa-stained blood

smears, *Babesia* spp. appear as small intra-erythrocyte ring forms. At times, tetrad forms may be present but these are relatively rare (Spach *et al.*, 1993). Malaria is the most important differential diagnosis as *Plasmodium* spp. may also show intraerythrocytic rings (Hunfeld *et al.*, 2008). Although microscopy is helpful for diagnosis, in an early infection it is difficult to identify the parasites as they are relatively few in circulation in the blood (Oliveira-Sequeira *et al.*, 2005). The immunofluorescence antibody test is commonly used but it is not able to differentiate whether an infection is recent or past. PCR-based assays are sensitive and are able to facilitate clinical diagnosis of human babesiosis (Persing *et al.*, 1992)**.**

2.4.4 Treatment and management of babesiosis

A combination of an anti-protozoal agent and an antibiotic such as clindamycin and quinine or atovaquone and azithromycin is used for treating human babesiosis. A few immunocompromised hosts do not clear infection for months or years despite multiple courses of antibiotics that can result in a mortality rate as high as 20%. The mechanism of parasitic persistence in the immunocompromised remains unknown (Pérez de León *et al.*, 2010) .

Antibiotic prophylaxis for human babesiosis has not been established and live vaccines are only available for animal babesiosis. In endemic areas public health awareness and screening of public blood supplies can help reduce the burden of disease in susceptible populations especially after tick bites or after blood transfusion (Hunfeld *et al.*, 2008).

2.5 Theileria

Theileriosis is a tick-borne disease caused by intracellular protozoan parasites which belong to the Genus *Theileria*, Family *Theileriidae*, Order *Piroplasmida*, Subclass P*iroplasmia* and Phylum *Apicomplexa*. Both *Theileria* and *Babesia* are members of the suborder *Piroplasmorina.* Although *Theileria* is not a target for this study, *Theileria* is a close relative of *Babesia* and can often be detected using the same primers. There are six *Theileria* species known to infect cattle, causing different infections, *Theileria parva, T. annulata, T. taurotragi, T. mutans, T. velifera* and *T. orientalis* (Belotindos *et al.*, 2014; Gebrekidan *et al.*, 2014). *Theileria* parasites can be broadly categorized into two groups, consisting of host-cell transforming and non-transforming species (Sivakumar *et al.*, 2014). One of the non-transforming species of interest, is the *Theileria orientalis* group, an emerging pathogen of cattle (Altangerel *et al.*, 2011). This group, consisting of the closely related parasites *T. orientalis*/ *T. buffeli*, and *T. sergenti. T. orientalis* has a worldwide distribution and can be separated into several genotypes, namely, type 1 (Chitose), type 2 (Ikeda), type 3 (Buffeli), types 4–8 and types N1-N3 (Hammer *et al.*, 2015) which can be identified by a type specific PCR. The type 2 (ikeda) genotype is pathogenic. Outbreaks of *T. orientalis* have been reported in cattle from Asia, Australia, (Kamau *et al.*, 2011), New Zealand, Japan (Ota *et al.*, 2009), China and Korea and in some cases resulted in economic losses. Human infection with *Theileria* spp. was reported in China, a hospitalized patient presenting with febrile illness. The species was closely related to *T. lunwenshuni* (Chen *et al.*, 2014).

2.5.1 Epidemiology and transmission risk factors for theileriosis

Theileria spp. are transmitted by ixodid ticks, and have complex life cycles in both vertebrate and invertebrate hosts. Ticks of the genus *Haemaphysalis* (Kakati, 2015) are biological vectors of *T. orientalis*, however other ticks may be involved such as *Rhipicephalus* and *Amblyomma* (Gebrekidan *et al.*, 2014; Zhang *et al.*, 2015). Mechanical transmission has been documented by intravenous inoculation or other biting arthropods such as the tabanids. Cattle with asymptomatic theileriosis become chronic carriers of the infection and hence sources of infection for tick vectors (Hammer *et al.*, 2016) (Altay *et al.*, 2008). In endemic areas where most adult cattle are likely to be immune, calves should be closely inspected for signs of anaemia, especially between the ages of 3-12 weeks. Early signs of disease are most likely to be apparent when the cattle are being shifted from one paddock to another. Introduced cattle should be examined closely for signs of the disease (Adjou Moumouni *et al.*, 2015).

2.5.2 Clinical manifestation of theileriosis

T. orientalis is a benign parasite but is known to cause anemia in the infected animal. The parasite enters the bloodstream and infects the red blood cells. When sufficient red blood cells are destroyed anemia results. This reduces the ability of blood to carry oxygen and makes the animal ill (Watts *et al.*, 2016). Other symptoms observed include fever, tachypnoea, depression, lethargy, jaundice, abortion, decreased milk production and occasionally death (Bogema *et al.*, 2015; Hammer *et al.*, 2015). Mortality, particularly in indigenous cattle, is rare, but infection can sometimes result in progressive chronic anemia.

2.5.3 Diagnosis of theileriosis

To diagnose this infection, other causes of disease with similar clinical manifestations have to be ruled out. The presence of the piroplasms are then demonstrated microscopically on a blood film. Serology tests such as ELISAs are preferred as they are cheap, fast and can be used to test a large number of samples. However, a PCR **test** is more reliable in identifying individual genotypes. From a laboratory perspective, the presence of both benign and pathogenic genotypes of *Theileria orientalis* complicates the diagnosis of clinical theileriosis (Mans *et al.*, 2015). Several PCR assays have been developed to test for *Babesia* and *Theileria* DNA (Gubbels *et al.*, 1999) because these related parasites sometimes occur in the same geographical areas.

2.5.4 Treatment and management of theileriosis

Treatment of theileriosis is limited to supportive care and symptomatic treatment. Buparvaquone, is used in the treatment of mildly affected animals but it must be administered early in the course of the disease as severely affected animals do not respond well to treatment (Kakati, 2015). Blood transfusion can be performed on valuable animals but the procedure is expensive and may not be practical if many animals are involved. Trace element deficiencies should be corrected to minimize susceptibility to theileriosis and also improve recovery rates of diseased animals (Watts *et al.*, 2016). Outbreaks have been reported in periods of drought when animals are moved in search of pasture. The spread of the disease can be prevented by minimizing stress and movement of affected cattle. The cattle should be rested, nursed and given high quality feed. A majority of outbreaks have been reported after introduction of

cattle with an unknown health status, new arrivals should be inspected for ticks and quarantined from the rest of the herd for a minimum of a month if practical (Kamau *et al.*, 2011). Overall, appropriate tick management practices should be adapted to prevent tick borne diseases (Jirapattharasate *et al.*,2016).

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CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Sites

The five sites included in this study were Garissa and Ijara, both located in Garissa County, Mai Mahiu located in Nakuru County, Marigat located in Baringo County and Isiolo in Isiolo County.

The study sites (Figure 3.1) were selected because they fall within the arid and semi-arid zones where most communities practice pastoral farming (Lwande *et al.*, 2013), and where some of the tick-borne diseases have been previously reported (Lwande *et al.*, 2012; Lutomiah *et al.*, 2014). Due to the low rainfall and dry climate, most of these communities keep livestock such as cattle, sheep and goats. However, in some of the semi-arid sites like Isiolo and Mai Mahiu land is cultivated. In Marigat small scale farming is carried out along the irrigation scheme on river Perkerra. Despite the fact that the land is arid, a deciduous forest is present in Mai Mahiu and a coastal forest (Boni forest) is found in Ijara.

3.1.1 Study design

A cross sectional study design was used.

3.1.2 Sample selection criteria

A simple random sampling procedure was used to select an equal number of samples per region, site and year to ensure that there was good geographical and temporal distribution. No sample was excluded even if already tested for other aetiologies.

3.1.3 Sample size determination

$$N = \frac{Z^2 P (1-P)}{e^2}$$

Where:

N = required sample size

Z = confidence level at 95% (standard value of 1.96)

P = estimated prevalence of the disease in the region

e = Margin of error at 5% (standard value of 0.05)(Fosgate, 2009).

It has been reported (Maamun *et al.*, 2010) that the prevalence of Babesiosis in non-human primates in Kenya is 22%. The prevalence of Spotted Fever group rickettsiosis in Kenya is estimated at 50 % (Prabhu *et al.*, 2011) and that of Q fever is approximately 12 % (Vanek, 1976). Using these prevalence rates as the P value in the formula above and substituting accordingly, the minimum sample size was determined. For Babesiosis, it was 264 samples; for Q fever, it was 162 samples and for Spotted Fever group rickettsiosis, it was 385. Eventually the actual sample size tested was: 278 for human samples and 380 for tick samples.

