Tick-Borne Pathogens as Etiologies of Undifferentiated Acute Febrile Illness in Kenya: Geographical Distribution, Risk Factors and The Tick Bacteriome as a Bio-Indicator of Tick - Borne Zoonosis

BETH KINYA MUTAI

H80/92845/2013

Thesis submitted in fulfillment for the award of Degree of the Doctor of Philosophy in Medical Microbiology

Faculty of Health Sciences

University of Nairobi.

Declaration

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

Signature _____ Date 29th March 2022

Beth Kinya Mutai

H80/928245/2013

This thesis has been submitted for our approval as supervisors

Signature _____ Date 31/3/2022

Kariuki Njaanake, PhD, University of Nairobi, Department of Medical Microbiology P.O. Box 19676-00202, Nairobi. Tel.: +254 721 354 630. E-mail: kn@uonbi.ac.ke

Signature _____ Date 31 MAR 2022

John N. Waitumbi, DVM, PhD, Research Laboratory Director, Kenya Medical Research Institute / Us Army Medical Research Directorate, Kisumu. Tel.: +254 733 616 548. Email: john.waitumbi@usamru-k.org

Signature _____ Date 29th March 2022

Prof. Benson Estambale, MBChB, PhD, Jaramogi Oginga Odinga University of Science and Technology Tel: +254 722 700 185. Email: bestambale@gmail.com.

Dedication

I dedicate this thesis to husband Edward, son Jeffrey and Jeff's nanny for accepting my absence for days, weeks and months while training to advance expertise need for my research.

Acknowledgement

I would like to appreciate my enthusiastic Mentor, Dr. John Waitumbi for the opportunity to join his laboratory and grow my career as a medical researcher. I wholeheartedly appreciate not only his mentorship but also many great opportunities that have greatly impacted my career.

I am grateful to my University supervisors Dr. Njaanake Kariuki and Prof. Estambale for their tremendous academic support and mentorship, they were truly dedicated mentors. I am indebted to the staff of UON Department of Medical Microbiology for their support.

My profound gratitude goes to the current and the Alumni team of KEMRI / USAMRD-A, Basic Science Laboratory, time spent with them in the lab and outside has been priceless. My PhD has been an amazing experience because of them.

I am very grateful to my forever interested, encouraging and always enthusiastic family, dad, mum and siblings for their support and encouragement. To my beloved husband and son who have been my anchor and source of strength throughout this experience.

Finally but no means least, I thank God Almighty for his unfailing love, provision and guidance through all my life and experiences. I will forever trust in him.

Table of Contents

List of Abbreviations

4-plex – Multiplex of four targets

ABI – Applied Biosystems

AFI - Acute Febrile Illness

Ct – Threshold cycle

CV – Coefficient of variation

DNA – Deoxyribonucleic acid

ELISA - Enzyme Linked Immunosorbent Assay

HGA – Human granulocytic anaplasmosis

HME – Human monocytic ehrlichiosis

IFA – Immunoflourescence assay

IS – Insertion sequences

kDa – Kilodalton

KEMRI – Kenya Medical Research Institute

LOD – Limit of detection

MSP2 – Major surface protein 2

NGS – Next generation sequencing

OUT – Operational taxonomic unit

PCoA – Principal coordinate analysis

PCR - Polymerase chain reaction

pM - Pico molar

Q fever – Query fever

qPCR – Real time PCR

rRNA –Ribosomal ribonucleic acid

SARS - Severe Acute Respiratory Syndrome

SD – Standard deviation

SERU / SSC – Scientific and ethical review unit

Spp. – Species

TBRF - Tick-borne relapsing fever

USA – United states of America

USAMRD-A – United State Medical Research Directorate - Africa

WHO – World Health Organization

WRAIR – Walter Reed Army Institute of Research

µL – Microliter

µM – Micromolar

List of tables

List of figures

Abstract

Background:

Vector-borne pathogens, which include viruses, bacteria and protozoa, are among the most common etiological agents of acute febrile illness (AFI) worldwide and have reportedly caused epidemics of human disease in recent decades. Ticks are important vectors and they are ranked only second to mosquitoes, in vector-borne disease transmission. Studies carried out at Kenya Medical Research Institute / United States Army Medical Research Directorate – Africa (KEMRI/USAMRD-A) have shown a wide distribution of ticks in Kenya indicating potential for transmission of tick-borne pathogens including *Rickettsia* spp., *Borrelia* spp., *Coxiella burnetii, Ehrlichia* spp., *Babesia* spp., *Anaplasma* spp. and Crimean-Congo virus. Although these pathogens have been reported in Kenya, there has been no systematic study of the disease burden in the country. Within Kenya, AFI is a reason common for patients to go to health care facilities and patients are often empirically treated without laboratory evaluation for the causal agent. It is therefore, imperative to determine etiologies of acute febrile illness other than malaria and associated risk factors.

Objective: The present study aimed at optimizing two multiplexed real time polymerase chain reaction assays for detection of eight tick-borne pathogens and using them to evaluate the distribution, risk factors and associated signs and symptoms of tick-borne pathogen infections as etiological agents of acute febrile illness in different geographical regions in Kenya. The study also aimed at determining whether ticks infesting domestic animals in Kenya harbor bacteria of zoonosis relevance that can be transmitted to human.

Methodology: The present study was conducted on 6,207 archived samples that were collected from patients seeking healthcare aged one year and above with fever of equal or

greater than 38ºC without a diagnosis of the causative agent of fever after routine clinical diagnosis. Samples used in this study were collected between 2008 and 2017 in county and sub-county hospitals located in diverse geographical regions in Kenya including Lake Victoria region (Kisumu County hospital, Kombewa sub-county hospital, Alupe sub-county hospital, Busia), Kisii Highlands (Kisii county hospital), Rift valley region (Marigat sub-county hospital Baringo, Lodwar county hospital and Gilgil sub-county hospital, Nakuru) North Eastern Region (Iftin sub-county hospital, Garissa, Garissa police line dispensary, Garissa and Isiolo county hospital), Coastal region (Malindi sub-county hospital, Kilifi) and Cosmopolitan urban region (Eastleigh health center, Nairobi). To determine bacteria community in ticks that can be potentially transmitted to humans, archived genomic DNA samples from 460 adult tick samples were used. These ticks were collected between 2007 and 2008 from domestic animals including cattle sheep and goats from diverse geographical regions in Kenya. Two multiplex Real Time PCR assays and 16s rRNA metagenomics were used for detection of tick-borne pathogens in the human and tick specimens, respectively. Demographic and clinical data including age, gender, place of residence, signs and symptoms, contact with animals and type of animals, tick bite, collected at the time of sample collection was used to determine risk factors for human tick-borne infections.

Results:

Two real time PCR assays in a multiplex format were optimized and evaluated for eight tick-borne zoonosis pathogens. The assays were organized in duo assays of 4-plex each. Assay 1 was optimized for *Anaplasma phagocytophilum, Coxiella burnetii, Borrelia burgdoferi and Ehrlichia chaffeensis.* Assay 2 was optimized for *Rickettsia* spp., *Bartonella* spp., other *Borrelia* spp. which are not *B. burgdoferi* and *Babesia* spp. Using synthetic plasmids it was

shown these assays can specifically detect all the target sequences in the same reaction tube. Assay 1 had a limit of detection of 2 copies for all target genes. Assay 2 was less sensitive and on average had a limit of detection of 18 copies of target genes. In replicate tests, both assays had intra-assay variation of less than two cycles. Using Bland-Altman analysis, the performance of the two 4-plex assay was similar to that of the singleplex assays qualifying them for simultaneous detection of four tick-borne zoonosis pathogens per assay. Whole blood samples collected from undifferentiated acute febrile illness patients were tested for presence of eight tick-borne zoonosis using the two 4-plex real time PCR. Of the patients accessed 3,082 (50.9%) were male and 2,979 949.2%) were female. The age of the patients ranged 1 to 80 years with a median of 5 years. Of these, all 6,207 were tested for *Rickettsia*, 4,017 for *Coxiella*, 3,615 for *A. phagocytophilum*, 3,659 for *E. chaffeensis*, and 3,657 for *B. burgdoferi*, 3,480 for *Babesia* spp., 3,463 for *Bartonella* spp and 3,463 for *Borrelia* spp. which are not *B. burgdoferi.* Overall, 1,017 (16%) were positive for at least 1 tick - borne zoonosis pathogen (confidence interval (CI :) 15.3 – 17.1). Of these 475 (8%) (CI: 7.9-8.3) were positive for *Rickettsia*, 271 (6%) were positive for *Coxiella* (CI: 5.6-7.0), 55 (1.5%) were positive for *A. phagocytophilum* (CI: 1.1 – 1.9), 111 (3%) were positive for *E. chaffeensis* (CI: 2.5 – 3.6), 56 (1.5%) were positive for *B. burgdoferi*, (CI: 1.1 – 1.9), 36 (1%) were positive for *Babesia* spp. (CI:: 0.2 – 1.4), 8 (0.2%) were positive for *Bartonella* spp. (CI: 0.07 – 0.4) and 135 (4%) were positive for other *Borrelia* spp. which are not *B. burgdoferi* (CI: 3.3 – 4.5). Out of the 1017 tick-borne zoonosis positive specimens 906 (89.1%) tested positive for a single tick-borne zoonosis pathogen and 99 (9.73 %) samples were positive for two tick-borne zoonosis pathogens, 11 (1.1%) samples were positive for three tick-borne zoonosis pathogens and 1 sample was positive for four tick-borne zoonosis pathogens. Majority of the co-infections

were combination of *Borrelia* spp. with *Rickettsia* spp. and *Coxiella burnetii* with *Rickettsia* spp. There was a higher prevalence of tick-borne zoonosis pathogen infection in the age categories 6 to 15 years (17.5%) and ≥ 16 years (19.4%) compared to 14.3% in the ≤ 5 years category (p-values = 0.02 and < 0.0001, respectively). Patients from a cosmopolitan urban region of Eastleigh, Nairobi County had higher prevalence of tick-borne zoonosis pathogens infections of 39% compared to Coastal region with 17%, Kisii highlands with12%, Lake Victoria region with 16%, and arid and semi – arid region with 17%. Patients who reported having contact with sheep and had a tick bite had higher odds of tick-borne zoonosis pathogen infection. In ticks collected from domestic animals, a total of 645 unique operational taxonomic units (OTUs) (bacteria genera) were detected and grouped into 27 bacteria phyla. Sequence reads in the phyla were skewed with four phyla contributing 96.2% of the sequences. Proteobacteria contributed the majority 61.2 % of the sequences that tarried to 33.8% OTUs, 15.9% for Firmicutes (23.4% OTUs), and 15.6% for Actinobacteria (20% OTUs), 4.7% for Bacteroidetes (11.6% OTUs). The remaining 22 phyla included 0.7% for Fusobacteria, 0.5% for TM7 (Saccharibacteria) 0.3% for Verrucomicrobia 0.2% for Acidobacteria, 0.2% for Deinococcus-Thermus, 0.2% for Planctomycetes, 0.1% for Chloroflexi, OD1 (Parcubacteria), Tenericutes and Gemmatimonadetes, 0.04% for Armatimonadetes, 0.03% for Spirochaetes, 0.02% for Aquificae, 0.01% for SR1 (Absconditabacteria), Lentisphaerae and BRC1, 0.004% for Chlamydiae and Nitrospira, 0.002% for Chlorobi, Synergistetes, Fibrobacteres, WS3 and Elusimicrobia contributed only 2.5 % of the sequences (11.2% OTUs). Potentially pathogenic bacterial genera identified include C*oxiella* 41.8%, *Corynebacterium* 13.6%, *Acinetobacter* 4.3%, *Staphylococcus* 3.9%, *Bacillus* 2.7%, *Porphyromonas* 1.6%, *Ralstonia* 1.5%, *Streptococcus* 1.3%, *Moraxella* 1.3%,

Cloacibacterium 1.3%, *Neisseria* 1.2%, *Escherichia_Shigella* 1.2% and *Proteus, Aerococcus, Alloiococcus, Stenotrophomonas* 1% each. *Coxiella genus was the* most abundant constituting 41.8% (15,445,204 out of 36,973,934 total sequences). Other less abundant (<0.1%) but potentially pathogenic genera included *Burkhoderia* (0.4%), *Klebsiella* (0.3%), *Escherichia-Shegella* (0.3%), *Achromobacter* (0.2%), *Rickettsia (*0.1%), *Haemophilus* (0.1%), *Legionella* (0.1%), *Campylobacter* (0.04%), *Treponema* (0.03%), *Francisella* (0.02), *Anaplasma* (0.01), *Elizabethkingia* (0.006%), *Mycoplasma* (0.006%), *Ehrlichia* (0.005%), *Bordetella* (0.004%), *Vibrio* (0.002%), *Borrelia* (0.0008%) *and Brucella* (0.0002%). By Shannon diversity index, *A. variegatum* carried less diverse bacteria (mean Shannon diversity index of 2.69 ± 0.92) compared to 3.79 ± 1.10 for *Amblyomma gemma*, 3.71 ± 1.32 for *A. hebraeum*, 4.15 ± 1.08 other *Amblyomma* spp, 3.79 ± 1.37 for *Hyalomma truncatum*, 3.67 ± 1.38 for other *Hyalomma* spp, 3.86 ± 1.27 for *Rhipicephalus* annulatus, 3.56 ± 1.21 for *Rh. appendiculatus*, 3.65 ± 1.30 for *Rh. Pulchellus*, but the difference was not statistically significant (p=0.443).

Conclusion:

Eight tick-borne pathogens were detected in undifferentiated acute febrile illness patients from different geographical regions in Kenya using the two 4-plex real time PCR assays optimized in this study. This study has shown up to 16.4 % prevalence of tick-borne zoonosis pathogens in acute febrile illness patients in Kenya. Prevalence of tick - borne zoonosis infection was significantly higher in patients of 6 years and above compare to patients of less than 5 years. Patients living in an overcrowded cosmopolitan urban region and reporting a tick bite were at higher risk of tick-borne zoonotic infections. Patients with tick-borne zoonosis infections reported non-descriptive signs and symptoms which can lead to difficulties in definitive diagnosis of tick-borne zoonosis. Adult ticks collected from livestock animals including cattle,

sheep and goats in the Kenya harbored an array of bacterial community including pathogenic bacteria genera which suggests potential risk of transmission of tick - borne zoonosis infections in the country. Pathogens harbored and transmitted by ticks (Tick-borne pathogens) should be added to the list of etiologies considered for differential diagnosis of acute febrile illness patients and laboratory capability for their diagnosis should be enhanced for early detection and appropriate treatment in Kenya. Tick control effort in Kenya should embrace one health approach to include human, animals and environment factors.

1 CHAPTER ONE: Introduction and literature review

1.1 Introduction

Many arthropods including ticks have been reported to transmit diseases that affect humans, companion animals, wildlife and livestock with the potential to cause morbidity and morbidity. Tick-borne pathogens that can either be viruses, bacteria or protozoa are common agents of acute febrile illness (AFI) worldwide and have been associated with multiple epidemics of human disease in the recent decades (Anderson et al., 1986; Cutler, 2010; Mackenzie et al., 2004; Ramos et al., 2010). Tick-borne zoonoses such as Lyme borreliosis and tick-borne rickettsioses can emerge in areas they were never reported when factors favoring their maintenance and transmission like presence of their arthropod vector(s), and their mammalian reservoir(s) occur (Fritz, 2009; Madison-antenucci et al., 2020). Diseases referred to as emerging or re-emerging are commonly of zoonotic in origin and have continually caused significant health problem to the society globally. Emergence of new and re-emergence of disease causing pathogens that had been controlled or eliminated globally can be attributed to human demographics and behavior, economical advancement, anthropogenic changes including land use and agriculture as well as microbes adapting to new hosts, vectors and among others (Greger, 2007; Lindahl and Grace, 2015; Stone et al., 2017). Rapid growth of human population has led to the need for more food production space as well as need to keep more animals for food production therein increasing the risk of emerging and re-emerging of zoonotic disease causing microbes. Increased emerging and re-emerging infectious diseases in the world have negatively affected human and animal lives due to a larger burden of infectious diseases. Mosquitos and biting flies are known to be major vectors of both known and novel pathogens but tick-transmitted disease have also significantly increased globally (Lindahl and Grace, 2015; Stone et al., 2017). Globalization, climate change and

international travel are other factors that have increased the mobility of these microbial agents and thus the risk of infections such as Lyme, borreliosis, tick borne relapsing fever, babesiosis, ehrlichiosis and Query fever (Q fever) (Coulter et al., 2005; Cutler, 2010; Jensenius et al., 2009). These factors have increased vulnerability to infections with vector-borne pathogens and specifically tick –borne pathogens to people living in any part of the globe (Cutler, 2010; Inci et al., 2016).

In Kenya acute febrile illness (AFI) is a common presentation in health care facilities with malaria being reported as the major cause (Whitty et al., 2008). However, with a reduction in malaria cases in the country as a result of control measures put in place it is apparent that other pathogens may also be important causes of acute febrile illness (Nyaoke et al., 2019; Okiro et al., 2007; Shillcutt et al., 2008). Previous zoonoses surveillance work in domestic animals showed a wide distribution of ticks known to be vectors of zoonoses with a 23.3% prevalence of rickettsiae infections, suggesting their possible role in transmission of tick-borne pathogens in the country (Mutai et al., 2013). Also sero-prevalence studies carried out in acute febrile illness patients in Kenya have shown possible exposure to tick-borne and other vector-borne pathogens in Kenya (Nyataya et al., 2020; Thiga et al., 2015). Bacterial agents of rickettsioses, Q fever, and Lyme disease have also been reported in different parts of Kenya, suggesting that the country's population is vulnerable to tick-borne epidemics (Jowi and Gathua, 2005; Richards et al., 2010a). Although these pathogens have been reported in the country there has been no systematic study to determine their prevalence and distribution in different regions using molecular methods that confirms presence of pathogen specific DNA in patient samples. There is also no reported of complete array of pathogen that can be harbored by ticks.

Lack of multi-pathogen detection method has posed as a challenge to pathogen surveillance effort especially in tropical region (Prasad et al., 2015). Multiplexed Taqman real time PCR assays can enable detection of multiple pathogens in a sample (Prasad et al., 2015; Pripuzova et al., 2012). Use of next generation sequencing allows unbiased detection of pathogens in a sample in a highly parallel sequencing approach (Motro and Moran-Gilad, 2017a). Targeted NGS metagenomics has been used to profile microbial communities in samples using gene markers and microbe databases. Detection of bacterial microbes involves amplification of 16s rRNA gene which has conserved and variable regions, sample bar-coding by PCR, parallel deep sequencing and bioinformatics sequence analysis (Couper and Swei, 2018a). This approach has been used for bacterial pathogen and symbionts detection in ticks (Bouquet et al., 2017).

The present study aimed to optimize two multiplex assays and use them to investigating the distribution of tick-borne pathogen infections in acute febrile illness patients from different regions in Kenya. This study also aimed to determine whether ticks infesting domestic animals in Kenya pose any risk of transmitting tick-borne zoonosis pathogens using 16s rRNA metagenomics unbiased next generation sequencing. Additionally this study aimed to establish factors that are associated with risk of tick - borne zoonosis infections as well as signs and symptoms associated with these infections in Kenya.

1.2 Literature review

1.2.1 Acute febrile illness

Acute febrile illness (AFI) is an illness that present with sudden onset internal body temperature above 38° C (Kashinkunti and Gundikeri 2013). Infectious diseases are common causes of acute febrile illness in the tropics although there are non-infectious cause of febrile illness such as malignancy, collagen vascular diseases, allergies among others (Chow and Robinson, 2011;

Cunha, 2007; Leelarasamee et al., 2004). In the tropical region there are limited laboratory diagnostic resources and presence of wide array of etiologies that cause acute febrile illness which hinder definitive diagnosis, timely admission of appropriate treatment as well as public health responses to endemic, epidemic, and novel diseases (Akpede and Akenzua, 2001). This is further confounded by the fact that most of the patients with acute febrile illness present with non-descriptive symptoms at the beginning of the illness such as fever, general malaise, headache, and myalgia (Kasper et al., 2012). Due to lack of pathogen specific diagnostic tools healthcare providers are usually unable to determine specific etiologies causing febrile illness and therefore diagnose patients presumptively based on clinical features and assumptions regarding locally circulating pathogens (Kasper et al., 2012). The etiologies of acute febrile illness vary by geographical location and presence or absence of vectors and reservoirs requiring diagnosis, treatment and control programs to be based on laboratory identification of specific etiologies in different locations (Kasper et al., 2012). In places where malaria was the main cause of fever improvement of malaria diagnosis and other control efforts have shown that most fevers are non-malaria (Bhatt et al., 2015; Maze et al., 2018). Making definitive diagnosis that can lead to appropriate treatment in acute febrile illness patients remains a challenge in many health care facilities in the tropics and sub-tropical regions (WHO, 2011). This challenge is due to the non-specific presentation of a broad variety of conditions causing acute febrile illness and unavailability of diagnostic tests (Maze et al., 2018). It is therefore important to determine prevalence and distribution of specific causes of fever in a country for better management of febrile patients.

1.2.2 Tick vector and tick- borne diseases of humans

Ticks are obligate hematophagous arthropods that parasitize every class of terrestrial vertebrates including mammals, reptiles and birds in almost every region of the world (Cupp, 1991; Heyman et al., 2010; Horak, 2014; Mediannikov et al., 2010b). There are over 900 species and sub species of ticks recorded globally belonging to 2 main tick families. Ixodidae family also referred to as hard ticks because of their sclerotized dorsal plate. Ticks belonging to this family are the most important in numerical terms and medical relevance (Filipe Dantas-Torres, 2020; Sonenshine, 1992) The Argasidae tick family also referred to as soft ticks because of their flexible cuticle is the second largest family (Filipe Dantas-Torres, 2020; Sonenshine, 1992). Nuttalliellidae is the third family of ticks represented with a single species and present only in South Africa (Horak, et al., 2002). Ixodids ticks have a number of attributes that enhance their vector potential compared to other tick families. They feed for relatively long periods of up to several days during which they remain firmly attached to their host (Estrada-Peña and Jongejan, 1999; Parola and Raoult, 2001a). Ixodids tick bites usually does not cause pain leading them to be go unnoticed for long period of time on their host. Each stage of the tick feeds only once but may feed on different vertebrate hosts found in the same location. Unlike the Ixodids ticks, Argasids feed for a short period of time on their host and they feed on a single species hosts (Sonenshine, 1992). These characteristics of ticks increase the chances of the tick to become infected at any stage of its lifecycle. The infection acquired can persists through each developmental stage through transovarial transmission (Socolovschi et al., 2009). Vertical transmission of pathogens between generations of ticks has been observed (transovarial transmission).Transovarial transmission is a widespread and efficient process through which pathogens can be passed between generations of arthropod vectors. Pathogens can utilize transovarial transmission as a means of persisting within the host when

classical horizontal transmission is not possible due to ecological constraints (Moore et al., 2018; Schwan and Raffel, 2021; Socolovschi et al., 2009b). Transstadial transmission of pathogens gives the ticks the opportunity to transmit the pathogens to a mammalian host during feeding. This is also true when a tick requires multiple days for blood feeding and increasing the opportunity for transfer of pathogens from tick to host within infected saliva. When different ticks feed on mammalian host found in the same location pathogens can also be transferred between ticks.(Karbowiak et al., 2016; Socolovschi et al., 2009).

After mosquitoes, ticks are the second in importance as vectors and/or reservoirs of human and animal infectious diseases in the world (Brites-Neto et al., 2015). The lifecycle of ticks generally involve the egg, larva, nymph and adult stages. To complete each stage in its life cycle the tick need to take a blood meal after hatching from the egg (Cupp, 1991). Some tick species feed on the same host during the whole of their life cycle while others require multiple hosts to complete their life cycle (Sonenshine, 1992). During blood meals ticks may ingest pathogens and act as vectors or reservoirs that transmit the pathogens transstadially and transovarially across generations (Sonenshine, 1992). Ticks that take blood meals from different hosts may spread the pathogens among vertebrate hosts a characteristic that makes ticks important source of zoonoses (Sonenshine, 1992). Each tick species has specific environmental condition requirements that determine where they are found and, consequently, the risk areas for tick-borne pathogens (Cortinas et al., 2002; Jongejan and Uilenberg, 2004). Known geographical distribution of ticks and tick-borne pathogens may however vary due to climatic changes, deforestation and the migration of humans and animals (Parola and Raoult, 2001a). People, animals and their products move around the world due to globalization which in turn increases the risk of tick-borne and other vector-borne diseases to emerge or re-emerge in regions they were not endemic (Colizza et al., 2006; Fèvre et al., 2006).

Other factors that may influence emergence of these pathogens include changes in land use and agriculture (Greger, 2007). Deforestation and human occupying and using habitants previously occupied by animals has been reported to contribute to emergence of zoonoses and vector-borne diseases (Morand and Lajaunie, 2021) .

All human tick borne pathogens are zoonosis and form an important proportion of emerging human pathogens in the world (Rabozzi et al., 2012). Generally emerging infectious diseases have been reported to correlate with ecological factors which can be used to identify regions with risk of these infections (Jones et al., 2008). Tick-borne pathogens including Rickettsia spp., *A. phagocytophilum*, *Babesia spp.*, *Bartonella spp*., and *Borrelia spp*. are considered as emerging human pathogens posing public health problem to any nation (Breitschwerdt et al., 2010; Hildebrandt et al., 2011, 2010; Lommano et al., 2012).

1.1.1.1 Rickettsiosis

Rickettsiosis is a cosmopolitan disease caused by bacteria belonging to rickettsiae family and infect human host through a bite by an arthropod vector such as ticks, fleas, lice and mites. Rickettsiae comprises of gram-negative bacteria belonging to the alpha sub-division of Proteobacteria phylum (Azad and Beard, 1998). They are divided, based on antigenicity, guanine and cytosine content, culture conditions and actin polymerization into 2 groups namely; typhus group and spotted fever group rickettsiae (Azad and Beard, 1998). The typhus group comprises of 2 species, *R. prowazekii* and *R. typhi* which are the causative agents of louse-borne epidemic and flea-borne murine typhus, respectively. The spotted fever group is made up of over 20 *Rickettsia* species which are the causative agents of either tick-, flea-, and mite-borne rickettsioses (Fournier and Raoult, 2009).

Human infection by rickettsiae starts at the site of arthropod vector bite and spreads throughout the body via the draining lymph nodes. The bacteria then infects endothelial cells that can result in

multi-organ pathological changes leading to potentially life-threatening disease (Azad and Beard, 1998). Clinical symptoms develop within 1 to 2 weeks of infection which vary with the causative agent. Symptoms commonly reported in rickettsial infections include fever, headache, malaise and sometimes nausea and vomiting (Biggs et al., 2016).

Early treatment of rickettsial infections with appropriate antibiotics is critical for rapid recovery. However this is hampered by lack of early diagnosis because early signs are non-specific making clinical diagnosis difficult especially in low-resource areas that lack laboratory diagnostic capacity (Van Eekeren et al., 2018). Serological tests are commonly used for diagnosis applying indirect immunofluorescence assay (IFA), enzyme- linked immunosorbent assays (ELISA) and Western blot (Richards et al , 1993). Nucleic acid-based antigen detection has been used to successfully identify rickettsia in various specimens ((Isyagi et al., 2007; Tzianabos et al., 1989; Webb et al; 1990).

Advanced molecular techniques have led to characterization of new *Rickettsia* species which are agents of human disease in different parts of the world where rickettsioses had not been previously reported. They include *R. japonica, R. honei, R. africae, R. felis, R. sibirica mongolotimonae strain, R. parkeri, R. heilongjiangensis* and *R. aeschlimannii* (Walker, 2007).

In Kenya, reported rickettsial diseases are Kenya tick typhus and African tick bite fever caused by *R. conorri* and *R. africae*, respectively (Macaluso et al., 2003; Rutherford et al., 2004). Flea-borne murine typhus has been reported in the country especially in urban areas where the house rat (*Rattus rattus*) is commonly found (Heisch et al., 1962; Heisch et al., 1957). Most recently, *R. felis* which causes flea-borne spotted fever has been reported in 3.7% patients with febrile illness from north eastern part of Kenya (Richards et al., 2010b). High sero-prevalence of spotted fever and scrub typhus was also reported in acute febrile illness patients from different parts of Kenya

(Thiga et al., 2015). These findings indicate Rickettsial infections maybe a cause of undifferentiated acute febrile illness in Kenya. Therefore, current Rickettsial distribution in Kenya is important for disease management and control strategies. This information is important in guiding differential diagnosis of undifferentiated acute febrile illness by health care providers. There is therefore need to determine Rickettsial distribution among the local population and establish the risk factors for these infections in Kenya.

1.1.1.2 Query fever (Q fever)

Q-fever is zoonosis caused by *Coxiella burnetii* which is a Gram-negative bacteria belonging to phylum Proteobacteria and the family *Coxiellaceae* family. *C. burnetii* is distributed worldwide in domestic animals, rodents and birds. Sheep, goats and cattle are its main domestic animals host (Steinmann et al., 2005). The bacteria are found in high concentrations in animal milk and fetal fluids during birth or abortions. Humans get infected via inhalation of contaminated dust and, to a lesser extent, ingestion of contaminated dairy products. Ticks are the main vectors between wild animals and livestock (Mediannikov et al., 2010b). *C. burnetii* can withstand harsh conditions and can be easily aerosolized making it a good biological weapon agent (Cyprian, 2012). *C. burnetii* is classified as a bioterrorist agent by World health organization (WHO) and Center for Disease Control (CDC) (Seitz, 2014).

Human infection with *C. burnetii* that cause Q fever disease can be asymptomatic or show unspecific clinical symptoms. Q fever has an incubation period of about 2–3 weeks and symptoms include atypical pneumonia, high fever, hepatitis- and flu-like symptoms, such as headache, epigastric pain and arthralgia (Mediannikov et al., 2010b). In some patients the disease progresses to a chronic stage characterized by severe endocarditis (Kampschreur et al., 2012). Q fever is diagnosed mainly by serology although antibodies are only detected 2 – 3 weeks after infection.

C. burnetii can also be detected directly by cell culture although it has low sensitivity and requires bio-safety enhanced laboratories (Musso and Raoult, 1995). Polymerase chain reaction (PCR) has also been used for direct detection of *C. burnetii* DNA in clinical specimen (Fenollar and Raoult, 2007).

High sero-prevalence of antibodies to *C. burnetii* in humans has been reported in countries neighboring Kenya. For example, sero-prevalence of 5% and 37% has been reported in Tanzania and Zimbabwe respectively (Prabhu et al., 2011). Despite these reports there is little information about the occurrence and burden of *C. burnetii* causative agent of Q fever among the Kenyan population. Determining distribution of *C. burnetii* in Kenya and the risk of infections among AFI patients will guide differential diagnosis of undifferentiated AFI, infection management and controls strategies.

1.1.1.3 Borreliosis

Borreliosis is a zoonotic disease caused by gram-negative spirochaetal bacteria of the genus *Borrelia* primarily transmitted by ticks to humans (Sparagano et al., 2015). There are 36 known *Borrelia* species 12 of which cause Lyme disease. Lyme borreliosis is an emerging zoonosis that occur widely in all the continents (Stone et al., 2017). The primary agents of Lyme borreliosis are *B. burgdorferii, B. afzelii, B. garinii* and *B. valaisiana*. Other tick-borne *Borrelia* species are *B. duttoni, B. parkeri, B. hermsii B. crocidurae* and *B. miyamoto* the causative agents of tick-borne relapsing fever. These pathogens are injected through the skin by the bite of *Ixodes* ticks, and then spread throughout the body via the blood stream causing a variety of symptoms which in the early stage include fever, headache, fatigue, depression and skin rash (Klindworth et al., 2013a; Rupprecht et al., 2008; Wilson and Smith, 2014). If left untreated the disease may involve the joints, heart and central nervous system (Feng et al., 2015).

Diagnosis of *Borrelia* disease can be done by serological tests such as Western blot or ELISA. Molecular techniques such as PCR can also be used to detect the spirochete deoxyribonucleic acid (DNA) (Coulter et al., 2005).

Little is known about Lyme disease and other tick borne borreliosis in sub-Saharan Africa. *B. burgdorferii sensu lato* have been identified in Morocco, Algeria, Egypt and Tunisia (Bouattour et al., 2004; Dsouli et al., 2006; Helmy, 2000). Tick borne *Borrelia spp*. has been reported in countries neighboring Kenya such as Tanzania and Ethiopia (Kisinza et al., 2003; Mitani et al., 2004; Socolovschi et al., 2012). Two cases of Lyme disease have also been reported in Kenya (Jowi and Gathua, 2005). These cases of *Borrelia spp*. infections in East African indicate possible transmission in Kenya making it important to determine the prevalence and distribution patterns

of *Borrelia* infections in the Kenya. This information is important for disease control and infection management among AFI patients in the Kenya.

1.1.1.4 Ehrlichiosis

Ehrlichiosis is a tick-borne disease caused by bacteria of the genus *Ehrlichia* which are obligate intracellular Gram negative cocci that particularly infect the leukocytes. Ehrlichiosis also known as human monocytic ehrlichiosis (HME) is caused by *E. canis, E. ewingii* and *E. chaffensis* is currently recognized as emerging tick-borne disease in humans (Dumler et al., 2007, Fishbein et al., 1994; Ismail et al., 2010; Walker DH, 1996). Human ehrlichiosis present commonly as undifferentiated illness with fever, headache, myalgia, anorexia and digestive, respiratory and neurological disturbances (Fishbein et al., 1994). The disease may be severe and even fatal particularly in patients with underlying immunosuppression (Dumler et al., 2007).

Diagnosis of ehrlichiosis is done either by microscopic observation of the organisms in leukocytes, serology to detect antibodies, cell culture isolation or by molecular methods (Comer et al., 1999; Goodman et al., 1996).

Reports of human ehrlichiosis are mainly from USA and Europe, with *Amblyomma americium* reported as the main vector. *Ehrlichia spp*. was reported in *Rhipicephalus* ticks from Cameroon suggesting *Rhipicephalus* ticks maybe vector of *Ehrlichia spp*. in Africa (Ndip et al., 2010, 2009). Little information is available on human ehrlichiosis in African countries although *E. canis, E. ewingii,* and *E. chaffeensis* have been reported in dogs and jackals in the region (Ndip et al., 2007; Pretorius and Kelly, 1998). A case of human infection with *E. chaffeensis* in Mali was serologically confirmed whereas in Kenya *E. canis* has been reported in puppies (Price and Karstad, 1980; Uhaa et al., 1992). In addition, there is high prevalent of *E. ruminantum* in domestic and wild animals in Kenya (Wesonga et al., 2006). This organism has been reported as an emerging pathogen causing

life-threatening disease in humans in Africa (Ndip et al., 2010). Understanding the distribution of *Ehrlichia spp.* in Kenyan will enhance knowledge needed for effective control and containment efforts in case of human disease epidemic in the country.

1.2.2.1 Anaplasmosis

Human anaplasmosis, also known as human granulocytic anaplasmosis (HGA), is an emerging tick-borne disease caused by a Gram-negative obligate intracellular bacterium, *Anaplasma phagocytophilum*, which is transmitted to humans by various *Ixodes* ticks (Dumler et al, 2005). HGA is characterized by symptoms such as malaise, fever, myalgia, headache, nausea, vomiting, diarrhea, cough and pulmonary infiltrates. Other signs include skin rash, leucopenia, thrombocytopenia, and damage to the liver (Dumler et al, 2005). Because of non-specific signs, laboratory confirmation for diagnosis is always required. Serological test, PCR and culturing of the organism from blood or cerebrospinal fluid are used for diagnosis (Dumler et al., 2005).