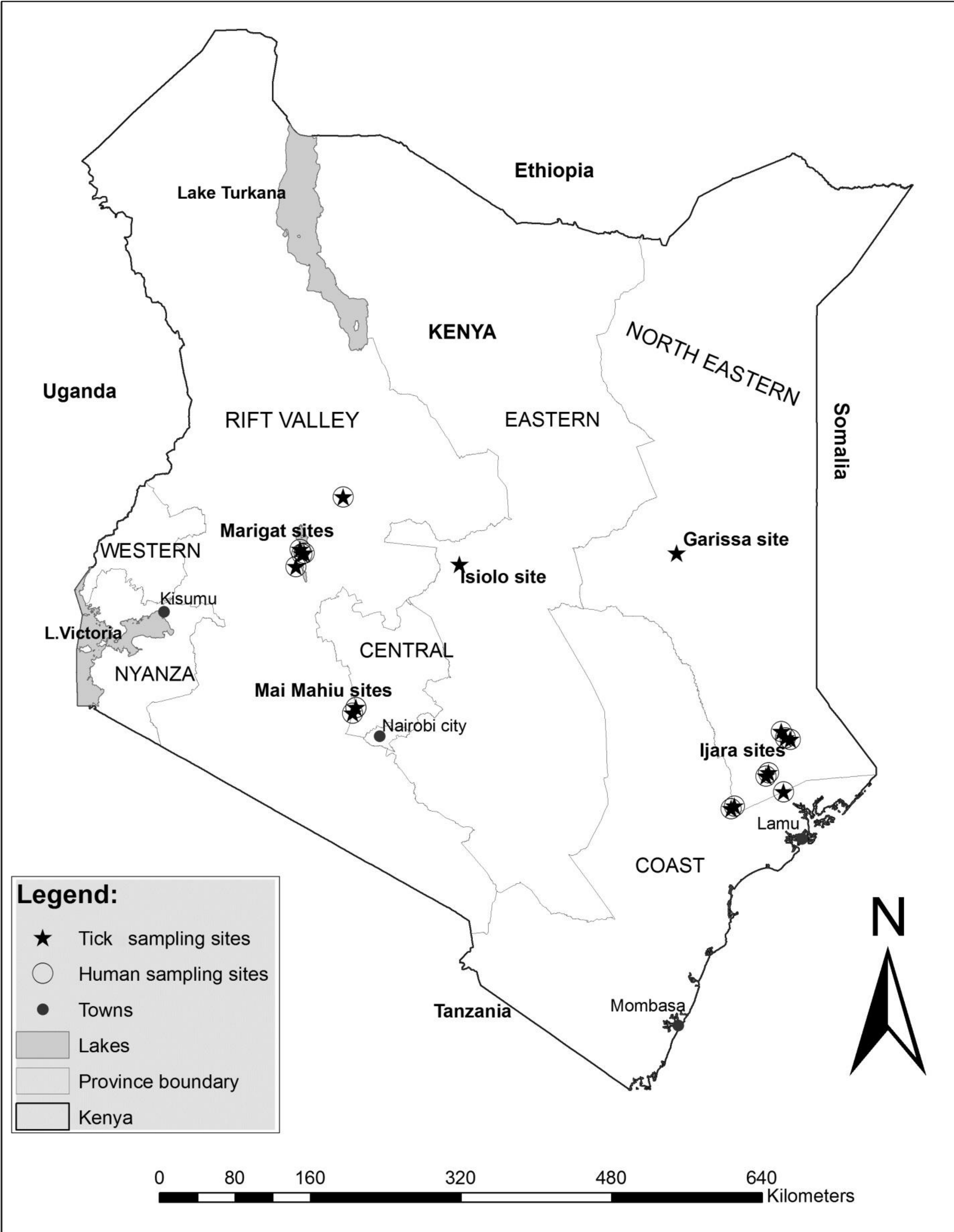

Figure 3.1: A map of Kenya indicating the sites where human blood and tick samples were collected

3.2 Human samples

Coded archived human whole blood samples from a previous parent study "*An integrated response system for emerging infectious diseases in East Africa*" KEMRI IRB #1560 were tested in this study. The samples had been kept in a -80^{0}C freezer at the KEMRI repository and were only obtained after approval was granted by the Principal Investigator of the study. The blood samples were obtained from three geographically dispersed pastoral communities. The samples had been collected from patients >1 year of age presenting with fever and attending dispensaries in Marigat, Mai Mahiu and Ijara from December 2011 to December 2012. The samples were collected to determine the cause of undiagnosed fevers. The main inclusion criteria that was used for patients to be recruited were fever >37.5^{o}C. Other symptoms that typically accompanied the fever included headache, arthralgia and myalgia, diarrhoea, chills, muscle aches, coughs, abdominal pain and vomiting.

3.3 Tick samples

All the tick tested had been collected from animals during the period 2006-2011 from five pastoral areas under the studies *"An integrated response system for emerging infectious diseases in East Africa*" IRB # 1560 and *Surrogate epidemiological methods to assess arboviral infection distribution by entomological surveillance*" IRB# 824 and WRAIR #1134. Some ticks had been identified, pooled and homogenized prior to storage at -70 to -80°C. These archived tick homogenates from Ijara, Isiolo and Garissa County were obtained for testing. A portion of archived whole adult ticks stored at -70 to -80°C from Marigat and Mai Mahiu districts were identified, pooled, homogenized and tested in this study.

3.4 Procedures

3.4.1Tick identification and homogenization

All the 380 tick pools tested in this study were collected in 2006-2011, from livestock in the five study sites. The archived tick homogenates and whole adult ticks were identified to the species level using two tick identification keys; Matthysse and Colbo 1987, Okello-Onen *et al.,* 1999 (Matthysse, 1987; Okello-Onen, 1999). The ticks were then pooled in groups of 1 to 8 according to sex, developmental stage, species, area, site, collection date and host**.** Each tick pool was placed in a pre-chilled sterile mortar and homogenized in 90-mesh alundum (Thermo Fisher Scientific, Fair Lawn, NJ) with a pestle. The homogenization media contained 2ml minimum essential medium, constituting 15% fetal bovine serum, 2% l-glutamine and 2% antibiotic and antimycotic (100 U/ml penicillin, 100µg/ml streptomycin and 1µl/ml fungizone) (Sigma-Aldrich, St. Louis, MO) (Lutomiah *et al.*, 2014). Homogenates were clarified by low speed centrifugation (5000rpm) and the supernatants stored at -70 to -80°C until tested.

3.4.2 DNA extraction

DNA was extracted from both tick homogenates and human whole blood samples using the Qiagen DNeasy Blood and Tissue kit (Qiagen Inc., Valencia, CA). Briefly, in a biological safety cabinet 200ul of tick homogenate was transferred to 1.5ml eppendorf tube and 20ul of proteinase K was added. An eppendorf thermomixer was used to incubate the sample at 56^{0}C for 1 hour. Buffer AL (200ul) was added to the sample, mixed and incubated at 56^{0}C for 10min. Absolute Ethanol (200ul) was added and the sample mixed. The mixture (about 630ul) was then pipetted into DNeasy mini spin column and centrifuged at 8000rpm for 1min. The flow through was discarded and the

collection column replaced. Buffer AW1 (500ul) was added to the column and the column centrifuged for 1 min. The collection tube containing the flow through was discarded and the column was placed in a new collection tube. Buffer AW2 (500ul) was added to the column and it was centrifuged for 3min at 14000rpm. The column was transferred to a 1.5ml centrifuge tube and 50ul of Buffer AE or water was added for elution. The column was incubated at room temperature for 1 min and then centrifuged for 1 min at 8000rpm. To extract human whole blood samples, the Qiagen DNeasy Blood and Tissue protocol for non-nucleated blood was used. The DNA obtained was quantified using a Nanodrop 2000 spectrophotometer (Thermo Fisher scientific) and stored at -70 to -80 ^{0}C.

3.4.3 PCR detection of *Rickettsia* spp. in human blood and tick samples

The spotted fever group of *Rickettsia* was detected by a single step PCR assay using the primers CS78 and CS323 targeting a 401 bp fragment of the citrate synthase gene (*gltA*) (Labruna *et al.*, 2004; Ndip *et al.*, 2004). The following PCR cycling conditions were used 95^{0}C for 3 min followed by 40 cycles of 95^{0}C for 15 s, 55^0 C for 30 s, 72^{0}C for 30 s and a final extension at 72^{0}C for 7 min. The DNA of *Rickettsia africae* was used as a positive control (Mutai *et al.*, 2013) and water as a negative control. Foetal bovine serum was also tested to determine that it was free of contaminating rickettsial bacteria DNA. Further analysis of the positive samples was performed by conventional PCR amplification of the rickettsial outer membrane protein genes using primer *ompA* and *rompB*. The additional primers were used to allow for more accurate speciation of the

Rickettsiae as species cannot be determined based on *gltA* gene-fragment alone (Table 3.1).

Table 3.1: Oligonucleotide primers and PCR product size used to detect Rickettsia spp., Coxiella burnetii and Babesia spp.