In Kenya there is limited information on human anaplasmosis although there have been reports of high sero-prevalence in domestic and wild animals (Ngeranwa et al., 2008). Wide distribution of the tick vector and frequent interactions between human and animals suggest the risk of transmission of anaplasmosis from animals to humans. There is therefore need to evaluate the distribution of human *A. phagocytophilum* in the Kenyan population as a possible etiological agent of undifferentiated AFI and also determine areas with risk of infection and the related risk factors. Understanding the distribution of *A. phagocytophilum* in Kenyan will enhance our knowledge needed for effective control and containment efforts in case of emergence of human anaplasmosis in the country.

1.1.1.5 Bartonellosis

Bartonellosis is an infectious disease caused by bacteria of the genus *Bartonella* which includes 19 distinct species of which at least 6, namely; *B. henselae, B. bacilliformis, B. quintana, B. elizabethae, B. vinsonii* and *B. koehlerae* are responsible for human disease (Florin et al., 2008). The bacteria are transmitted to human by blood suckling arthropods such as ticks, fleas, lice, and sand flies with mammals as reservoirs (Angelakis et al., 2010; Billeter et al., 2011). Following an infection, the bacteria colonize endothelial cells and after every five days they are released into the blood stream where they infect erythrocytes and multiply (Maurin et al., 1997). Early symptoms of *Bartonella* infection are fever, fatigue, headache and poor appetite. Patients infected with *Bartonella* also show unusual streaked rash and lymphadenitis especially around the neck, head and arms. Infections with *Bartonella spp.* are responsible for a broad range of clinical syndromes, such as Carrion's disease, Trench fever, and cat scratch disease (Maurin et al., 1997). Human cases have been diagnosed based on PCR of tissue biopsy and serology using specific antigens (Henri-Jean et al., 2005).

In the recent years data on *Bartonella spp*. has increased rapidly after this group of organisms were found to cause a spectrum of emerging and re-emerging diseases ranging from self-limiting, short term fever to potentially fatal systemic disease (Anderson and Neuman, 1997). A case of *B. quintana* reported in a patient from Ethiopia and high prevalence of *Bartonella spp.* reported in bats sampled from different locations in Kenya suggests *Bartonella spp.* are endemic in East Africa (Kosoy et al., 2010; Tasher et al., 2017). There is therefore a need to investigate whether the agents might be responsible for human illness in Kenya. Understanding of the distribution and risk of *Bartonella spp*. in Kenyan population will enhance our knowledge which is important for effective control and containment efforts in case of human disease emergence in the country.

1.1.1.6 Babesiosis

Babesia parasites are transmitted by a bite of Ixodidae ticks and are the causative agent of human babesiosis. Babesia parasites are zoonotic as they can be transmitted between animals and humans. The animal hosts include rodents, cattle and deer (Young et al., 2019). Babesiosis is a malaria-like parasitic disease caused by intra-erythrocytic protozoa of genus *Babesia*. It is a zoonotic disease maintained by the interaction of tick vectors, transport hosts, and animal reservoirs (Vannier and Gewurz, 2008). The *Ixodidae* ticks ingest *Babesia* parasites when taking a blood meal from the vertebrate host and are transferred to a next vertebrate host through a tick bite. Multiplication of the blood stage parasites takes place in red blood cells which leads to hemolytic anemia, thrombocytopenia, and atypical lymphocyte formation (Kjemtrup and Conrad, 2000). Alterations in red blood cells membranes cause decreased conformability and increased red cell adherence, which can lead to development of non-cardiac pulmonary edema and acute respiratory distress syndrome in heavy infections (Homer et al., 2000). The signs and symptoms mimic those of malaria and range in severity from asymptomatic to septic shock. These include fever, chills, sweating, myalgia, fatigue, hepatosplenomegaly and hemolytic anemia. Symptoms typically occur after an incubation period of 1 to 4 weeks, and can last several weeks. The disease is more severe in patients who are immunosuppressed, splenectomized, and/or the elderly. Babesiosis is usually suspected in a patient with fever, hemolytic anemia, and history of exposure to vector tick bites (Homer et al., 2000). Diagnosis of *Babesia* infections is made through identification of the parasites on thin blood smears with Geisma or Wright staining. Serological testing and PCR testing can also be used for diagnosis (Homer et al., 2000).

Infections with *Babesia* parasites in natural hosts like cattle causes bovine babesiosis result in significant economic losses while in humans these infections can cause severe disease. In humans

the cases are increasing where transmission is primarily due to a tick bite but also infections resulting from blood transfusion with infected blood and mother to child during pregnancy have been reported (Schnittger et al., 2012; Zintl et al., 2003). Four Babesia species have been confirmed to infect humans include *B. microti, B. divergens*, *B. duncani*, and *B. venatorum* (Louise and Lobo, 2015). Babesiosis caused by the rodent parasite *B. microti*, which has emerged as an important human pathogen in recent years has been reported in non-human primates living in close proximity to humans in Kenya (Maamun et al., 2011; Westblade et al., 2017). Together with the bovine parasite *B. divergens* the two are now recognized as important zoonotic agents of human babesiosis (Tabara et al., 2007; Zhou et al., 2014; Zintl et al., 2003). However, no epidemiologic studies have been carried out to evaluate human babesiosis in Africa despite the importance and risk for human infections. These studies are necessary because understanding of distribution and risk factors of *Babesia spp*. infections in Kenya is imperative for effective infection control and management.

1.2.3 Detection methods for tick borne pathogens

Containment of diseases in the population and environment largely depends on early detection and reporting which is enabled by timely diagnosis (Halliday et al., 2007; Hitchcock et al., 2007). Detection of tick – borne pathogens in potential vectors and reservoirs has been used in studies to assess and determine prevalence of these disease causing pathogens in nature and the risk of infections in the human population as well as to detect emerging and re-emerging pathogens (Courtney et al., 2004). Numerous methods including antibody and antigen detection assays such as IFA, ELISA and Western blot, culture isolation and PCR based assays for the detection of tick borne pathogens have been described (Parola and Raoult, 2001a, 2001b).

Conventional PCR assays for tick – borne pathogen detection have been shown to be pathogen specific with higher sensitivity compared to culture detection methods. Conventional PCR can be time-consuming and labor-intensive especially when there is need to test multiple pathogens for differential diagnosis (Courtney et al., 2004). A real-time Taqman based PCR assay that uses fluorogenic 5' nuclease methods can overcome the challenges of serological and conventional PCR assays. Lack of multi-pathogen detection methods is a challenge to pathogen surveillance effort especially in tropical region where there is a wide array of infectious agents (Prasad et al., 2015). There is, therefore, need for a faster and easier multiplexed method for screening patients and other samples from vectors and reservoirs for pathogens that cause infections displaying non-specific symptoms. Laboratories need to test increasing number of pathogens from patients' samples especially when handling increased number of samples during outbreaks and epidemics. Combining multiple individual pathogen assays into a single diagnostic assay that is capable of detecting and differentiating multiple pathogens can provide a timely, cost effective means of pathogen identification in samples. In addition, such an assay if used for routine diagnosis would significantly increase the number of tests performed on each sample without increasing the cost of overall tests and the time to results. Multiplexing capabilities of Taqman based real time PCR (qPCR) enables high-throughput method for the detection of more than one pathogen simultaneously in a single tube assay. (Pripuzova et al., 2012). Real time PCR technology bases on Taqman® probe–based assays allows multiplexing of multiple targets. Each target is labelled with a unique fluorescent dye emitting different color at varying wavelength that can be observed simultaneously. Real time PCR instruments are equipped with different wavelength filters and can discriminate between the different dyes. The amount of signal from each dye is used to separately determine the amount of each target detected in a assay (Henegariu et al., 1997).

Most of the molecular and microbiology techniques used in detection of microbes in humans, animals and vectors are limited by targeting only known pathogens. Advent of next generation sequencing (NGS) allows unbiased open-ended detection of pathogens in a sample in a highly parallel sequencing approach (Motro and Moran-Gilad, 2017a). NGS emerged during the past decade which involves high-throughput massively parallel sequencing of clonally amplified DNA molecules on a flow cell or a chip. A single NGS run can generate hundreds of millions to gigabases of nucleotide sequence data depending upon platform configuration, chemistry, and flow cell or chip capacity (Behjati and Tarpey, 2013). NGS therefore enables simultaneous sequencing of all the genomic material in a sample whether clinical, vector, animal or environmental allowing comprehensive study of microbial population. This method of microbial detection is referred to as metagenomics next generation sequencing (mNGS) (Duan et al., 2020). Targeted NGS metagenomics has been used to profile microbial communities in samples using gene markers and microbe databases. Detection of bacterial microbes involves amplification of 16s rRNA gene which has conserved and variable regions, sample bar-coding by PCR, parallel deep sequencing and bioinformatics sequence analysis (Couper and Swei, 2018a). This approach has been used for bacterial pathogen and symbionts detection in ticks (Bouquet et al., 2017). NGS can therefore be used for unbiased identification of bacterial community in tick vectors. This information will enhance understanding of the risk of tick-borne zoonosis pathogens transmission by ticks to humans.

1.3 Study Justification

Although commonly caused by malaria parasites, not all acute febrile illness cases are caused by these parasites. There is need to determine what other pathogens are responsible for acute febrile illness (Leslie et al., 2012). Cases of tick-borne pathogens such as Q fever and spotted fever

rickettsial being reported in travelers returning from Kenya coupled with a wide distribution of ticks known to be vectors and reservoirs of various pathogens is an indication of possible risk of infections in Kenya. The emergence of tick-borne diseases is attributed to human activities like living in close proximity to animals, deforestation and outdoor activities which are common among Kenya population (Chaput et al., 2002). Identification of factors associated with these infections and their geographical distribution in different parts of Kenya is essential for effective infection control. However, the distribution and risks factors of tick borne infections are not well understood in Kenya. When specific etiologies of acute febrile illness are not known, accurate diagnosis, effective treatment and implementation of targeted public health disease control measures is difficult. This situation can lead to morbidity and mortality among acute febrile illness patients due to delayed or inappropriate treatment. Determining current prevalence, distribution and risk factors of tick borne pathogens among the indigenous population may inform decisions on differential diagnosis of acute febrile illness and efforts in developing point of care diagnostics platforms as well as planning for disease control measures.

1.4 Null Hypothesis

1. Tick-borne pathogens are not etiological agents of undifferentiated acute febrile illness in Kenya
2. Tick-borne pathogens are not distributed in different ecological zones in Kenya and there is no risk of human infections.

1.5 Problem statement

It is known that most of the emerging tick-borne zoonoses are accidentally transmitted to humans by tick vectors and humans are mostly dead-end host for these pathogens (Cristina Socolovschi et al., 2009). In Kenya there is high prevalence of diseases of veterinary importance that are

transmitted to animals through tick bites. These reports are common in areas where human live close to their domestic animals and wildlife which increases the risk of human infections with tick-borne pathogens. Information on the risks and burden of tick-borne zoonosis in human is unknown in Kenya, although cases have been reported. Tick-borne pathogens are common causes of acute febrile illness globally which is a common presentation in Kenyan health facilities. Acute febrile illness is commonly present with fever (defined as temperature >38˚C (Elshout et al., 2011) without localizing features and is among the most common reasons for persons to seek health care in low and middle-income countries (Nyaoke et al., 2019). Data from community-based infection prevalence in childhood above 34–37% show that 50% or more of fevers are likely to be associated with malaria infection (Okiro et al., 2007). For patients who present with fever without localizing features, clinical diagnosis is difficult and malaria may be the default diagnosis (Nyaoke et al., 2019). Limited laboratory diagnostic resources and the great diversity of acute febrile illness etiologies in tropical regions hinder determination of specific etiologies of acute febrile illness leaving most of the etiological agents of acute febrile illness undetected. Health care providers without proper diagnostic tools are usually unable to determine specific etiologies and undifferentiated acute febrile illness patients are often diagnosed presumptively based on clinical features and assumptions regarding circulating pathogens (Kasper et al., 2012). However this can be challenging when knowledge of full spectrum of circulating pathogens is unknown. Information on risk factors, prevalence, distribution and signs and symptoms of these infections among the Kenyan population is scarce making infection diagnosis, treatment and control difficult.

1.6 Objectives

1.6.1 General objective

To optimize and validate multiplex based assays for *Rickettsia, Borrelia, Coxiella, Ehrlichia, Babesia, Anaplasma and Bartonella* and use this assays to determine the distribution of human tick-borne pathogens as etiological agents of acute febrile illness of unknown origin, their associated signs and symptoms and their risk factors in Kenya. Determine potentially pathogenic bacteria harbored by ticks infesting domestic animals in Kenya

1.6.2 Specific objectives

1. To develop and validate multiplex based assays for *Rickettsia, Borrelia, Coxiella, Ehrlichia, Babesia, Anaplasma and Bartonella*
2. To determine the prevalence, distribution, signs and symptoms and risk factors of tick borne pathogens in patients with acute febrile illness in Kenya
3. Determine potentially pathogenic tick-borne bacteria community in ticks collected from domestic animals in Kenya.

2 CHAPTER TWO: Material and methods

2.1 Conceptual framework of the study

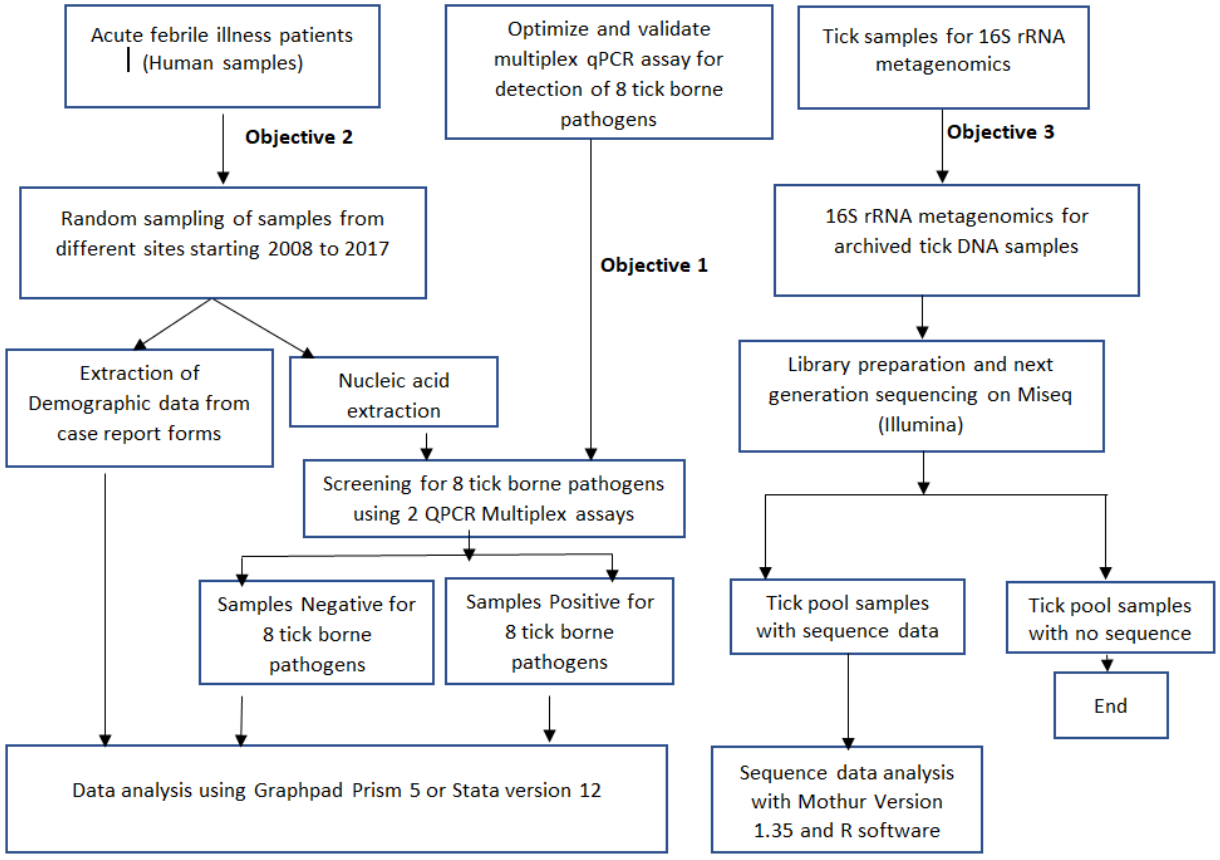


Figure 1: Study work strategy on sample acquisition and analysis

2.2 Study design and site for acute febrile illness patients

This was a laboratory-based study where archived whole blood samples were used that had been collected in a surveillance study between March 2008 and December 2017. The study that samples and patient data were collected from aimed at determining endemic etiologies of acute febrile illness in Kenya. Diseases targeted by this study included *malaria*, *Leptospirosis*, *Brucellosis*, *Salmonella typhi*, *Dengue*, *Leishmaniasis*, measles, West Nile virus, Influenza and Hepatitis E. Recruitment was done from healthcare facilities located in Lake Victoria region (Kisumu county

hospital, Kombewa sub-county hospital, Alupe sub-county hospital, Busia), Kisii Highlands (Kisii county hospital), Rift valley region (Marigat sub-county hospital, Baringo, Lodwar county hospital, Gilgil sub-county hospital, Nakuru), North Eastern Region (Iftin sub-county hospital, Garissa police line dispensary and Isiolo county hospital), Coastal region (Malindi sub-county hospital, Kilifi) and Cosmopolitan urban region (Eastleigh health center, Nairobi). These facilities serve both urban and rural areas except Eastleigh health center in Nairobi that mainly serve an urban area. The health care facilities were chosen because of their geographical diversity and capture areas of Kenya in which 2 major epidemiologic forms of malaria transmission exists; stable endemic transmission and epidemic transmission. The health care facilities also had already established KEMRI / USAMRD – K surveillance site with a clinical team to recruit the patients and process and ship the samples. The health facilities also were located in areas with a network of courier service to enable sample shipment from the site to the Laboratory. Figure 2 shows a map of Kenya showing the location of health facilities where samples used in this study originated.

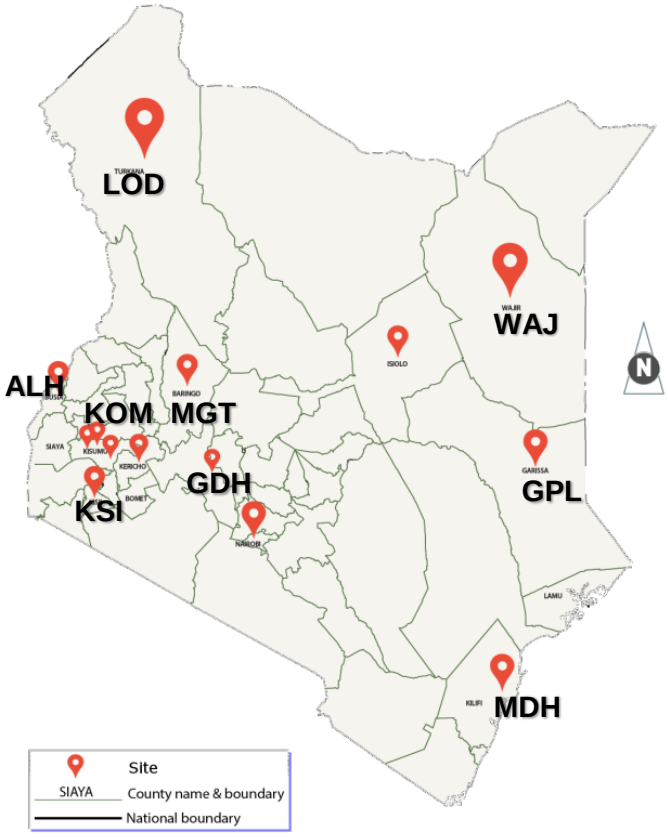


Figure 2: Map of Kenya showing healthcare facilities from where patients were recruited. LOD – Lowdar county hospital, WAJ – Wajir county hospital, ALH – Alupe sub-county hospital, KOM – Kombewa sub-county hospital, KDH – Kisumu county hospital, GPL - Garissa Police line, MGT – Marigat sub-county hospital, GDH – Gilgil sub-county hospital, KSI – Kisii county hospital, MDH – Malindi sub-county hospital, IFT - Iftin sub-county hospital, Eastleigh health center, Nairobi and ISL - Isiolo county hospital

2.3 Tick sample acquisition and study site for profilling bacteria commenity in ticks

Archived tick genomic DNA samples used for profiling tick-borne bacterial community in ticks were collected from domestic animals including cattle, sheep and goats at major slaughter houses in Nairobi and Mombasa originating from sites shown in Figure 3. Adult ticks were collected from domestic animals presented for slaughter to major slaughterhouses in Nairobi (Athi River Kenya Meat Commission [KMC] slaughter house) and Mombasa (KMC Kibarani, Uwanja wa Ndege, Mariakani and Kasemeni) between November, 2007 and September, 2008. These slaughterhouses receive animals from nearly all counties in the country. Animal movement

permits issued by the local veterinarian from the animal's area of origin were used to determine the animals' county of origin. At least 10 adult ticks were also collected from each animal found to have ticks. The collected ticks were placed in 1.5-mL vials containing 70% ethanol and transported to the laboratory for archiving and testing.

Ticks were identified taxonomically using the standard taxonomic key (Estrada-peña et al., 2003). Ticks of the same species and from the same domestic animal were pooled together and placed in a 1.5-mL vial containing 70% ethanol and stored at -80°C. Each vial containing classified pooled ticks was labeled with information of the tick species and identification of the tick's animal host.

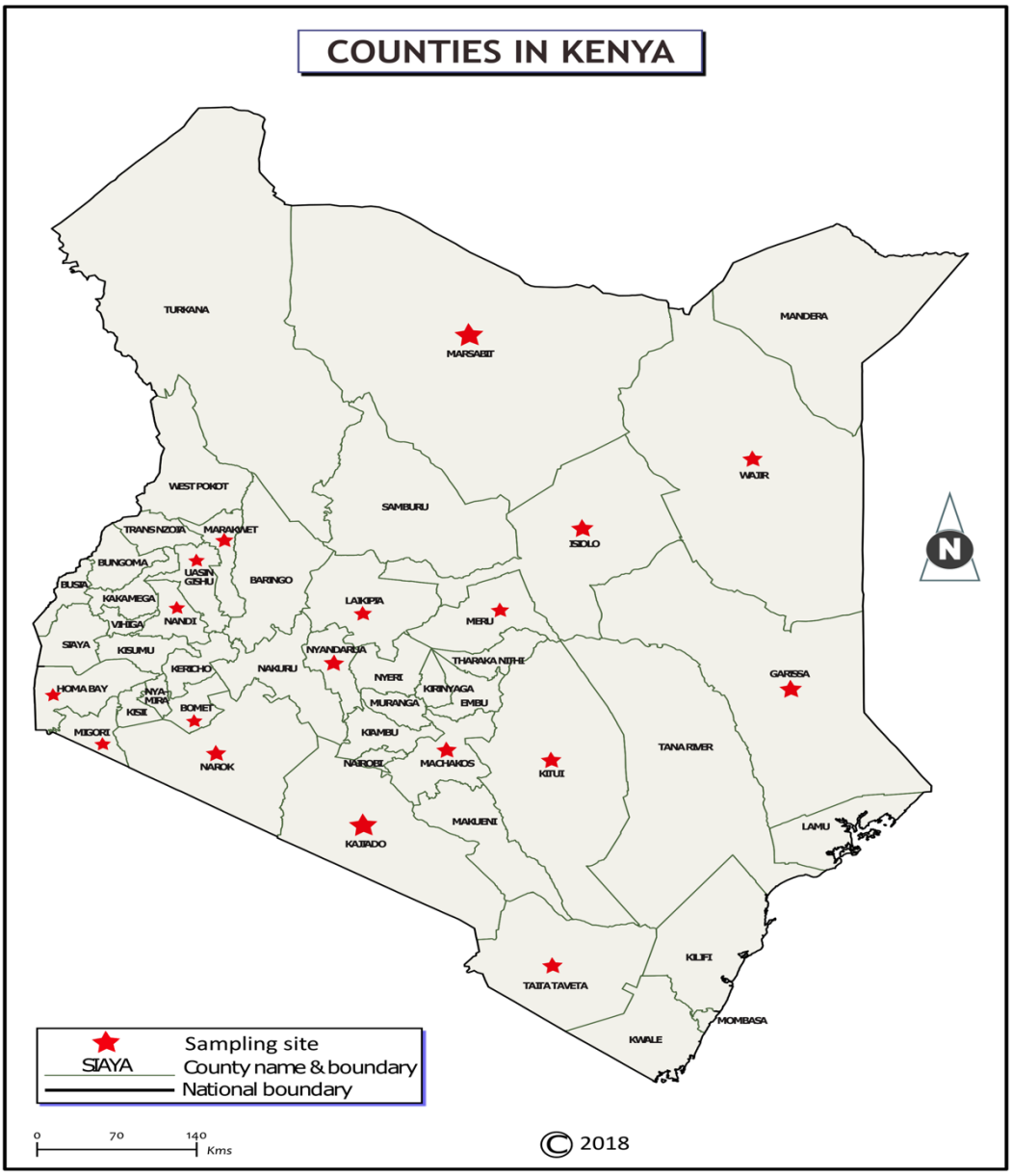


Figure 3: Counties of origin of animals presented at slaughter houses from which ticks were collected

2.4 Ethical consideration

This study used archived samples collected under an acute febrile surveillance study approved by scientific and ethical review committee of the Kenya Medical Research Institute, Nairobi (KEMRI SERU/SSC # 1282) and the Walter Reed Army Research Institute human use protection board, Silver Spring, Maryland USA (WRAIR HSPB # 1402) (Appendix 1). To determine the distribution

and risk factors of tick-borne zoonotic infections in Kenya the primary protocol revised in continual approval to include tick-borne zoonoses in the list of pathogens to be tested. The principal investigator of the study authorized use of the archived samples to investigate tick - borne zoonosis and their risk factors in Kenya (Appendix 2_Letter). Eligible patients were voluntarily enrolled upon signing an informed consent or assent (Appendix 3). To determine the bacterial microbial community in ticks, archived genomic DNA samples from ticks collected from domestic animals presented for slaughter were used. The tick samples had been collected under protocol SERU/SSC # 1248 that was reviewed and approved by the Animal Use Committee of the Kenya Medical Research Institute (Appendix 4).

2.5 Undifferentiated acute febrille illness patient sample acquisition and sample size calculation

At the time of this study 7000 samples were collected and data entered in a database in Microsoft access 2007. Samples were randomly selected using a computer random sampling application in Stata data analysis and statistical software version 12.0 (StataCorp LP, Texas USA). The suitable sample size for this study was based on studies carried out to determine tick - borne zoonosis infections in Cameroon that reported prevalence of 10% (Ndip et al., 2009). The formula N = Z2 P (1-P) / d2 Where N = sample size, Z = statistic for a level of confidence of 95 %, p is the expected prevalence was used (Naing et al., 2006). Using expected prevalence or proportion of 10% and d = 0.05 sample size (N) was obtained (N = {1.962 * 0.1 (1-0.1)} / 0.052 = 138.29). At least 139 whole blood samples required to be tested for each of the eight ticks – borne pathogens. The minimum number of samples tested for any of the 8 tick – borne pathogens was 3615.

2.6 Study population, clinical, demographic and epidemiology data

Samples used in the present study comprised of archived 6,207 patients' blood samples collected in an ongoing research study aimed at investigating the endemic pathogens that are common causes of Acute Febrile Illness (AFI) in Kenya. An individual was included in this study upon signing an informed consent or assent form to participate in all study related procedures. A guardian was required to give consent for their children to participate in the study. Children between 12 years and 18 years were required to sign an assent form to participate in the study (Appendix 3). Patients of both genders aged one year and above presenting with fever (≥38ºC) of unknown origin after routine clinical examination were eligible for participation in the study. Patients who declined to consent or assent to participate in the study were excluded from participating in the study activities. If they were not willing to give blood for use in the study or refuse their blood to be stored for future use they were also excluded from the study. Individuals enrolled within the previous seven days were also excluded. This was because there would be high likelihood the same infection would be causing them febrile illness and go back to seek health care. During recruitment patient's clinical, epidemiology and demographic information was obtained according to patient data sheet (Appendix 5). Data recorded from the patients included, age, gender, place of residence, clinical signs and symptoms they were showing at the time of recruitment, any contact with animals and the type of animal and whether they had a tick bite. This information was used to determine factors associated with getting a tick–borne infection. Data collected from the patient was used to determine risk factors and sings and symptoms for tick-borne infections.

2.7 Undifferentiated actute febrile illness patients sample collection

During sample collection approximately 5 mL of blood was collected from each participant by venipuncture and labeled with identifiers to distinguish them by site and date of collection.

Specimens were stored in aliquots of 200 microliter (µl) in liquid nitrogen in dry shippers and then shipped overnight by a courier service to KEMRI / USAMRD-A, Kisumu laboratory. On receiving the samples, sample quality and proper labeling assessment to accept or reject the samples was done. Samples were then archived at -80° C and entered in an access controlled inventory until processing and testing was done. Participants' data sheets were received in the Laboratory where data were entered in an access controlled database on Microsoft Access 2007.

2.8 Optimization and evaluation of two multiplex qPCR for detection of eight tick borne pathogens

To optimize and validate real time polymerase chain reaction (qPCR) for detection of tick–borne zoonosis, artificial linearized plasmids containing a fragment of the gene sequences targeted by the primers and probes listed in Table 1 were commercially synthesized by Genscript (NJ, USA) were used. Plasmids were supplied lyophilized at 4ng per plasmid. They were reconstituted with 20 microliter (µl) of nuclease free water as was recommended by the manufacturer. Plasmid copy numbers were computed based on average base pair weight (650 Daltons), length of the plasmid, size of the vector used to clone the plasmids and number of base pairs using an online calculator (Staroscik, 2004).

Single target (single-plex) assays were optimized on Applied Biosystems 7,500 fast Real time PCR system which has 5 detection channels to allow simultaneous detection of 4 targets and one channel for normalizing fluorescence (Applied Biosystems, CA. USA). The assay was optimized in a total of 10 µl reaction volume that contained 5 µl of Sensifast probe Lo-rox PCR master mix comprising of the enzymes and salts required for PCR amplification (Bioline, USA Inc), 0.5 micro molar (µm) of each forward and reverse primers, 0.4 µm of the probe and 3.0 µl of plasmid (6.15x10^6 copies/ µl) and nuclease-free water to a final volume of 10 µl. Primers and probes were selected from

published assays and only those with data on specificity against related pathogens were used (Courtney et al., 2004; Jiang et al., 2012, 2003; Loftis et al., 2003; Radzijevskaja et al., 2008; Schneeberger et al., 2010). For *Bartonella* spp., the primers and probe used were modified to target a short region (200 bp) and included a probe using primer express software (Applied Biosystems, CA USA) from a published conventional PCR assay (Billeter et al., 2011). The specificity of all primers and probes was tested by homology searches in the NCBI nucleotide database (Wheeler et al., 2004). Probes were labeled at the 5' end with a fluorescent reporter dye and an appropriate non-fluorescent quencher at the 3' end. Reporter dyes combinations were selected to allow multiplexing of four targets in a 7500 ABI platform. Primers and probes used for the assays are listed in Table 1.

Two 4-plex assays were optimized following the initial optimization of single-plex assays. The primers and probes were pooled into two 4-plex assays since the 7500 fast Real time PCR machine has only 5 channels. The reaction mix for assay one was optimized to detect *C. burnetii*, *A. phagocytophilum*, *B. burgdoferi* and *E. chaffeensis* at cycling conditions of 50ºC hold for 2 minutes, 95ºC initial denaturation for 10 minutes followed by 45 cycles of 95ºC denaturation for 15 seconds, and 60ºC annealing for 1 minute. Assay two was optimized to detect *Rickettsia* spp., *Babesia* spp., *Bartonella* spp. and other *Borrelia* spp. at cycling conditions of 50 ºC hold for 2 minutes, 95ºC denaturation for 10 minutes followed by 45 cycles of 95ºC denaturation for 15 seconds, and 56ºC annealing for 1 minute. After the run, each target result was analyzed separately to set appropriate threshold level (Ct) which is the amplification cycle where fluorescence increases significantly above the background fluorescence. For both assays, qPCR was performed in a total of 10 µl reaction volume that contained 5 µl of 2X Sensifast probe Lo-ROX PCR master

mix (Bioline, NJ, USA Inc), 0.5 µm of each primer, 0.4 µm of each probe and 3.0 µl of plasmid and nuclease-free water was added to a final volume of 10 µl.

Table 1: Primers and probes used for the duo 4-plex qPCR for detection 8 Tick Borne Zoonoses

Target	Sequence of primer or probe (5'-3')	Annealing temparature (ºC)	Target gene	Reference
Coxiella burnetii	Forward primer AAAACGGATAAAAAGAGTCTGTGGTT Reverse Primer - CCACACAAGCGCGATTCA T Probe - [FAM]AAAGCACTCATTGAGCGCCGCG[MGB]	60	IS1111 transposase	(Schneeberger et al., 2010)
Borrelia burgdorferi	Forward primer CGAGTCTTAAAAGGGCGATTTAGT Reverse Primer -GCTTCAGCCTGGCCATAAATAG Probe - [NED]-AGATGTGGTAGACCCGAAGCCGAGTG[MGB]	60	23S rRNA	(Courtney et al., 2004)
Anaplasma phagocytophilum	Forward primer ATGGAAGGTAGTGTTGGTTATGGTATT Reverse Primer -TTGGTCTTGAAGCGCTCGTA Probe - [JOE]TGGTGCCAGGGTTGAGCTTGAGATTG[BHQ-1]	60	MSP2	(Courtney et al., 2004)
Ehrlichia Chaffeensis	Forward primer - GCGGCAAGCCTAACACATG Reverse Primer -CCCGTCTGCCACTAACAATTATT Probe - [CY-5]AGTCGAACGGACAATTGCTTATAACCTTTTGGT [BBQ650]	60	16S rRNA	(Loftis et al., 2003)
Bartonella spp	Fwd primer - TGCTTCGACATCCACTGTACGTC Rev Primer -CACCTGCTGCAATACATGCAAATG Probe - [CY-3]TTGCAGGTTCATCAGGTGCTAATC [BHQ-2]	60	Citrate synthase	(Billeter et al., 2011)
Rickettsia spp	Fwd primer- ATGAATAAACAAGGKACNGGHACAC Rev Primer -AAGTAATGCRCCTACACCTACTC Probe - [FAM] CCGAATTGAGAACCAAGTAATGC[BHQ-1]	60	17 KDa antigen	(Jiang et al., 2012)
Babesia spp	Forward primer- CAGCTTGACGGTAGGGTATTGG Reverse Primer AGATGTGGTAGACCCGAAGCCGAGTG Probe - [CY5]CGAGGCAGCAACGG[BBQ]	56	18S rRNA	(Radzijevskaja et al., 2008)
Borrelia spp	Forward primer GCTGAAGAGCTTGGAATGCAAC Reverse Primer -GCTTCATCCTGATTTGCACCAAC Probe - [JOE]CGCGATACACCAGCATCATTATCTGAATCACAATCGCG[BHQ-1]	56	Flagellin	(Jiang et al., 2003)

In brackets are reporter and quencher fluorescent dye: FAM - 6-carboxyfluorescein, CY-5 – cyanine -5, JOE - 6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein, VIC - 2′-chloro-7′phenyl-1,4-dichloro-6-carboxy-fluorescein, NED – Naphthyl EthyleneDiamine), BHQ - Black Hole Quencher, BBQ – Black berry quencher, MGB – Minor groove binder

2.8.1 Determining assay limit of detection (LOD) for single-plex and 4-plex assays

Each plasmid was diluted serially 10-fold over a range of 6.15x10^6 to 6.15x10^-1 copies/ µl. Each concentration was tested eight times with single-plex and 4-plex assays. For 4-plex assays, plasmids were mixed together to mimic co-infections then diluted in 10-fold increments. Limit of detection (LOD) for each assay was determined as the lowest plasmid concentration that was amplified for all targets. LOD was then reported as equivalent pathogen detectable by the assay depending on copy number of each target gene present in its genome. *B. burgdoferi* has 2 copies of 23S rRNA gene (Wheeler et al., 2004), *A. phagocytophilum* has 1 copy of major surface protein 2 (MSP2) gene (Lin et al., 2004), *E. chaffeensis* has 1 copy of 16S rRNA gene (Loftis et al., 2003), *Coxiella burnetii* has 2 to 20 copies of insertion sequences (IS111) gene (Seshadri et al., 2003), *Rickettsia* has 1 copy of 17 kilodalton (kDa) gene (Reif et al., 2008), *Borrelia* has 1 copy of flagelin gene (Wallich et al., 1990), *Bartonella* has 1 copy of citrate synthase gene (Guy et al., 2013) and *Babesia* has 2 copies of 18S rRNA gene . Sensitivity of co-amplifications was also tested using low concentrations (6.15x10^-1 copies/µL) of each target in presence of high concentrations (6.15x10^6 copies/µL) of other non-targets.

2.8.2 Determining Efficiency, linearity and precision of single-plex and 4-plex assays

To determine linearity and Efficiency of the assays, standard curves of threshold cycle (Ct) versus copy numbers were generated over the 6.15x10^6 to 6.15x10^-1 copies/µL test range. qPCR reaction Efficiency was calculated from the slope of the standard curve according to the equation Log E = $10^{(-1/slope)-1}$ (Ian M. Mackay, 2007). To determine the reproducibility (precision) of the assays, three concentrations (6.15x10^6, 6.15x10^3 and 6.15x10^1 copies/µL) of plasmid were

assayed in triplicates five times on separate days. Coefficient of variation (CV) and Standard Deviation (SD) were calculated at each concentration for all targets.

2.8.3 Pairwise comparison between single-plex and multiplex assays

Bland-Altman analysis was used to depict the magnitude of disagreement between single-plex and multiplex assays. In Bland-Altman analysis, the Ct value difference (ΔCt) between single-plex and multiplex assays are plotted against the mean of the Ct values of the two assays (Altman and Bland, 1983). Pairwise comparison was performed using Ct values from single-plex and 4-plex assays at various dilutions.

2.8.4 Use of the two-multiplex qPCR for detection of tick–borne zoonosis pathogens

Nucleic acids were extracted from 200 µl whole blood using ZR-Duet DNA/RNA mini prep kit (ZYMO Research, CA USA). Briefly, 200 µl of whole blood was lysed by adding 600 µl of DNA/RNA lysis buffer. The lysed sample was vortexed to mix thoroughly and then transferred to a spin column with a collection tube. This was followed by centrifuging at 12000 ×g for 1 minute.The flow-through was discarded. The spin column was transferred to a new collection tube and 400 µl of DNA/RNA buffer added and followed by centrifuging at 12000 ×g for 1 minute. The flow-through was discarded and 700 µl of DNA/RNA wash buffer added into the spin column then centrifuged at 12000 ×g for 30 seconds. The flow-through was discarded and 700 µl of DNA/RNA wash buffer added into the spin column then centrifuged at 12000 ×g for 2 minutes to ensure complete removal of wash buffer. The spin column was transferred to a clean DNase / RNase free micro-centrifuge tube. 50 µl of DNase / RNase free water was added to the column and let to stand for 5 minutes and then centrifuged at 10000 ×g for 30 seconds. The eluted DNA was stored at ≤ 20 ° C until testing was done. Eight tick–borne zoonosis pathogens were tested using the two

multiplex assays optimized as described above using three microliters of the eluted nucleic acid extracted. A specimen was scored positive for any of the tick-borne zoonosis pathogen if it was amplified with a Ct value of less than 40 otherwise the specimen was scored negative.

2.9 16s rRNA amplicons metagenomics for detection of bacterial pathogens in tick specimen

Tick pools were homogenized using a pestle and mortar and then resuspended in 1 mL of Phosphate buffered saline. DNA was extracted from 200 µl of the homogenate using a Qiagen QIAamp DNA Mini Kit according to manufacturer's instructions (Qiagen Inc., Valencia, CA). Briefly, 20µl of Qiagen protease was added into a 1.5ml micro-centrifuge tube followed by 200µl of the homogenate and mixed thoroughly by vortexing. 200 µl of buffer of lysis buffer was added and mix by pulse vortexing for 15 seconds. The solution was then incubated at 56 ºC for 10 minutes and briefly centrifuged to remove drops from the inside of the lid. 200 µl of absolute ethanol (100%) was added to the solution and mixed again by pulse vortexing for 15 seconds. The solution was transferred into a QIAamp mini spin column (in a 2 ml collection tube) without wetting the rim then centrifuged at 8000rpm for 1 minute. The spin column was transferred in a clean 2 ml collection tube and the tube containing the filtrate discarded. This was followed by adding 500 µl of wash buffer (AW1) without wetting the rim and centrifuged at 8000 rpm for 1 minute. The spin column was placed in a clean 2ml collection tube and the tube containing the filtrate discarded. 500 µl of the second wash buffer (AW2) was added into spin column without wetting the rim and centrifuge at 14,000 rpm for 3 minutes. The spin column was placed in a new 2ml collection tube and the collection tube with the filtrate discarded followed by centrifuging at 14,000rpm for 1 minute. The spin column was placed in a clean 1.5ml micro-centrifuge tube and the collection tube containing the filtrate discarded. 200µl of elusion buffer (AE) was added to the spin column

and incubated at room temperature for 1 minute. This was followed by centrifuging at 8000rpm for 1 minute. The spin column was discarded and the micro-centrifuge tube containing the eluted DNA was stored at -80°C until testing was done.

The presence of bacteria in ticks genomic DNA were detected by PCR amplification using Illumina barcode tagged primers targeting V3–V4 hyper variable region of the 16s rRNA gene as described earlier (Klindworth et al., 2013b). PCR was performed with 2.5 µl of genomic DNA, 5 µl of forward and reverse primers each at a final concentration of 1 µM, and 12.5 µl of NEBNext High-Fidelity 2X PCR Master Mix (New Englands BioLabs, MA, USA) at 95 °C initial denaturation for 3 min, followed by 25 cycles of 95 °C for 30 s, 62.3 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 5 min. Reactions were cleaned up with Agencourt AMPure XP beads (Beckman Coulter Genomics, CA, USA) according to the manufacturer's instructions. Dual indexing to allow multiplexing of samples was done using 5 µl of purified amplicons, 5 µl of Nextera XT Index Primer 1, 5 µl of Nextera XT Index Primer 2 (Illumina), 25 µl of NEBNext High-Fidelity 2X PCR Master Mix (New Englands BioLabs MA, USA) and 10 µL of PCR grade water (Thermo Fisher Scientific, MA, USA), with thermo cycling at 95 °C for 3 min, followed by 12 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 5 min. Constructed 16S metagenomic libraries were purified with Agencourt AMPure XP beads ((Beckman Coulter Genomics, CA, USA). The libraries were quantified on Qubit Flourometer 2.0 using Qubit dsDNA HS Assay kit (ThermoFsher Scientific, MA, USA). Libraries were normalized and pooled to 4 nM based on Qubit values. Pooled samples were denatured and diluted to a final concentration of 10 Pico molar (pM) and spiked with 10 % PhiX (Illumina, CA, USA). Ninety-six (96) samples were multiplexed for each sequencing run and sequenced using MiSeq Reagent Kit V3 on the Illumina MiSeq System.

2.10 Data management and analysis

The data obtained from patients' records were entered in a Microsoft access 2007 and analysis performed with either Graphpad Prism 5 (Graphpad Software INC San Diego CA USA) and Stata version 12 (StataCorp, College station, Texas USA). Prevalence was calculated as overall percentage of positive patients to each tick–borne zoonosis pathogen tested and compared using Fisher's exact or Chi-square test as appropriate to determine significant differences. In this present study we defined risk for tick–borne zoonoses infections as age, gender, contact with animals and a tick bite. Associations between patients' demographic characteristics (age, gender and region), clinical signs and symptoms and contact with any animal and tick–borne zoonosis results were determined using bivariate logistic regression. Statistically significant levels were set at alpha 0.05 and 95% confidence interval.

For 16s rRNA metagenomics data analysis, de-multiplexing and adapter trimming of the Miseq sequences output was done using the Miseq reporter software version 2.6.3 (Illumina, CA USA). Mothur version 1.35 pipeline was used for paired end reads contig assembly, sequence quality filtering, chimera removal and taxonomic identification (Schloss et al., 2009). In brief, contigs containing ambiguous bases, and those with lengths < 350 base pairs (bp) or greater than 466bp were discarded. Sequences were then aligned to a customized 16s rRNA gene V3-V4 region from the SILVA database (Quast et al., 2013), followed by merging sequences that were less than 2 bp different of each other using the pre.cluster command in Mothur. The merged sequences were then filtered for chimeric sequences and the chimera free sequences used for taxonomic assignment against the customized V3-V4 SILVA database (Schloss et al., 2009) using Bayesian classifier with 80% confidence (Quast et al., 2013;). Unassigned OTUs and those assigned to *Chloroplast*, *Mitochondria*, *Archaea*, and *Eukaryote* were discarded. Tick pools with < 1000 (n=40) sequences

were excluded from downstream analysis as small library sizes are often a cofounding factor that conceals biologically meaningful results (Weiss et al., 2017). Taxa detected in the non-template control were censored from the tick sample dataset(Davis et al., 2017).

Statistical analysis and data visualization were done using OUT tables generated by Mothur on R software environment version 4 with Phyloseq, vegan and ggplot2 statistical adds-on (McMurdie et al., 2013; Oksanen et al., 2014; R Core Team, 2018 and Wickham, 2009). To down sample the data for alpha diversity analysis and account for unequal sequencing between samples, rarefaction was done using rarefy_even_depth command in phloseq with replacement (Weiss et al., 2015). The rarefied data was used to determine Shannon diversity index by first determining bacterial diversity for each tick pool and then aggregated to genus level before computing the mean (Shannon, 1948).

3 CHAPTER 3: Results

3.1 Performance of duo 4-plex qPCR assays

Assay specificity: The primer and probe specificities were first evaluated *in silico* by aligning the nucleotide sequences on the NCBI Blast database that contain nucleotide sequences of all sequenced organisms. No complete matches to the primer and probe sequences were found other than those corresponding to the target genes of each of the eight tick borne zoonosis. To validate the *in silico* results, the specificity of the primers and probes were evaluated by real time PCR in the presence of the 8 targeted and non-target plasmids. As shown in Table 2, there was no cross-amplification or interference in product formation. Ct values were fairly similar between corresponding single-plex and 4-plex targets. High target plasmid concentration did not have significant effect on detection level of any target.

Table 2: Target specific with no cross amplification for mono-plex and 4-plex assays

	Assay one mean Ct values with mixed targets			
Target at 6.15x10^6 plasmid copies per µL	*Coxiella*	*A. phagocytophilum*	*B. burgdoferi*	*E. chaffeensis*
Coxiella	12.83 (12.35)	–	–	–
A. phagocytophilum	–	13.03 (13.13)	–	–
B. burgdoferi	–	–	11.69 (12.96)	–
E. chaffeensis	–	–	–	12.89 (12.1)
	Assay two mean Ct values with mixed target			
Target at 6.15x10^6 plasmid copies per µL	*Rickettsia* spp.	Other *Borrelia* spp.	*Bartonella* spp.	Babesia spp.
Rickettsia	11.13 (10.33)	–	–	–
Other Borrelia	–	10.39 (10.88)	–	–
Bartonella	–	–	12.08 (12.1)	–
Babesia	–	–	–	24.20 (24.53)

– Target not detected by the assay
– values in brackets are Ct values from the 4-plex assay

3.1.1 Assay limit of detection and dynamic range

As shown in Figure 3, in 8 repeat experiments, the linear range of Ct values was from 12 to 40 corresponding to 6.15*10^6 to < 3 copy/µL) for most of the targets in assay 1.This limit of detection was based on the copy number of the target genes corresponding to 3 copy of each pathogen targeted by assay one. Assay two was less sensitive and all targets could consistently be detected at Ct values of 11 to 37 corresponding to 6.15*10^6 to ~18 copies/ µL. This limit of detection based on the copy number of the target genes corresponding to 9 copies of *Babesia*, 18 copies each of *Rickettsia*, *Borrelia* and *Bartonella* (Figure 4 and 5).

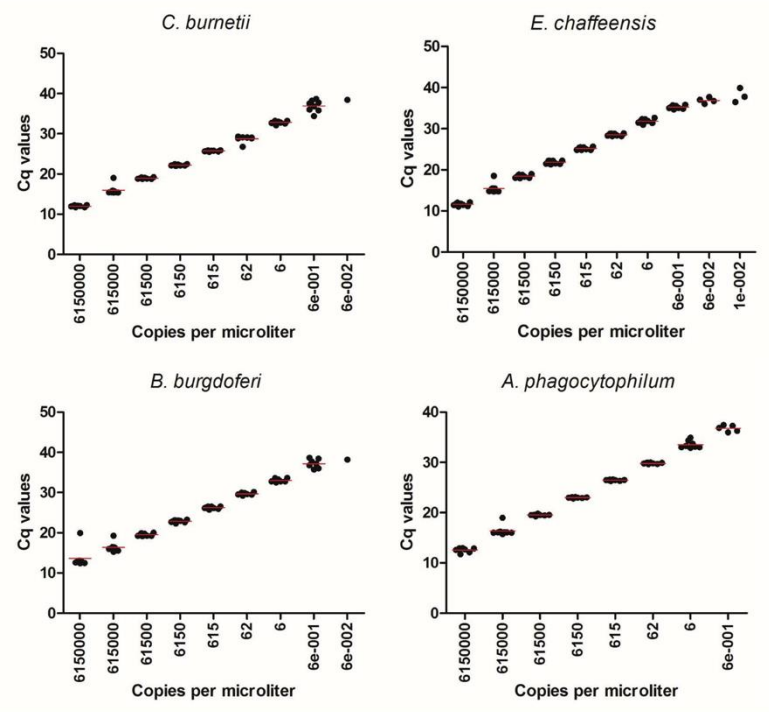


Figure 4: Ct values plotted against copy numbers of serially diluted plasmids for the 4-plex assay one. In 8 repeat experiments, the linear range of Ct values was from 12 to 40 corresponding to 6.15*10^6 to <3 copy/µL) for most of the targets in assay one. All targets had a coefficient of variation of less than 0.1. The Line crossing the data points indicates the mean Ct for each dilution.

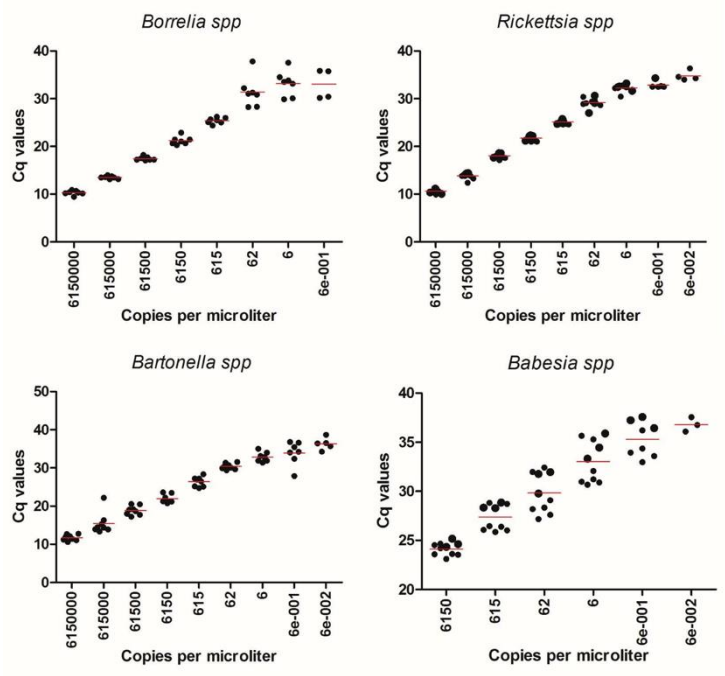


Figure 5: Ct values plotted against Copies per microliter of serially diluted plasmid DNA with 4-plex assay two. In 8 repeat experiments, the linear range of 11 to 36 for Rickettsia spp. and Borrelia spp. 11 to 38 for Bartonella spp. and 23 to 37 for Babesia Ct values corresponding to 6.15*10^6 to ~18 copies/ µL. Line crossing data points indicates the mean Ct value at each dilution.

3.1.2 Efficiency, linearity and precision of optimized multiplex assays:

The duo 4-plex assays had strong linear correlation to input DNA template (shown in Figure 4 and 5). The assay's efficiencies ranged from 94 to 100 with correlation coefficient (R^2) values of 0.995 to 0.999 indicating high linearity (Table 3). All assays had a standard deviation of one cycle or below indicating high precision for all targets at different concentrations. Coefficients of variation for replicate Ct values at each concentration for all targets were below 0.1 with a median value of 0.01 for assay one and 0.02 for assay two for all the concentration tested.

Table 3: Duo 4-plex qPCR showing amplification linearity and Efficiency for target pathogens

Assay	Target pathogen	Standard curve Slope	PCR Efficiency	R^2
Panel one	*C. burnetii*	-3.442	95.96	0.99
	A. phagocytophilum	-3.468	94.23	0.998
	B. burgdoferi	-3.464	94.40	0.998
	E. chaffeensis	-3.436	95.46	0.998
Panel two	*Rickettsia*	-3.736	95.00	0.997
	Other Borrelia	-3.462	94.00	0.996
	Bartonella	-3.678	97.00	0.995
	Babesia	-2.595	100.00	0.997

3.1.3 Comparison of multiplex and single-plex qPCR assays

Bland – Altman analysis was used to depict the magnitude of disagreement between multiplex and single-plex assays. The difference in Ct values (ΔCt) between multiplex and single-plex assays were plotted against the mean of the Ct values of the serially diluted plasmid for multiplex and single-plex assays. As shown in Figure 6, the ΔCt for the assays were within a range of -1.7 to 0.8 for *Coxiella*, -1.8 to 2.9 for *A. phagocytophilum*, 03 to 3.4 for *B. burgdoferi*, -1.3 to 2.9 for *E. chaffeensis*, -1.3 to 0.4 for *Rickettsia*, -0.8 to 0.1 for *Bartonella*, -0.3 to 2.3 for *Babesia* and -1.7 to 1.7 for other *Borrelia*. Mean ΔCt are very close to zero indicating small differences between the multiplex and single-plex assays.

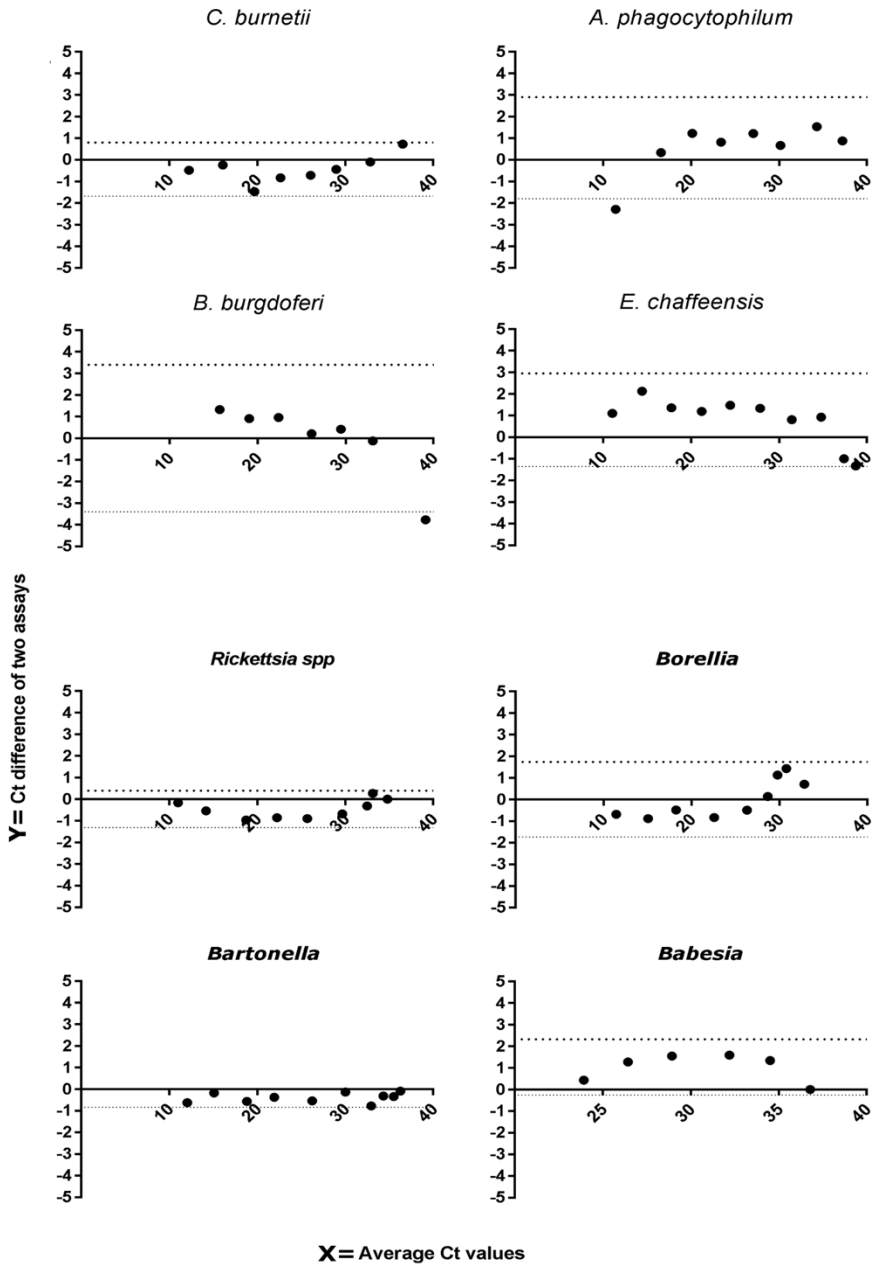


Figure 6: Bland – Altman plots showing differences between single-plex and multiplex assays. The difference in Ct values (ΔCt) between multiplex and single-plex assays against the mean of the Ct values of the serially diluted plasmid for multiplex and single-plex assays (dots) with representation of the limits of agreement (dotted line) within -1.96 SD to +1.96 SD. The reference line (solid line) on the plot indicates the ideal zero difference. X axis = average Ct values and y axis = Ct differences of the two assays.

3.2 Tick-borne zoonotic pathogens in acute febrile illness patients

3.2.1 Study participants

A total of 6,207 whole blood samples collected between the period of February 2008 and December 2017 were tested for presence of tick–borne zoonosis pathogens using the duo 4-plex real time PCR. Of these, 6,207 were tested for *Rickettsia*, 4,289 for *Coxiella*, 3,615 for *A. phagocytophilum*, 3,659 for *E. chaffeensis*, 3,657 for *B. burgdoferi*, 3,480 for *Babesia* spp., 3,463 for *Bartonella* spp. and *Borrelia* spp. that are not *B. burgdoferi.* For patients whose demographic data were available 3,082 (50.9%) were males and 2,979 (49.2%) were females. The ages ranged from 1 to 80 years with a median of 5 years.

3.2.2 Prevalence of tick–borne zoonosis pathogens

Overall 16.4%, C.I 15.3-17.1 was positive for at least 1 tick–borne zoonosis pathogen. This comprised of 7.7%, CI: 7.9-8.3 for *Rickettsia* spp., 6%, CI: 5.6-7.0 for *C. burnetii*, 1.5%, CI: 1.1-1.9 for *A. phagocytophilum*, 3%, CI: 2.5-3.6 for *E. chaffeensis*, 1.5%, CI: 1.1-1.9 for *B. burgdoferi*, 1%, CI: 0.2-1.4 for *Babesia* spp., 0.2%, CI: 0.07-0.4 for *Bartonella* spp. and 4%, CI: 3.3-4.5 for other *Borrelia* which are not *B. burgdoferi.* The number of tick-borne zoonosis positive samples was similar in males 16.6%, CI: 14.92–17.53 and females 16.2%, CI: 15.28–17.95. Tick–borne zoonosis prevalence was significantly higher in 6 to 15 year olds 17.5%, CI: 15.49-19.44, p = 0.02 and >16-year olds 19.2%, CI: 17.12-21.35, p=0.001 categories compared to the <5-year olds category 14.8%, CI: 13.57-15.99. For different regions in the country, urban cosmopolitan region showed highest prevalence of 39.4%, CI: 31.72-47.03, p=< 0.0001 compared to Coastal region 17.0%, CI: 12.61-21.43, Kisii highlands 12.5%, CI: 10.60-14.38, Lake Victoria region 15.5%, CI: 14.31-17.06, Semi-arid region 17.1%, CI: 15.40-18.76 as shown in Table 4.

Table 4: Demographic characteristics of acute febrile illness patients tested for tick-borne zoonosis

Patient parameter	% (number positive/N)	P-value
Age		
< 5 year olds	14.78 (489/3,309)	Ref
6 - 15 year olds	17.46 (248/1,420)	**0.02***
> 16 year olds	19.24 (257/1,336)	**0.001****
Gender		
Male	16.62 (500/3,082)	Ref
Female	16.22 (495/2,979)	0.7052
Region of residence		
Semi-arid region	17.08 (331/1,938)	Ref
Coastal region	17.02 (48/282)	0.3198
Urban cosmopolitan	39.37 (63/160)	**<0.0001*****
Kisii highland	12.49 (147/1,177)	0.1014
Lake Victoria region	15.94 (421/2,684)	0.3017

Boldface indicates statistical significance. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Statistical significance was tested using either Fisher's exact or Chi-square test (χ^2) where applicable.

3.2.2.1 Prevalence of Rickettsia spp.

Out of the 6,207 AFI participants tested for Rickettsia 7.7%, CI: 7.9-8.3 were positive. This comprised of 7.6 % in males and 7.9 % in females (Table 5). Although tick–borne zoonosis prevalence was higher in >15-year olds (9.2%) compared to 7.10% and 8.0% in <5-year olds and 6 to 15 year olds, respectively, the difference was not statistically significant (p = 0.792) (Table 6). There was higher prevalence of *Rickettsia* spp. in the urban cosmopolitan region (24.5%) (p=<0.0001) compared to Coastal region (8.5 %), Kisii highlands (4.59 %), Lake Victoria region (7.26 %) and Semi-arid region 8.50 %, (Table 7).

3.2.2.2 Prevalence of *C. burnetii*

Out of the 4,289 AFI participants tested for *C. burnetii* 6.3%, CI: 5.59-7.05 were positive. This comprised of 6.3 % in males and 6.5 % in females (Table 5). There was no significant statistical difference in the *C. burnetii* prevalence among the different age categories (p=0.7880) with <5 year olds had a prevalence of 6.2% compared to 6.4% in the 6 to 15 year olds and 6.9 % in the >15 year olds (Table 6). With regard to different regions in Kenya, the prevalence of *C. burnetii* showing no significant statistical different (p=0.9150) among the five regions that were investigated with the urban cosmopolitan showing a prevalence of 5.0% while Coastal region, Kisii highlands, Lake Victoria region and Semi-arid region had prevalence of 7.1%, 6.5%, 6.2% and 6.4%, respectively (Table 7).

3.2.2.3 Prevalence of A. phagocytophilum

Out of the 3,615 AFI participants tested for *A. phagocytophilum* 1.5%, CI: 1.12-1.92 were positive. This comprised of 1.5% in males and 1.6% in females (Table 5). There was no significant statistical difference among the various age categories (p=0.7349) with 1.4% in <5 year olds, 1.5% in 6 to 15 year olds, and 1.8% in >15 year olds (Table 6). Different regions investigated showed no significant statistical difference in prevalence (p=0.3457) with urban cosmopolitan reporting a prevalence of 3.1%, 1.3% in Coastal region, 0.7% in Kisii highlands, 1.5% in Lake Victoria region and 1.93 % in Semi-arid region (Table 7).

3.2.2.4 Prevalence of *E. chaffeensis*

Out of the 3,510 AFI participants tested for *E. chaffeensis* 3.1 %, CI: 1.1-1.9 were positive. This comprised of 2.8% males and 3.3% in females (Table 5). Different age categories showed no significant difference (p=0.1843) with 2.6%, in <5 year olds, 3.2% in 6 to 15 year olds, and > 3.9% in 15 year olds (Table 6). Similarly, the prevalence was not significantly different among the

different regions (p=0.4948) with 1.9% in urban cosmopolitan, 4.0% in Coastal region, 3.6% in Kisii highlands, 2.6% in Lake Victoria region and 3.4% in Semi-arid region (Table 7).

3.2.2.5 Prevalence of *B. burgdoferi*

Out of the 3615 AFI participants tested for *B. burgdoferi* 1.5%, CI: 1.13-1.93 were positive. This comprised of 1.6% in male and 1.4% in female (Table 5). Prevalence was not significantly different among the age categories (p=0.0593) with <5 year olds 1.1%, >6 to 15 year olds 1.7% and >15 year olds 2.3% (Table 6). The prevalence was not significantly different in the different regions, with Urban cosmopolitan 3.1%, Coastal region 1.3%, Kisii highlands 0.91, Lake Victoria region 1.5% and Semi-arid region 1.6% (p-value 0.3756) (Table 7).

3.2.2.6 Prevalence of *Borrelia* spp.

Out of the 3,464 AFI participants tested for *Borrelia* spp. 135/3364 (3.89 % CI: 3.25-4.54) were positive. This comprised of 4.3% in males and 3.8% in females (Table 5). Different age categories did not show significant statistical difference in prevalence (p=0.7583) with <5 year olds 3.9%, >6 to 15 year olds 4.5% and >15 year olds 3.9 % (Table 6). *Borrelia* spp. prevalence was not significantly different in different regions (p=0.2342) with urban cosmopolitan region 6.2%, Coastal region 3.7%, Kisii highlands 3.8%, Lake Victoria region 4.5% and Semi-arid region 3.0% (Table 7).

3.2.2.7 Prevalence of *Babesia* spp.

Out of the 3,480 AFI participants tested for *Babesia* spp. 1.0% CI: 0.7-1.37 were positive. This comprised of 1.2% in males and 0.8% in females (Table 5). Although low prevalence was observed, there was significant statistical difference in the prevalence of *Babesia* spp. among the different age categories (p=0.0324) with <5 year olds 1.5%, >6 to 15 year olds 0.7% and >15 year olds 0.4% (Table 6). The prevalence was significantly different in different regions in Kenya (p=0.0126) with higher prevalence in Kisii highlands at 2.0%, Lake Victoria region 1.3%, Coastal region 0.7%, Semi-arid region 0.4%, and no *Babesia* spp. pathogen detected in urban cosmopolitan (Table 7).

3.2.2.8 Prevalence of *Bartonella* spp.

Out of the 3463 AFI participants tested for *Bartonella* spp. 0.2% CI: 0.07-0.39 were positive. This comprised of 0.2% in males and 0.3% in females (Table 5). There was no significant statistical difference in prevalence among the different age categories (p=0.8157) with <5 year olds 0.3%, >6 to 15 year olds 0.2% and >5 year olds 0.1% (Table 6). The prevalence were low in all zones with no *Bartonella* spp. detected in the coastal and urban cosmopolitan regions, Kisii highlands 0.38%, Lake Victoria region 0.2% and Semi-arid region 0.27% (Table 7).

Table 5: Tick–borne zoonosis pathogens with gender

	Number positive/Number tested (Percentage, CI :)	
Tick–borne zoonosis pathogens	**Male**	**Female**
C. burnetii	135/2080 (6.26, CI: 5.23-7.28)	36/2159 (6.54 CI: 5.48-7.60 %)
A. phagocytophilum	26/1767 (1.47, (CI:. 0.91-2.033)	27/1698 ((1.59, CI: 0.99-2.19)
E. chaffeensis	50/1792% (2.79 (CI:. 2.03-3.55)	57/1718 ((3.33, CI: 2.47-4.17)
B. burgdoferi	29/1791 (1.62 (CI:. 1.03-2.20)	24/1716 ((1.4, CI: 0.84-1.96)
Rickettsia spp.	235/3072 (7.6, CI: 6.71-8.39)	235/2971 (7.9, CI: 6.93-8.88)
Borrelia spp.	72/1688 (4.27, (CI:. 3.3-5.23)	61/1625 ((3.75, CI: 2.83-4.68)
Bartonella spp.	4/1688 (0.24 (CI:. 4.78e-005-0.47)	4/1625 0.25, CI: 4.97e-005-0.49)
Babesia spp.	21/1727 (1.22, CI:. 0.7-1.73)	14/1670 ((0.83, CI: 0.40-1.28)

Table 6: Tick–borne zoonosis pathogens with age categories

	Number positive/Number tested (Percentage, CI :)		
Tick–borne zoonosis pathogens	**< 5 year olds**	**6 - 15 year olds**	**> 16 year olds**
C. burnetii	141/2280 (6.18% CI: 5.19-7.17)	60/941 (6.38 % CI: 4.81-7.94)	70/1020 (6.86 % CI: 5.31-8.42)
A. phagocytophilum	26/1845 (1.41, CI: 0.87-1.948)	13/854 (1.52, CI: 0.7-2.35)	14/765 (1.83, CI: 0.878-2.78)
E. chaffeensis	48/1859 (2.58, CI: 1.86 – 3.30)	48/1487 (3.23, CI: 2.33-4.13)	31/787 (3.94, CI: 2.58-5.30)
B. burgdoferi	20/1857 (1.08, CI: 0.61-1.55)	15/862 (1.74, CI: 0.87-2.62)	18/787 (2.29, CI: 1.24-3.33)
Rickettsia spp.	234/3295 (7.10, CI: 6.22-7.98)	113/1417 (7.98, CI: 6.56-9.39)	123/1337 (9.21, CI: 7.66 - 10.77)
Borrelia spp.	68/1757 (3.87, CI: 2.97-4.77)	37/827 (4.47, CI: 3.06-5.89)	28/728 (3.85, CI: 2.45-5.25)
Bartonella spp.	5/1757 (0.28, CI: 0.035-0.53)	2/827 (0.24, CI: 0.01-0.58)	1/728 (0.14, CI: -0.13-0.41)
Babesia spp.	**26/1790 (1.45, CI: 0.9-2.01)**	**6/854 (0.7, CI: 0.14-1.28)**	**3/762 (0.39, CI: -0.05-0.84)***

Boldface indicates statistical significance. **p* value = < 0.0324

Table 7: Tick–borne zoonosis pathogens in different regions of Kenya**.**

Number positive/Number tested (Percentage, CI :)					
Tick–borne zoonosis pathogens	**Coastal region**	**Kisii highlands region**	**Lake Victoria region**	**Arid and semi-arid region**	**Cosmopolitan Urban region**
C. burnetii	3/184 (7.07 % CI: 3.328-10.80)	57/882 (6.46 % CI: 4.84-8.09)	108/1740 (6.21 % CI: 5.07-7.34)	86/1341 (6.41% (CI: 71.35-8.66)	7/140 (5.00 % CI: 1.35-8.66)
A. phagocytophilum	2/151 (1.33, CI: 0.52-3.17)	4/551(0.73, CI: 0.015-1.44)	24/1602 (1.5, CI: 0.90-2.09)	18/934 (1.93, (CI: -0.52-3.17)	5/160 (3.13, CI: 0.4--5.85)
E. chaffeensis	6/150 (4.0 , CI: 0.83-7.17)	20/551(3.63, CI: 2.06-5.20)	42/1632 (2.57, CI: 1.81-3.34)	40/1165 (3.43, (CI: 2.39-4.48)	3/160 (1.88, CI: -0.25-4.0)
B. burgdoferi	2/151 (1.33, CI: 0.52-3.170	5/551(0.91, CI: 0.11-1.7)	25/1631 (1.53, CI: 0.94-2.13)	19/1164 (1.63, (CI: -0.90-2.36)	5/160 (3.13, CI: 0.4-5.85)
Rickettsia spp	**24/282 (8.5, CI: 5.23-11.79)**	**54/1177 (4.59 , CI: 3.39-5.79)**	**193/2684 (7.26, CI: 6.27-8.25)**	**164/1938 (8.50, CI: 7.25-9.74)**	**39/160 (24.53, CI: 17.77-31.29)***
Borrelia spp	5/135 (3.7, CI: 0.48-6.93)	20/532 (3.76, CI: 2.14-5.38)	68/1526 (4.45, CI: 3.42-5.49)	33/1122 (2.94, (CI: 1.95-3.93)	9/146 (6.16, CI: 2.22-10.11)
Bartonella spp	None detected	2/532 (0.38, CI: -0.15-1)	3/1527 (0.2, CI: -0.02-0.42)	3/1122 (0.27, (CI: -0.04-0.57)	None detected
Babesia spp	**1/140 (0.71, CI: -0.7-2.13)**	**11/540 (2.04, CI: 0.84-3.23)**	**20/1527 (1.31, CI: 0.74-1.88)**	**4/1113 (0.36, (CI: 7.28e-005-0.71)**	**None detected****

Boldface indicates statistical significance. **p* value = < 0.0001, ***p* value = 0.0126

3.2.3 Mixed tick–borne zoonosis infections

Tick–borne pathogens investigated in this study can be transmitted by the same tick species. Out of the 1,017 patients with tick–borne zoonosis infections, 906 (89.1%) had mono-infection with a tick–borne zoonosis pathogen and 99 (9.7%) had an infection with two tick–borne zoonosis pathogens, 11 (1.1%) patients had an infection with three tick–borne zoonosis pathogens while 1 patient (<1%) had an infection with four tick–borne zoonosis pathogens as shown in Table 8. Co-infections with *Rickettsia* spp., *C. burnetii*, *Rickettsia* spp. and *Borrelia* spp which are not *B. burgdoferi* were more common (Table 8).