Primer	Nucleotide Sequence	Expe cted size	Pathogen
CS78	GCAAGTATCGGTGAGGATGTA	401bp	Rickettsia
CS323	GCTTCCTTAAAATTCAATAAATC		
OmpAF	ATGGCGAATATTTCTCCAAAA	532bp	Spotted fever Rickettsia
OmpAR	AGTGCAGCATTCGCTCCCCCT		
rompB OF	GTAACCGGAAGTAATCGTTTCGTAA	420bp	Spotted fever Rickettsia
rompB OR	GCTTTATAACCAGCTAAACCACC		
rompB SFG IF	GTTTAATACGTGCTGCTAACCAA	420bp	Spotted fever Rickettsia
rompB SFG/TG IR	GGTTTGGCCCATATACCATAAG		
rompB TG IF	AAGATCCTTCTGATGTTGCAACA	230bp	
TRANS1	TATGTATCCACCGTAGCCAGTC	687bp	Coxiella-Q Fever
TRANS2	CCCAACAACACCTCCTTATTC		
F34	TGTGGTAACCAGAT(t/c)GG(a/t)GCCAA	310-460bp	Babesia spp
R323	TCnGT(a/g)TA(a/g)TGnCC(t/c)TT(a/g)GCCCA		
F79	GA(a/g)CA(t/c)GGnATnGA(t/c)CCnGTAA	169-319bp	Babesia spp
R206	AC(a/t/g)GA(a/g)TCCATGGT(a/t/g)CCnGG(t/c)T		

For the *ompA* primer the following conditions were used, 94^{0}C for 5 min followed by 94^{0}C for 30 s, 58^{0}C for 1 min, 72^{0}C for 2 min sec, for 35 cycles and a final extension at 72^{0}C for 5 min (Ndip *et al.*, 2004) (Regnery *et al.*, 1991). The *rompB* primer was a nested primer set for the *ompB* gene. The following conditions were used in the first PCR reaction. An initial denaturation step at 95°C for 5 min, followed by 35 cycles of

95°C for 15 s, 54°C for 15 s and 72°C for 30 s, and a final extension at 72°C for 3 min. The nested PCR cycling conditions included an initial denaturing step of 95°C for 5 min, subjected to 35 cycles of 95°C for 15 s, 56°C for 15 s and 72°C for 30 s, and a final extension at 72°C for 3 min (Choi *et al.*, 2005).

The PCR assays were performed in a Gene Amp 9700 thermocycler (Applied Biosystems) using a Taq PCR master mix kit (Qiagen Inc.,Valencia, CA), 1ng of template DNA and 1µl of 20 µM of *gltA, ompA* and outer r*ompB* primers pairs in a 25µl reaction mix. The nested PCR reaction mixture contained 1µl of 10 µM of the inner *ompB* primers in a 25µl reaction mix. PCR products were separated on a 2% agarose gel and visualized with ethidium bromide on a UV transilluminator. Products were sized using an O'rangeRuler 100bp DNA ladder (Thermo Fisher Scientific).

3.4.4 PCR detection of *Coxiella burnetii* in human blood and tick samples

Coxiella burnetti was detected using a single step conventional PCR assay using the primers Trans 1 and Trans 2 (Fard and Khalili, 2011). The primers were designed to amplify a 687-bp fragment which recognizes the repetitive, transposon-like IS1111 region. The PCR amplification conditions for the Trans primer included an initial denaturation step at 95° C for 2 min, followed by five cycles at 94° C for 30 s, 66 to 61° C (the temperature was decreased by 1° C between consecutive steps) for 1 min, and 72°C for 1 min. These cycles were followed by 35 cycles of 94° C for 30 s, 61° C for 30 s, and 72° C for 1 min and then a final extension step of 10 min at 72° C. *Coxiella burnetii* DNA was used as a positive control and water was used as a negative control. The positive control was provided by Dr John Waitumbi (Walter Reed Project-Kisumu).

The PCR assays were performed in a Gene Amp 9700 thermocycler (Applied Biosystems) using a Taq PCR master mix kit (Qiagen Inc.,Valencia, CA), 1ng of template DNA and 1µl of 50 µM of the Trans primer in a 25µl reaction mix. PCR products were separated on a 2% agarose gel visualized with ethidium bromide on a UV transilluminator. Products were sized using an O'rangeRuler 100bp DNA ladder (Thermo Fisher Scientific).

3.4.5 PCR detection of *Babesia* spp. in human blood and tick samples

Babesia spp. were detected by a nested PCR of the conserved β-tubulin gene that is able to detect seven *Babesia* species including *B.microti* and *B. divergens* that are known to infect humans (Caccio *et al.*, 2000). The primary primer is also able to detect piroplasms of the genus *Theileria*. This primer set is strongly conserved and is able to detect the genomic DNA of several hosts including man and cattle and thus a nested PCR amplification is recommended. The PCR assays were performed in a 7500 fast thermocycler (Applied Biosystems) using a Taq PCR master mix kit (Qiagen Inc.,Valencia, CA), 1ng of template DNA and 20 µM of the Babesia primer pairs. The primary PCR thermocycling conditions consisted of an initial denaturation step at 94^0C for 5 min, followed by 35 cycles each consisting of 94^0C for 30 s, 62^0C for 30 s and 72^0C for 60 s with a final extension cycle at 72^0C for 5 min. The nested PCR cycling conditions were similar to the primary PCR conditions. However, the number of cycles were reduced to 30. Expected amplicon sizes for *B. microti* were 310 bp - 460 bp for *Babesia* spp. and 367 bp for *Theileria* spp. *B. microti* DNA was used as a positive

control (Maamun *et al.*, 2010) and water was used as a negative control. PCR products were separated on a 2% agarose gel visualized with ethidium bromide.

3.4.6 Sequencing

The PCR products obtained from samples that were positive for *Rickettsia* spp., *Coxiella burnetii* and *Babesia* spp. were purified using the QIAquick PCR purification kit (Qiagen Inc., Valencia, CA), according to manufacturer's instructions and sequenced directly using the Sanger chain termination method. The resulting sequence fragments were assembled into contigs using DNA baser version 3.2 (Heracle, 2012). Preliminary identification of specific species obtained were performed using the online Basic Local Alignment Search Tool (BLASTn) (NCBI, 2016).

The obtained nucleotide sequences encoding *gltA*, *ompA* and *ompB* proteins of *Rickettsia* spp., were separately aligned by Muscle v3.8 software (Edgar, 2004) and compared to reference sequences from GenBank. Nucleotide sequences from this study were deposited in the GenBank.

3.4.7 Data management and analysis

The tick pools and human blood samples selected from the archived samples for this study were entered in an Excel database (Microsoft Corporation, Redmond, WA, USA) which included relevant information pertaining to the study. For the tick samples, this included the region, collection date, animal host, site name, site number, tick pool

number, sex, species and PCR result. For the human samples, this included date of collection, village, age, sex, occupation, animals the subject was in contact with and the PCR results. The archived human samples had codes that de-identified the patients thus maintaining confidentiality. Statistical analysis was done using the R statistical software -R version 3.1.0. R core Team (2014) Vienna, Austria. Group comparisons were carried out using the Chi-square test. Outcomes were considered significant if the p-value was < 0.05 (RCoreTeam, 2014).

3.4.8 Ethical consideration

This study was approved by the Scientific Steering Committee (SSC) and the Ethical Review Committee (ERC) at Kenya Medical Research Institute (KEMRI), protocol# 2454 (Appendix I and II) and at Walter Reed Army Institute of Research (WRAIR), protocol #2099. Collection of human samples and ticks had been previously approved under KEMRI protocols #1560 - *An integrated response system for emerging infectious diseases in East Africa*" and #824 -"*Surrogate epidemiological methods to assess arboviral infection distribution by entomological surveillance*" (WRAIR #1134).

CHAPTER FOUR

RESULTS

4.1 Prevalence of Rickettsia spp. in human blood samples from Ijara, Mai Mahiu and Marigat

Overall, 39/278 (14.03%, 95% CI 10.17-18.68%) of the human blood samples were positive for *Rickettsia* using the *gltA* primer set (Figure 4.1). All the human blood samples that were positive in the initial screening by *gltA* gene tested negative using the outer membrane protein primers (*ompA* and *ompB*). The only statistically significant association with *Rickettsia* spp. infection was with blood of patients with no report of tick bites (χ^2=5.65, df =1, p=0.017) (Table 4.1). The 39 patients whose blood was positive for rickettsia were between 2-72 years of age. A majority of the patients (35.9 %) had a fever that was $\geq$ 38.0 ^{0}C but no rash and 41% were housewives. Malaria was commonly diagnosed in these patients and many of them reported coming into contact with domestic animals (Table 4.2).

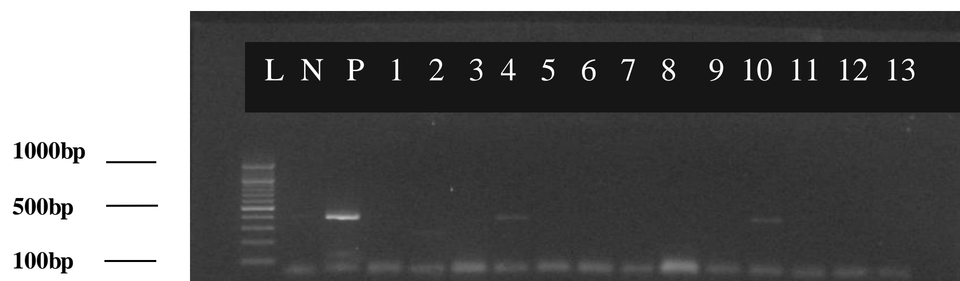

Figure 4.1: Agarose gel image of PCR results of the Rickettsia positive human blood samples using the *gltA* primer. L- GeneRuler DNA 100 bp ladder, N- negative control (water), P- positive control (*R. africae*) expected size 401bp, Lane 1-13 samples tested. Rickettsiae DNA positive samples are in lane 4, 10.