Table 8: Co-infections of tick–borne zoonosis pathogens detected in clinical samples

Pathogen associations	**Number of** tick–borne zoonosis **infections**
Rickettsia spp. + *C. burnetii*	25
Rickettsia spp. + *B. Burgdoferi*	3
Rickettsia spp.+ *A. phagocytophilum*	4
Rickettsia spp. + *E. chaffeensis*	9
Rickettsia spp. + *Babesia* spp.	6
Rickettsia spp. + *Other Borrelia* spp.	27
Rickettsia spp. + *Bartonella* spp.	1
C. burnetii + *E. chaffeensis*	3
C. burnetii + *B. Burgdoferi*	1
C. burnetii + *A. phagocytophilum*	1
C. burnetii + *Other Borrelia* spp.	2
A. phagocytophilum + *E. chaffeensis*	1
A. phagocytophilum + *other Borrelia* spp.	2
A. phagocytophilum + *B. burgdoferi*	2
E. chaffeensis + *B. burgdoferi*	1
E. chaffeensis + *Other Borrelia* spp.	5
E. chaffeensis + *Babesia* spp.	2
B. burgdoferi + *other Borrelia* spp.	2
Babesia spp. *and other Borrelia* spp.	2
Rickettsia spp. + *Babesia* spp. + *other Borrelia* spp.	2
Rickettsia spp. + *Babesia* spp. + *C. burnetii*	3
Rickettsia spp. + *A. phagocytophilum* + *E. chaffeensis*	1
A. phagocytophilum + *B. burgdoferi* + *Borrelia* spp.	1
Rickettsia spp.+ *C. burnetii* + *A. phagocytophilum*	1
A. phagocytophilum + *C. burnetii* + *E. chaffeensis*	1
Rickettsia spp.+ *C. burnetii* + *B. burgdoferi*	1
E. chaffeensis + *C. burnetii* + *other Borrelia* spp.	1
C. burnetii + *A. phagocytophilum* + *Babesia* spp.+ *Borrelia* spp.	1

3.3 Determination of risk factors for tick–borne infections in acute febrile illness patients in Kenya

To understand which factors are associated with tick–borne infections, this study investigated age, gender, region of residence, contact with animals and a tick bite variables as risk indicator. As shown in Table 5, prevalence of tick–borne infection in males and females were similar showing no significant statistical difference (p=0.7052) when assessed generally or for each individual tick–borne zoonosis pathogen.

In general prevalence of tick–borne zoonosis infections was significantly higher in patients aged >6 to 15 (17.5%, p=0.02) and >15 years (19.2%, p=0.001) compared to patients aged less than 5 years (14.8%) as shown in Table 4. Prevalence of *C. burnetii, A. phagocytophilum, B. burgdoferi, E. chaffeensis, Rickettsia, Borrelia* and *Bartonella infections* shown no significant statistical different in the age categories investigated (Table 6). However prevalence of *Babesia* infections was significantly higher in patients below 5 years old (p = 0.03) compared to patients aged >6 to 15 and >15 years old (Table 6). Urban cosmopolitan region had significantly higher prevalence of tick–borne zoonosis (39.4%, p<0.0001) compared to other regions investigated.

As shown in table 9 and 10, being in contact with sheep was associated with higher odds of a tick–borne zoonosis infection (p =0.033, OR 1.44 CI: 1.03 - 2.02). Generally, contact with cows, donkeys, cats, dogs, goats, camels, pigs, chicken, ducks and geese had no association with tick–borne zoonosis infection status (Table 9).When assessed individually for each tick–borne zoonosis pathogen contact with sheep was associated with higher odds of having *Rickettsia* spp. infection (p<0.0001, OR 2.21CI: 1.47-3.31) whereas being contact with geese was associated with higher odds of *B. burgdoferi* infection (p=0.026, OR 19.18 CI: 1.4 30 - 257.14) and *Babesia* spp. (p=0.027, OR 22.03 CI: 1.415- 343.013). Being in contact with a cat was associated with higher

odds of *Borrelia* spp. infection (p = 0.029, OR 2.4 CI: 1.093 - 5.277) (Table 10). Some patients also reported tick bites, which can potentially lead to a tick–borne zoonosis infection. A tick bite can be indicative of a tick–borne zoonosis infection. Generally patients who reported a tick bite had higher odds of being positive for a tick–borne zoonosis infection (p = 0.008) compared to those not reporting a tick bite (Table 11 and 12).

Table 9: Risk of tick–borne zoonosis infection due to animal contact in acute febrile illness patients

Animal contact	% (number of patients / total number)	Odds ratio	P value	95% Conf. Interval
Goat				
No contact	74.93 (5935/7921)	1		
Contact	25.07 (1986/7921)	0.998	0.986	0.842 - 1.184
Cow				
No contact	66.19 (5243/7921)	1		
Contact	33.81 (2678/7921)	0.883	0.139	0.748 - 1.042
Cat				
No contact	95.83 (7591/7921)	1		
Contact	4.17 (330/7921)	0.9781	0.903	0.684 - 1.398
Sheep				
No contact	96.31(7629/7921)	1		
Contact	**3.69 (292/7921)**	**1.442**	**0.033**	**1.0298 - 2.020ł**
Dog				
No contact	98.7 (7639/7921)	1		
Contact	1.3 (101/7921)	1.139	0.666	0.63 - 2.062
Camel				
No contact	97.21 (7700/7921)	1		
Contact	2.79 (221/7921)	0.913	0.71	0.566 - 1.473
Pig				
No contact	98.61 (7811/7921)	1		
Contact	1.39 (110/7921)	0.716	0.27	0.396 - 1.296
Donkey				
No contact	98.66 (7815/7921)	1		
Contact	1.34 (106/7921)	1.659	0.084	0.935 - 2.944
Geese				
No contact	99.82 (7907/7921)	1		
Contact	0.18 (14/7921)	1.237	0.788	0.2623 - 5.840
Duck				
No contact	98.68 (1769/7921)	1		
Contact	1.92 (152/7921)	0.821	0.465	0.485 - 1.393
Chicken				
No contact	50.98 (4038/7921)	1		
Contact	49.02 (3882/7921)	0.998	0.985	0.855 - 1.166

Table 10: Animal contact with significantly increased risk of tick–borne zoonosis infection

TBZ	Animal contact	Odds Ratio	P value	95% Conf. Interval
Rickettsia spp.	Sheep	2.21	<0.001	1.474 - 3.31
B. burgdoferi	Geese	19.18	0.026	1.4 30 - 257.143
Babesia spp.	Geese	22.033	0.027	1.415- 343.013
Borrelia spp.	Cat	2.4	0.029	1.093 - 5.277

3.4 Signs and symptoms associated with tick–borne zoonosis infection

As shown in Table 11, in addition to having fever, clinical signs and symptoms that the AFI patients with or without tick–borne infections reported included muscle aches, joint aches, headache, runny nose, cough, chills, sore throat, difficulty in breathing abdominal pains, vomiting, blood in stool, rash, bleeding, painful eyes, and seizures. Some patients also reported tick bites, which can potentially lead to a tick–borne zoonosis infection. This study sought to determine which signs and symptoms are likely indicators of tick–borne zoonosis infection. Of the signs and symptoms accessed headache, cough, chills, runny nose, joint aches, muscle aches, abdominal pains, sore throat, rash, and difficulty in breathing did not show association with tick–borne zoonosis infections (Table 11). As shown in Table 11 and 12, patients who reported a tick bite (OR 1.24, p=0.008 CI: 1.06-1.45) and blood in stool (OR 1.31, p=0.034 CI: 1.02-1.68) had higher odds of having a tick–borne zoonosis infection. When signs and symptoms were considered for each individual tick–borne zoonosis tested, patients who reported blood in stool were associated with higher odds ratio of *Borrelia* spp. infection (OR 1.8, p=0.036 CI: 1.04-3.03) and *Rickettsia* spp. (OR 1.7, p<0.0001 CI: 1.27-2.23) infections. Patients with *Babesia* spp. infections were associated with higher odds of vomiting (OR 2.2, p<0.033 CI: 1.06-4.48) and bruising (OR 1.57, p<0.021 CI: 1.57-230.37). Patients with *Bartonella* spp. infections were associated with higher odds of seizures (OR 10.27, p<0.033 CI: 1.21-86.98) and bleeding (OR 27.89, p<0.006 CI: 2.58-301.53). Patients who reported bleeding were also associated with *B. burgdoferi* infection. In addition to reporting blood in stool patients with *Rickettsia* spp. infections had significantly higher odds ratio for a tick bite (OR 1.3, p<0.022 CI: 1.04-1.59) and painful eyes (OR 1.4, p<0.002 CI: 1.13-1.70).

Table 11: Signs and symptoms associated with tick–borne zoonosis infections

Signs and symptoms in tick–borne zoonosis positive infections				
Clinical characteristics	**% (positive with sign/Total positive)**	**OR**	**P value**	**CI:**
Chills	61.29(581/948)	1.01	0.887	0.89-1.15
Cough	49.24 (489/993)	1.04	0.631	0.89-1.23
Difficulty_breathing	5.96(59/990)	1.17	0.258	0.90-1.54
Sore_throat	17.06 (151/885)	0.10	0.111	0.79-1.03
Headache	66.67(564/846)	0.83	0.011	0.73-0.96
Runny_nose	41.51 (411/990)	0.885297	0.132	0.76-1.04
Painful eyes	12.11(116/958)	1.12	0.167	0.95-1.31
Seizures	2.44(23/944)	0.98	0.871	0.80-1.21
Tick_bites	**2.59(23/889)**	**1.24**	**0.008**	**1.06-1.45**
Abdominal_pain	44.38 (411/926)	1.01	0.839	0.89-1.15
Vomiting	32 (319/997)	0.92	0.262	0.79-1.07
Diarrhoea	14.16(141/996)	0.91	0.384	0.74-1.12
Blood_in_stool	**2.37 (23/972)**	**1.31**	**0.034**	**1.02-1.68**
Bleeding	1.81 (18/993)	1.57	0.054	0.998-2.49
Bruising	0.20 (2/992)	0.81	0.515	0.429-1.55
Rash	3.47(34/980)	0.96	0.791	0.74-1.26
Joint_aches	45.9(375/817)	1.12	0.15	0.96-1.31
Muscle_aches	35.71(286/801)	0.99	0.92	0.86-1.15
Dark_urine	1.21 (11/907)	0.74	0.01	0.59-0.93
Jaundice	0.77 (7/907)	1.10	0.436	0.86-1.41
Boldface indicates statistically significant association				

Table 12: Signs and symptoms with significant association with tick–borne zoonosis infections in acute febrile illness patients

Pathogen	Clinical signs	% (positive with sign/Total positive)	Odds Ratio	P value	95% Conf. Interval
	Tick bites	2.59(23/889)	1.24	0.008	1.06-1.45
TBZ	Blood_in_stool	2.37 (23/972)	1.31	0.034	1.02-1.68
	Vomiting	54.29(19/35)	2.2	0.033	1.06-4.48
Babesia spp.	Bruising	2.86(1/35)	19	0.021	1.57-230.37
	Seizures	12.50 (1/8)	10.27	0.033	1.21-86.98
Bartonella spp.	Bleeding	12.50 (1/8)	27.89	0.006	2.58-301.53
Borrelia spp.	Blood_in_stool	2.36 (3/127)	1.8	0.036	1.04-3.03
B. burgdoferi	Bleeding	7.55 (4/53)	7.6	0.001	2.34-24.85
E. chaffeensis	Chills	76.92 (80/104)	1.5	0.047	1.00 -2.19
	Blood_in_stool	2.64(12/454)	1.7	0	1.27-2.32
Rickettsia	Tick bites	3.10 (13/419)	1.3	0.022	1.04-1.59
spp.	Eye pain	15.83 (64/450)	1.4	0.002	1.13-1.70

3.5 Bacterial community in ticks collected from livestock in Kenya using 16s rRNA metagenomics

3.5.1 16s rRNA metagenomics sequencing result

Of the 463 tick pools used for sequencing library prepared, 400 pool samples had their paired end sequences assembled to yield a total of 97,993,917 sequence contigs, with the sample with the least number of sequences having 237, while that with the most sequences having 4,252,150 sequence contigs. The median sequence count was 196,400 sequences per sample. After quality filtering steps described above 41,500,930 unique sequences ranging from 2 to 2,240,157 with a median of 70,929 sequences per pool remained which were used for picking operational taxonomic units (OTUs) and taxonomic assignment. Of the 400 tick pools that yielded contigs, 40 pools with small library size and poor quality sequences were dropped from further analysis. Sequences were rarefied to 1,210 sequences which was the smallest number of sequences in a pool to overcome

effects of PCR bias and unequal sequencing between samples. Using prevalence method in the "decontan" command within R package 6 OTUS were identified as contaminants and removed from the tick dataset. 40 tick pools with library size of <1000 sequences were dropped from downstream analysis and a total of 360 tick pools remained with 36, 973,934 sequences.

3.5.2 Bacterial community composition across tick genera

A total of 645 unique OTUs classified to genus level were identified across tick pools representing over 90% (36,973,934 sequences) of the sequence reads obtained after quality filtering. The bacteria genera grouped into 27 phyla. As shown in Figure 7, Proteobacteria contributed the majority 61.2 % of the sequences that tarried to 33.8% OTUs, 15.9% for Firmicutes (23.4% OTUs), and 15.6% for Actinobacteria (20% OTUs), 4.7% for Bacteroidetes (11.6% OTUs). The remaining 22 phyla included 0.7% for Fusobacteria, 0.5% for TM7 (Saccharibacteria) 0.3% for Verrucomicrobia 0.2% for Acidobacteria, 0.2% for Deinococcus-Thermus, 0.2% for Planctomycetes, 0.1% for Chloroflexi, OD1 (Parcubacteria), Tenericutes and Gemmatimonadetes, 0.04% for Armatimonadetes, 0.03% for Spirochaetes, 0.02% for Aquificae, 0.01% for SR1 (Absconditabacteria), Lentisphaerae and BRC1, 0.004% for Chlamydiae and Nitrospira, 0.002% for Chlorobi, Synergistetes, Fibrobacteres, WS3 and Elusimicrobia each constituting 11.2% OTUs (Figure 8).

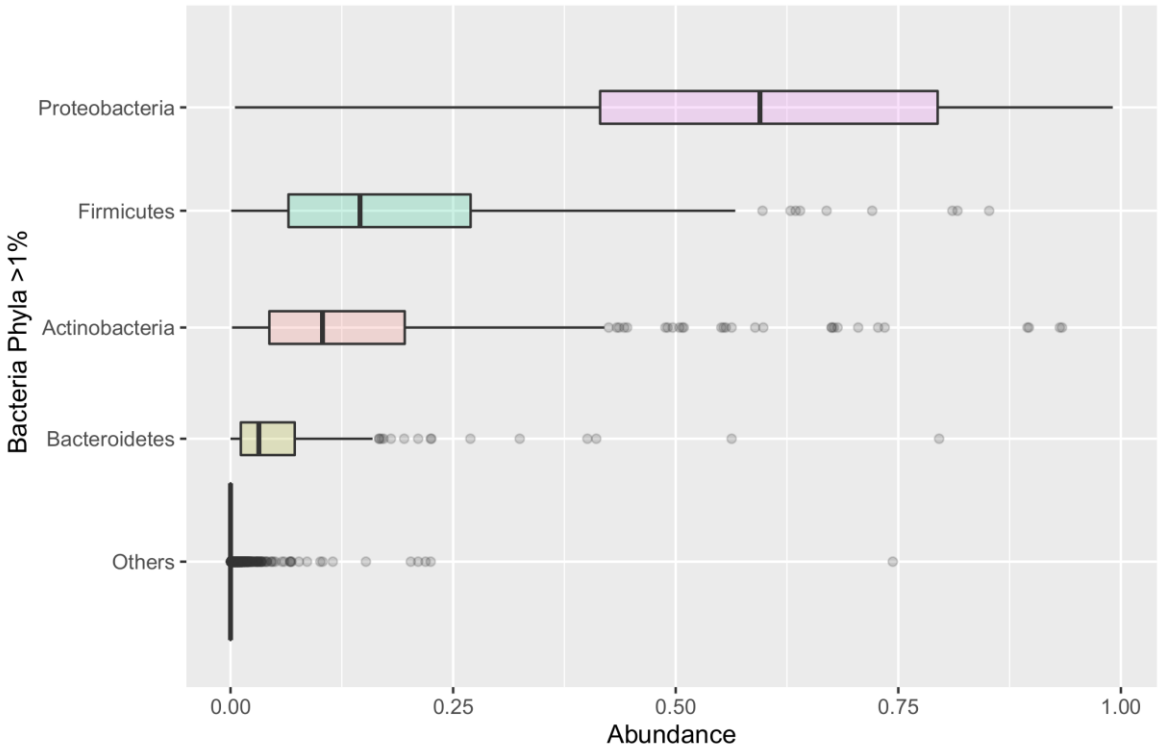


Figure 7: Box plots of bacterial phylum with greater than 0.1 relative abundance. X axis shows relative abundance while the y axis shows bacteria phylum.

Of the 645 genera, the dominant genera was C*oxiella* contributing 41.8% (15,445,204 out of 36,973,934), *Corynebacterium* 13.6%, *Acinetobacter* 4.3%, *Staphylococcus* 3.9%, *Bacillus* 2.7%, *Porphyromonas* 1.6%, *Ralstonia* 1.5%, *Streptococcus* 1.3%, *Moraxella* 1.3%, *Cloacibacterium* 1.3%, *Neisseria* 1.2%, *Escherichia_Shigella* 1.2%, *Proteus* 1.0%, and *Aerococcus, Alloiococcus, Stenotrophomonas* 1% each of the sequences (Figure 8). Other bacterial genera known to carry potentially medical relevant species were also detected in ticks although in relative abundances of less than 0.1. These genera included *Burkhoderia* (0.4%), *Klebsiella* (0.3%), *Escherichia-Shegella* (0.3%), *Achromobacter* (0.2%), *Rickettsia (*0.1%), *Haemophilus* (0.1%), *Legionella* (0.1%), *Campylobacter* (0.04%), *Treponema* (0.03%), *Francisella* (0.02), *Anaplasma* (0.01), *Elizabethkingia* (0.006%), *Mycoplasma* (0.006%), *Ehrlichia* (0.005%), *Bordetella* (0.004%), *Vibrio* (0.002%), *Borrelia* (0.0008%) *and Brucella* (0.0002%). (Figure 8 and Table 13)

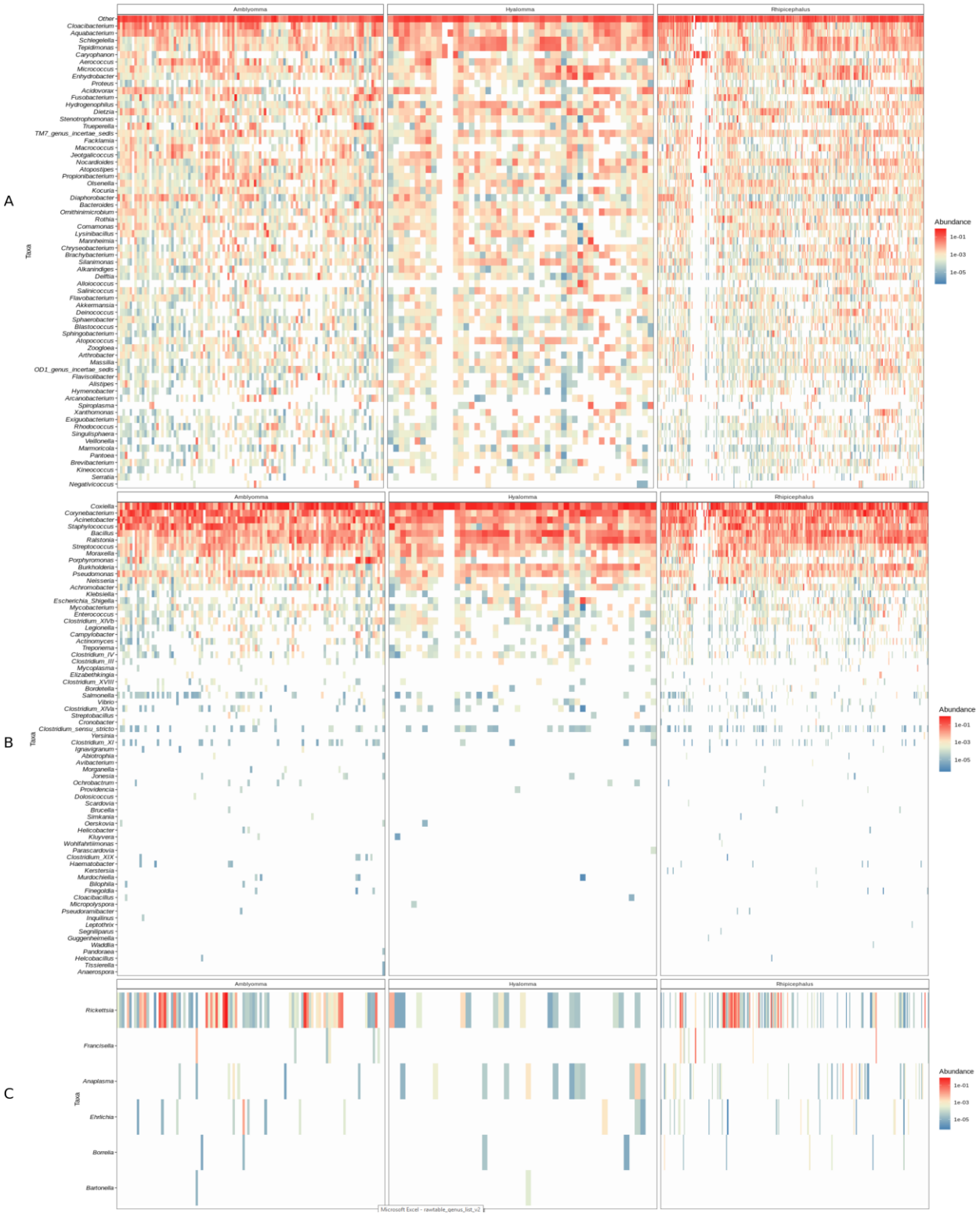


Figure 8: Heat map showing the three groups of bacteria that were identified in different tick genera. Panel A shows taxa that are probably commensals/symbiotic or from the environment appearing at abundance >1%). The commensals/symbionts group dominated, contributing 93.6%

of the OTUs, but their individual sequence contribution was very low. Group two comprised genera that are known to have pathogenic species (Panel B): *Coxiella* dominated in this group at 41.8%, followed by *Corynebacterium* (13.6%), *Acinetobacter* (4.3%), *Staphylococcus* (3.9%), *Bacillus* (2.7%) and *Porphyromonas* (1.6%), *Ralstonia* (1.5%), *Streptococcus* (1.3%), *Moraxella* (1.3%), amongst others. Group three comprised tick borne zoonotic pathogens (Panel C): *Rickettsiae*, *Anaplasma*, *Francisella*, *Ehrlichia*, *Bartonella* and *Borrelia* and individually contributed <1% of the sequences. The X-axis represents the samples from the different tick genus. The Y-axis represents microbial taxa at the genus level, ordered by hierarchical clustering. Red color indicates a greater number of reads of that bacterial genus and dark blue color indicate less reads. White boxes indicate samples without sequence reads

Table 13: Bacteria taxa with potentially tick-borne pathogens species identified in ticks

Potentially Pathogenic		**Known tick-borne zoonotic**	
Bacterial taxa	**Percentage abundance**	**Bacterial taxa**	**Percentage abundance**
Coxiella	41.77332	*Rickettsia*	0.11211
Corynebacterium	13.62813	*Anaplasma*	0.00666
Acinetobacter	4.31421	*Francisella*	0.00533
Staphylococcus	3.88176	*Ehrlichia*	0.00294
Bacillus	2.70122	*Borrelia*	0.00144
Porphyromonas	1.61327	*Bartonella*	0.00002
Ralstonia	1.48149		
Streptococcus	1.33425		
Moraxella	1.29198		
Neisseria	1.23056		
Escherichia_Shigella	1.16413		
Pseudomonas	0.44633		
Burkholderia	0.3833		
Klebsiella	0.32713		
Enterococcus	0.14419		
Campylobacter	0.07846		
Achromobacter	0.05374		
Mycobacterium	0.05267		
Clostridium_XlVb	0.05216		
Actinomyces	0.035		
Legionella	0.0252		
Treponema	0.0194		
Clostridium_IV	0.01561		
Clostridium_III	0.00415		
Clostridium_XVIII	0.00292		
Vibrio	0.00268		
Bordetella	0.00262		
Mycoplasma	0.00208		
Clostridium_XlVa	0.0018		
Elizabethkingia	0.00169		
Salmonella	0.00148		
Streptobacillus	0.00106		
Cronobacter	0.00096		
Clostridium_sensu_stricto	0.00079		
Clostridium_XI	0.00021		
Yersinia	0.00008		
Brucella	0.00002		

3.5.3 Diversity of tick bacterial community

A total of 37,344,683 sequences from 360 samples were rarefied to 1220 reads per samples resulting to 381, 726 sequences that were used to determine the Shannon diversity index. Rarefaction curves for the samples approached saturation at 1,210 reads. As, shown in Figure 9, the Shannon diversity index of bacteria detected among the tick species ranges from 2.69 to 4.15 suggestive of low diversity. *A. variegatum* carried less diverse bacteria (mean Shannon diversity index of 2.69 ± 0.92) compared to 3.79 ± 1.10 for *Amblyomma gemma*, 3.71 ± 1.32 for *A. hebraeum*, 4.15 ± 1.08 other *Amblyomma* spp, 3.79 ± 1.37 for *Hyalomma truncatum*, 3.67 ± 1.38 for other *Hyalomma* spp, 3.86 ± 1.27 for *Rhipicephalus annulatus*, 3.56 ± 1.21 for *Rh. appendiculatus*, 3.65 ± 1.30 for *Rh. Pulchellus*, but the difference was not significant (p=0.443)

The dominant phyla were present in all tick species with Proteobacteria, Firmicutes and Actinobacteria present in all 360 samples (Figure 10). Most abundant genera were identified across all tick genera and in most of the samples. *Coxiella* genus was identified in 96.9% (349 out of 360) samples, followed by *Corynebacterium* present in 96.3% (347 out of 360), *Acinetobacter* 95.6% (344 out of 360), *Staphylococcus* 89.4% (322 out of 360) and *Bacillus* 96.9% (349 out of 360).

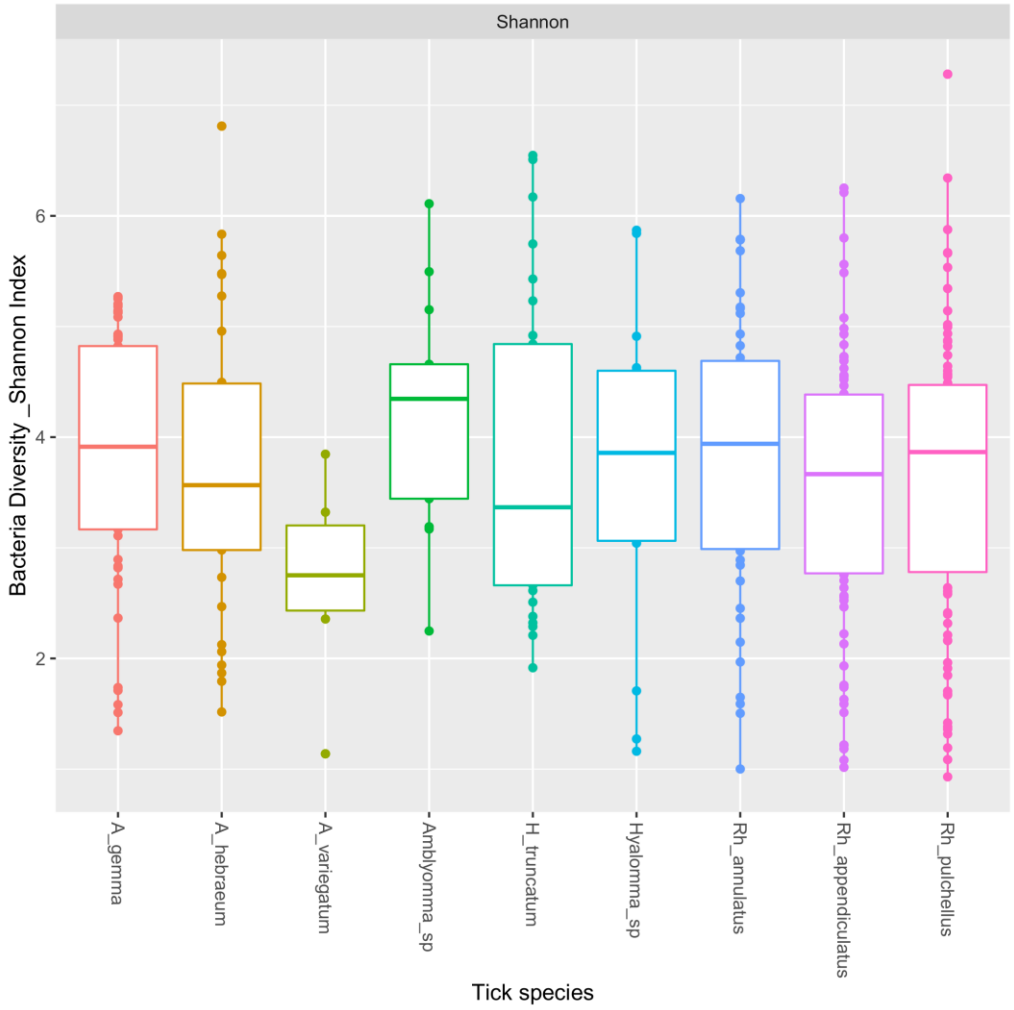


Figure 9: Boxplot showing median bacteria diversity in tick species measured by Shannon diversity index. *A. variegatum* carried less diverse bacteria (mean Shannon diversity index (2.69 ± 0.92 standard deviation) compared to the other tick species, but the difference was not significant (p=0.443).

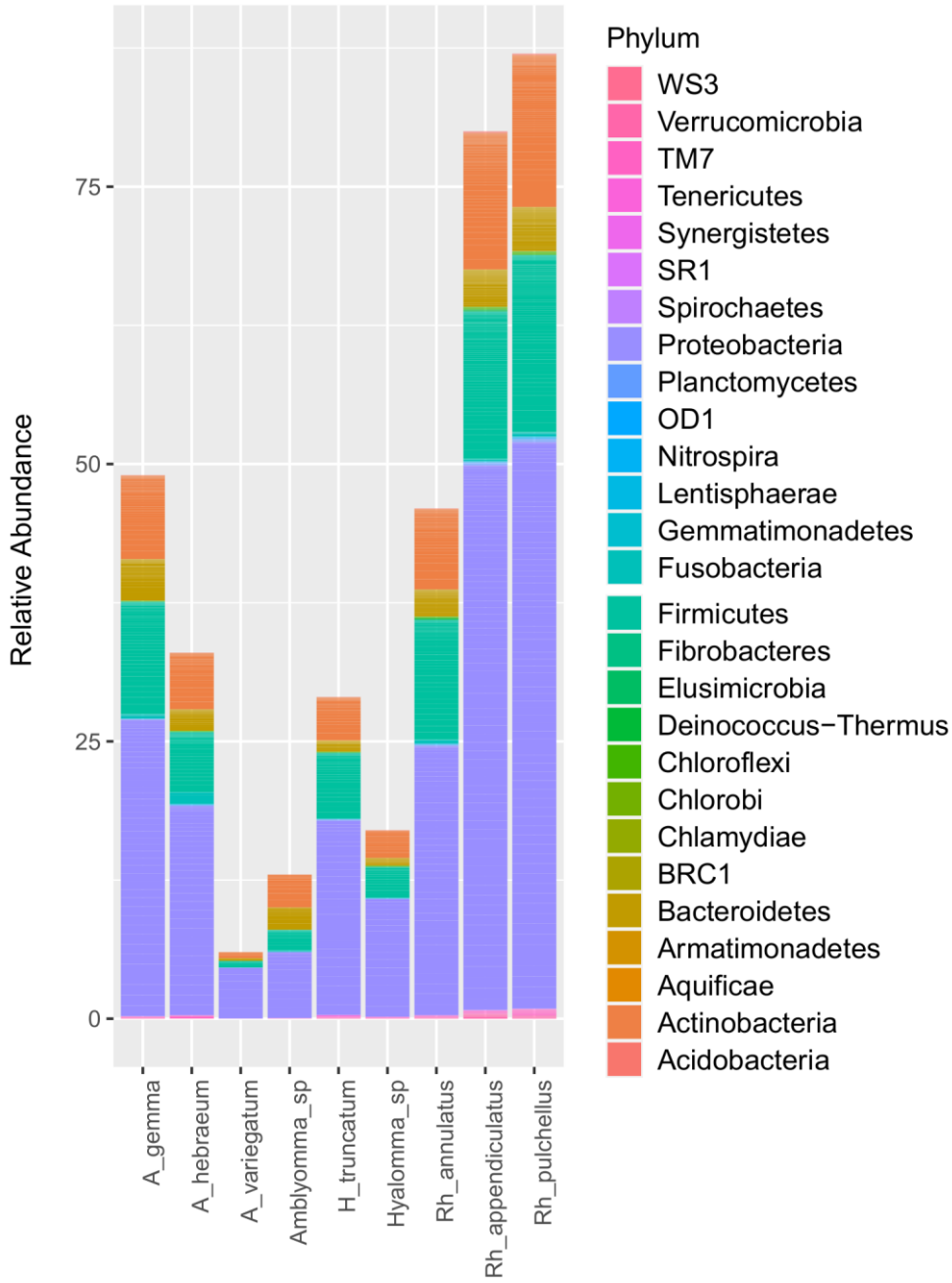


Figure 10: Bar plot showing bacteria phyla in tick species based on relative abundance of sequences. Most abundant bacteria phyla including Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes were shared across tick species.