Table 4.1: Demographic characteristics of Rickettsiae positive subjects and prevalence of Rickettsia spp. by PCR targeting the gltA fragment.

Variable		Subjects	Rickettsia		x^2	P value
			# positive	% positive		
All		278	39	14.03		
Sex						
	Female	174	26	14.94		
	Male	98	12	12.24		
	Missing	6	1	16.67	0.414	0.813
Age (years)						
	< 20	94	12	12.77		
	20-29	62	11	17.74		
	30-39	54	9	16.67		
	40-49	14	1	7.14		
	50+	46	5	10.87		
	Missing	8	1	12.50	2.091	0.836
Occupation						
	House wife	95	16	16.84		
	Farmer/herdsman	54	8	14.81		
	Teacher/student/sheik	81	9	11.11		
	Business	20	4	20.00		
	Retired	12	1	8.33		
	Other	16	1	6.25	2.940	0.709
Location						
	Ijara	182	27	14.84		
	Mai Mahiu	32	4	12.5		
	Marigat	64	8	12.5	0.284	0.868
Had tick bite						
	No	232	37	15.95		
	Yes	43	1	2.33		
	Unknown	3	1	33.33		0.038

N.B: Demographic data obtained from the parent study #1560

4.2. Distribution of tick species for each study site

In total 380 tick pools were sampled, **76** from each of the five sites. Some of the tick species could not be fully identified to species level as some body parts were missing. In the ticks tested, 12 species were identified from the five sites. Ijara had the greatest diversity of tick species (8) followed by Garrisa (7), Isiolo (4), Mai Mahiu (5) and Marigat (5) (Table 4.3). Only two species (*Rhipicephalus pulchellus* and *Amblyomma gemma*) were found across all five sites. Half (53%, n=201) of all ticks sampled were *Rh. pulchellus*, the most abundant species.

Table 4.3: Distribution of tick species for each collection site

Species	Garissa		Ijara		Isiolo		Mai mahiu		Marigat	
	#ticks	%ticks	#ticks	%ticks	#ticks	%ticks	#ticks	%ticks	# ticks	%ticks
Rh. pulchellus	34	44.7	55	72.4	68	89.5	1	1.3	43	56.6
Rh. evertsi evertsi	0	0.0	0	0.0	0	0.0	65	85.5	3	3.9
H. truncatum	21	27.6	4	5.3	3	3.9	0	0.0	1	1.3
Rh. appendiculatus	0	0.0	1	1.3	0	0.0	0	0.0	28	36.8
A. gemma	6	7.9	5	6.6	4	5.3	1	1.3	1	1.3
H. marginatum	5	6.6	2	2.6	0	0.0	8	10.5	0	0.0
Hyalomma spp	7	9.2	1	1.3	0	0.0	0	0.0	0	0.0
A. lepidum	0	0.0	7	9.2	0	0.0	0	0.0	0	0.0
A. variegatum	2	2.6	0	0.0	0	0.0	1	1.3	0	0.0
A. hebraeum	0	0.0	0	0.0	1	1.3	0	0.0	0	0.0
Amblyomma spp	1	1.3	0	0.0	0	0.0	0	0.0	0	0.0
Rh. annulatus	0	0.0	1	1.3	0	0.0	0	0.0	0	0.0

A: Amblyomma, H: Hyalomma, Rh: Rhipicephalus

4.2.1 Prevalence of *Rickettsia* spp. in ticks

Of the 380 tick pools tested, 25% (95% CI 20.72-29.67%) were positive for *Rickettsia* using the *gltA* primer set (Table 4.4) (Fig 4.2). The prevalence of *Rickettsia* varied significantly across the sites (χ^2=42.25, df =4, P<0.0001), with Garissa recording the highest prevalence (52.63%), followed by Isiolo (25%), Marigat (19.7%), Ijara (14.5%), and Mai Mahiu (13.16%). The number of tick pools positive for *Rickettsia* also varied significantly with animal host (χ^2=34.21, df =3, P<0.0001) (Table 4.4). The highest number of tick pools positive for *Rickettsia* were collected from camels (60%), followed by cattle (31.37%), sheep (16.67%), and goats (13.74%). *Rickettsiae* positive ticks were detected predominantly in three genera; *Hyalomma* (76.9 %), *Amblyomma* (44.8%) and *Rhipicephalus* (14 %). All *gltA* positives tick samples were subsequently tested using the outer membrane protein primers (*ompA* and *ompB*). A total of 21 (22.1%) were positive for the *ompA* gene and 27 (28.4%) were positive for the *ompB* primer set and 16.8% of these tick samples were both *ompA* and *ompB* positive.

Table 4.4: Prevalence of Rickettsia spp. and C. burnetii in tick pools collected from different hosts in the study sites (gltA gene)

Species	# tick pools per animal host				Total	*Rickettsia*		*Coxiella*	
	Camel	Cattle	Goat	Sheep		n	% positive	n	% positive
A. gemma	1	11	4	1	**17**	7	41.2	1	5.9
A. hebraeum	0	0	1	0	**1**	0	0.0	0	0.0
A. lepidum	0	3	4	0	**7**	3	42.9	0	0.0
A. variegatum	0	1	2	0	**3**	3	100.0	0	0.0
Amblyomma spp.	0	1	0	0	**1**	0	0.0	0	0.0
Rh. annulatus	0	0	0	1	**1**	0	0.0	0	0.0
H. marginatum	1	13	1	0	**15**	13	86.7	0	0.0
H. truncatum	7	14	4	4	**29**	20	69.0	0	0.0
Hyalomma spp.	6	2	0	0	**8**	7	87.5	0	0.0
Rh. appendiculatus	0	4	17	8	**29**	7	24.1	2	6.9
Rh. pulchellus	15	79	79	28	**201**	33	16.4	8	4.0
Rh. evertsi evertsi	0	25	19	24	**68**	2	2.9	10	14.7
Total	**30**	**153**	**131**	**66**	**380**				
N	18	48	18	11	**95**				
%Positive	60.0	31.37	13.74	16.67	25.0				

A: Amblyomma, H: Hyalomma, Rh: Rhipicephalus, n: number positive tick pools

Figure 4.2: Agarose gel image of PCR results of tick samples that were positive for Rickettsia DNA using the *gltA* primer. L- GeneRuler DNA 100 bp ladder, N- negative control (water), P- positive control expected size 401bp *(R. africae)*, Lane 1-13 samples tested. Positive samples are in lane 11, 12, 13.

4.3 *Rickettsia* spp. identified from the PCR positive tick and human blood samples

A total, of 12 PCR positive amplicons from human blood samples were successfully sequenced using the *gltA* primer and compared to those available in GenBank database using BLASTn analyses. Three *Rickettsia* spp.; *R. aeschlimannii (*n=8)*, R. africae* (n=3) and *R. raoultii* (n=1) were detected in the human blood samples (98% homology). However, none of the human blood samples tested positive for *Rickettsia* spp. using either the *ompA* or *ompB* primer.

A total of 23 *gltA*, 21 *OmpA* and 27 *OmpB* amplicons from tick pools were successfully sequenced and compared to those available in GenBank database using BLASTn analyses. The *Rickettsia* spp. detected in the *gltA* positive tick pools were predominantly *R. africae* or *R. aeschlimannii,* and *R. raoultii*, with single *R. sibirica*, and *R. conorii* subsp. *israelensis.* A BLASTn search showed that the *OmpA* sequences of the tick pools exhibited homology to those of *R. aeschlimannii* (n=14), *R. africae* (n=6) and *R. monglotimonae* (n=1) species available in GenBank. Likewise O*mpB* sequences of the tick pools showed homology to *R. aeschlimannii* (n=14), *R. raoultii (n=1)*, *R. montanensis (n=1), R. africae* (n=7) and *R. parkeri* (n=4)(Sequence homologies ranged from 97- 100 %). Only seven (7) tick pool samples were successfully amplified using the three primers (*gltA, ompA* and *ompB*). Using several genes targets clarifies the taxonomic classification of species within the spotted fever group *Rickettsia*. However, consensus sequence identity was not obtained for two of the seven tick pool samples, different species were detected with each of the three primer sets (Table 4.5). For five of the tick pools sequences, a consensus identity was obtained with sequence homology to

R. aeschlimannii and *R. africae*. Nucleotide sequences of *gltA*, *OmpA* and *OmpB* genes reported in this study are available in the GenBank under accession numbers: KX227761 to KX227792. Those for reference strains were: NC_010263, NC_017044, NC_012633, NZ_AABW01000001, NC_016639, NC_015866, NC_016050, NC_003103, CP001227, CP010969, NC_017043, NZ_CP013133, NZ_CM001467, NC_009881 NC_017058, NC__009900, HM050277, HM050278 and HM050289.

4.4. Prevalence of *Coxiella burnetii* in ticks

All the human blood samples tested negative for *Coxiella burnetii* DNA. On the contrary, *Coxiella burnetii* was detected in 5.53% (95% CI 3.45-8.32) of the tick pools tested (Figure 4.3). The prevalence of *Coxiella burnetii* varied significantly (p= 0.006) across the sites with Mai Mahiu recording the highest prevalence at 13.16%; followed by Marigat (7.89%), Isiolo and Ijara (both 2.63%), and Garissa recording only a single positive tick. The number of tick pool samples positive for *C. burnetii* was not significant with respect to animal host (p= 0.152): sheep (10.61%), goat (5.34%), cattle (4.58%) and no tick from camels was positive. *C. burnetii* was predominantly detected in *Rhipicephalus* species (95.2%)(See section 4.2.1, Table 4.4).

4.4.1 *Coxiella burnetii* identified from the PCR positive tick samples

Four *C. burnetii* positive samples from ticks were sequenced and compared with sequences in GenBank. Two of the tick pools sequenced were *Rh. evertsi* from Mai Mahiu, one from sheep and one from cattle. The other two were *Rh. pulchellus* tick pools, one each, from Garissa and Isiolo collected from a goat and cattle respectively.

The *C. burnetii* positive samples were 94-97% identical to *C. burnetii* strain Cb175 from Guyana that was deposited in the GenBank.

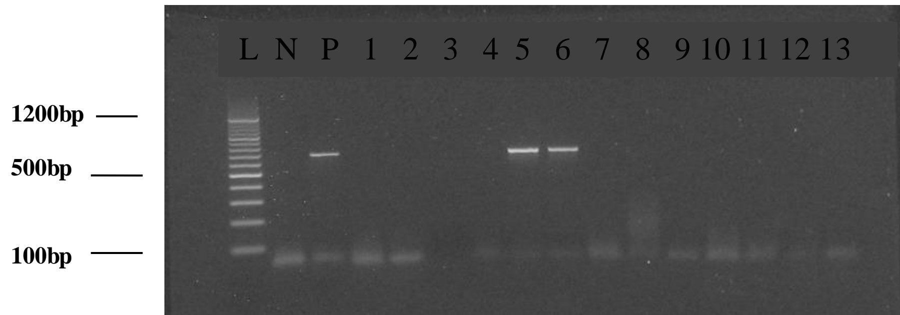

Figure 4.3: Agarose gel image of PCR results of Coxiella burnetii positive tick samples. L- GeneRuler DNA 100 bp ladder, N- negative control (water), P- positive control (*Coxiella burnetii*) expected size 687bp, Lane 1-13 samples tested. Positive samples in lane 5, 6.

4.5 Prevalence of *Theileria spp* using the ubiquitous *Babesia* primer set in ticks

All the human blood samples tested negative with the *Babesia* primer set. Nonetheless, one tick pool (0.26%), tested positive with the *Babesia* primer set (Figure 4.4). The tick pool, a *Rhipicephalu*s *pulchellus* species had been collected from a goat in the Marigat site. The amplicon was sequenced and found to have a 99% sequence homology to *Theileria orientalis* which is closely related to the Kenya *Theileria parva* strain Muguga sequence in the GenBank.

Figure 4.4: Agarose gel image of PCR results of a Babesia positive tick pool. L- GeneRuler DNA 100 bp ladder, N- negative control (water), P- positive control (*Babesia*) expected size 310bp, Lane 1-13 samples tested. Positive sample on lane 4 (367bp).

CHAPTER 5

DISCUSSION

This study set out to determine if rickettsiosis, Q fever and babesiosis were a cause of acute febrile illness in populations at increased risk for tick transmitted zoonotic diseases due to their pastoral lifestyle. The specific objectives were to determine the prevalence of *Rickettsia* spp., *Coxiella burnetii* and *Babesia* spp. infection in acute febrile patients and in ticks from pastoralist communities and identify the important tick species and reservoir host animals involved in transmission.

5.1 Rickettsia spp.

5.1.1 *Rickettsia* spp. prevalence in humans

Rickettsial DNA was detected in 14% of the blood of febrile patients. However, the DNA was only detected using the *gltA* gene specific primers and not the outer membrane protein primers indicating that the *gltA* primers were more sensitive than the outer membrane protein primers in detecting the rickettsia present in the human blood samples. A study comparing the performance of the single stage (16S *r*DNA, *gltA*, *htrA*) and sequential (*ompB*, *gltA*, *ompA*) PCR assays in the diagnosis of human rickettsioses also reported that the single stage PCR for the *gltA* primer was more sensitive in detecting *Rickettsia* (Santibáñez *et al.*, 2013). In contrast, another study found the conventional PCR assay for the *gltA* primer to be less sensitive than the *OmpA* primer for testing blood and tissue samples from infected laboratory animals (Zemtsova *et al.*, 2015). It is unclear what factors contribute to the variability observed between sensitivities of these assays, but this study would suggest that multiple gene loci should be utilized to conduct molecular screening for rickettsial infections in humans.

Sixty four percent of the patients whose blood was positive for *Rickettsia* by the *gltA* PCR had been diagnosed as having malaria. The non-specific clinical characteristic presented by the patients in this study and especially the lack of a rash or eschar could have led to the undiagnosed cases of rickettsiosis. Our findings suggest that *Rickettsia* is a significant contributor to undiagnosed fevers and should be included in the differential diagnosis of febrile illnesses in pastoral communities. Clinicians should also be educated on some of the clinical features that distinguish malaria from Rickettioses. The rickettsial infections in this study are likely related to exposure to bites from anthropophagic ticks that were collected in the five sites (Gleim *et al.*, 2016). The only statistically significant association observed between the different groups studied and their characteristics was that the rate of rickettsial infection was higher in those who indicated that they had experienced no tick bites. This is a fairly counter-intuitive association given that *Rickettsiae* are tick transmitted. However, a history of no tick bite is generally reported by patients diagnosed with tick-borne diseases. This is because the hard ticks do not cause pain when feeding and in most cases the immature stages of the ticks go undetected (Parola and Raoult, 2001). This fact probably allowed the ticks to feed longer and thus transmit the bacteria more efficiently. It is also possible that feeding ticks are not the only mode of transmitting the infection but the bacterium is transmitted by people inadvertently scratching crushed ticks or infectious tick feces into the skin (Parola *et al.*, 2005). A known tick bite was protective and this was probably because prompt removal of attached ticks prevents or shortens the time for feeding and therefore reduces opportunity for bacterial transmission.

Three spotted fever group rickettsiae: *R. africae, R. aeschlimannii* and *R. raoultii*, were detected in the human blood samples that were sequenced. Importantly, *R. aeschlimannii* and *R. raoultii* have not been reported before in human samples from Kenya. In fact, very few acute clinical cases of *Rickettsia* infection have been reported in Kenya (Wanzala and Ondiaka, 2013) and diagnosed cases of acute rickettsiosis are mainly reported by travelers returning from sub-Saharan African countries (Raoult *et al.*, 2001; Jensenius *et al.*, 2003). Unfortunately, only a few positive amplicons were sequenced due to depletion of the sample and thus it is possible that other pathogenic rickettsial species would have been detected if all the positive blood samples had been sequenced. *Rickettsia africae* is known to cause African tick bite fever (ATBF) while *R. aeschlimannii* causes a clinical syndrome that is similar to the Mediterranean spotted fever (MSF) also known as the Kenya typhus fever that is caused by *R. conorii* (Pretorius and Birtles, 2002). ATBF is a mild illness characterized by fever, headache, myalgia, but no prominent rash (Kelly *et al.*, 1996). Infection with *R. aeschlimannii* is characterized by fever, maculopapular rash, an inoculation eschar at the tick bite site and is normally mistaken for an *R. conorii* infection (Raoult *et al.*, 2002). *R. raoultii* causes scalp eschar and neck lymphadenopathy after tick bite (SENLAT) (El Karkouri *et al.*, 2016). However, an eschar or rash was not seen in any of the patients that tested positive for *Rickettsia* in this study. This is not surprising given that in early stages of rickettsiosis these symptoms are not always present and even if present there is a possibility of lack of clinical recognition by the physicians (Halajian *et al.*, 2015).

5.1.2 *Rickettsia* spp. prevalence in ticks

The study was able to demonstrate a high prevalence (25%) of rickettsial infection in tick pools obtained from livestock, with positive ticks being found at every site. This prevalence level is similar to that found in a study which examined ticks from livestock originating from counties across Kenya at two major slaughter houses (Mutai *et al.*, 2013). This confirms that *Rickettsia* will be present where cattle and other domestic animals which harbor ticks are found (Okabayashi *et al.*, 1999) (Brouqui *et al.*, 2004). The greatest diversity of ticks was collected from Ijara followed by Garissa and Isiolo. Furthermore, ticks from Garissa (52.63%) and Isiolo (25%) had a significantly higher *Rickettsia* infection rate than in Ijara, Marigat and Mai Mahiu. Although, there was no human blood samples from Garissa and Isiolo to test, which was a limitation of this study, the results suggest that the populations in these communities may be at a greater risk of rickettsiosis.

H. marginatum, H. truncatum, Rh. pulchellus, Rh. appendiculatus, Rh. evertsi and *A. gemma,* the main tick species infected with *Rickettsia* in this study, predominantly feed on cattle, sheep, goats, camels and large wild ruminants such as giraffe, buffaloes and rhinoceroses (Walker *et al.*, 2003). The larvae and nymph stages also feed on humans and small mammals such as hares, rabbits, hedgehogs and birds (Walker *et al.*, 2003). The wide host range of these ticks at each developmental stage may increase their ability to acquire and transmit *Rickettsia* spp. infections to human populations living in Isiolo, Garissa, Marigat, Ijara and Mai Mahiu.