4 CHAPTER 4: Discussion

Ticks transmit a wide diversity of pathogens to humans and animals compared to other vectors which has led tick–borne zoonosis to form a large proportion of emerging infections (Fritz, 2009; Parola and Raoult, 2001b, 2001a). Tick–borne zoonosis can therefore emerge in previously non-endemic areas where factors favorable for their maintenance and transmission occur. Tick–borne zoonosis have also been report as a common cause of febrile illness globally (Cutler et al., 2017; Kulkarni et al., 2015). In the developing tropical and sub-tropical countries acute febrile illness is a common cause of hospital admission and death (Zerfu et al., 2018). In Kenya acute febrile illness is a common presentation in health care facilities although it is commonly attributed to malaria infections (Nyaoke et al., 2019; Whitty et al., 2008). In places where malaria was the main cause of fever improvement of malaria diagnosis and other control efforts have shown that most fevers are non-malaria (Bhatt et al., 2015; Maze et al., 2018). Making definitive diagnosis that can lead to appropriate treatment in acute febrile illness patients remains a challenge in many health care facilities in the tropics and sub-tropical regions (WHO, 2011). This challenge is due to the non-specific presentation of a broad variety of conditions causing acute febrile illness and unavailability of diagnostic tests (Maze et al., 2018). Therefore understanding the epidemiology of causes of fever has important implications for management of febrile patients.

This study aimed to ascertain the presence of tick–borne zoonosis pathogens, their importance as etiologies of acute febrile illness and the risk factors of tick–borne zoonosis infections in Kenya using molecular methods. Two multiplex real time PCR assays were optimized to test a panel of 8 tick–borne zoonosis pathogens. In total 6,207 undifferentiated febrile illness patients from different geographical regions of Kenya were used for this study. Male (16.72%) and female (16.22%) patients had similar presentation to the study. The patients ranged 1 year to 80 years of age with a

median of 5 years. Data generated in this study demonstrate presence of tick–borne zoonosis pathogens that are potential etiologies of undifferentiated acute febrile illness in Kenya.

Lack of multi pathogen diagnostics have been cited as a major limitation to surveillance efforts in Africa that has led to poor epidemiological information and a narrow list of differential diagnosis of infectious pathogens (Prasad et al., 2015). As has been reported earlier, assay multiplexing simplifies workflow, is amenable to high throughput, conserves specimen volumes, reduces reagent costs and person time (Wang et al., 2014; Yu et al., 2012). In the present study, multiplex real time PCR assays were designed for 8 tick–borne zoonosis pathogens and thereafter used to evaluate their presence in clinical samples obtained from febrile illness patients. Real time PCR assays have gained popularity as a tool for detection of disease causing pathogens in clinical patient samples (Espy et al., 2006). These assays are limited by the quality of primers and probes used to target specific pathogens. The target primer and probe sequences must be unique in order to identify a specific organism and must be able to identify the organism with high efficiency and specificity in the specimen of interest.(Espy et al., 2006; Lemmon and Gardner, 2008). Homology search of databases such as National Center for Biotechnology Information (NCBI) can reveal cross-reactivity (Espy et al., 2006; Lemmon and Gardner, 2008). Primers and probes used in this study showed no cross reactivity by homology search of NCBI database. In addition, primer and probes were selected from published assays that had tested them against related organisms (Table 1).

Real time PCR instruments have limited number of signal acquisition channels, which limits the number of targets that can be multiplexed in an assay. Therefore, for the 8 pathogens, the 5 channel 7500 Fast Real time PCR system (Applied Biosystems. Foster city CA USA) could only accommodate detection of 4 targets at a time because one channel is assigned a reference dye to

allow normalization of fluorescence. The two 4-plex assays performed as well as the single-plex assays qualifying them for simultaneous detection of four pathogens each in a sample. As shown in Figure 5, Bland – Altman pairwise comparison of the 2 assay formats shown small differences in threshold cycles.

Many of the tick-borne bacteria targeted by the two 4-plex assays do not generate high bacteremia and have fastidious growth requirement in culture, thus making convectional diagnostic methods less sensitive and time consuming (Fenollar and Raoult, 2007). An assay that can detect low copies of bacterial DNA in samples during early stage of the infections is critical for early diagnosis and targeted treatment (Nilsson et al., 2010). The two 4-plex real time PCR assays have limit of detection of 1.80 gene copies for assay one and 18.45 gene copies for assay two. The copy number of the target gene in each of the tick–borne zoonosis tested was used to determine equivalent number of pathogens detectable by these assays. The optimized assays were robust with high linearity and efficiency and they can be used for detection of the 8 common tick–borne zoonosis pathogens.

The overall prevalence of tick–borne zoonosis infections among the evaluated undifferentiated febrile illness patients was 16.38%. Earlier studies on tick-borne zoonosis in febrile illness patients have focused on specific pathogens using serological methods but the present study tested a panel of 8 tick-borne zoonosis pathogens using molecular methods (Heinrich et al., 2015; Mediannikov et al., 2010a; Thiga et al., 2015). As shown in Table 5, 6 and 7, prevalence of infections with *Rickettsia* spp. (7.8%) and that of *Coxiella burnetii* (6%), the causative agents of spotted fever and Q fever, respectively were more common than other tick-borne zoonosis pathogens. Prevalence of *Rickettsia* spp and *Coxiella burnetii* are similar to earlier reports of high sero-prevalence to spotted fever *rickettsia* (10%) and *Coxiella burnetii* (11%) among acute febrile illness patients in Kenya

(Lemtudo et al., 2021; Thiga et al., 2015). *Borrelia* spp. the causative agent of tick-borne relapsing fever (TBRF) was present in 4% of the patients tested. *E. chaffeensis* the causative agent of human monocytic ehrlichiosis was present in 4% of the patients tested. *B. burgdoferi* the causative agent of Lyme disease and *A. phagocytophilum* the causative agent of human granulocytic anaplasmosis were present in 1.5% of patients tested. *Babesia* spp. the causative agent of human babesiosis and *Bartonella* spp. the causative agent agents of bartonelosis were present in 1% and 0.2% of patients tested, respectively. Higher prevalence of *Rickettsia* spp. and *Coxiella burnetii* could be due to similar high prevalence in animals and ticks which are reservoirs and vectors in Kenya and therefore increasing risk of human infections for people living in close proximity with the animals. (Koka et al., 2017; Maina et al., 2014; Muema et al., 2017; Mutai et al., 2013). This is enhanced by frequent humans, domestic animals and wildlife direct and indirect contact in the country (Leeuw et al., 1991; Ottichilo et al., 2000). Humans living in close proximity with animals increases their chances of getting a tick bite that also bite animals leading to increased risk of tick–borne zoonosis infections (Fischhoff et al., 2019). The prevalence of *Borrelia* spp. The agent that causes tick-borne relapsing fever was 4% which is similar to that reported among febrile children in Tanzania where tick-borne relapsing fever is endemic (Kisinza et al., 2003). This is supported by a report of house infestation with *Ornithodorus* spp. ticks which are vectors of tick-borne relapsing fever in Kenya and other East Africa countries (Walton, 1962). Although no recent cases of tick-borne relapsing fever have been reported in Kenya, tick-borne relapsing fever was reported in Meru County of Kenya six decades ago with case fatality rate of 8.1% (Bell, 1953). The present study shows there potential transmission of tick-borne relapsing fever with can lead to human infections in Kenya. There were two reported cases of Lyme disease caused by *B. burgdoferi* in Kenya after which no other reports have reported (Jowi and Gathua, 2005). The present study has

reported *B. burgdoferi* infections in Kenya with 1.5% prevalence. This finding is supported by recent report on high sero–prevalence (21%) of antibodies against Lyme disease caused by *B. burgdoferi* in acute febrile illness patients in Kenya (Nyataya et al., 2020). These findings confirm possible transmission of *B. burgdoferi* in Kenya.

No human clinical case associated with *A. phagocytophilum* has been published in Africa although it has a worldwide distribution and high sero-prevalence in animals have been reported in Kenya and Morocco (Njiiri et al., 2015; Teshale et al., 2018; Khatat et al., 2016). This present study, a prevalence of 1.5% is reported which, although low, is an indication of on-going *A. phagocytophilum* transmission in Kenya. This study has reported 3.1% prevalence of *E. chaffeensis*. This is the first report of human infections with *E. chaffeensis* in Kenya. Reports of *Ehrlichia* spp. infections in ticks and animals in Kenya exist underlining the risk of this tick–borne zoonosis in the country (Omondi et al., 2017; Oswe et al., 2018). Low (0.2%) prevalence of *Bartonella* spp. and *Babesia* spp. (1%) were observed showing low risk of infection to these two tick–borne zoonosis pathogens. This report is contrary to recent report of high prevalence of Bartonella species in rodent and other animals respectively in Kenya (Halliday et al., 2015; Liyai et al., 2021; Maamun et al., 2011; Omondi et al., 2017; Oswe et al., 2018).

Although low tick–borne zoonosis prevalence was observed, the detection of the pathogen DNA suggests that these pathogens could be present and associated with febrile illness. Besides ability of the multiplex real time PCR method to detect low copies of targeted pathogens, the timing of the sample collection after tick bites and start of an antibiotic treatment strongly affects the ability of pathogen detection (Jahfari et al., 2016).

Although many of tick–borne zoonosis pathogens can be transmitted by the same tick species and infection with multiple pathogens can alter clinical sequelae (Swanson et al., 2006), risk of co-

infections with tick-borne zoonosis pathogens is dependent on the prevalence of the pathogens in the tick vectors and/or antagonistic effect of one bacterium on to another when they co-exist in a tick vector. This effect can inhibit transmission of a second bacteria agent after a tick bite leading to frequent infections with single pathogens (Swanson et al., 2006; Levin and Fish, 2001). Majority of tick–borne zoonosis infections were mono-infections (84.3%) and declined progressively to 13.7% for dual infections, and <1% for three or four pathogen infections (Table 8). Common co-infections in this study were dual infections with *C. burnetii* and *Rickettsia* and *Rickettsia* with *Borrelia* (Table 8). This corroborates high prevalence of *Rickettsia* and *C. burnetii* in animals and ticks in Kenya (Koka et al., 2018, 2017; Mutai et al., 2013).

Unlike human diseases that are transmitted from person to person and those caused by genetic anomalies vector–borne diseases can be predicted from factors that impact presence of vectors and reservoirs carrying pathogens. Human behavior that impact the likelihood of being infected with these pathogens can also be used as predictive tool for getting vector-borne infections (Hofhuis et al., 2017). Risk of exposure to tick-borne infections can be determined by presence of tick and reservoirs infected with pathogens which is determined by climate and land use of a region. Human behavior and demographic characteristic determines whether a person will be exposed to a tick-borne infection given entry into tick habitat or being in contact with animals infested by ticks (Fischhoff et al., 2019; Mahachi et al., 2020). The present study established patient age, gender, region of origin, contact with animals and a tick bite as potential risk factors for tick-borne zoonosis infection in Kenya.

Risk factors for tick-borne zoonosis infections in this study that shown significant statistical association with tick-borne zoonosis infections were age, being in contact with sheep and geographical region of sample collection. Tick-borne zoonosis infections increased with increase

in age where patients older than 16 years had the highest percentage of tick-borne zoonosis infections followed by patients of 6 to 15 years old compared to patients who were less than 5 years of age (Table 4 and 6). This finding is similar to earlier report of higher sero-positivity against Rickettsia and Q-fever in older people in Kenya (Thiga et al., 2015; Njeru et al., 2016). These findings suggest that human behavior and activities they are involved could be risk factors of tick-borne zoonosis infections where young adults and older adults are likely to interact with animals leading to prolonged exposure to tick vectors more than children below 5 years old. These age categories are likely to be involved in livestock herding in the country an activity that increases their risk of encountering a tick bite (Roberts, 1996). Being in contact with goat, cow, dog, camel, pig, donkey duck and chicken shown no association with testing positive for a tick-borne zoonosis pathogen. As shown in Table 9, being in contact with sheep was associated with higher odds of a tick-borne zoonosis infection compared to patients with no contact with sheep. For example when specific tick-borne zoonosis pathogens were considered, contact with sheep compared to having no contact showed higher odds of being positive for *Rickettsia* spp. A study on ticks collected from domestic animals in Kenya showed up to 15 % prevalence of *Rickettsia* in tick collected from sheep suggesting that sheep act as reservoir for this pathogen (Mutai et al., 2013). This finding further corroborates an earlier report of 7.9% prevalence of *Rickettsia* infections in sheep from Tunisia (Belkahia et al., 2021). Contact with geese compared to having no contact showed higher odds of infections with *B. burgdoferi* and *Babesia* spp. while contact with cat showed higher odds of infections with *Borrelia* spp. compared to patients with no contact with a cat. It is noteworthy that *Babesia* spp. and *B. burgdoferi* share tick vectors thus contact with geese resulted in higher odd of infections with the two pathogens (Knapp and Rice, 2015; Anderson et al., 1986). Geese have been reported to be vertebrate reservoirs of *Borrelia* and *Babesia* spp and harbors ticks that

can transmit multiple pathogens including *A. phagocytophilum*, *Babesia* and *Ehrlichia* species (Jaenson and Wilhelmsson, 2019; Werther et al., 2017). Contact with cat showed higher odds of infections with *Borrelia* spp. compared to having no contact with a cat, an indication that cats harbor tick vectors of *Borrelia* spp. as earlier reported (Lappin, 2018). This finding is supported by earlier reports of *Borrelia* species the causative agents of tick-borne relapsing fever in cats (Baneth et al., 2016). Cats spend substantial time outdoors as well as sharing shelter with human situations that are likely to increase their exposure to ticks and the pathogens they carry which can be transmitted to human therein (Duplaix et al., 2021).

This study has reported higher tick-borne zoonosis pathogen infections in the urban cosmopolitan area of Eastleigh, Nairobi County compared to the Lake Victoria, Kisii highlands, semi-arid and coastal regions of Kenya. This is similar to an earlier report of high prevalence of *Bartonella* spp. in small mammals sampled from Kibera an urban informal settlement in Nairobi, Kenya (Halliday et al., 2015). These finding can be attributed to uncontrolled urban setting that promote environmental degradation and decreased biological diversity which can led to blooming of small mammals including rodents, shrews, dogs and cats that can serve as tick-borne zoonosis pathogen reservoirs (Costa et al., 2017). As a consequence urban settings has increased risk of human exposure to rodent-borne tick vectors and tick borne pathogens (Oechslin et al., 2017).

Signs and symptoms reported by patients with tick-borne infections are non-specific with common reports of fever, chills, aches, pains and rashes vomiting (Krause et al., 2002). Therefore definitive clinical diagnosis of tick-borne infections can be challenging resulting in underreporting of these pathogens. In the present study, all patients had fever greater than 38 ºC. As shown in Table 11, other signs and symptoms reported and assessed were non-specific showing no significant statistical association with tick-borne infections. This finding indicates difficulties on clinical

diagnosis of tick-borne zoonosis infections in Kenya as reported elsewhere (Biggs et al., 2016). As shown in Table 11 and 12, a tick bite was associated with higher odds of tick-borne zoonosis infections, which can be used as a guide for differential diagnosis of tick-borne pathogen infections. This is supported by the fact that tick-borne zoonosis pathogens are transmitted to humans through a tick bite (Eisen, 2018). Ixodid ticks which are vectors of most tick-borne pathogens have attributes that enhance their vector capability such as feeding and attaching to the host for prolonged period of time (Parola and Raoult, 2001a). Their bite is usually painless and can go undetected for long period of time increasing the risk of tick-borne zoonosis pathogen transmission to the host (Parola and Raoult, 2001a). However some of these pathogens such as *Coxiella burnetii* can also be transmitted to humans through contaminated water, soil and animals products (Aslam et al., 2015; Dworkin et al., 2008). However it is worthy to note some of tick-borne pathogens can also be transmitted to humans and animals by other vectors such as fleas, mites, and lice (Oguntomole et al., 2018).

Presence of blood in stool was associated with higher odds of tick-borne zoonosis infections (Table 11). This is similar to earlier reports of massive hemorrhage in the gastrointestinal tract in patients with Rickettsial disease (Middleton, 1978). Tick-borne zoonosis infection can be suspected when a patient reports blood in stool which can guide differential clinical diagnosis. Epileptic seizures, among other neurological disorders, have been reported in patients with *Bartonella* infections (Puligheddu et al., 2004; Stek et al., 2011). In this study having seizure was associated with higher odds of *Bartonella* spp infection suggesting undifferentiated acute febrile illness patients with seizure can be suspected of having *Bartonella* infection. Differential clinical diagnosis of tick-borne zoonosis therefore is difficult especially in resource-limited regions where there is need for enhancement of laboratory capabilities (Prasad et al., 2015).

The present study presents a survey of bacterial communities associated with *Rhipicephalus* spp., *Amblyomma* spp. and *Hyalomma* spp. using 16s rRNA deep sequencing. The ticks were collected from domestic animals (cows, goats and sheep) at major slaughter houses in Nairobi and Mombasa. Ticks were collected from domestic animals presented for slaughter at the slaughterhouses between November, 2007 and September, 2008 as shown in Figure 3. These slaughterhouses receive animals from nearly all counties in the country. Animal movement permits issued by the local veterinarian from the animal's area of origin were used to determine the animals' county of origin. This study assumed ticks collected from the animals were due to tick infestation at place of origin. However, the animals could get infested with ticks on transit or at the slaughterhouse waiting yards limiting this study ability to report geographical distribution of bacteria detected in the ticks. Ticks were identified taxonomically using the standard taxonomic key (Estrada-peña et al., 2003). Ticks of the same species and from the same domestic animal were pooled together to form the tick pools.

Ticks belonging to the three genera *Rhipicephalus, Amblyomma,* and *Hyalomma* have been reported to have affinity for humans and are vectors of multiple pathogens that cause diseases in human and animals that can pose serious threat to human and veterinary health (Parola and Raoult, 2001a). In addition to pathogens, tick microbiota is composed of other microorganisms which are non-pathogenic such as commensals and mutualistic and have been reported to affect tick development and their capability for pathogen transmission (Bonnet et al., 2017; Couper and Swei, 2018a; Narasimhan and Fikrig, 2015). Also the list of known and potential tick-borne pathogens is constantly increasing and includes viruses, protozoan and bacteria affecting human and animals globally (Bonnet et al., 2017). Therefore are broad pathogen detection that detect both known and

novel pathogens is very desirable for identification of all microbes present in the tick vectors (Miller et al., 2013).

The advent of next-generation sequencing approaches has enabled rapid, highly parallel sequencing of diverse microbial communities (Duan et al., 2020; Motro and Moran-Gilad, 2017a). Targeting the highly-conserved 16S rRNA gene has facilitated characterizations of bacteria microbes present within vectors. This technique involves amplification of the 16S rRNA gene, sample barcoding via PCR, loading samples onto a flow cell for sequencing, and bioinformatics approaches to match sequence data with those in databases like SILVA database (Klindworth et al., 2013a; Quast et al., 2013). This method is therefore suitable for charactering bacteria microbes in ticks allowing identification to genus level.

The present study reports, a diverse array of bacterial communities detected in these tick samples of which 706 bacterial taxa were detected and identified to genus level with 528 detected in *Amblyomma* spp., 641 in *Rhipicephalus* spp. and 447 in *Hyalomma* spp. Bacterial genera detected in this study may comprise bacteria genera found internally and on exoskeleton of ticks. Tick samples used in this study were collected and preserved in absolute ethanol before nucleic acid extraction with no further tick surface sterilization. Previous studies have shown differences in bacterial communities between sodium hypochlorite surface sterilized ticks with and non-sterilized ticks (Menchaca et al., 2013)

As shown in figure 7, the commonly identified bacteria belonged to the phylum Proteobacteria, Firmicutes, Bacteroidetes and Actinobacteria. Proteobacteria was the most abundant phylum accounting for 61.2% of the total sequences and 33.3% of the 645 unique OTUs. The phylum comprises of Gram negative bacteria and contains genera of most known pathogenic bacteria to humans and animals (Gupta, 2000). Dominance of Proteobacteria phylum reported in this study

agrees with previous studies of ticks collected from elsewhere that reported high dominance of Proteobacteria phylum (Thapa et al., 2019; Treuren et al., 2015). However, most of these studies have been reported in western countries where Lyme disease is a serious public health problem with scarce reports of tick microbiome in African countries (Bonnet et al., 2017; Chandra et al., 2021; Brinkerhoff et al., 2020; Thapa et al., 2019; Treuren et al., 2015) Firmicutes phylum that majorly comprise of Gram-positive bacteria were second abundant with 15.9% of the sequences. Previous studies of tick microbiome have reported Firmicutes as second abundance phylum after Proteobacteria and they are also found in different environments and includes notable pathogens (Govil et al., 2021; Kisten et al., 2021; Segura et al., 2020; Varela-Stokes et al., 2017). Other less abundant phyla detected includes Fusobacteria, TM7 (Saccharibacteria), Verrucomicrobia, Acidobacteria, Deinococcus-Thermus, Planctomycetes, Chloroflexi, OD1 (Parcubacteria), Tenericutes, Gemmatimonadetes, Armatimonadetes, Spirochaetes, Aquificae, SR1 (Absconditabacteria), Lentisphaerae, BRC1, Chlamydiae, Nitrospira, Chlorobi, Synergistetes, Fibrobacteres, WS3 and Elusimicrobia. This finding corroborates other tick microbiome studies that have reported these phylum to be the most common (Chandra et al., 2021; Segura and de Juan, 1966; Thapa et al., 2019).

As shown in Table 13, Coxiella was the most abundant genera comprising of 41.8 % of the sequences and 33.3 % of the 645 OTUs. This finding is similar to earlier reports on tick microbiome studies that reported *Coxiella* to dominate tick microbiome (Clay and Fuqua, 2010; Brinkerhoff et al., 2020). *Coxiella* was present in all tick genera accessed in this study. Hard and soft ticks including *Amblyomma, Hyalomma* and *Rhipicephalus* species have been documented to harbor *c. burnetii* the causative agent of Q fever (Cooper et al., 2013; Khoo et al., 2016; Mediannikov et al., 2010b). Mainly human infections with *C. burnetii* are due to contact with infected body fluids and

products of infected animals via the inhalation of contaminated aerosols, high prevalence of *C. burnetii* in ticks in endemic regions indicate a role for ticks in the epidemiology of Q fever.

Rickettsia was present in *Hyalomma* ticks at lower than 0.1 relative abundance which we used as our cut-off to plot the abundance. Our earlier report showed 35.9 % of *Rickettsia* infection in *Hyalomma* ticks by PCR (Mutai et al., 2013). This finding could be due to less sensitivity of 16s rRNA metagenomics using next generation sequencing in detection of pathogens compared to target specific PCR (Prachayangprecha et al., 2014; Thorburn et al., 2015). Due to insufficient resolution for species delimitation with the short fragment of 16s rRNA (v3-v4 variable region) used in this study, it was not possible to determine whether the *Coxiella, Francisella* and *Rickettsia* identified are *Coxiella*-like endosymbionts, *Francisella*-like endosymbionts and *Rickettsia* –like endosymbionts respectively. However our earlier report shown high prevalence of *Rickettsia* spp in ticks which were identified as *Rickettsia africae, R. aeschlimannii, R. mongolotimonae, R. conorii subsp. israelensis), Candidatus Rickettsia kulagini, and Rickettsia* spp (Kimita et al., 2016; Mutai et al., 2013). Other bacteria genera with high relative abundance (>1) identified in ticks included *Corynebacterium, Acinetobacter, Staphylococcus, Bacillus, Porphyromonas, Ralstonia, Streptococcus, Moraxella, Cloacibacterium, Neisseria, Escherichia_Shigella, Proteus* which are made up of both potentially pathogenic and nonpathogenic bacteria species. *Corynebacterium* the second most abundant genus belongs to phyla Actinobacteria and is widely distributed in nature as part of animal and human microbiota. *Corynebacterium* commonly exists in commensal relationship with the host while *Corynebacterium diphtheria* causes human infections. As shown in Figure 8, bacteria genera, *Staphylococcus, Klebsiella, Acitenobacter* and *Psuedomonas* (ESKAPE) that have species grouped as pathogens with high rates of antibiotic resistance were identified in the ticks (Santajit and Indrawattana, 2016). Ticks may therefore not only act as vectors

of pathogens but also as reservoirs for dissemination of antimicrobial resistance genes to pathogenic bacteria. As shown in table 1, other bacteria genera of potential medical relevance were presence in ticks though in relative abundance of less than 0.1 including *Burkhoderia, Klebsiella, Escherichia-Shegella, Achromobacter, Rickettsia, Haemophilus, Legionella, Campylobacter, Treponema, Francisella, Anaplasma, Elizabethkingia, Mycoplasma, Ehrlichia, Bordetella, Vibrio, Borrelia and Brucella.* This study has therefore reported an array of bacteria genera similar to other previous studies globally with the potential of transmission to human. (Ahantarig et al., 2013; Saldaña et al., 2017; Taylor et al., 2012). In this study, commonly tick endosymbionts reported as part of tick microbiome elsewhere including *Midichloria mitochloria, Wolbachia* spp, and Neoehrlichia spp were not identified (Ahantarig et al., 2013; Hoffmann et al., 2021; Saldaña et al., 2017; Thapa et al., 2019).

In summary, more than 15 new tick-borne bacterial diseases have been described in the world in places they were previously unknown or re-emerging in areas that had reported significant declined incidences and an increase in their geographical range (Rochlin and Toledo, 2020). Several factors have been reported to drive this emergence and re-emergence of these pathogens including human behavior, globalization, and change in land use, climate change, tick and tick-borne pathogen evolution. Therefore tick-borne disease prevalence, risk factors and transmission pattern are important public health issue in any part of the world. For any country to control these emerging diseases, tick population control as well as detection and treatment of infections by pathogens they transmit are imperative. Healthcare workers should be aware of signs and symptoms of tick-borne pathogens infections to reduce morbidity and mortality among the patients. Advance in molecular techniques for pathogens detection have facilitated detection of these pathogens globally and this capability should be enhance in health facilities in the country. Tick-borne bacterial infections are

easily treatable by use of antibiotics if detected early in the illness by can be fatal if there are delays in diagnosis and treatment. Tick-borne infections in humans can easily be prevented by avoiding contact with tick infested animals and areas. In Kenya animal keeping is very common and avoiding contact with tick infested animals and areas may not be possible. However, tick bites can be prevented by use of repellants or wearing protective clothing which has been effective elsewhere (Fritz, 2009).

Also, understanding the composition of tick microbiome will not only led to identification of pathogens of veterinary and human health relevance but also how it may impact transmission and maintenance of pathogens. Additionally understanding of the tick microbial composition will add knowledge on how manipulation of the microbial community may serve as an avenue for tick or pathogen control (Narasimhan and Fikrig, 2015; Varela-Stokes et al., 2017). Interestingly, *Wolbachia* spp. and *Candidatus Midichloria mitochondrii* endosymbionts that are reported as part of tick microbiome in other studies were absent in ticks from domestic animals in Kenya (Taylor et al., 2012). This is worth noting as use of endosymbionts is gaining popularity in new approaches to control ticks and tick borne pathogens.

One limitation of the present study is the lack of confirmed tick-borne zoonosis positive or negative clinical samples for evaluation of analytical sensitivity of the two multiplex assays. These assays were used for detection of pathogen DNA in patient samples which is indicative of an infection that can cause febrile illness. Because clinical samples have complex sample matrix, analytical sensitivities based on artificial plasmids could differ from those of vectors, patients or other reservoir hosts.

Secondly, the v3-v4 variable region of 16s rRNA used in detection of bacteria community in ticks is 460 base pairs with insufficient resolution of taxa detected to species. The length of the

nucleotide sequence size is determined by the NGS sequencing platform. The Miseq (Illumina, CA USA) used in this study allows pair end sequencing of up to 300 base pair compared to other technologies that sequence shorted fragments. Therefore bacteria detected in ticks collected from domestic animals in Kenya were classified to genus level.

5 CHAPTER 5: Conclusions and Recommendations

5.1 Conclusions

1. The duo 4-plex qPCR assays that were optimized and used in the present study can simultaneously detect 4 tick-borne pathogens per assay. The assays showed high precision and PCR efficiency and could detected low copies of targeted pathogens. The assays identified eight targeted tick-borne zoonosis pathogens in samples collected from patients with undifferentiated febrile illness from different regions of Kenya at a prevalence of 16.4 %. These pathogens are neither routinely diagnosed in hospitals in Kenya nor are they in the differential diagnosis list, in patients with febrile illnesses. This demonstrates that tick-borne zoonosis pathogens are present in some acute febrile illness patients in Kenya.
2. Risk factors for tick-borne zoonosis pathogen infections in Kenya include age, animal contact and residing in urban region. Patients aged six years and older had higher prevalence of tick-borne zoonosis infections compared to patients less than 6 years. Patients residing in urban region of Eastleigh in Nairobi County had higher prevalence of tick-borne zoonosis infections compared to other regions accessed. This is presumably because older people look after animals and uncontrolled urban environment encourages blooming of small mammals that act as pathogen reservoirs that can be transmitted to humans.
3. Clinical symptoms such as headache, muscle pain and joint pain are non-descriptive for tick-borne zoonosis infections and are similar to malaria symptoms making clinical diagnosis difficult. However having a tick bites was associated with higher odds of tick-borne zoonosis infection and can be regarded as an indicator of tick-borne zoonosis infection in acute febrile illness patients.

4. *Ambloyomma* spp., *Hylomma* spp., and *Rhipicephalus* spp. ticks collected from Domestic animals including cattle, sheep and goats in Kenya harbor a wide array of bacterial community which includes genera of medical relevance posing a risk of tick-borne pathogens transmission between domestic animals and humans. Proteobacteria is the most dominant bacteria phyla and *Coxiella* is the most dominant bacterial genus in ticks infesting domestic animals in the country. These findings suggest Kenyan ticks could serve as reservoirs and/or vectors for tick borne pathogens.

5.2 Recommendations

The present study has shown presence of tick-borne zoonosis pathogens in acute febrile illness and ticks collected from domestic animals from different regions in Kenya. There is therefore risk of infections with tick-borne zoonosis pathogens that maybe etiology of undifferentiated acute febrile illness in the country. For holistic knowledge on tick-borne zoonosis in Kenya this study recommends that:

1. Tick-borne zoonosis pathogens as etiologies of acute febrile illness in Kenya and therefore should be considered in the differential diagnosis and diagnostic capability be established.
2. Future investigations of tick-borne zoonosis pathogens in Kenya should include identification of other tick-borne zoonosis vectors including fleas, mites, lice and reservoirs, which will inform control strategies.
3. Molecular laboratory capability for diagnosis of tick-borne zoonosis pathogens should be enhanced for early detection and appropriate treatment in Kenya
4. Tick control strategies in Kenya should focus not only on veterinary health but also human health with a multi-sectorial one health approach to include animal, human and environmental elements.

6 CHAPTER 6: References

1. Ahantarig, A., Trinachartvanit, W., Baimai, V., Grubhoffer, L., 2013. Hard ticks and their bacterial endosymbionts (or would be pathogens). Folia Microbiol. (Praha). https://doi.org/10.1007/s12223-013-0222-1
2. Akpede, G.O., Akenzua, G.I., 2001. Management of children with prolonged fever of unknown origin and difficulties in the management of fever of unknown origin in children in developing countries. Paediatr. Drugs. https://doi.org/10.2165/00128072-200103040-00002
3. Altman, D.G., Bland, J.M., 1983. Measurement in Medicine: The Analysis of Method Comparison Studies. Stat. 32, 307. https://doi.org/10.2307/2987937
4. Anderson, B.E., Neuman, M.A., 1997. Bartonella spp. as emerging human pathogens. Clin. Microbiol. Rev. 10, 203–219. https://doi.org/10.1128/cmr.10.2.203
5. Anderson, J.F., Johnson, R.C., Magnarelli, L.A., Hyde, F.W., 1986. Involvement of birds in the epidemiology of the Lyme disease agent Borrelia burgdorferi. Infect. Immun. 51, 394–396.
6. Andreotti, R., De León, A.A.P., Dowd, S.E., Guerrero, F.D., Bendele, K.G., Scoles, G.A., 2011. Assessment of bacterial diversity in the cattle tick Rhipicephalus (Boophilus) microplus through tag-encoded pyrosequencing. BMC Microbiol. 11, 6. https://doi.org/10.1186/1471-2180-11-6
7. Angelakis, E., Billeter, S.A., Breitschwerdt, E.B., Chomel, B.B., Raoult, D., 2010. Potential for tick-borne bartonelloses. Emerg. Infect. Dis. https://doi.org/10.3201/eid1603.081685
8. Aslam, S., Latif, M., Daud, M., Rahman, Z., Tabassum, B., Riaz, M., Khan, A., Tariq, M., Husnain, T., 2015. Crimean-Congo hemorrhagic fever: Risk factors and control measures for the infection abatement (Review). Biomed. Reports. https://doi.org/10.3892/br.2015.545
9. Azad, A.F., Beard, C.B., 1998. Rickettsial pathogens and their arthropod vectors. Emerg. Infect. Dis. https://doi.org/10.3201/eid0402.980205
10. Baneth, G., Nachum-Biala, Y., Halperin, T., Hershko, Y., Kleinerman, G., Anug, Y., Abdeen, Z., Lavy, E., Aroch, I., Straubinger, R.K., 2016. Borrelia persica infection in dogs and cats: Clinical manifestations, clinicopathological findings and genetic characterization.