In the tick positive pools confirmed by sequencing, *R. aeschlimannii* was predominant, followed by *R. africae.* The study detected *R. aeschlimannii* in *H. marginatum, H.*

truncatum, Rh. pulchellus and *Hyalomma spp* ticks. This *Rickettsia* species has been reported before in Kenya in *H. truncatum* ticks (Mutai *et al.*, 2013) but not in *H. marginatum* and *Rh. pulchellus* ticks. *R. aeschlimannii* was first described in *Hyalomma marginatum* tick species from Morrocco (Sarih *et al.*, 2008) and has also been reported in *Rhipicephalus appendiculatus* ticks from South Africa (Pretorius and Birtles, 2002). This is the first description of *R. aeschlimannii* being identified in *H. marginatum* and *Rh. pulchellus* ticks in Kenya indicating a broader range of widely distributed ticks that could transmit this infection. In this study, *R. africae* was predominantly detected in *Amblyomma* tick species which correlates well with other studies which show a strong link between the distribution of *R. africae* and the geographical distribution of *Amblyomma* tick species.

Rickettsia species found in ticks are non-pathogenic, but others including *R. aeschlimannii, R. africae, R. sibirica, R. raoultii,* and *R. conorii* subsp. *israelensis* that were identified in this study have been reported to cause disease in humans (Fournier and Raoult, 2009). However, the presence of these *Rickettsia* species which cause human illness in the ticks, is not predictive of transmission to humans, as not every tick species will feed on humans (Dupont *et al.*, 1995).

One limitation of this study is that not all *gltA* positive samples were sequenced and as a result only seven tick pools had sequences for all three gene targets. In two of the tick pools tested, different *Rickettsia* species were obtained with each of the primers used. Although we used three target genes to clarify the taxonomic classification of the species (Fournier and Raoult, 2009), the lack of phylogenetic resolution in some of our

nucleotide sequences could have been due to a mixed infections of *Rickettsia* species in the tick pools.

5.1.3 The camel could be an important reservoir

Ticks collected from camels in Garissa and Isiolo had a higher *Rickettsia* infection rate than those from other animals. The high infection rate suggests the involvement of these animals in the maintenance of SFG *rickettsia.* Camels and ticks infesting camels have also been found infected with spotted fever group *Rickettsia* in Israel (Kleinerman *et al.*, 2013) and in Egypt respectively (Abdel-Shafy *et al.*, 2012). However, no study has shown whether they develop rickettsiosis. In some of these communities, camels are important for long distance travel and are sometimes traded across borders. Therefore, we suggest that the role of the camel in the transmission of *Rickettsia* should be a focus of future studies.

5.1.4 Evidence of tick to human transmission

In Ijara, *R. aeschlimannii* and *R. africae* were detected in the tick pools tested and in the human blood samples sequenced. Secondly, in Mai Mahiu, *R. africae* was detected in human blood samples tested and in a tick pool tested. In Marigat, *R. aeschlimannii* was detected in human blood samples, yet only *R. africae*, *R. conorii* subsp. *israelensis* and *R. raoultii* were detected in the tick pool samples. Though, the ticks and human blood samples were collected at different times, there is a possibility that direct transmission of *Rickettsia* may occur in some of the sites (Raoult *et al.*, 2001).

5.2 *Coxiella burnetii*

5.2.1 Prevalence of *Coxiella burnetii* in human blood

Coxiella burnetii was not detected in the human blood samples tested. In spite of activities such as herding, slaughtering of cattle and milking that allow contact with infected animals occurring in these communities (Lwande *et al.*, 2012), no human transmission of Q fever was reported. Thus, it is likely that none of the patients came into contact with an infected animal. Only, a small percent (2-5%) of acute cases of Q fever require hospitalization (Knobel *et al.*, 2013). As a result, it is also possible that people in these communities did not seek medical care as Q fever is mainly asymptomatic or may cause a mild self-limiting disease. In addition, being an occupational disease, it may be, that it is confined to certain populations that may be at risk such as abbatoir workers (Maurin and Raoult, 1999). On the other hand, an outbreak of Q fever in humans reported by the Zoonotic Disease Unit in Kenya in March 2014 indicated that exposure may have occurred when people came into contact with an aborted foetus, when animals were being assisted to deliver or a retained placenta was being removed (ZDU, 2014). Therefore, another probable reason for not detecting *C. burnetii* in humans could be that these high risk behaviours were absent in our sample population. Unfortunately, in most countries an outbreak of Q fever in human is what leads to investigation of livestock (Cutler *et al.*, 2007). In fact, an outbreak in the human population can be prevented by monitoring domestic animals for signs of abortion or delivery of weak offspring which are indicators of Q fever infection (Honarmand, 2012).

5.2.2 Prevalence of *Coxiella burnetii* in tick samples

C. burnetii was detectable in a small percent of ticks (5.53%) indicating that very few domestic animals were infected. Furthermore, tick samples from Mai Mahiu (13.16%) and Marigat (7.89%), particularly from sheep and goats were significantly more infected with *C. burnetii* than those from other sites. This is consistent with a study done in rural Western Kenya that showed that *C. burnetii* infections were detected in goats and sheep with still births (Knobel *et al.*, 2013). These domestic ruminants are the primary reservoirs of the infection and could play a key role in the transmission of Q fever to humans in these sites. In most cases, a natural infection with *C. burnetii* will cause an abortion in goats and sheep and females tend to shed the organism during parturition. The high titres of *C. burnetii* shed during the lambing and calving season (Cutler *et al.*, 2007) (Woldehiwet, 2004) become a source of infection to the tick vectors.

Fourty species of ticks are known to be infected with *C. burnetii* worldwide (Sprong *et al.*, 2012). In our study, *Rh. evertsi evertsi* was the predominant tick species with a *C. burnetii* infection. This tick is widespread but confined to the tropical region of the sub Saharan Africa. It prefers to feed on donkeys, horses, cattle and sheep (Walker *et al.*, 2003). The tick may be involved in the transmission cycle of *C. burnetii* between domestic animals and wildlife. Another tick, *Rh. pulchellus* was also found infected with *C. burnetii* in this study. Its preferred hosts are cattle, camels and sheep but it also feeds on wild hosts such as zebras, black rhinoceroses and elands. *Rh. appendiculatus* and *A. gemma* tick species that were also found infected with *C. burnetii* feed on cattle, camels, goats, giraffes and buffaloes (Walker *et al.*, 2003). In ticks, *C. burnetii* can multiply to very high titers, remaining viable during their entire life cycle. Ticks expel heavy loads

of *C. burnetii* with their feces onto the skin of the animal host at the time of feeding. Therefore, tick control programs would effectively reduce this infection as tick serve as an indication of the infection in nature (Norlander, 2000; Fard and Khalili, 2011).

5.3 Theileria orientalis

5.3.1 Detection of *Theileria orientalis* in a tick pool

Babesia spp. DNA was not detected in the tick and human blood samples tested although competent vertebrate and invertebrate host were present in all the sites (Homer *et al.*, 2000; Adjou Moumouni *et al.*, 2015). However, a related parasite, *Theileria orientalis* was detected by the *Babesia* primer set in one tick pool of *Rhipicephalu*s *pulchellus* species collected from a goat in the Marigat site. The Ikeda type 2 genotype of *Theileria orientalis* is known to be pathogenic. However, the sample was not sub-typed thus its pathogenicity remains unknown. *Rh. pulchellus* species are also known to transmit *Theileria taurotragi* which causes benign bovine theileriosis (Walker *et al.*, 2003). This tick species prefers to feed on cattle, camels, sheep, goats, hares, zebras, black rhinoceroses and elands. Consequently, having a 3-host life cycle, *Rh. pulchellus* is able to spread the infection between domestic and wild animals. To prevent the spread of theileriosis reliable diagnostic methods such as PCR and sequencing are useful in the identification of carrier animals which exhibit low parasitemia and may be asymptomatic (Gebrekidan *et al.*, 2014). Therefore, we suggest that the role of small ruminants as carriers of *Theileria* spp. in Kenya be investigated at the Marigat site in Baringo County.

5.4 Conclusion

1. The findings of this study suggests that *Rickettsia* spp. contribute to a significant proportion of febrile illness in Kenya with multiple rickettsial species circulating among tick and human populations in pastoral communities. The prevalence rates are 14% in humans and 25% in tick samples. *R. aeschlimannii* and *R. raoultii,* which have never been reported in Kenya, were detected in the human blood samples tested. In addition, the tick collected from camels indicate that this animal host may be involved in the maintenance of SFG rickettsia in Kenya.
2. The human blood samples were negative for *C. burnetii* in all the pastoral communities. However, the prevalence of *C. burnetii* was reported at 5.53% in ticks indicating a natural infection present in the vector in all the sites. Goats and sheep significantly more infected than other hosts.
3. All the tick and human samples tested negative for *Babesia* spp. However, *Theileria orientalis* a related species was detected in a tick pool from the Marigat site collected from a goat.