Parasites and Vectors 9, 1–10. https://doi.org/10.1186/s13071-016-1530-5

11. Barka, E.A., Vatsa, P., Sanchez, L., Nathalie Gaveau-Vaillant, C.J., Klenk, H.-P., Clément, C., Ouhdouch, Y., P. van Wezeld, G., 2016. Taxonomy, Physiology .... Am. Soc. Microbiol. 80, 1–43. https://doi.org/10.1128/MMBR.00019-15.Address
12. Behjati, S., Tarpey, P.S., 2013. What is next generation sequencing? Arch. Dis. Child. Educ. Pract. Ed. 98, 236–238. https://doi.org/10.1136/archdischild-2013-304340
13. Belkahia, H., Selmi, R., Zamiti, S., Daaloul-Jedidi, M., Messadi, L., Ben Said, M., 2021. Zoonotic Rickettsia Species in Small Ruminant Ticks From Tunisia. Front. Vet. Sci. 8, 1–11. https://doi.org/10.3389/fvets.2021.676896
14. Bell, S., 1953. Predisposing factors in tick-borne relapsing fever in Meru district, Kenya. Trans. R. Soc. Trop. Med. Hyg. 47, 309–317. https://doi.org/10.1016/0035-9203(53)90053-4
15. Bhatt, S., Weiss, D.J., Cameron, E., Bisanzio, D., Mappin, B., Dalrymple, U., Battle, K.E., Moyes, C.L., Henry, A., Eckhoff, P.A., Wenger, E.A., Briët, O., Penny, M.A., Smith, T.A., Bennett, A., Yukich, J., Eisele, T.P., Griffin, J.T., Fergus, C.A., Lynch, M., Lindgren, F., Cohen, J.M., Murray, C.L.J., Smith, D.L., Hay, S.I., Cibulskis, R.E., Gething, P.W., 2015. The effect of malaria control on Plasmodium falciparum in Africa between 2000 and 2015. Nature 526, 207–211. https://doi.org/10.1038/nature15535
16. Biggs, H.M., Behravesh, C.B., Bradley, K.K., Dahlgren, F.S., Drexler, N.A., Dumler, J.S., Folk, S.M., Kato, C.Y., Lash, R.R., Levin, M.L., Massung, R.F., Nadelman, R.B., Nicholson, W.L., Paddock, C.D., Pritt, B.S., Traeger, M.S., 2016. Diagnosis and management of tickborne rickettsial diseases: Rocky mountain spotted fever and other spotted fever group rickettsioses, ehrlichioses, and anaplasmosis - United States a practical guide for health care and public health professionals. MMWR Recomm. Reports. https://doi.org/10.15585/mmwr.rr6502a1
17. Billeter, S.A., Gundi, V.A.K.B., Rood, M.P., Kosoy, M.Y., 2011. Molecular detection and identification of Bartonella species in Xenopsylla cheopis fleas (Siphonaptera: Pulicidae) collected from Rattus norvegicus rats in Los Angeles, California. Appl. Environ. Microbiol. 77, 7850–7852. https://doi.org/10.1128/AEM.06012-11
18. Bonnet, S.I., Binetruy, F., Hernández-Jarguín, A.M., Duron, O., 2017. The Tick Microbiome: Why Non-pathogenic Microorganisms Matter in Tick Biology and Pathogen

Transmission. Front. Cell. Infect. Microbiol. 7. https://doi.org/10.3389/fcimb.2017.00236

19. Bouattour, A., Ghorbel, A., Chabchoub, A., Postic, D., 2004. Lyme borreliosis situation in North Africa. Arch. Inst. Pasteur Tunis 81, 13–20.
20. Bouquet, J., Melgar, M., Swei, A., Delwart, E., Lane, R.S., Chiu, C.Y., 2017. Metagenomic-based Surveillance of Pacific Coast tick Dermacentor occidentalis Identifies Two Novel Bunyaviruses and an Emerging Human Ricksettsial Pathogen. Sci. Rep. 7. https://doi.org/10.1038/s41598-017-12047-6
21. Breitschwerdt, E.B., Maggi, R.G., Chomel, B.B., Lappin, M.R., 2010. Bartonellosis: An emerging infectious disease of zoonotic importance to animals and human beings. J. Vet. Emerg. Crit. Care. https://doi.org/10.1111/j.1476-4431.2009.00496.x
22. Brites-Neto, J., Duarte, K.M.R., Martins, T.F., 2015. Tick-borne infections in human and animal population worldwide. Vet. World 8, 301–315. https://doi.org/10.14202/vetworld.2015.301-315
23. Budachetri, K., Browning, R.E., Adamson, S.W., Dowd, S.E., Chao, C.-C., Ching, W.-M., Karim, S., 2014. An Insight Into the Microbiome of the Amblyomma maculatum (Acari: Ixodidae) NIH Public Access. J Med Entomol 51, 119–129.
24. Bukin, Y.S., Galachyants, Y.P., Morozov, I. V., Bukin, S. V., Zakharenko, A.S., Zemskaya, T.I., 2019. The effect of 16s rRNA region choice on bacterial community metabarcoding results. Sci. Data 6, 1–14. https://doi.org/10.1038/sdata.2019.7
25. Carpi, G., Cagnacci, F., Wittekindt, N.E., Zhao, F., Qi, J., Tomsho, L.P., Drautz, D.I., Rizzoli, A., Schuster, S.C., 2011. Metagenomic profile of the bacterial communities associated with Ixodes ricinus ticks. PLoS One 6. https://doi.org/10.1371/journal.pone.0025604
26. Chakravorty, S., Helb, D., Burday, M., Connell, N., Alland, D., 2007. A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. J. Microbiol. Methods 69, 330–339. https://doi.org/10.1016/j.mimet.2007.02.005
27. Chandra, S., Harvey, E., Emery, D., Holmes, E.C., Šlapeta, J., 2021. Unbiased Characterization of the Microbiome and Virome of Questing Ticks. Front. Microbiol. 12, 1–15. https://doi.org/10.3389/fmicb.2021.627327
28. Chaput, E.K., Meek, J.I., Heimer, R., 2002. Spatial Analysis of Human Granulocytic Ehrlichiosis near Lyme, Connecticut, Emerging Infectious Diseases •.

29. Chow, A., Robinson, J.L., 2011. Fever of unknown origin in children: A systematic review. World J. Pediatr. 7, 5–10. https://doi.org/10.1007/s12519-011-0240-5
30. Clay, K., Fuqua, C., 2010. The Tick Microbiome: Diversity , Distribution and Influence of the Internal Microbial Community for a Blood-Feeding Disease Vector. "Critical Needs Gaps Underst. Prev. Amelior. Resolut. Lyme Other Tick-Borne Dis. Short-Term Long-Term Outcomes" Washington, D. C., Oct. 11-12, 2010 1–22.
31. Colizza, V., Barrat, A., Barthélemy, M., Vespignani, A., 2006. The modeling of global epidemics: Stochastic dynamics and predictability. Bull. Math. Biol. https://doi.org/10.1007/s11538-006-9077-9
32. Comer, J.A., Nicholson, W.L., Sumner, J.W., Olson, J.G., Childs, J.E., 1999. Diagnosis of human ehrlichiosis by PCR assay of acute-phase serum. J. Clin. Microbiol. https://doi.org/10.1128/jcm.37.1.31-34.1999
33. Cooper, A., Stephens, J., Ketheesan, N., Govan, B., 2013. Detection of coxiella burnetii dna in wildlife and ticks in Northern Queensland, Australia. Vector-Borne Zoonotic Dis. 13, 12–16. https://doi.org/10.1089/vbz.2011.0853
34. Cortinas, M.R., Guerra, M.A., Jones, C.J., Kitron, U., 2002. Detection, characterization, and prediction of tick-borne disease foci. Int. J. Med. Microbiol. https://doi.org/10.1016/S1438-4221(02)80003-0
35. Costa, F., Carvalho-Pereira, T., Begon, M., Riley, L., Childs, J., 2017. Zoonotic and Vector-Borne Diseases in Urban Slums: Opportunities for Intervention. Trends Parasitol. https://doi.org/10.1016/j.pt.2017.05.010
36. Coulter, P., Lema, C., Flayhart, D., Linhardt, A.S., Aucott, J.N., Auwaerter, P.G., Dumler, J.S., 2005. Two-year evaluation of Borrelia burgdorferi culture and supplemental tests for definitive diagnosis of lyme disease. J. Clin. Microbiol. https://doi.org/10.1128/JCM.43.10.5080-5084.2005
37. Couper, L., Swei, A., 2018a. Tick Microbiome Characterization by Next-Generation 16S rRNA Amplicon Sequencing. J. Vis. Exp. https://doi.org/10.3791/58239
38. Couper, L., Swei, A., 2018b. Tick microbiome characterization by next-generation 16S rRNA amplicon sequencing. J. Vis. Exp. 2018, 1–10. https://doi.org/10.3791/58239
39. Couper, L.I., Kwan, J.Y., Ma, J., Swei, A., 2019. Drivers and patterns of microbial community assembly in a Lyme disease vector. Ecol. Evol. 9, 7768–7779.

https://doi.org/10.1002/ece3.5361

40. Courtney, Joshua W, Kostelnik, L.M., Zeidner, N.S., Massung, R.F., 2004. Multiplex Real-Time PCR for Detection of Anaplasma phagocytophilum and Borrelia burgdorferi Multiplex Real-Time PCR for Detection of Anaplasma phagocytophilum and Borrelia burgdorferi. J. Clin. Microbiol. Vol. 42, N, 3164–3168. https://doi.org/10.1128/JCM.42.7.3164
41. Courtney, Joshua W., Kostelnik, L.M., Zeidner, N.S., Massung, R.F., 2004. Multiplex real-time PCR for detection of Anaplasma phagocytophilum and Borrelia burgdorferi. J. Clin. Microbiol. 42, 3164–3168. https://doi.org/10.1128/JCM.42.7.3164-3168.2004
42. Cunha, B.A., 2007. Fever of Unknown Origin: Clinical Overview of Classic and Current Concepts. Infect. Dis. Clin. North Am. 21, 867–915. https://doi.org/10.1016/j.idc.2007.09.002
43. Cupp, E.W., 1991. Biology of ticks. Vet. Clin. North Am. Small Anim. Pract. https://doi.org/10.1016/S0195-5616(91)50001-2
44. Cutler, S.J., 2010. Relapsing fever - A forgotten disease revealed. J. Appl. Microbiol. https://doi.org/10.1111/j.1365-2672.2009.04598.x
45. Cutler, S.J., Ruzic-Sabljic, E., Potkonjak, A., 2017. Emerging borreliae – Expanding beyond Lyme borreliosis. Mol. Cell. Probes. https://doi.org/10.1016/j.mcp.2016.08.003
46. Cyprian, E.O., 2012. Biological Weapons-agents for Life and Environmental Destruction. Res. J. Environ. Toxicol. https://doi.org/10.3923/rjet.2012.65.87
47. Davis, N.M., Proctor, D.M., Holmes, S.P., Relman, D.A., Callahan, B.J., 2017. Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. Simple Stat. Identif. Remov. Contam. Seq. marker-gene metagenomics data 221499. https://doi.org/10.1101/221499
48. de la Fuente, J., Estrada-Pena, A., Venzal, J.M., Kocan, K.M., Sonenshine, D.E., 2008. Overview: Ticks as vectors of pathogens that cause disease in humans and animals. Front. Biosci. 13, 6938–46.
49. Dsouli, N., Younsi-Kabachii, H., Postic, D., Nouira, S., Gern, L., Bouattour, A., 2006. Reservoir role of lizard Psammodromus algirus in transmission cycle of Borrelia burgdorferi sensu lato (Spirochaetaceae) in Tunisia. J. Med. Entomol. 43, 737–42. https://doi.org/10.1603/0022-2585(2006)43

50. Duan, H., Li, Xuan, Mei, A., Li, P., Liu, Y., Li, Xiaofeng, Li, W., Wang, C., Xie, S., 2020. The diagnostic value of metagenomic next-generation sequencing in infectious diseases 1–13. https://doi.org/10.21203/rs.3.rs-18934/v1
51. Dumler, J.S. et al, 2005. Human Granulocytic Anaplasmosis and Anaplasma phagocytophilum. Emerg. Infect. Dis. 11.
52. Dumler, J.S., Choi, K.S., Garcia-Garcia, J.C., Barat, N.S., Scorpio, D.G., Garyu, J.W., Grab, D.J., Bakken, J.S., 2005. Human granulocytic anaplasmosis and Anaplasma phagocytophilum. Emerg. Infect. Dis. 11, 1828–1834. https://doi.org/10.3201/eid1112.050898
53. Dumler, J.S., Madigan, J.E., Pusterla, N., Bakken, J.S., 2007. Ehrlichioses in humans: Epidemiology, clinical presentation, diagnosis, and treatment. Clin. Infect. Dis. 45, 45–51. https://doi.org/10.1086/518146
54. Duplaix, L., Wagner, V., Gasmi, S., Lindsay, L.R., Dibernardo, A., Thivierge, K., Fernandez-Prada, C., Arsenault, J., 2021. Exposure to Tick-Borne Pathogens in Cats and Dogs Infested With Ixodes scapularis in Quebec: An 8-Year Surveillance Study. Front. Vet. Sci. https://doi.org/10.3389/fvets.2021.696815
55. Dworkin, M.S., Schwan, T.G., Anderson, D.E., Borchardt, S.M., 2008. Tick-Borne Relapsing Fever. Infect. Dis. Clin. North Am. https://doi.org/10.1016/j.idc.2008.03.006
56. Eisen, L., 2018. Pathogen transmission in relation to duration of attachment by Ixodes scapularis ticks. Ticks Tick. Borne. Dis. https://doi.org/10.1016/j.ttbdis.2018.01.002
57. Elbir, H., Almathen, F., Alhumam, N.A., 2019. A glimpse of the bacteriome of Hyalomma dromedarii ticks infesting camels reveals human Helicobacter pylori pathogen. J. Infect. Dev. Ctries. 13, 1001–1012. https://doi.org/10.3855/JIDC.11604
58. Elshout, G., Monteny, M., Van Der Wouden, J.C., Koes, B.W., Berger, M.Y., 2011. Duration of fever and serious bacterial infections in children: A systematic review. BMC Fam. Pract. 12. https://doi.org/10.1186/1471-2296-12-33
59. Espy, M.J., Uhl, J.R., Sloan, L.M., Buckwalter, S.P., Jones, M.F., Vetter, E.A., Yao, J.D.C., Wengenack, N.L., Rosenblatt, J.E., Cockerill, F.R., Smith, T.F., 2006. Real-time PCR in clinical microbiology: Applications for routine laboratory testing. Clin. Microbiol. Rev. https://doi.org/10.1128/CMR.19.1.165-256.2006
60. Estrada-Peña, A., Jongejan, F., 1999. Ticks feeding on humans: a review of records on

human-biting Ixodoidea with special reference to pathogen transmission Climate, niche, ticks, and models: what they are and how we should interpret them. Exp. Appl. Acarol. 23, 685–715.

61. Estrada-peña, A., Walker, A.., Bouattour, A., Camicas, J.., Horak, I.., Latif, A.., Pegram, R.., Preston, P.., 2003. Ticks of domestic animals in Africa: a guide to identification of species, The University of Edinburgh.
62. Feng, J., Auwaerter, P.G., Zhang, Y., 2015. Drug combinations against Borrelia burgdorferi persisters in vitro: Eradication achieved by using daptomycin, cefoperazone and doxycycline. PLoS One 10, 1–15. https://doi.org/10.1371/journal.pone.0117207
63. Fenollar, F., Raoult, D., 2007. Molecular diagnosis of bloodstream infections caused by non-cultivable bacteria. Int. J. Antimicrob. Agents 30, 7–15. https://doi.org/10.1016/j.ijantimicag.2007.06.024
64. Fèvre, E.M., Bronsvoort, B.M.D.C., Hamilton, K.A., Cleaveland, S., 2006. Animal movements and the spread of infectious diseases. Trends Microbiol. https://doi.org/10.1016/j.tim.2006.01.004
65. Filipe Dantas-Torres, D.O., 2020. Ixodid and Argasid Ticks. Ref. Modul. Biomed. Sci.
66. Fischhoff, I.R., Keesing, F., Ostfeld, R.S., 2019. Systematic Reviews and Meta- and Pooled Analyses Risk Factors for Bites and Diseases Associated With Black-Legged Ticks : A Meta-Analysis 188, 1742–1750. https://doi.org/10.1093/aje/kwz130
67. Fishbein, D.B., Dawson, J.E., Robinson, L.E., 1994. Human ehrlichiosis in the United States, 1985 to 1990. Ann. Intern. Med. https://doi.org/10.7326/0003-4819-120-9-199405010-00003
68. Florin, T.A., Zaoutis, T.E., Zaoutis, L.B., 2008. Beyond cat scratch disease: Widening spectrum of Bartonella henselae infection. Pediatrics 121. https://doi.org/10.1542/peds.2007-1897
69. Fournier, P.E., Raoult, D., 2009. Current knowledge on phylogeny and taxonomy of rickettsia spp. Ann. N. Y. Acad. Sci. https://doi.org/10.1111/j.1749-6632.2009.04528.x
70. Fritz, C.L., 2009. Emerging Tick-borne Diseases. Vet. Clin. North Am. - Small Anim. Pract. 39, 265–278. https://doi.org/10.1016/j.cvsm.2008.10.019
71. Fuks, G., Elgart, M., Amir, A., Zeisel, A., Turnbaugh, P.J., Soen, Y., Shental, N., 2018. Combining 16S rRNA gene variable regions enables high-resolution microbial community

profiling. Microbiome 6, 1–13. https://doi.org/10.1186/s40168-017-0396-x

72. Galperin, M.Y., 2015. Genome diversity of spore-forming firmicutes bacterial systematics from gram stain to 16S rRNA. Microbiol Spectr. 1, 1–27. https://doi.org/10.1128/microbiolspectrum.TBS-0015-2012.Genome
73. Gerhart, J.G., Moses, A.S., Raghavan, R., 2016. A Francisella-like endosymbiont in the Gulf Coast tick evolved from a mammalian pathogen. Sci. Rep. 6, 1–6. https://doi.org/10.1038/srep33670
74. Goodman, J.L., Nelson, C., Vitale, B., Madigan, J.E., Dumler, J.S., Kurtti, T.J., Munderloh, U.G., 1996. Direct Cultivation of the Causative Agent of Human Granulocytic Ehrlichiosis. N. Engl. J. Med. https://doi.org/10.1056/nejm199601253340401
75. Govil, T., Paste, M., Samanta, D., David, A., Goh, K.M., Li, X., Salem, D.R., Sani, R.K., 2021. Metagenomics and culture dependent insights into the distribution of firmicutes across two different sample types located in the black hills region of South Dakota, USA. Microorganisms 9, 1–24. https://doi.org/10.3390/microorganisms9010113
76. Greger, M., 2007. The human/animal interface: Emergence and resurgence of zoonotic infectious diseases. Crit. Rev. Microbiol. https://doi.org/10.1080/10408410701647594
77. Guizzo, M.G., Parizi, L.F., Nunes, R.D., Schama, R., Albano, R.M., Tirloni, L., Oldiges, D.P., Vieira, R.P., Oliveira, W.H.C., Leite, M.D.S., Gonzales, S.A., Farber, M., Martins, O., Vaz, I.D.S., Oliveira, P.L., 2017. A Coxiella mutualist symbiont is essential to the development of Rhipicephalus microplus. Sci. Rep. 7, 1–10. https://doi.org/10.1038/s41598-017-17309-x
78. Gupta, R.S., 2000. The phylogeny of proteobacteria: Relationships to other eubacterial phyla and eukaryotes. FEMS Microbiol. Rev. 24, 367–402. https://doi.org/10.1016/S0168-6445(00)00031-0
79. Guy, L., Nystedt, B., Toft, C., Zaremba-Niedzwiedzka, K., Berglund, E.C., Granberg, F., Näslund, K., Eriksson, A.S., Andersson, S.G.E., 2013. A Gene Transfer Agent and a Dynamic Repertoire of Secretion Systems Hold the Keys to the Explosive Radiation of the Emerging Pathogen Bartonella. PLoS Genet. 9. https://doi.org/10.1371/journal.pgen.1003393
80. Halliday, J.E.B., Knobel, D.L., Agwanda, B., Bai, Y., Breiman, R.F., Cleaveland, S., Njenga, M.K., Kosoy, M., 2015. Prevalence and Diversity of Small Mammal-Associated

Bartonella Species in Rural and Urban Kenya. PLoS Negl. Trop. Dis. 9. https://doi.org/10.1371/journal.pntd.0003608

81. Halliday, J.E.B., Meredith, A.L., Knobel, D.L., Shaw, D.J., Bronsvoort, B.M.D.C., Cleaveland, S., 2007. A framework for evaluating animals as sentinels for infectious disease surveillance. J. R. Soc. Interface 4, 973–984. https://doi.org/10.1098/rsif.2007.0237
82. He, X.M., Cheng, T.Y., 2018. Tick saliva microbiomes isolated from engorged and partially fed adults of Haemaphysalis flava tick females. J. Appl. Entomol. https://doi.org/10.1111/jen.12427
83. Heinrich, N., Dill, T., Dobler, G., Clowes, P., Kroidl, I., Starke, M., Ntinginya, N.E., Maboko, L., Löscher, T., Hoelscher, M., Saathoff, E., 2015. High Seroprevalence for Spotted Fever Group Rickettsiae, Is Associated with Higher Temperatures and Rural Environment in Mbeya Region, Southwestern Tanzania. PLoS Negl. Trop. Dis. 9. https://doi.org/10.1371/journal.pntd.0003626
84. Heisch, R.B., Grainger, W.E., Harvey, A.E.C., Lister, G., 1962. Feral aspects of Rickettsial infections in Kenya. Trans. R. Soc. Trop. Med. Hyg. https://doi.org/10.1016/0035-9203(62)90048-2
85. HEISCH, R.B., McPHEE, R., RICKMAN, L.R., 1957. The epidemiology of tick-typhus in Nairobi. East Afr. Med. J.
86. Helmy, N., 2000. Seasonal abundance of Ornithodoros (O.) savignyi and prevalence of infection with Borrelia spirochetes in Egypt. J. Egypt. Soc. Parasitol. 30, 607--619 LA--English.
87. Henegariu O, Heerema NA, Dlouhy SR, Vance GH, V.P., 1997. Multiplex PCR: Critical Parameters and Step-by-Step Protocol. Biotechniques 23, 504–11.
88. Henri-Jean Boulouis , Chao-Chin Chang, Jennifer B Henn, Rickie W Kasten, B.B.C., 2005. Factors associated with the rapid emergence of zoonotic Bartonella infections. Vet. Res. 36.
89. Heyman, P., Cochez, C., Hofhuis, A., Van Der Giessen, J., Sprong, H., Porter, S.R., Losson, B., Saegerman, C., Donoso-Mantke, O., Niedrig, M., Papa, A., 2010. A clear and present danger: Tick-borne diseases in Europe. Expert Rev. Anti. Infect. Ther. 8, 33–50. https://doi.org/10.1586/eri.09.118

90. Hildebrandt, A., Fritzsch, J., Franke, J., Sachse, S., Dorn, W., Straube, E., 2011. Co-circulation of emerging tick-borne pathogens in middle Germany. Vector-Borne Zoonotic Dis. https://doi.org/10.1089/vbz.2010.0048
91. Hildebrandt, A., Fritzsch, J., Franke, J., Sachse, S., Straube, E., 2010. Interesting insights into the co-circulation of pathogens in Ixodes ricinus ticks from rodents, birds and in questing stages in middle Germany. Clin. Microbiol. Infect.
92. Hitchcock, P., Chamberlain, A., Van Wagoner, M., Inglesby, T. V., O'Toole, T., 2007. Challenges to global surveillance and response to infectious disease outbreaks of international importance. Biosecurity and Bioterrorism 5, 206–227. https://doi.org/10.1089/bsp.2007.0041
93. Hoffmann, A., Müller, T., Fingerle, V., Noll, M., 2021. Presence of human pathogens of the borrelia burgdorferi sensu lato complex shifts the sequence read abundances of tick microbiomes in two german locations. Microorganisms 9. https://doi.org/10.3390/microorganisms9091814
94. Hofhuis, A., Van De Kassteele, J., Sprong, H., Van Den Wijngaard, C.C., Harms, M.G., Fonville, M., Van Leeuwen, A.D., Simões, M., Van Pelt, W., 2017. Predicting the risk of Lyme borreliosis after a tick bite, using a structural equation model. PLoS One 12, 1–15. https://doi.org/10.1371/journal.pone.0181807
95. Homer, M.J., Aguilar-Delfin, I., Telford, S.R., Krause, P.J., Persing, D.H., 2000. Babesiosis. Clin. Microbiol. Rev. 13, 451–469. https://doi.org/10.1128/CMR.13.3.451-469.2000
96. Horak, I. G., Camicas, J.-L., & Keirans, J.E., 2002. The Argasidae, Ixodidae and Nuttalliellidae (Acari: Ixodida): A World List of Valid Tick Names. Exp. Appl. Acarol. 28, 27–54.
97. Horak, A.A.G.G.R.A.A.N.P.E.-P.G., 2014. The Hard Ticks of the World (Acari: Ixodida: Ixodidae).
98. Hugo, C.J., Jooste, P.J., Segers, P., Vancanneyt, M., Kersters, K., 1999. A polyphasic taxonomic study of chryseobacterium strains isolated from dairy sources. Syst. Appl. Microbiol. 22, 586–595. https://doi.org/10.1016/S0723-2020(99)80012-5
99. Ian M. Mackay (Ed.), 2007. Real-time PCR in microbiology: From diagnosis to characterization, 1st ed. Caister Academic Press, Poole, UK.

100. Inci, A., Yildirim, A., Duzlu, O., Doganay, M., Aksoy, S., 2016. Tick-Borne Diseases in Turkey: A Review Based on One Health Perspective. PLoS Negl. Trop. Dis. https://doi.org/10.1371/journal.pntd.0005021

101. Ismail, N., Bloch, K.C., McBride, J.W., 2010. Human ehrlichiosis and anaplasmosis. Clin. Lab. Med. https://doi.org/10.1016/j.cll.2009.10.004

102. Isyagi, N.A., Veverica, K.L., Asiimwe, R., Daniels, W.H., 2007. Manual for the Commercial Pond Production of the African Catfish in Uganda. Water Qual. Gen. Pond Manag. 70–96.

103. Jaenson, T.G.T., Wilhelmsson, P., 2019. First records of tick-borne pathogens in populations of the taiga tick Ixodes persulcatus in Sweden. Parasites and Vectors 12, 1–12. https://doi.org/10.1186/s13071-019-3813-0

104. Jahfari, S., Hofhuis, A., Fonville, M., van der Giessen, J., van Pelt, W., Sprong, H., 2016. Molecular Detection of Tick-Borne Pathogens in Humans with Tick Bites and Erythema Migrans, in the Netherlands. PLoS Negl. Trop. Dis. 10. https://doi.org/10.1371/journal.pntd.0005042

105. Jensenius, M., Davis, X., Von Sonnenburg, F., Schwartz, E., Keystone, J.S., Leder, K., Lopéz-Véléz, R., Caumes, E., Cramer, J.P., Chen, L., Parola, P., 2009. Multicenter GeoSentinel analysis of rickettsial diseases in international travelers, 1996-2008. Emerg. Infect. Dis. https://doi.org/10.3201/eid1511.090677

106. Jiang, J., Stromdahl, E.Y., Richards, A.L., 2012. Detection of Rickettsia parkeri and Candidatus Rickettsia andeanae in Amblyomma maculatum Gulf Coast Ticks Collected from Humans in the United States. Vector-Borne Zoonotic Dis. 12, 175–182. https://doi.org/10.1089/vbz.2011.0614

107. Jiang, J., Temenak, J.J., Richards, A.L., 2003. Real-time PCR duplex assay for Rickettsia prowazekii and Borrelia recurrentis. Ann. N. Y. Acad. Sci. 990, 302–310. https://doi.org/10.1111/j.1749-6632.2003.tb07380.x

108. Jones, K.E., Patel, N.G., Levy, M.A., Storeygard, A., Balk, D., Gittleman, J.L., Daszak, P., 2008. Global trends in emerging infectious diseases. Nature. https://doi.org/10.1038/nature06536

109. Jongejan, F., Uilenberg, G., 2004. The global importance of ticks. Parasitology. https://doi.org/10.1017/S0031182004005967

110. Jory Brinkerhoff, R., Clark, C., Ocasio, K., Gauthier, D.T., Hynes, W.L., 2020. Factors affecting the microbiome of Ixodes scapularis and Amblyomma americanum. PLoS One 15, 1–20. https://doi.org/10.1371/journal.pone.0232398

111. Jowi, J.O., Gathua, S.N., 2005. Lyme disease: Report of two cases. East Afr. Med. J. 82, 267–269. https://doi.org/10.4314/eamj.v82i5.9318

112. Kampschreur, L.M., Dekker, S., Hagenaars, J.C.J.P., Lestrade, P.J., Renders, N.H.M., de Jager-Leclercq, M.G.L., Hermans, M.H.A., Groot, C.A.R., Groenwold, R.H.H., Hoepelman, A.I.M., Wever, P.C., Oosterheert, J.J., 2012. Identification of risk factors for chronic Q fever, the Netherlands. Emerg. Infect. Dis. https://doi.org/10.3201/eid1804.111478

113. Karbowiak, G., Biernat, B., Werszko, J., Rychlik, L., 2016. The transstadial persistence of tick-borne encephalitis virus in Dermacentor reticulatus ticks in natural conditions. Acta Parasitol. 61, 201–203. https://doi.org/10.1515/ap-2016-0028

114. Kashinkunti MD, Gundikeri SK, D.M., 2013. Acute undifferentiated febrile illness-clinical spectrum and outcome from a tertiary care teaching hospital of north karnataka. Int J Biol Med Res. 4, 3399–3402.

115. Kasper, M.R., Blair, P.J., Touch, S., Sokhal, B., Yasuda, C.Y., Williams, M., Richards, A.L., Burgess, T.H., Wierzba, T.F., Putnam, S.D., 2012. Infectious etiologies of acute febrile illness among patients seeking health care in South-Central Cambodia. Am. J. Trop. Med. Hyg. 86, 246–253. https://doi.org/10.4269/ajtmh.2012.11-0409

116. Khatat, S.E., Sahibi, H., Hing, M., Moustain, I.A., El Amri, H., Benajiba, M., Kachani, M., Duchateau, L., Daminet, S., 2016. Human exposure to anaplasma phagocytophilum in two cities of northwestern Morocco. PLoS One 11. https://doi.org/10.1371/journal.pone.0160880

117. Khoo, J.J., Lim, F.S., Chen, F., Phoon, W.H., Khor, C.S., Pike, B.L., Chang, L.Y., Abubakar, S., 2016. Coxiella Detection in Ticks from Wildlife and Livestock in Malaysia. Vector-Borne Zoonotic Dis. 16, 744–751. https://doi.org/10.1089/vbz.2016.1959

118. Kimita, G., Mutai, B., Nyanjom, S.G., Wamunyokoli, F., Waitumbi, J., 2016. Phylogenetic Variants of Rickettsia africae, and Incidental Identification of “Candidatus Rickettsia Moyalensis” in Kenya. PLoS Negl. Trop. Dis. 10. https://doi.org/10.1371/journal.pntd.0004788

119. Kisinza, W.N., McCall, P.J., Mitani, H., Talbert, A., Fukunaga, M., 2003. A newly identified tick-borne Borrelia species and relapsing fever in Tanzania. Lancet 362, 1283–1284. https://doi.org/10.1016/S0140-6736(03)14609-0

120. Kisten, D., Brinkerhoff, J., Tshilwane, S.I., Mukaratirwa, S., 2021. A pilot study on the microbiome of amblyomma hebraeum tick stages infected and non-infected with rickettsia africae. Pathogens 10, 1–16. https://doi.org/10.3390/pathogens10080941

121. Kjemtrup, A.M., Conrad, P.A., 2000. Human babesiosis: An emerging tick-borne disease. Int. J. Parasitol. 30, 1323–1337. https://doi.org/10.1016/S0020-7519(00)00137-5

122. Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., Glöckner, F.O., 2013a. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res. 41, e1. https://doi.org/10.1093/nar/gks808

123. Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., Glöckner, F.O., 2013b. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res. 41. https://doi.org/10.1093/nar/gks808

124. Knapp, K.L., Rice, N.A., 2015. Human coinfection with Borrelia burgdorferi and Babesia microti in the United States. J. Parasitol. Res. https://doi.org/10.1155/2015/587131

125. Koka, H., Sang, R., Kutima, H.L., Musila, L., 2018. Coxiella burnetii Detected in Tick Samples from Pastoral Communities in Kenya. Biomed Res. Int. 2018. https://doi.org/10.1155/2018/8158102

126. Koka, H., Sang, R., Kutima, H.L., Musila, L., Macaluso, K., 2017. The detection of spotted fever group rickettsia DNA in tick samples from pastoral communities in Kenya. J. Med. Entomol. 54, 774–780. https://doi.org/10.1093/jme/tjw238

127. Kosoy, M., Bai, Y., Lynch, T., Kuzmin, I. V., Niezgoda, M., Franka, R., Agwanda, B., Breiman, R.F., Rupprecht, C.E., 2010. Bartonella spp. in bats, Kenya. Emerg. Infect. Dis. 16, 1875–1881. https://doi.org/10.3201/eid1612.100601

128. Krause, P.J., McKay, K., Thompson, C.A., Sikand, V.K., Lentz, R., Lepore, T., Closter, L., Christianson, D., Telford, S.R., Persing, D., Radolf, J.D., Spielman, A., 2002. Disease-Specific Diagnosis of Coinfecting Tickborne Zoonoses: Babesiosis, Human Granulocytic Ehrlichiosis, and Lyme Disease. Clin. Infect. Dis. 34, 1184–1191.

https://doi.org/10.1086/339813

129. Kulkarni, M.A., Berrang-Ford, L., Buck, P.A., Drebot, M.A., Lindsay, L.R., Ogden, N.H., 2015. Major emerging vector-borne zoonotic diseases of public health importance in Canada. Emerg. Microbes Infect. https://doi.org/10.1038/emi.2015.33

130. Lappin, M.R., 2018. Update on flea and tick associated diseases of cats. Vet. Parasitol. 254, 26–29. https://doi.org/10.1016/j.vetpar.2018.02.022

131. Leelarasamee, A., Chupaprawan, C., Chenchittikul, M., Udompanthurat, S., 2004. Etiologies of acute undifferentiated febrile illness in Thailand. J. Med. Assoc. Thail.

132. Leeuw, P.N. de, Grandin, B.E., Bekure, S., 1991. Chapter 2: Introduction to the Kenyan rangelands and Kajiado district, in: Maasai Herding - An Analysis of the Livestock Production System of Maasai Pastoralists in Eastern Kajiado District, Kenya. p. 172.

133. Lejal, E., Estrada-Peña, A., Marsot, M., Cosson, J.F., Rué, O., Mariadassou, M., Midoux, C., Vayssier-Taussat, M., Pollet, T., 2020. Taxon Appearance From Extraction and Amplification Steps Demonstrates the Value of Multiple Controls in Tick Microbiota Analysis. Front. Microbiol. 11, 1–10. https://doi.org/10.3389/fmicb.2020.01093

134. Lemmon, G.H., Gardner, S.N., 2008. Predicting the sensitivity and specificity of published real-time PCR assays. Ann. Clin. Microbiol. Antimicrob. 7. https://doi.org/10.1186/1476-0711-7-18

135. Lemtudo, A.P., Mutai, B.K., Mwamburi, L., Waitumbi, J.N., 2021. Seroprevalence of Coxiella burnetii in patients presenting with acute febrile illness at Marigat District Hospital , Baringo 1–7. https://doi.org/10.1002/vms3.493

136. Leslie, T., Mikhail, A., Mayan, I., Anwar, M., Bakhtash, S., Nader, M., Chandler, C., Whitty, C.J.M., Rowland, M., 2012. Overdiagnosis and mistreatment of malaria among febrile patients at primary healthcare level in Afghanistan: Observational study. BMJ 345, 5–9. https://doi.org/10.1136/bmj.e4389

137. Levin, M.L., Fish, D., 2001. Interference Between the Agents of Lyme Disease and Human Granulocytic Ehrlichiosis in a Natural Reservoir Host. Vector-Borne Zoonotic Dis. 1, 139–148. https://doi.org/10.1089/153036601316977741

138. Lin, Q., Rikihisa, Y., Felek, S., Wang, X., Massung, R.F., Woldehiwet, Z., 2004. Anaplasma phagocytophilum has a functional msp2 gene that is distinct from p44. Infect. Immun. 72, 3883–3889. https://doi.org/10.1128/IAI.72.7.3883-3889.2004

139. Lindahl, J.F., Grace, D., 2015. The consequences of human actions on risks for infectious diseases: a review. Infect. Ecol. Epidemiol. 5, 30048. https://doi.org/10.3402/iee.v5.30048

140. Liyai, R., Kimita, G., Masakhwe, C., Abuom, D., Mutai, B., Onyango, D.M., Waitumbi, J., 2021. The spleen bacteriome of wild rodents and shrews from Marigat , Baringo County , Kenya 1–17. https://doi.org/10.7717/peerj.12067

141. Loftis, A.D., Massung, R.F., Levin, M.L., 2003. Quantitative real-time PCR assay for detection of Ehrlichia chaffeensis. J. Clin. Microbiol. 41, 3870–3872. https://doi.org/10.1128/JCM.41.8.3870-3872.2003

142. Lommano, E., Bertaiola, L., Dupasquier, C., Gern, L., 2012. Infections and coinfections of questing Ixodes ricinus ticks by emerging zoonotic pathogens in Western Switzerland. Appl. Environ. Microbiol. https://doi.org/10.1128/AEM.07961-11

143. Louise Ord, R., Lobo, C.A., n.d. Human Babesiosis: Pathogens, Prevalence, Diagnosis and Treatment. https://doi.org/10.1007/s40588-015-0025-z

144. Maamun, J.M., Suleman, M. a, Akinyi, M., Ozwara, H., Kariuki, T., Carlsson, H.-E., 2011. Prevalence of Babesia microti in free-ranging baboons and African green monkeys. J. Parasitol. 97, 63–7. https://doi.org/10.1645/GE-2391.1

145. Macaluso, K.R., Davis, J., Alam, U., Korman, A., Rutherford, J.S., Rosenberg, R., Azad, A.F., 2003. Spotted fever group Rickettsiae in ticks from the masai mara region of Kenya. Am. J. Trop. Med. Hyg. https://doi.org/10.4269/ajtmh.2003.68.551

146. Mackenzie, J.S., Gubler, D.J., Petersen, L.R., 2004. Emerging flaviviruses: The spread and resurgence of japanese encephalitis, west nile and dengue viruses. Nat. Med. https://doi.org/10.1038/nm1144

147. Madison-antenucci, S., Kramer, L.D., Gebhardt, L.L., 2020. crossm Emerging Tick-Borne Diseases 33, 1–34.

148. Mahachi, K., Kontowicz, E., Anderson, B., Toepp, A.J., Lima, A.L., Larson, M., Wilson, G., Grinnage-Pulley, T., Bennett, C., Ozanne, M., Anderson, M., Fowler, H., Parrish, M., Saucier, J., Tyrrell, P., Palmer, Z., Buch, J., Chandrashekar, R., Scorza, B., Brown, G., Oleson, J.J., Petersen, C.A., 2020. Predominant risk factors for tick-borne co-infections in hunting dogs from the USA. Parasites and Vectors 13, 1–12. https://doi.org/10.1186/s13071-020-04118-x

149. Maina, A.N., Jiang, J., Omulo, S.A., Cutler, S.J., Ade, F., Ogola, E., Feikin, D.R., Njenga, M.K., Cleaveland, S., Mpoke, S., Ng'ang'a, Z., Breiman, R.F., Knobel, D.L., Richards, A.L., 2014. High Prevalence of *Rickettsia africae* Variants in *Amblyomma variegatum* Ticks from Domestic Mammals in Rural Western Kenya: Implications for Human Health. Vector-Borne Zoonotic Dis. 14, 693–702. https://doi.org/10.1089/vbz.2014.1578

150. Maurin, M., Birtles, R., Raoult, D., 1997. Current knowledge of Bartonella species. Eur. J. Clin. Microbiol. Infect. Dis. 16, 487–506. https://doi.org/10.1007/BF01708232

151. Maze, M.J., Bassat, Q., Feasey, N.A., Mandomando, I., Musicha, P., Crump, J.A., 2018. The epidemiology of febrile illness in sub-Saharan Africa: implications for diagnosis and management. Clin. Microbiol. Infect. https://doi.org/10.1016/j.cmi.2018.02.011

152. McMurdie, P.J., Holmes, S., 2013. Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. PLoS One 8. https://doi.org/10.1371/journal.pone.0061217

153. Mediannikov, O., Diatta, G., Fenollar, F., Sokhna, C., Trape, J.F., Raoult, D., 2010a. Tick-borne rickettsioses, neglected emerging diseases in rural Senegal. PLoS Negl. Trop. Dis. 4. https://doi.org/10.1371/journal.pntd.0000821

154. Mediannikov, O., Fenollar, F., Socolovschi, C., Diatta, G., Bassene, H., Molez, J.F., Sokhna, C., Trape, J.F., Raoult, D., 2010b. Coxiella burnetii in humans and ticks in rural Senegal. PLoS Negl. Trop. Dis. https://doi.org/10.1371/journal.pntd.0000654

155. Menchaca, A.C., Visi, D.K., Strey, O.F., Teel, P.D., Kalinowski, K., Allen, M.S., Williamson, P.C., 2013. Preliminary Assessment of Microbiome Changes Following Blood-Feeding and Survivorship in the Amblyomma americanum Nymph-to-Adult Transition using Semiconductor Sequencing. PLoS One 8. https://doi.org/10.1371/journal.pone.0067129

156. Middleton, D.B., 1978. Rocky mountain spotted fever: Gastrointestinal and laboratory manifestations. South. Med. J. 71, 629–632. https://doi.org/10.1097/00007611-197806000-00007

157. Miller, R.R., Montoya, V., Gardy, J.L., Patrick, D.M., Tang, P., 2013. Metagenomics for pathogen detection in public health. Genome Med. 5. https://doi.org/10.1186/gm485

158. Mitani, H., Talbert, A., Fukunaga, M., 2004. New world relapsing fever Borrelia found in Ornithodoros porcinus ticks in Central Tanzania. Microbiol. Immunol. https://doi.org/10.1111/j.1348-0421.2004.tb03545.x

159. Moore, T.C., Pulscher, L.A., Caddell, L., von Fricken, M.E., Anderson, B.D., Gonchigoo, B., Gray, G.C., 2018. Evidence for transovarial transmission of tick-borne rickettsiae circulating in Northern Mongolia. PLoS Negl. Trop. Dis. 12, 1–13. https://doi.org/10.1371/journal.pntd.0006696

160. Morand, S., Lajaunie, C., 2021. Outbreaks of Vector-Borne and Zoonotic Diseases Are Associated With Changes in Forest Cover and Oil Palm Expansion at Global Scale. Front. Vet. Sci. 8, 1–11. https://doi.org/10.3389/fvets.2021.661063

161. Motro, Y., Moran-Gilad, J., 2017a. Next-generation sequencing applications in clinical bacteriology. Biomol. Detect. Quantif. 14, 1–6. https://doi.org/10.1016/j.bdq.2017.10.002

162. Motro, Y., Moran-Gilad, J., 2017b. Next-generation sequencing applications in clinical bacteriology. Biomol. Detect. Quantif. https://doi.org/10.1016/j.bdq.2017.10.002

163. Moutailler, S., Valiente Moro, C., Vaumourin, E., Michelet, L., Tran, F.H., Devillers, E., Cosson, J.F., Gasqui, P., Van, V.T., Mavingui, P., Vourc'h, G., Vayssier-Taussat, M., 2016. Co-infection of Ticks: The Rule Rather Than the Exception. PLoS Negl. Trop. Dis. 10. https://doi.org/10.1371/journal.pntd.0004539

164. Muema, J., Thumbi, S.M., Obonyo, M., Wanyoike, S., Nanyingi, M., Osoro, E., Bitek, A., Karanja, S., 2017. Seroprevalence and Factors Associated with Coxiella burnetii Infection in Small Ruminants in Baringo County, Kenya. Zoonoses Public Health 64, e31–e43. https://doi.org/10.1111/zph.12342

165. Musso, D., Raoult, D., 1995. Coxiella burnetii blood cultures from acute and chronic Q-fever patients. J. Clin. Microbiol. 33, 3129–3132. https://doi.org/10.1128/jcm.33.12.3129-3132.1995

166. Mutai, B.K., Wainaina, J.M., Magiri, C.G., Nganga, J.K., Ithondeka, P.M., Njagi, O.N., Jiang, J., Richards, A.L., Waitumbi, J.N., 2013. Zoonotic surveillance for rickettsiae in domestic animals in Kenya. Vector-Borne Zoonotic Dis. 13. https://doi.org/10.1089/vbz.2012.0977

167. Naing L, Winn T, R.B.., 2006. Practical Issues in Calculating the Sample Size for

Prevalence Studies. Arch. Orofac. Sci. 1, 9–14.

168. Narasimhan, S., Fikrig, E., 2015. Tick microbiome: The force within. Trends Parasitol. https://doi.org/10.1016/j.pt.2015.03.010

169. Ndip, L.M., Labruna, M., Ndip, R.N., Walker, D.H., McBride, J.W., 2009. Molecular and clinical evidence of Ehrlichia chaffeensis infection in Cameroonian patients with undifferentiated febrile illness. Ann. Trop. Med. Parasitol. 103, 719–725. https://doi.org/10.1179/000349809X12554106963753

170. Ndip, L.M., Ndip, R.N., Esemu, S.N., Walker, D.H., McBride, J.W., 2010. Predominance of Ehrlichia chaffeensis in Rhipicephalus sanguineus ticks from kennel-confined dogs in Limbe, Cameroon. Exp. Appl. Acarol. https://doi.org/10.1007/s10493-009-9293-8

171. Ndip, L.M., Ndip, R.N., Ndive, V.E., Awuh, J.A., Walker, D.H., McBride, J.W., 2007. Ehrlichia species in Rhipicephalus sanguineus ticks in Cameroon. Vector-Borne Zoonotic Dis. 7, 221–227. https://doi.org/10.1089/vbz.2006.0608

172. Ngeranwa, J.J.N., Shompole, S.P., Venter, E.H., Wambugu, A., Crafford, J.E., Penzhorn, B.L., 2008. Detection of anaplasma antibodies in wildlife and domestic species in wildlife-livestock interface areas of Kenya by major surface protein 5 competitive inhibition enzyme-linked immunosorbent assay. Onderstepoort J. Vet. Res. 75, 199–205. https://doi.org/10.4102/ojvr.v75i3.95

173. Nilsson, A.C., Björkman, P., Welinder-Olsson, C., Widell, A., Persson, K., 2010. Clinical severity of Mycoplasma pneumoniae (MP) infection is associated with bacterial load in oropharyngeal secretions but not with MP genotype. BMC Infect. Dis. 10, 39. https://doi.org/10.1186/1471-2334-10-39

174. Njeru, J., Henning, K., Pletz, M.W., Heller, R., Neubauer, H., 2016. Q fever is an old and neglected zoonotic disease in Kenya: A systematic review. BMC Public Health. https://doi.org/10.1186/s12889-016-2929-9

175. Njiiri, N.E., Bronsvoort, B.M. de C., Collins, N.E., Steyn, H.C., Troskie, M., Vorster, I., Thumbi, S.M., Sibeko, K.P., Jennings, A., van Wyk, I.C., Mbole-Kariuki, M., Kiara, H., Poole, E.J., Hanotte, O., Coetzer, K., Oosthuizen, M.C., Woolhouse, M., Toye, P., 2015. The epidemiology of tick-borne haemoparasites as determined by the reverse line blot hybridization assay in an intensively studied cohort of calves in western Kenya. Vet.

Parasitol. 210, 69–76. https://doi.org/10.1016/j.vetpar.2015.02.020

176. Nyaoke, B.A., Mureithi, M.W., Beynon, C., 2019. Factors associated with treatment type of non-malarial febrile illnesses in under-fives at Kenyatta National Hospital in Nairobi, Kenya. PLoS One 14, 1–16. https://doi.org/10.1371/journal.pone.0217980

177. Nyataya, J., Maraka, M., Lemtudo, A., Masakhwe, C., Mutai, B., Njaanake, K., Estambale, B.B., Nyakoe, N., Siangla, J., Waitumbi, J.N., 2020. Serological Evidence of Yersiniosis, Tick-Borne Encephalitis, West Nile, Hepatitis E, Crimean-Congo Hemorrhagic Fever, Lyme Borreliosis, and Brucellosis in Febrile Patients Presenting at Diverse Hospitals in Kenya. Vector-Borne Zoonotic Dis. 20, 348–357. https://doi.org/10.1089/vbz.2019.2484

178. Oechslin, C.P., Heutschi, D., Lenz, N., Tischhauser, W., Péter, O., Rais, O., Beuret, C.M., Leib, S.L., Bankoul, S., Ackermann-Gäumann, R., 2017. Prevalence of tick-borne pathogens in questing Ixodes ricinus ticks in urban and suburban areas of Switzerland. Parasites and Vectors 10. https://doi.org/10.1186/s13071-017-2500-2

179. Oguntomole, O., Nwaeze, U., Eremeeva, M., 2018. Tick-, Flea-, and Louse-Borne Diseases of Public Health and Veterinary Significance in Nigeria. Trop. Med. Infect. Dis. 3, 3. https://doi.org/10.3390/tropicalmed3010003

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

181. Oksanen, J., Blanchet, F.G., Kindt, R., Legendre, P., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H. and Wagner, H., 2014. Vegan: Community Ecology Package. R Package Version 2.2-0. [WWW Document].

182. Omondi, D., Masiga, D.K., Fielding, B.C., Kariuki, E., Ajamma, Y.U., Mwamuye, M.M., Ouso, D.O., Villinger, J., 2017. Molecular Detection of Tick-Borne Pathogen Diversities in Ticks from Livestock and Reptiles along the Shores and Adjacent Islands of Lake Victoria and Lake Baringo, Kenya. Front. Vet. Sci. 4. https://doi.org/10.3389/fvets.2017.00073

183. Oswe, M., Odhiambo, R., Mutai, B., Nyakoe, N., Awinda, G., Waitumbi, J.N., 2018. Zoonotic Pathogens in Ticks Collected from Livestock in Kenya. Open J. Prev. Med. 08, 248–259. https://doi.org/10.4236/ojpm.2018.88021

184. Ottichilo, W.K., Grunblatt, J., Said, M.Y., Wargute, P.W., 2000. Wildlife and Livestock Population Trends in the Kenya Rangeland, in: Wildlife Conservation by Sustainable Use. pp. 203–218. https://doi.org/10.1007/978-94-011-4012-6_10

185. Parola, P., Raoult, D., 2001a. Ticks and tickborne bacterial diseases in humans: an emerging infectious threat. Clin. Infect. Dis. 32, 897–928. https://doi.org/10.1086/319347

186. Parola, P., Raoult, D., 2001b. [Molecular tools in the epidemiology of tick-borne bacterial diseases]. Ann. Biol. Clin. (Paris). 59, 177–182.

187. Perveen, N., Muzaffar, S. Bin, Vijayan, R., Al-Deeb, M.A., 2020. Microbial communities associated with the camel tick, Hyalomma dromedarii: 16S rRNA gene-based analysis. Sci. Rep. 10, 1–11. https://doi.org/10.1038/s41598-020-74116-7

188. Prabhu, M., Nicholson, W.L., Roche, A.J., Kersh, G.J., Fitzpatrick, K.A., Oliver, L.D., Massung, R.F., Morrissey, A.B., Bartlett, J.A., Onyango, J.J., Maro, V.P., Kinabo, G.D., Saganda, W., Crump, J.A., 2011. Q fever, spotted fever group, and typhus group rickettsioses among hospitalized febrile patients in Northern Tanzania. Clin. Infect. Dis. 53. https://doi.org/10.1093/cid/cir411

189. Prachayangprecha, S., Schapendonk, C.M.E., Koopmans, M.P., Osterhaus, A.D.M.E., Schürch, A.C., Pas, S.D., Van Der Eijk, A.A., Poovorawan, Y., Haagmans, B.L., Smits, S.L., 2014. Exploring the potential of next-generation sequencing in detection of respiratory Viruses. J. Clin. Microbiol. 52, 3722–3730. https://doi.org/10.1128/JCM.01641-14

190. Prasad, N., Murdoch, D.R., Reyburn, H., Crump, J.A., 2015. Etiology of severe febrile illness in low- and middle-income countries: A systematic review. PLoS One. https://doi.org/10.1371/journal.pone.0127962

191. Pretorius, A.M., Kelly, P.J., 1998. Serological survey for antibodies reactive with Ehrlichia canis and E. chaffeensis in dogs from the Bloemfontein area, South Africa. J. S. Afr. Vet. Assoc. https://doi.org/10.4102/jsava.v69i4.840

192. Price, J.E., Karstad, L.H., 1980. Free-living jackals (Canis mesomelas)-potential reservoir hosts for Ehrlichia canis in Kenya. J. Wildl. Dis. https://doi.org/10.7589/0090-3558-16.4.469

193. Pripuzova, N., Wang, R., Tsai, S., Li, B., Hung, G.C., Ptak, R.G., Lo, S.C., 2012. Development of real-time PCR array for simultaneous detection of eight human blood-

borne viral pathogens. PLoS One 7, e43246. https://doi.org/10.1371/journal.pone.0043246

194. Puligheddu, M., Giagheddu, A., Genugu, F., Giagheddu, M., Marrosu, F., 2004. Epilepsia partialis continua in cat scratch disease. Seizure 13, 191–195. https://doi.org/10.1016/S1059-1311(03)00159-6

195. Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner, F.O., 2013. The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. Nucleic Acids Res. 41. https://doi.org/10.1093/nar/gks1219

196. R Core Team, 2018. R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing; 2018. R Foundation for Statistical Computing. R Found. Stat. Comput. Vienna, Austria. https://doi.org/ISBN 3-900051-07-0

197. Rabozzi, G., Bonizzi, L., Crespi, E., Somaruga, C., Sokooti, M., Tabibi, R., Vellere, F., Brambilla, G., Colosio, C., 2012. Emerging zoonoses: The "one health approach." Saf. Health Work. https://doi.org/10.5491/SHAW.2012.3.1.77

198. Radzijevskaja, J., Paulauskas, A., Rosef, O., 2008. Prevalence of Anaplasma phagocytophilum and Babesia divergens in Ixodes ricinus ticks from Lithuania and Norway. Int. J. Med. Microbiol. 298, 218–221. https://doi.org/10.1016/j.ijmm.2008.01.008

199. Raileanu, C., Moutailler, S., Pavel, I., Porea, D., Mihalca, A.D., Savuta, G., Vayssier-Taussat, M., 2017. Borrelia Diversity and Co-infection with Other Tick Borne Pathogens in Ticks. Front. Cell. Infect. Microbiol. 7. https://doi.org/10.3389/fcimb.2017.00036

200. Ramos, R., Ramos, C., Araújo, F., Oliveira, R., Souza, I., Pimentel, D., Galindo, M., Santana, M., Rosas, E., Faustino, M., Alves, L., 2010. Molecular survey and genetic characterization of tick-borne pathogens in dogs in metropolitan Recife (north-eastern Brazil). Parasitol. Res. https://doi.org/10.1007/s00436-010-1979-7

201. Regier, Y., Komma, K., Weigel, M., Kraiczy, P., Laisi, A., Pulliainen, A.T., Hain, T., Kempf, V.A.J., 2019. Combination of microbiome analysis and serodiagnostics to assess the risk of pathogen transmission by ticks to humans and animals in central Germany 11 Medical and Health Sciences 1108 Medical Microbiology. Parasites and Vectors 12, 1–16. https://doi.org/10.1186/s13071-018-3240-7

202. Reif, K.E., Stout, R.W., Henry, G.C., Foil, L.D., Macaluso, K.R., 2008. Prevalence and infection load dynamics of Rickettsia felis in actively feeding cat fleas. PLoS One 3. https://doi.org/10.1371/journal.pone.0002805

203. Richards, A. L., Malone, J. D., Sheris, S., Weddle, J. R., Rossi, C. A., Ksiazek, T. G., LeDuc, J. W., Dasch, G. A., Hyams, K. C., Richards, A. L., Malone, J. D., Sheris, S., Weddle, J. R., Rossi, C. A., Ksiazek, T. G., LeDuc, J. W., Dasch, G. A., Hyams, J.W., 1993. Arbovirus and Rickettsial Infections among Combat Troops during Operation Desert Shield/Desert Storm. J. Infect. Dis. 168, 1080–1081.

204. Richards, A.L., Jiang, J., Omulo, S., Dare, R., Abdirahman, K., Ali, A., Sharif, S.K., Feikin, D.R., Breiman, R.F., Njenga, M.K., 2010a. Human infection with Rickettsia felis, Kenya. Emerg. Infect. Dis. 16, 1081–1086. https://doi.org/10.3201/eid1607.091885

205. Richards, A.L., Jiang, J., Omulo, S., Dare, R., Abdirahman, K., Ali, A., Sharif, S.K., Feikin, D.R., Breiman, R.F., Njenga, M.K., 2010b. Human infection with Rickettsia felis, Kenya. Emerg. Infect. Dis. https://doi.org/10.3201/eid1607.091885

206. Roberts, B.D., 1996. Livestock production, age, and gender among the Keiyo of Kenya. Hum. Ecol. 24, 215–230. https://doi.org/10.1007/BF02169127

207. Rochlin, I., Toledo, A., 2020. Emerging tick-borne pathogens of public health importance: A mini-review. J. Med. Microbiol. 69, 781–791. https://doi.org/10.1099/jmm.0.001206

208. Ross, B.D., Hayes, B., Radey, M.C., Lee, X., Josek, T., Bjork, J., Neitzel, D., Paskewitz, S., Chou, S., Mougous, J.D., 2018. Ixodes scapularis does not harbor a stable midgut microbiome. ISME J. 12, 2596–2607. https://doi.org/10.1038/s41396-018-0161-6

209. Ruiling, Z., Zhendong, H., Guangfu, Y., Zhong, Z., 2019. Characterization of the bacterial community in Haemaphysalis longicornis (Acari: Ixodidae) throughout developmental stages. Exp. Appl. Acarol. https://doi.org/10.1007/s10493-019-00339-7

210. Rupprecht, T.A., Koedel, U., Fingerle, V., Pfister, H.W., 2008. The pathogenesis of lyme neuroborreliosis: From infection to inflammation. Mol. Med. 14, 205–212. https://doi.org/10.2119/2007-00091.Rupprecht

211. Rutherford, J.S., Macaluso, K.R., Smith, N., Zaki, S.R., Paddock, C.D., Davis, J., Peterson, N., Azad, A.F., Rosenberg, R., 2004. Fatal Spotted Fever Rickettsiosis, Kenya. Emerg. Infect. Dis. https://doi.org/10.3201/eid1005.030537

212. Saldaña, M.A., Hegde, S., Hughes, G.L., 2017. Microbial control of arthropod-borne disease. Mem. Inst. Oswaldo Cruz 112, 81–93. https://doi.org/10.1590/0074-02760160373

213. Santajit, S., Indrawattana, N., 2016. Mechanisms of Antimicrobial Resistance in ESKAPE Pathogens. Biomed Res. Int. https://doi.org/10.1155/2016/2475067

214. Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski, R.A., Oakley, B.B., Parks, D.H., Robinson, C.J., Sahl, J.W., Stres, B., Thallinger, G.G., Van Horn, D.J., Weber, C.F., 2009. Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl. Environ. Microbiol. 75, 7537–7541. https://doi.org/10.1128/AEM.01541-09

215. Schneeberger, P.M., Hermans, M.H.A., Van Hannen, E.J., Schellekens, J.J.A., Leenders, A.C.A.P., Wever, P.C., 2010. Real-time PCR with serum samples is indispensable for early diagnosis of acute Q fever. Clin. Vaccine Immunol. 17, 286–290. https://doi.org/10.1128/CVI.00454-09

216. Schnittger, L., Rodriguez, A.E., Florin-Christensen, M., Morrison, D.A., 2012. Babesia: A world emerging. Infect. Genet. Evol. 12, 1788–1809. https://doi.org/10.1016/j.meegid.2012.07.004

217. Schwan, T.G., Raffel, S.J., 2021. Transovarial transmission of borrelia hermsii by its tick vector and reservoir host ornithodoros hermsi. Microorganisms 9. https://doi.org/10.3390/microorganisms9091978

218. Segura, E.T., de Juan, A., 1966. Electroencephalographic studies in toads. Electroencephalogr. Clin. Neurophysiol. 21, 373–380. https://doi.org/10.1016/0013-4694(66)90043-5

219. Segura, J.A., Isaza, J.P., Botero, L.E., Alzate, J.F., Gutiérrez, L.A., 2020. Assessment of bacterial diversity of Rhipicephalus microplus ticks from two livestock agroecosystems in Antioquia, Colombia. PLoS One 15, 1–18. https://doi.org/10.1371/journal.pone.0234005

220. Seitz, R., 2014. Coxiella burnetii - Pathogenic Agent of Q (Query) Fever. Transfus. Med. Hemotherapy 41, 60–72. https://doi.org/10.1159/000357107

221. Seshadri, R., Paulsen, I.T., Eisen, J.A., Read, T.D., Nelson, K.E., Nelson, W.C.,

Ward, N.L., Tettelin, H., Davidsen, T.M., Beanan, M.J., Deboy, R.T., Daugherty, S.C., Brinkac, L.M., Madupu, R., Dodson, R.J., Khouri, H.M., Lee, K.H., Carty, H.A., Scanlan, D., Heinzen, R.A., Thompson, H.A., Samuel, J.E., Fraser, C.M., Heidelberg, J.F., 2003. Complete genome sequence of the Q-fever pathogen Coxiella burnetii. Proc Natl Acad Sci U S A 100, 5455–5460. https://doi.org/10.1073/pnas.0931379100

222. Shannon, C.E., 1948. A Mathematical Theory of Communication. Bell Syst. Tech. J. 27, 623–656. https://doi.org/10.1002/j.1538-7305.1948.tb00917.x

223. Shillcutt, S., Morel, C., Goodman, C., Coleman, P., Bell, D., Whitty, C.J.M., Mills, A., 2008. Cost-effectiveness of malaria diagnostic methods in sub-Saharan Africa in an era of combination therapy. Bull. World Health Organ. https://doi.org/10.2471/BLT.07.042259

224. Shivlata, L., Satyanarayana, T., 2015. Thermophilic and alkaliphilic Actinobacteria: Biology and potential applications. Front. Microbiol. 6, 1–29. https://doi.org/10.3389/fmicb.2015.01014

225. Smith, T.A., Driscoll, T., Gillespie, J.J., Raghavan, R., 2015. A Coxiella-like endosymbiontis a potential vitamin source for the lone star tick. Genome Biol. Evol. 7, 831–838. https://doi.org/10.1093/gbe/evv016

226. Socolovschi, C., Bitam, I., Raoult, D., Parola, P., 2009a. Transmission of Rickettsia conorii conorii in naturally infected Rhipicephalus sanguineus. Clin. Microbiol. Infect. 15, 319–321. https://doi.org/10.1111/j.1469-0691.2008.02257.x

227. Socolovschi, C., Honnorat, E., Consigny, P.H., Dougados, J., Passeron, A., Parola, P., Raoult, D., 2012. Tick-borne relapsing fever with cutaneous eschar and radiculopathy, ethiopia. J. Travel Med. https://doi.org/10.1111/j.1708-8305.2012.00625.x

228. Socolovschi, C., Huynh, T.P., Davoust, B., Gomez, J., Raoult, D., Parola, P., 2009b. Transovarial and trans-stadial transmission of Rickettsiae africae in Amblyomma variegatum ticks. Clin. Microbiol. Infect. 15, 317–318. https://doi.org/10.1111/j.1469-0691.2008.02278.x

229. Socolovschi, Cristina, Mediannikov, O., Raoult, D., Parola, P., 2009. The relationship between spotted fever group rickettsiae and ixodid ticks. Vet. Res. 40. https://doi.org/10.1051/vetres/2009017

230. Sonenshine, D.E., 1992. Biology of ticks., 1st ed. Oxford University press.

231. Sparagano, O., George, D., Giangaspero, A., Špitalská, E., 2015. Arthropods and associated arthropod-borne diseases transmitted by migrating birds. The case of ticks and tick-borne pathogens. Vet. Parasitol. 213, 61–66. https://doi.org/10.1016/j.vetpar.2015.08.028

232. Špitalská, E., Sparagano, O., Stanko, M., Schwarzová, K., Špitalský, Z., Škultéty, Ľ., Havlíková, S.F., 2018. Diversity of Coxiella-like and Francisella-like endosymbionts, and Rickettsia spp., Coxiella burnetii as pathogens in the tick populations of Slovakia, Central Europe. Ticks Tick. Borne. Dis. 9, 1207–1211. https://doi.org/10.1016/j.ttbdis.2018.05.002

233. Srinivasan, R., Karaoz, U., Volegova, M., MacKichan, J., Kato-Maeda, M., Miller, S., Nadarajan, R., Brodie, E.L., Lynch, S. V., 2015. Use of 16S rRNA gene for identification of a broad range of clinically relevant bacterial pathogens. PLoS One. https://doi.org/10.1371/journal.pone.0117617

234. Stackebrandt, E., Murray, R.G.E., Truper, H.G., 1988. Proteobacteria classis nov., a name for the phylogenetic taxon that includes the "purple bacteria and their relatives." Int. J. Syst. Bacteriol. 38, 321–325. https://doi.org/10.1099/00207713-38-3-321

235. Staroscik, A., 2004. Calculator for determining the number of copies of a template [WWW Document]. URL http://cels.uri.edu/gsc/cndna.html (accessed 4.9.18).

236. Steinmann, P., Bonfoh, B., Péter, O., Schelling, E., Traoré, M., Zinsstag, J., 2005. Seroprevalence of Q-fever in febrile individuals in Mali. Trop. Med. Int. Heal. https://doi.org/10.1111/j.1365-3156.2005.01420.x

237. Stek, C.J., Van Eijk, J.J.J., Jacobs, B.C., Enting, R.H., Sprenger, H.G., Van Alfen, N., Van Assen, S., 2011. Neuralgic amyotrophy associated with Bartonella henselae infection. J. Neurol. Neurosurg. Psychiatry. https://doi.org/10.1136/jnnp.2009.191940

238. Stone, B.L., Tourand, Y., Brissette, C.A., 2017. Brave New Worlds: The Expanding Universe of Lyme Disease. Vector-Borne Zoonotic Dis. https://doi.org/10.1089/vbz.2017.2127

239. Swanson, S.J., Neitzel, D., Reed, K.D., Belongia, E.A., 2006. Coinfections acquired from Ixodes ticks. Clin. Microbiol. Rev. https://doi.org/10.1128/CMR.00011-06

240. Tabara, K., Arai, S., Kawabuchi, T., Itagaki, A., Ishihara, C., Satoh, H., Okabe, N., Tsuji, M., 2007. Molecular survey of Babesia microti, Ehrlichia species and Candidatus

neoehrlichia mikurensis in wild rodents from Shimane Prefecture, Japan. Microbiol. Immunol. 51. https://doi.org/10.1111/j.1348-0421.2007.tb03923.x

241. Tasher, D., Raucher-Sternfeld, A., Tamir, A., Giladi, M., Somekh, E., 2017. Bartonella quintana, an unrecognized cause of infective endocarditis in children in Ethiopia. Emerg. Infect. Dis. 23, 1246–1252. https://doi.org/10.3201/eid2308.161037

242. Taylor, M., Mediannikov, O., Raoult, D., Greub, G., 2012. Endosymbiotic bacteria associated with nematodes, ticks and amoebae. FEMS Immunol. Med. Microbiol. https://doi.org/10.1111/j.1574-695X.2011.00916.x

243. Teshale, S., Geysen, D., Ameni, G., Dorny, P., Berkvens, D., 2018. Survey of Anaplasma phagocytophilum and Anaplasma sp. "Omatjenne" infection in cattle in Africa with special reference to Ethiopia. Parasites and Vectors 11. https://doi.org/10.1186/s13071-018-2633-y

244. Thapa, S., Zhang, Y., Allen, M.S., 2019. Bacterial microbiomes of Ixodes scapularis ticks collected from Massachusetts and Texas, USA. BMC Microbiol. 19, 1–12. https://doi.org/10.1186/s12866-019-1514-7

245. Thiga, J.W., Mutai, B.K., Eyako, W.K., Ng'Ang'A, Z., Jiang, J., Richards, A.L., Waitumbi, J.N., 2015. High seroprevalence of antibodies against spotted fever and scrub typhus bacteria in patients with febrile illness, Kenya. Emerg. Infect. Dis. 21. https://doi.org/10.3201/eid2104.141387

246. Thomas, F., Hehemann, J.H., Rebuffet, E., Czjzek, M., Michel, G., 2011. Environmental and gut Bacteroidetes: The food connection. Front. Microbiol. 2, 1–16. https://doi.org/10.3389/fmicb.2011.00093

247. Thorburn, F., Bennett, S., Modha, S., Murdoch, D., Gunson, R., Murcia, P.R., 2015. The use of next generation sequencing in the diagnosis and typing of respiratory infections. J. Clin. Virol. 69, 96–100. https://doi.org/10.1016/j.jcv.2015.06.082

248. Trout Fryxell, R.T., DeBruyn, J.M., 2016. The microbiome of Ehrlichia-infected and uninfected lone star ticks (Amblyomma americanum). PLoS One 11, 1–19. https://doi.org/10.1371/journal.pone.0146651

249. Tzianabos, T., Anderson, B.E., McDade, J.E., 1989. Detection of Rickettsia rickettsii DNA in clinical specimens by using polymerase chain reaction technology. J. Clin. Microbiol. https://doi.org/10.1128/jcm.27.12.2866-2868.1989

250. Uhaa, I.J., MacLean, J.D., Greene, C.R., Fishbein, D.B., 1992. A case of human ehrlichiosis acquired in Mali: Clinical and laboratory findings. Am. J. Trop. Med. Hyg. https://doi.org/10.4269/ajtmh.1992.46.161

251. van Eekeren, L.E., de Vries, S.G., Wagenaar, J.F.P., Spijker, R., Grobusch, M.P., Goorhuis, A., 2018. Under-diagnosis of rickettsial disease in clinical practice: A systematic review. Travel Med. Infect. Dis. https://doi.org/10.1016/j.tmaid.2018.02.006

252. van Treuren, W., Ponnusamy, L., Brinkerhoff, R.J., Gonzalez, A., Parobek, C.M., Juliano, J.J., Andreadis, T.G., Falco, R.C., Ziegler, L.B., Hathaway, N., Keeler, C., Emch, M., Bailey, J.A., Roe, R.M., Apperson, C.S., Knight, R., Meshnick, S.R., 2015. Variation in the microbiota of Ixodes ticks with regard to geography, species, and sex. Appl. Environ. Microbiol. 81, 6200–6209. https://doi.org/10.1128/AEM.01562-15

253. Vannier E, Gewurz B, E.K.P.J., 2008. Human babesiosis. Infect. Dis. Clin. North Am. 22.

254. Varela-Stokes, A.S., Park, S.H., Kim, S.A., Ricke, S.C., 2017. Microbial communities in North American Ixodid ticks of veterinary and medical importance. Front. Vet. Sci. 4, 1–7. https://doi.org/10.3389/fvets.2017.00179

255. Walker, D.H., 2007. Rickettsiae and rickettsial infections: The current state of knowledge. Clin. Infect. Dis. https://doi.org/10.1086/518145

256. Walker DH, D.J., 1996. Emergence of ehrlichioses as human health problems. Emerg Infect Dis 2, 18–19.

257. Wallich, R., Moter, S.E., Simon, M.M., Ebnet, K., Heiberger, A., Kramer, M.D., 1990. The Borrelia burgdorferi Flagellum-associated 41-kilodalton antigen (Flagellin): Molecular cloning, expression, and amplification of the gene. Infect. Immun. 58, 1711–1719.