5.5 Recommendations

1. The study demonstrates that *Rickettsia*, Q fever and *Theileria* are circulating in these communities .Therefore, the Ministry of Health in Kenya, through the Zoonotic Disease Unit should put up a surveillance system to monitor these pathogens in the animal and human population.
2. *Rickettsia* spp. should be included in the differential diagnosis of febrile illnesses and that clinicians should also be educated on some of the clinical features that distinguish malaria and other febrile illnesses from Rickettioses.

3. Further studies to establish the role of camels and emerging rickettsial strains in febrile illness should be done.
4. More studies focus on *Theileria* spp. in small ruminants to identify carrier animals for treatment.
5. Acaricides should be used to control ticks in the domestic animals.

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APPENDICES

Appendix I: Table 4.2: Clinical and demographic data of *Rickettsia* spp. PCR positive patients and diagnosis

Patient No	Sex	Age	Sites	Occupation	Contact Description	Tick Bite	Reason for visiting the hospital	Clinical Temp	Diagnosis
1	F	7	Marigat	Student	Farming, casual contact	No	Fever	38.1	Pyrexia of unknown origin, clinical malaria
2	F	26	Ijara	Farmer	N/A	No	Fever	37.8	Malaria
3	F	40	Ijara	Sheikh	N/A	No	Malaise	38.1	Malaria
4	M	37	Ijara	Herdsman	N/A	No	Passing loose bloody stool	38.3	Dysentry
5	M	31	Ijara	Herdsman	N/A	No	Fever,lack of appepite,vomiting	38.7	Typhoid fever
6	M	32	Ijara	Farmer	Farming	No	Abdominal pain	38.4	Brucellosis
7	F	18	Ijara	House wife	Farming, food preparation	No	Vomiting blood	38.1	Upper GIT bleeding
8	F	25	Ijara	House wife	Food preparation	No	Fever	38	Enteric fever
9	M	20	Ijara	Herdsman	Farming	No	Headache	38.7	Malaria
10	F	36	Ijara	House wife	N/A	No	Leg pain	37.8	Malaria, tonsilitis
11	F	29	Mai Mahiu	Business	Farming, casual contact	No	Malaise	37.8	Acute febrile illness, malaria
12	M	12	Marig	Studen	Farming, casual contact	No	Fever	39	Pyrexia of

			at	t					unknown origin, clinical malaria
13	F	24	Mai Mahiu	Business	Neighbour keeps donkeys	No	Malaise	38.6	Typhoid fever
14	F	28	Marigat	Business	N/A	No	Fever	38.8	Pyrexia of unknown origin, clinical malaria
15	M	4	Ijara	Student	N/A	No	Fever,lack of appepite,vomiting	38	Malaria
16	M	21	Marigat	Student	Casual contact	Yes	Fever,vomiting,headache,body weakness	38	Acute febrile illness, malaria
17	M	56	Ijara	Farmer	Contact with goats, cows and sheep	No	Fever	39	Malaria,upper tract infection
18	F	72	Ijara	House wife	Farming, food preparation	No	Abdominal pain	38.1	Malaria
19	M	2	Ijara	Child	N/A	No	Fever	40	Pneumonia, malaria
20	F	23	Ijara	House wife	N/A	No	Joint pains	39	Malaria
21	F	35	Mai Mahiu	House wife	Farming	No	Malaise	37.6	Malaria in pregnancy
22	F	19	Marigat	Student	Farming, casual contact	No	Malaise	38	Pyrexia of unknown origin, clinical malaria, epistaxis
23	F	35	Ijara	House wife	N/A	No	Backache	38	Gastroenteretis , malaria
24	M	8	Ijara	Student	N/A	No	Bloody urine	39	Malaria
25	F	18	Ijara	House	Farming	No	Malaise	38	Typhoid fever

				wife					
26	F	22	Marigat	House wife	Casual contact	No	Fever	38.7	Pyrexia of unknown origin, clinical malaria
27	F	50	Ijara	Retired	N/A	No	Malaise	38.2	Malaria
28	F	20	Ijara	House wife	Farming, food preparation, casual contact	No	Malaise	38.4	Gastroenteritis
29	F	19	Ijara	House wife	N/A	No	Headache	37.9	Malaria
30	F	32	Mai Mahiu	Business	Neighbour keeps chicken and donkeys	No	Malaise	37.9	Enteric fever
31	F	70	Ijara	House wife	Farming	No	Malaise	37.9	Gastroenteritis
32	F	35	Ijara	House wife	N/A	No	Fever	41	Pneumonia, malaria
33	M	60	Marigat	Farmer	Farming, casual contact	No	Fever	39	Pyrexia of unknown origin, clinical malaria, anaemia
34	N/I	N/I	Ijara	N/I	N/I	N/I	Malaise	N/I	N/I
35	F	19	Ijara	House wife	Slaughter, food preparation, casual contact	No	Malaise	39	Malaria
36	F	20	Ijara	House wife	Farming	No	Headache	38.3	Malaria
37	M	13	Ijara	Herdsman	Farming	No	Malaise	37.7	Gastroenteritis
38	F	10	Marig	Studen	Farming, casual contact	No	Fever	38	Pyrexia of

			at	t					unknown origin, lumbago
39	F	35	Ijara	House wife	Food preparation	No	Coughing	37.8	Respiratory tract infection

N/I-Not indicated
N/A-Not available
GIT-Gastrointestinal tract

Appendix II: Table 4.5: Rickettsial species detected in human blood samples and tick pools by sequencing of gltA, OmpA and OmpB genes PCR products

Sample Tested	Site	Host	*gltA*	*OmpA*	*OmpB*
H. truncatum	Garissa	Camel	ns	*R. africae*	*R. parkeri*
H. truncatum	Garissa	Camel	ns	*R. aeschlimannii*	*R. aeschlimannii*
H. truncatum	Garissa	Cattle	*R. aeschlimannii*	*R. aeschlimannii*	*R. aeschlimannii*
H. truncatum	Garissa	Cattle	ns	*R. aeschlimannii*	*R. aeschlimannii*
H. truncatum	Garissa	Camel	ns	*R. aeschlimannii*	*R. aeschlimannii*
H. truncatum	Garissa	Camel	*R. aeschlimannii*	*R. aeschlimannii*	*R. aeschlimannii*
H. truncatum	Garissa	Cattle	ns	*R. aeschlimannii*	*R. aeschlimannii*
Hyalomma spp	Garissa	Camel	ns	*R. aeschlimannii*	*R. aeschlimannii*
Hyalomma spp	Garissa	Camel	ns	*R. monglotimonae*	*R. parkeri*
H. truncatum	Garissa	Cattle	ns	*R. aeschlimannii*	*R. aeschlimannii*
Hyalomma spp	Garissa	Camel	*R. aeschlimannii*	*R. aeschlimannii*	na
A. gemma	Garissa	Camel	*R. africae*	*R. africae*	*R. africae*
H. truncatum	Garissa	Camel	ns	na	*R. africae*
H. truncatum	Garissa	Sheep	ns	na	*R. parkeri*
H. truncatum	Garissa	Cattle	ns	na	*R. africae*
H. truncatum	Garissa	Cattle	ns	na	*R. aeschlimannii*
H. truncatum	Garissa	Cattle	ns	na	*R. aeschlimannii*
H. marginatum	Garissa	Cattle	ns	na	*R. aeschlimannii*
Rh. appendiculatus	Garissa	Cattle	ns	na	*R. montanensis*
H. truncatum	Garissa	Camel	*R. aeschlimannii*	na	*R. aeschlimannii*
Rh. pulchellus	Garissa	Cattle	*R. aeschlimannii*	na	na
H. truncatum	Garissa	Camel	*R. aeschlimannii*	na	na
Rh. pulchellus	Garissa	Cattle	*R. raoultii*	na	na
A. lepidum	Ijara	Goat	*R. africae*	*R. africae*	*R. africae*
Rh. pulchellus	Ijara	Camel	ns	*R. africae*	*R. parkeri*

Hyalomma spp	Ijara	Camel	*R. sibirica*	*R. africae*	*R. aeschlimannii*
H. marginatum	Ijara	Cattle	*R. aeschlimannii*	*R. aeschlimannii*	*R. aeschlimannii*
H. marginatum	Ijara	Cattle	*R. raoultii*	*R. aeschlimannii*	na
A. lepidum	Ijara	Goat	*R. africae*	na	na
A. gemma	Isiolo	Camel	*R. aeschlimannii*	*R. africae*	*R. africae*
A. gemma	Isiolo	Cattle	*R. africae*	*R. africae*	na
A. gemma	Isiolo	Sheep	*R. raoultii*	na	*R. africae*
Rh. pulchellus	Isiolo	Goat	*R. aeschlimannii*	na	na
Rh. pulchellus	Isiolo	Goat	*R. raoultii*	na	na
H. marginatum	Mai Mahiu	Cattle	*R. aeschlimannii*	na	na
H. marginatum	Mai Mahiu	Cattle	*R. aeschlimannii*	*R. aeschlimannii*	na
A. variegatum	Mai Mahiu	Cattle	*R. aeschlimannii*	na	*R. africae*
H. marginatum	Mai Mahiu	Goat	ns	*R. aeschlimannii*	na
Rh. appendiculatus	Marigat	Sheep	ns	na	*R. raoultii*
Rh. appendiculatus	Marigat	Sheep	*R. coronii subsp Israelensis*	na	na
A. gemma	Marigat	Cattle	*R. africae*	na	na

A: Amblyomma, H: Hyalomma, Rh: Rhipicephalus, R: Rickettsia, na –no amplification, ns-not sequenced

Appendix III: A screen shot of blast alignment indicating Rickettsia spp. species obtained

Descriptions

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Rickettsia africae isolate ATHO01824 citrate synthase (gltA) gene, partial cds	717	717	96%	0.0	99%	KX227776.1
Rickettsia africae strain GL055 citrate synthase (gltA) gene, partial cds	717	717	96%	0.0	99%	JN043505.1
Rickettsia africae citrate synthase (gltA) gene, partial cds	717	717	96%	0.0	99%	HM050288.1
Rickettsia africae ESF-5, complete genome	717	717	96%	0.0	99%	CP001612.1
Rickettsia africae ESF-5 citrate synthase (gltA) gene, partial cds	712	712	96%	0.0	99%	U59733.1
Rickettsia sibirica isolate Xinjiang-citrate citrate synthase (gltA) gene, partial cds	706	706	96%	0.0	99%	MF002541.1
Rickettsia sibirica isolate Xinjiang-HBH citrate synthase (gltA) gene, partial cds	706	706	96%	0.0	99%	MF002540.1
Rickettsia slovaca isolate Xinjiang-EM citrate synthase (gltA) gene, partial cds	706	706	96%	0.0	99%	MF002529.1
Rickettsia slovaca isolate Xinjiang-citrate citrate synthase (gltA) gene, partial cds	706	706	96%	0.0	99%	MF002528.1
Rickettsia raoultii isolate Xinjiang-citrate citrate synthase (gltA) gene, partial cds	706	706	96%	0.0	99%	MF002517.1
Rickettsia raoultii isolate Xinjiang-EM citrate synthase (gltA) gene, partial cds	706	706	96%	0.0	99%	MF002516.1
Rickettsia raoultii citrate synthase (gltA) gene, partial cds	706	706	96%	0.0	99%	KX258621.1
Rickettsia sibirica isolate ATHO02750 citrate synthase (gltA) gene, partial cds	706	706	96%	0.0	99%	KX227778.1
Rickettsia africae isolate RQB010790 citrate synthase (gltA) gene, partial cds	706	706	95%	0.0	99%	KX227767.1
Rickettsia raoultii isolate Crimea-2 type II citrate synthase (gltA) gene, complete cds	706	706	96%	0.0	99%	KU961538.1

Appendix IV: Scientific Steering Committee Approval letter

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

KEMRI/SSC/102567 6th March, 2014

Hellen Koka

Thro'
Director, CCR
NAIROBI

Forwarded 10 Mar. 2014

REF:SSC No.2454 (Amendment) – A survey of non-viral tick-borne febrile illnesses in archived human and tick samples from high-risk pastoral communities in Kenya

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 **212th** meeting held on **4th March, 2014** and has since been approved for implementation by the SSC.

Kindly submit 4 copies of the amended protocol to SSC within 2 weeks from the date of this letter i.e, 21st March, 2014.

We advise that work on this project can only start when ERC approval is received.

FOR: **Sammy Njenga, PhD**
SECRETARY, SSC

In Search of Better Health

Appendix V: Ethical Committee Approval letter

KENYA MEDICAL RESEARCH INSTITUTE

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KEMRI/RES/7/3/1 **February 26, 2013**

TO: **HELLEN KOKA,**
PRINCIPAL INVESTIGATOR

THE DIRECTOR, CCR,
NAIROBI

Forwarded 7/3/2013

RE: **SSC PROTOCOL NO. 2454 (*INITIAL SUBMISSION*): A SURVEY OF NON-VIRAL TICK-BORNE FEBRILE ILLNESSES IN ARCHIVED HUMAN AND TICK SAMPLES FROM HIGH RISK PASTORAL COMMUNITIES IN KENYA**

This is to inform you that during the 212th meeting of the KEMRI/ERC meeting held on 26th February 2013, the above study was reviewed.

The Committee notes that the above referenced study aims to investigate the cause of non viral febrile illness in tick-exposed communities in Kenya and the potential vectors involved in transmission.

The above referenced study is entirely lab based, using samples that obtained from SSC 1560 and 824. Permission has been granted by both the Principal Investigator(PI) of the study and the research participants for further studies for SSC 1560 and SSC 824.

There being no human contact no ethical issues arise the study is therefore **granted approval** for implementation effective this **26th day of February 2013,** for a period of twelve (12) months.

Please note that authorization to conduct this study will automatically expire on **25th February 2014.** 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 **13th January 2014**.

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,

DR. ELIZABETH BUKUSI,
ACTING SECRETARY,
KEMRI/ETHICS REVIEW COMMITTEE

In Search of Better Health

Appendix VI: Publication in a peer review journal

Journal of Medical Entomology Advance Access published January 10, 2017

Journal of Medical Entomology, 2017, 1–7
doi: 10.1093/jme/tjw238
Research article

Vector-Borne Diseases, Surveillance, Prevention

OXFORD

The Detection of Spotted Fever Group Rickettsia DNA in Tick Samples From Pastoral Communities in Kenya

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This work is presented with the permission of the Director, KEMRI. The work presented here represents the opinions of the authors and should not be seen to represent the policy of the Kenya Medical Research Institute, US Army Medical Research Directorate—Kenya, Walter Reed Army Institute of Research, US Department of the Army, or the US Department of Defense.

Subject Editor: Kevin Macaluso

Received 26 September 2016; Editorial decision 8 December 2016

Abstract

In this study, ticks from pastoral communities in Kenya were tested for *Rickettsia* spp. infections in geographical regions where the presence of tick-borne arboviruses had previously been reported. Rickettsial and arbovirus infections have similar clinical features which makes differential diagnosis challenging when both diseases occur. The tick samples were tested for *Rickettsia* spp. by conventional PCR using three primer sets targeting the *gltA*, *ompA*, and *ompB* genes followed by amplicon sequencing. Of the tick pools screened, 25% (95/380) were positive for *Rickettsia* spp. DNA using the *gltA* primer set. Of the tick-positive pools, 60% were ticks collected from camels. *Rickettsia aeschlimannii* and *R. africae* were the main *Rickettsia* spp. detected in the tick pools sequenced. The findings of this study indicate that multiple *Rickettsia* species are circulating in ticks from pastoral communities in Kenya and could contribute to the etiology of febrile illness in these areas. Diagnosis and treatment of rickettsial infections should be a public health priority in these regions.

Key words: *Rickettsia*, pastoral, tick, *gltA*, diagnosis

Rickettsioses are caused by bacteria in the order Rickettsiales and the genus *Rickettsia*. The *Rickettsia* genus is divided into four groups: spotted fever group (SFG), the transitional group (TRG), typhus group (TG), and the ancestral group (AG; Gillespie et al. 2008). Rickettsioses mainly cause fever, malaise, arthralgia, lymphadenopathy, myalgia, and headaches in humans (La Scola and Raoult 1997). An inoculation eschar may be absent or present and clinical manifestations vary between species of *Rickettsia* (Faccini-Martínez et al. 2014). The same spectrum of clinical symptoms are observed in other diseases such as malaria, influenza, and typhoid fever (Richards et al. 2010), making empirical diagnosis of *Rickettsia* infection difficult. Rickettsial infections are easily and affordably treated with antibiotics such as doxycycline or chloramphenicol (Huntzinger 2007), so accurate diagnosis can lead to significant reduction in the associated morbidity. Ticks are the main vectors and reservoirs of the SFG *Rickettsiae*, and they transmit the infection to domestic animals, wildlife, and humans (Raoult and Roux 1997). There is a growing body of evidence indicating the widespread presence of *Rickettsia* spp. in ticks, vertebrate hosts, and humans in Kenya. Antibodies against *R. conorii* were detected in rodents from Machakos, Kerugoya, Kisumu, Nairobi and the Rift Valley (Heisch et al. 1962). In addition, several *Rickettsia* spp. have been detected in ticks and whole blood collected from domestic animals at two major slaughter houses in Kenya (Mutai et al. 2013). *Rickettsia africae* was detected in ticks collected from domestic animals and wildlife in Kenya at the Maasai Mara and the Shimba hill reserves (Macaluso et al. 2003, Mwamuye et al. 2016) and *R. africae* variants detected in ticks from rural Western Kenya. In a recent study, the heterogeneity of *R. africae* was confirmed and a new *Rickettsia* species identified that had been collected from the northern part of Kenya where communities are mainly nomads (Kimita et al. 2016). Nomadic communities in Kenya live in arid and semiarid zones and practice pastoralism with herds predominantly consisting of sheep, goats, and camels. Ticks found on these livestock potentially play a role in the transmission dynamics of tick-borne diseases (Pfaffle et al. 2013). Since these nomadic communities in Kenya interact closely with their livestock and are at risk of exposure to ticks, they could also be at high risk of acquiring tick-

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