258. WALTON, G.A., 1962. The Ornithodorus moubata superspecies problem in relation to human relapsing fever epidemiology. Symp. Zool. Soc. London 83–156.

259. Wang, H.-Y., Kim, S., Kim, J., Park, S.-D., Uh, Y., Lee, H., 2014. Multiplex real-time PCR assay for rapid detection of methicillin-resistant staphylococci directly from positive blood cultures. J. Clin. Microbiol. 52, 1911–20. https://doi.org/10.1128/JCM.00389-14

260. Wang, S., Hua, X., Cui, L., 2021. Characterization of microbiota diversity of

engorged ticks collected from dogs in China. J. Vet. Sci. 22, 1–14. https://doi.org/10.4142/JVS.2021.22.E37

261. Webb.; Carl;, Azad, M.D., 1990. Detection of murine typhus infection in fleas by using the polymerase chain reaction. J Clin Microbiol 530–534.

262. Weiss, S., Xu, Z., Amir, A., Peddada, S., Bittinger, K., Gonzalez, A., Lozupone, C., Zaneveld, J., Vazquez-Baeza, Y., Birmingham, A., Knight, R., 2015. Effects of library size variance, sparsity, and compositionality on the analysis of microbiome data. PeerJ Prepr. https://doi.org/10.7287/peerj.preprints.1157

263. Weiss, S., Xu, Z.Z., Peddada, S., Amir, A., Bittinger, K., Gonzalez, A., Lozupone, C., Zaneveld, J.R., Vázquez-Baeza, Y., Birmingham, A., Hyde, E.R., Knight, R., 2017. Normalization and microbial differential abundance strategies depend upon data characteristics. Microbiome 5, 1–18. https://doi.org/10.1186/s40168-017-0237-y

264. Werther, K., Luzzi, M. de C., Gonçalves, L.R., de Oliveira, J.P., Alves Junior, J.R.F., Machado, R.Z., André, M.R., 2017. Arthropod-borne agents in wild Orinoco geese (Neochen jubata) in Brazil. Comp. Immunol. Microbiol. Infect. Dis. 55, 30–41. https://doi.org/10.1016/j.cimid.2017.09.003

265. Wesonga, F., Wesongah, J., Chemuliti, J., Wanjala, K., Munga, L., Gitau, P., 2006. Seroprevalence of *Ehrlichia ruminantium* (heartwater) in small ruminants in a pastoral production system in Narok district, Kenya. Bull. Anim. Heal. Prod. Africa. https://doi.org/10.4314/bahpa.v54i1.32727

266. Westblade, L.F., Simon, M.S., Mathison, B.A., Kirkman, L.A., 2017. crossm Babesia microti : from Mice to Ticks to an 55, 2903–2912.

267. Wheeler, D.L., Church, D.M., Edgar, R., Federhen, S., Helmberg, W., Madden, T.L., Pontius, J.U., Schuler, G.D., Schriml, L.M., Sequeira, E., Suzek, T.O., Tatusova, T.A., Wagner, L., 2004. Database resources of the National Center for Biotechnology Information: update. Nucleic Acids Res. 32, D35-40. https://doi.org/10.1093/nar/gkh073

268. Whitty, C.J.M., Chandler, C., Ansah, E., Leslie, T., Staedke, S.G., 2008. Deployment of ACT antimalarials for treatment of malaria: Challenges and opportunities. Malar. J. https://doi.org/10.1186/1475-2875-7-S1-S7

269. WHO, 2011. Non-Malarial Febrile Illness Study Final Review Workshop Cambodia and Lao People's Democratic Republic, Vientiane, Lao People's Democratic

Republic, 12-13 January 2011 : report [WWW Document].

270. Wickham, H., 2009. ggplot2: elegant graphics for data analysis. . New York: Springer-Verlag.

271. Wilson, E.R., Smith, K.J., 2014. Lyme disease Lyme Disease : ecology , epidemiology and prevention.

272. Winand, R., Bogaerts, B., Hoffman, S., Lefevre, L., Delvoye, M., Van Braekel, J., Fu, Q., Roosens, N.H.C., De Keersmaecker, S.C.J., Vanneste, K., 2020. Targeting the 16s rRNA gene for bacterial identification in complex mixed samples: Comparative evaluation of second (illumina) and third (oxford nanopore technologies) generation sequencing technologies. Int. J. Mol. Sci. 21, 1–22. https://doi.org/10.3390/ijms21010298

273. Wu-Chuang, A., Hodžić, A., Mateos-Hernández, L., Estrada-Peña, A., Obregon, D., Cabezas-Cruz, A., 2021. Current debates and advances in tick microbiome research. Curr. Res. Parasitol. Vector-Borne Dis. 1, 100036. https://doi.org/10.1016/j.crpvbd.2021.100036

274. Young, K.M., Corrin, T., Wilhelm, B., Uhland, C., Greig, J., Mascarenhas, M., Waddell, L.A., 2019. Zoonotic Babesia: A scoping review of the global evidence, PLoS ONE. https://doi.org/10.1371/journal.pone.0226781

275. Yu, D., Chen, Y., Wu, S., Wang, B., Tang, Y.W., Li, L., 2012. Simultaneous Detection and Differentiation of Human Papillomavirus Genotypes 6, 11, 16 and 18 by AllGlo Quadruplex Quantitative PCR. PLoS One 7. https://doi.org/10.1371/journal.pone.0048972

276. Zerfu, B., Medhin, G., Mamo, G., Getahun, G., Tschopp, R., Legesse, M., 2018. Community-based prevalence of typhoid fever, typhus, brucellosis and malaria among symptomatic individuals in Afar Region, Ethiopia. PLoS Negl. Trop. Dis. https://doi.org/10.1371/journal.pntd.0006749

277. Zhang, Y.K., Yu, Z.J., Wang, D., Bronislava, V., Branislav, P., Liu, J.Z., 2019. The bacterial microbiome of field-collected Dermacentor marginatus and Dermacentor reticulatus from Slovakia. Parasites and Vectors 12, 1–10. https://doi.org/10.1186/s13071-019-3582-9

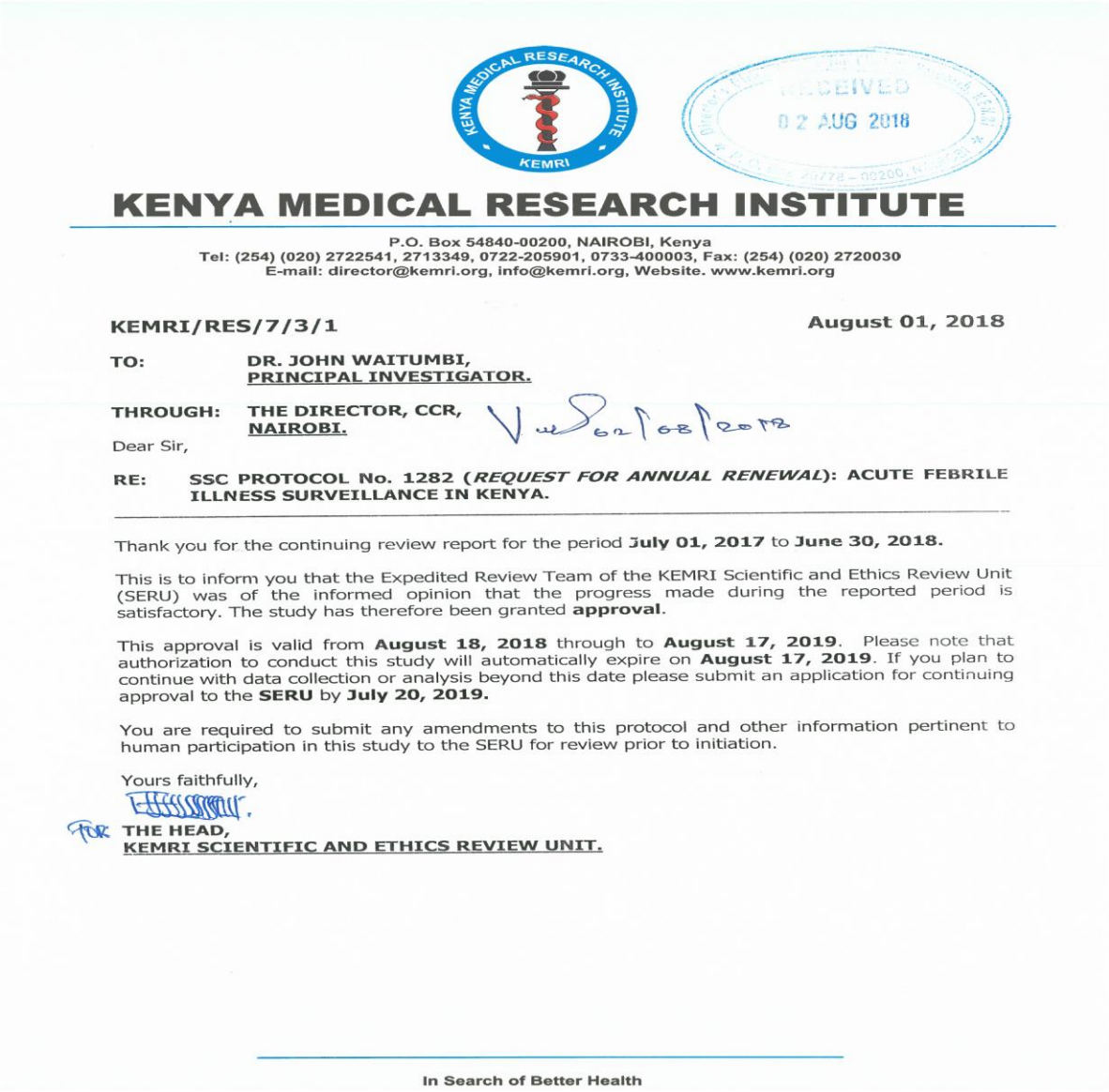
278. Zhou, X., Xia, S., Huang, J.L., Tambo, E., Zhuge, H.X., Zhou, X.N., 2014. Human babesiosis, an emerging tick-borne disease in the people's republic of China. Parasites and

Vectors 7, 1–10. https://doi.org/10.1186/s13071-014-0509-3

279. Zintl, A., Mulcahy, G., Skerrett, H.E., Taylor, S.M., Gray, J.S., 2003. Babesia divergens, a Bovine Blood Parasite of Veterinary and Zoonotic Importance. Clin. Microbiol. Rev. https://doi.org/10.1128/CMR.16.4.622-636.2003

7 CHAPTER 7: Appendices

7.1 Appendix 1: The Kenya Medical Research Institute, Nairobi (KEMRI SERU/SSC # 1282) and The Walter Reed Army Research Institute human use research committee, Silver Spring, Maryland USA (WRAIR # 1402)



KENYA MEDICAL RESEARCH INSTITUTE

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

RECEIVED
02 AUG 2018

KEMRI/RES/7/3/1 August 01, 2018

TO: DR. JOHN WAITUMBI,
PRINCIPAL INVESTIGATOR.

THROUGH: THE DIRECTOR, CCR,
NAIROBI.

Dear Sir,

RE: SSC PROTOCOL No. 1282 (*REQUEST FOR ANNUAL RENEWAL*): ACUTE FEBRILE ILLNESS SURVEILLANCE IN KENYA.

Thank you for the continuing review report for the period **July 01, 2017** to **June 30, 2018.**

This is to inform you that the Expedited Review Team of the KEMRI Scientific and Ethics Review Unit (SERU) was of the informed opinion that the progress made during the reported period is satisfactory. The study has therefore been granted **approval**.

This approval is valid from **August 18, 2018** through to **August 17, 2019**. Please note that authorization to conduct this study will automatically expire on **August 17, 2019**. If you plan to continue with data collection or analysis beyond this date please submit an application for continuing approval to the **SERU** by **July 20, 2019.**

You are required to submit any amendments to this protocol and other information pertinent to human participation in this study to the SERU for review prior to initiation.

Yours faithfully,

For THE HEAD,
KEMRI SCIENTIFIC AND ETHICS REVIEW UNIT.

In Search of Better Health

MCMR-UWZ-C 3 January 2018

MEMORANDUM FOR Director, Human Subjects Protection Branch (HSPB), Walter Reed Army Institute of Research (WRAIR), 503 Robert Grant Avenue, Silver Spring, Maryland 20910-7500

SUBJECT: Continuing Review Report Acceptance for the Minimal Risk Human Subjects Research Protocol, **WRAIR #1402,** HRPO Log Number A-14327.2

1. The continuing review report, dated 17 November 2017, for the protocol, **WRAIR #1402,** HRPO Log Number A-14327.2, entitled, "Acute Febrile Illness Surveillance in Kenya," (Version 20.7, dated 15 September 2016), submitted by John Waitumbi, DVM, PhD, Laboratory Director, Kondele Research Unit, United States Army Medical Research Unit—Kenya (USAMRU-K), is accepted.

2. The continuing review report covers the reporting period from 3 November 2016 to 31 October 2017. The enrollment for this study is ongoing.

3. As this is a minimal risk protocol, the continuing review report was reviewed by expedited review procedures according to 32 CFR 219.110. In addition, 45 CFR 46.404 continues to apply to this study as research not involving greater than minimal risk to children. Pregnant women may be enrolled and blood collected by venipuncture; therefore, 45 CFR 46.204 also applies. This study continues to meet the requirements for approval under 32 CFR 219.111.

4. The study is sponsored by WRAIR and funded by the Department of Defense Global Emerging Infections Surveillance and Response System (GEIS).

5. The Kenya Medical Research Institute (KEMRI) Scientific and Ethics Review Unit (SERU) reviewed and approved the continuation of this protocol on 14 August 2017 with an expiration date of 18 August 2018.

6. The following documents are approved for continuation:

a. Continuing Review Report, dated 17 November 2017;
b. Protocol, version 20.7, dated 15 September 2016;
c. Informed Consent, version 20.8, dated 18 October 2016;
d. Assent Form, version 20.8, dated 18 October 2016; and
e. AR Clinical Data Sheet, version 20.7, dated 15 September 2016.

7. Per the current WRAIR Policy #11-49, "Initial and Continuing Human Subjects Protection Education and Training Requirements", an 80% grade on each individual module must be obtained. The Principal investigator is responsible for ensuring each research team member's, to include those listed on the protocol, as well as those who are not explicitly listed but may be providing study/laboratory support, human subjects protection training is current. Additionally, the PI must maintain records of documentation of this training (i.e., a staff log and training files).

8. The expiration date of this study at the WRAIR is **9 January 2019.** A closeout report is due on **9 January 2023**. No changes, amendments, or addenda may be made to the protocol

MCMR-UWZ-C
SUBJECT: Continuing Review Report Acceptance for the Minimal Risk Human Subjects Research Protocol, **WRAIR #1402,** HRPO Log Number A-14327.2

without prior review and approval by the WRAIR IRB, the KEMRI SERU, and the USAMRMC Office of Research (ORP) Human Subjects Protection Office (HRPO).

9. The point of contact for this action is Michelle Block, MS, CIP at 301-319-9535 or michelle.e.block.civ@mail.mil.

for Dr. Lee

LISA M. LEE, PHD, MA, MS
Chair, Institutional Review Board
Walter Reed Army Institute of Research

CF:
Victor Melendez, COL, MS
Douglas Shaffer, PhD
John Waitumbi, DVM, PhD
Stacy Gondi
KEMRI SERU
MCMR-RP

7.2 Appendix 2: Authorization for use of archived samples

USA MEDICAL RESEARCH DIRECTORATE
KENYA MEDICAL RESEARCH INSTITUTE
Basic Science Laboratory
Kakamega Highway
Kisumu, Kenya

P.O. Box 54, 40100 Kisumu, Kenya Tel: +254 717174935 E-mail: kisumu.admin@usamru-

19 January 2022

TO:
THE REGISTRAR
GRADUATE SCHOOL
UNIVERSITY OF NAIROBI
P.O BOX 30197-00100
NAIROBI

FROM: JOHN N. WAITUMBI, DVM, PhD
DIRECTOR, KISUMU FIELD STATION
AND DIRECTOR, BASIC SCIENCE LAB
KENYA MEDICAL RESEARCH INSTITUTE / US ARMY MEDICAL
RESEARCH DIRECTORATE-AFRICA/KENYA
KISUMU KENYA
MOBILE: +254 733616548
HTTP://WWW.USAMRUKENYA.ORG/#

Dear Sir / Madam,

RE: AUTHORIZATION FOR USE OF ARCHIVED SAMPLES

This is to confirm that Ms Beth Kinya Mutai (H80/928245/2013) a student at the Department of Medical Microbiology, School of Health Science of University of Nairobi was authorized by the undersigned to formulate a PhD research question based on an ongoing surveillance study protocol titled "Acute febrile illness surveillance in Kenya" (KEMRI SERU/ SSC # 1282). Ms Mutai research study examined the proportion of tick'borne zoonoses in patients with febrile illnesses and bacterial community in ticks collected from domestic animals in Kenya (KEMRI SSC # 1248). The research projects involved design of multiplexed PCR assay to diagnose the eight common Tick-Borne Pathogens. She thereafter assessed their distribution and the associated risk factors.

Yours faithfully,

John Waitumbi

John N. Waitumbi, DVM, PhD
Principal Investigator KEMRI SERU/ SSC # 1282 and KEMRI SSC # 1248

Page 1 of 2

7.3 Appendix 3: Consent and Assent forms

Acute Febrile Illness Surveillance in Kenya KEMRI SSC # 1282 WRAIR # 1402
V20.8 dated 18October2016

INFORMED CONSENT AGREEMENT

What is the study called: Acute febrile illness surveillance in Kenya.

What is this study about: This is a research study where we are trying to find out the causes of illnesses in adults and children who have fever but no other signs of being sickness. We want to draw a small amount of blood and swab the inside of your nose and test it in the lab to see if we can find the germ that is causing your illness. Additionally the study is testing a new method of diagnosing malaria.

Who is running the study: The study is being run by Dr John Waitumbi from United States Army Medical Research Unit-Kenya(USAMRU-K)/Kenya Medical Research Institute, Nairobi, Kenya.

Do I have to participate: Participation in this study is voluntary. There is no penalty for refusing to participate. If you (your child) decide not to be in the study you (your child) may still receive medicine to help with your fever. If you start the study you (your child) may stop the study at any time. If you (your child) stop the study after we draw your (your child's) blood or take the swab from your (your child's) nose, we will destroy these samples so that no future testing can be done.

Do I have other choices: You have the option of not participating in the study and just continuing with the health care plan as directed by the health care provider at the clinic.

What will happen to me if I participate in the study: You will be asked some questions about where you live, your illness and any medications you may have taken recently. Then about 1-2 tablespoons of blood will be taken from a vein in your arm. The blood will be put into small tubes so we can test for germs that may be causing your illness. We will also take a swab from your nose to test for germs that may be causing your illness.

The information collected about your illness and where you live will be used by study team to determine your home's location on an electric map (called Global Positioning System -GPS) and assign it a unique house number. The GPS mapping provides information about how diseases spread in communities. By documenting the location of everyone's home, we can better understand how diseases spread in your community and can contribute to understanding the best way to monitor for emerging infections, outbreaks and epidemics.

Are there any risks if I participate in the study: There is the possibility of mild discomfort, bruising and very rarely infection at the site of needle injection.
We will protect all personal information we collect. You (your child) will be assigned a study number that will be used on all study documents. All study documents will be secured and will only be accessible to authorized study personnel.

Are there any benefits from the study: Because we are conducting this study at this health care facility, free malaria testing and treatment is available. If you want to, you can receive medication to treat your (your child's) fever.

Additionally, the study can lead to a better understanding of the causes of fever in Kenya and improve the medical care in your (your child's) area. Your hospital may learn about new or emerging diseases that cause fever. There are no direct benefits to you for participating in the study.

Will there be any compensation for being in the study: There is no compensation to volunteers for their participation. But, should you (your child) be injured as a direct result of participating in this research project, you (your child) will be provided medical care, at no cost to you (your child), for that injury. You (your child) will not receive any injury compensation, only medical care. You (your child) should also understand that this is not a waiver or release of your (your child's) legal rights.

How long does the study last: This study requires only completion of the questionnaire, one blood draw and nasal swab. There is no follow-up or further information needed. The questionnaire and other study procedures will take about 30 minutes.

Who can participate in this study: Anyone (including pregnant women) can participate in the study if you have a fever without a source after evaluation by the clinician. If there is an obvious source of infection causing the fever, like a skin infection or lung infection, then you (your child) should not participate.

Who will be able to see my information or lab results: Any information about you (your child) will remain confidential. Only the people involved in the study will be able to see your information. We will protect all your information. The information and specimens we collect may also be reviewed by representatives of the Ministry of Health, Kenya Medical Research Institute, the U.S. Army Medical Research and Materiel Command (USAMRMC), and the Walter Reed Army Institute of Research as part of their responsibility to oversee this research, for legal reasons or to investigate an outbreak. Federal Regulatory Agencies from United States and other local regulatory agencies may review the study records. In the event of an outbreak, the Kenya MoH will be provided with information about the location (and/or the individual) to investigate the outbreak of an illness that is considered reportable to the Kenyan MoH. Federal Regulatory Agencies from United States and other local regulatory agencies may review the study records.
Any report from this study will refer to you/your child only by a study identification number and not by a name. All blood samples, and nasal swabs collected will be labeled with a study identification number; no names will be used. The lab tests will take a long time to complete and will not be available to you or your health care provider to use in your care.

What will happen to my blood: Your (your child's) blood will be tested for things that could cause fever. A sample of your blood will be kept frozen in case we want to do more testing on it in the future. Future testing may include evaluating new disgnostics tests or looking for new or emerging diseases. These samples, will be labeled with only your study number. They will be

secured in freezers at WRP/KEMRI facilities and only study investigators and their authorized staff will have access. All safeguards ensuring privacy that are in place during this study period will also continue to be in place for the long-term storage of samples. Some blood tests planned for this trial will be done with our collaborating laboratories abroad. Before shipment of samples to such collaborating laboratories, authority for sample shipment and testing will be sought from KEMRI ERC.

If samples are sent outside of Kenya for additional lab testing, no personal identifiers will be included.

If we do need to use the stored blood samples in the future we will first get permission from the Walter Reed Army Institute of Research (WRAIR) Institutional Review Board (IRB) and Kenya National Ethical Review Committee.

Who can I contact about the study or my rights as a volunteer in this research study: If during the course of this study, you have questions concerning the nature of the research or you believe you have sustained a research-related injury, you should contact:

Dr. John N. Waitumbi
USAMRU-K /Walter Reed Project, PO Box 54,
Kisumu
Tel. 0733 333 530 or +254 57 20 22 942

The Chairman of the Kenya National Ethical Review Committee
C/o Kenya Medical Research Institute
P.O. Box 54840, Nairobi, Kenya
Tel. 254-20-2722541

IF THERE IS ANY PORTION OF THIS CONSENT AGREEMENT THAT YOU DO NOT UNDERSTAND, PLEASE TALK TO SOMEONE ON THE STUDY TEAM BEFORE SIGNING.

You will receive a copy of the consent form to take home with you.

Subject's Name: _____

If Subject a minor
Parent/Guardian's Name: _____

Subject's or Parent/Guardian's Signature: _____ Date: _____

Permanent Address: _____

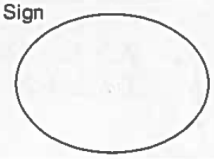
If Adult Subject or Parent/Guardian Illiterate

Witness's Name: _____

Witness's Signature: _____ Date: _____

Study Number: _____

Thumbprint of Volunteer or Volunteer's Parent /Guardian if Unable to Sign



INDIVIDUAL OBTAINING CONSENT: I certify that I have explained to the above individual the nature and purpose of this study, potential benefits, and possible risks associated with participation in this study. I have answered any questions that have been raised.

Name:_____

Signature: _____ Date: _____

ASSENT FORM FOR INDIVIDUALS 12 THROUGH 17 YEARS OF AGE

What is the study called: Acute febrile illness surveillance in Kenya.

What is this study about: This is a research study where we are trying to find out the causes of illnesses in adults and children who have fever but no other signs of being sick. . We want to draw a small amount of blood and test it in the lab to see if we can find the germ that is causing your illness.

Who is running the study: The study is being run by Dr Waitumbi of United States Army Medical Research Unit- Kenya (USAMRU-K).

Do I have to be in the study: No you do not have to be in the study. You will not get in trouble for refusing to be in the study. If you start the study you may stop the study at any time. If you stop the study after we draw your blood, or take the swab from your nose, we will destroy these samples so that no future testing can be done.

Do I have other choices:
You have the option of not participating in the study and just continuing with the health care plan as directed by the health care provider at the clinic.

What will happen to me if I participate in the study: You and the adult you are with will be asked some questions about where you live, your illness and any medications you may have taken recently. Then about a tablespoon of blood will be taken from a vein in your arm. The blood will be put into small tubes so we can test for germs that may be causing your illness. We will not test for HIV. We will also take a swab from your nose to test for germs that may be causing your illness.

The information collected about your illness and where you live will be used by study team to determine your home's location on an electric map (called Global Positioning System -GPS) and assign it a unique house number. The GPS mapping provides information about how diseases spread in communities. By documenting the location of everyone's home, we can better understand how diseases spread in your community and can contribute to understanding the best way to monitor for emerging infections, outbreaks and epidemics.

Are there any risks if I participate in the study: Having your blood drawn may hurt a little. You may have some bleeding or a bruise after the blood is drawn. We will protect all personal information we collect. You will be assigned a study number that will be used on all study documents. All study documents will be kept in a safe cabinet at Walter Reed Project and will only be accessible to authorized study personnel.

Are there any benefits from participating in the study: Free malaria testing and malaria treatment is available at this health care facility because this study is being conducted here. If you want to, you can receive medication to treat your fever.

Additionally, the study can lead to a better understanding of the causes of fever in Kenya and improve the medical care in your area. Your hospital may learn about new or emerging diseases that cause fever. There are no direct benefits to you for participating in the study

Will I get anything for being in the study: No, you do not receive anything for being in the study. But, should you be injured by being in this study, you will be provided medical care, at no cost to you, for that injury.

How long does the study last: Answering the questions, blood draw, and obtaining a swab from your nose will take about 30 minutes.

Who can be in this study: Anyone can be in the study if you have a fever without a source after evaluation by the people who work in the hospital.

Who will be able to see my information or lab results: Only people who are involved in the study, such as study doctors and the staff will have access to your information. Your lab tests will take a long time to complete and will not be given to you or your doctor. In the event of an outbreak, the Kenya MoH will be provided with information about the location (and/or the individual) to investigate the outbreak of an illness that is considered reportable to the Kenyan MoH. Your information or lab results may also be seen by representatives from federal regulatory agencies from the United States and other local regulatory agencies as part of their responsibility in ensuring your protection. Federal Regulatory Agencies from United States and other local regulatory agencies may review the study records.

What will happen to my blood: Your blood will be tested for things that could cause your fever. A sample of your blood will be kept frozen in case we want to do more testing on it in the future. Future testing may include evaluating new diagnostics tests or looking for new diseases.
Some blood tests planned for this trial will be done with our collaborating laboratories abroad. Before shipment of samples to such collaborating laboratories, authority for sample shipment and testing will be sought from KEMRI ERC.

Who can I contact about the study or my rights as a volunteer in this research study: If you have any question you or your parent should contact:

Dr. John N. Waitumbi
Walter Reed Project, PO Box 54,
Kisumu
Tel. 0733 333 530 or +254 57 20 22 942

The Chairman of the Kenya National Ethical Review Committee
C/o Kenya Medical Research Institute
P.O. Box 54840, Nairobi, Kenya
Tel. 254-20-2722541

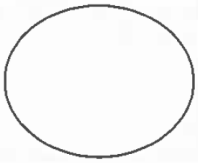
IF THERE IS ANY PORTION OF THIS CONSENT AGREEMENT THAT YOU DO NOT UNDERSTAND, PLEASE ASK STUDY TEAM BEFORE SIGNING.

Subject's Name: _____

Subject's Signature: _____ Date: _____

WITNESS: I have witnessed the explanation of the research study to the participant. The participant was given an opportunity to ask questions, and the participant's questions, if any, were answered.

Thumbprint of Volunteer is unable to Sign



Witness's Name: _____

Witness's Signature: _____ Date: _____

INDIVIDUAL OBTAINING ASSENT: I certify that I have explained to the above individual the nature and purpose of this study, potential benefits, and possible risks associated with participation in this study. I have answered any questions that have been raised.

Name:_____

Signature: _____ Date: _____

7.4 Appendix 4: KEMRI SERU/SSC Animal use committee approval (SERU/SSC # 1248)

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840 - 00200 NAIROBI, Kenya
Tel: (254) (020) 2722541, 2713349, 0722-205901, 0733-440003; Fax: (254) (020) 2720030
E-mail: kemri-hq@nairobi.mimcom.net, director@kemri.org; Website: www.kemri.org

KEMRI/RES/7/3/1 August 24, 2007

FROM: SECRETARY, KEMRI/National Ethical Review Committee

THROUGH: THE DIRECTOR, CCR Forwarded 27/8/07

TO: Dr. John N. Waitumbi (PRINCIPAL INVESTIGATOR)
Director, Kondele Research Laboratories
WRP,KISUMU

RE: SSC NO. 1248 (REVISED): ZOONOTIC DISEASE SURVEILLANCE OF *RICKETTSIA* INFECTIONS IN DOMESTIC ANIMALS PRESENTED AT DAGORETTI AND ATHI RIVER SLAUGHTER HOUSES, NAIROBI, KENYA (Version #3, 25 May 2007)

Dear Sir,

This is to inform you that, during the 146th meeting of KEMRI/National Ethical Review Committee held on August 21, 2007, the above referenced proposal was discussed. We acknowledge receipt of a copy of the approval letter from the KEMRI Animal Care and Use Committee (ACUC) dated 7th May 2007.

The Committee notes that the proposed animals study aims to complement USAMRU-K's Global Emerging Infections and Surveillance and Response System (GEIS) human surveillance program for *Rickettsia* by screening animals presenting with tick-borne Rickettsial diseases at Dagoretti and Athi River slaughter houses in Nairobi.

The Committee acknowledges that efforts made in the development and testing of effective strategies for the management of Ricketsial diseases will be beneficial to Kenyan communities.

The study is hereby granted approval for a period of one (1) year effective today, the 24th day of August 2007 to 23rd August 2008. Please note that you are responsible for reporting any changes to the research study design or procedures to the ACUC and to the ERC prior to implementation.

Sincerely,
C. Wasunna,
For: Secretary,
KEMRI/NATIONAL ETHICAL REVIEW COMMITTEE

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: kemri-hq@nairobi.mimcom.net; director@kemri.org; Website: www.kemri.org

KEMRI/RES/7/3/1 **NOVEMBER 5th, 2008**

FROM: **SECRETARY, KEMRI/National Ethical Review Committee**

THRO': **DR. RJ RASHID,**
CENTRE DIRECTOR,CCR,
NAIROBI.

Forwarded 7/11/08

TO: **DR. JOHN WAITUMBI, (PRINCIPLE INVESTIGATOR)**

RE: **SSC NO. 1248 (AM): ZOONOTIC DISEASES SURVEILLANCE OF RICKETSSIA INFECTIONS IN DOMESTIC ANIMAL PRESENTED AT DAGORETTI, ATHI RIVER SLAUGHTER HOUSES, NAIROBI, KENYA (V 4.0)**

Dear Sir,

This is to inform you that during the 160th meeting of KEMRI/National Ethical Review Committee held on 4th NOVEMBER 2008, the requested amendments to the abovementioned study were reviewed.

The Committee notes that the requested amendment is to add Mombasa Slaughter houses i.e. Uwanja wa Ndege, Mombasa slaughter house, Mariakani slaughet house and Kasemini slaughter house as additional sites for collecting blood and ticks for identification of Rickettsia infections. This is because it has become clear from data collected that you are missing vital data from the Coastal and North Eastern parts of the country.

The amendments do not alter the original design of the study and are granted approval. You may continue with your study.

Respectfully,

RCKithinji

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

16 JAN 2019

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/RES/7/3/1 January 8, 2019

TO: **DR. JOHN WAITUMBI,**
PRINCIPAL INVESTIGATOR.

THROUGH: **THE DIRECTOR, CCR,**
NAIROBI.

16/01/2019

Dear Sir,

RE: **SSC PROTOCOL NO. 1248 *(REQUEST FOR ANNUAL RENEWAL)*: ZOONOTIC DISEASE SURVEILLANCE OF RICKETTSIA INFECTIONS IN DOMESTIC ANIMALS PRESENTED AT DAGORETTI AND ATHI RIVER SLAUGHTER HOUSES, KENYA.**

Thank you for the continuing review report for the period **November 21, 2017** to **November 20, 2018.**

This is to inform you that the Expedited Review Team of the KEMRI Scientific and Ethics Review Unit (SERU) conducted the annual review of the above referenced application and was of the informed opinion that the progress made during the reported period is satisfactory. The study has therefore been granted **approval**.

This approval is valid from **January 19, 2019** through to **January 18, 2020**. Please note that authorization to conduct this study will automatically expire on **January 18, 2020**. If you plan to continue with data collection or analysis beyond this date please submit an application for continuing approval to the **SERU** by **December 07, 2019.**

You are required to submit any amendments to this protocol and any other information pertinent to human participation in this study to the SERU for review prior to initiation.

You may continue with the study.

Yours faithfully,

ENOCK KEBENEI,
ACTING HEAD,
KEMRI SCIENTIFIC AND ETHICS REVIEW UNIT.

7.5 Appendix 5: Case report form for demographical and clinical data collected for acute febrile illness patients recruited to the study

Name of Interviewer _____

Acute Febrile Illness Study Number _____

1. Sample Collection date _____ (Day/month/year)
2. Gender: Male Female
3. Age: _____Years _____Months_____
4. Where do you or your child reside?
5. Village: _____District:_____Province:_____
6. How long have you or your child resided in this location? _____years ____Months_____
7. Where have you or your child resided in the last 5 days? (Check one)
   - City or Village of residence _____
   - In Kenya, but not in your residence, where? _____
   - Outside Kenya, where? _____
     - Have you or your child traveler outside your usual residence?_____ How many times in the last two months: ___
8. For how long ago: Less than 2 weeks 2-4 weeks 1-2 months
9. What is your occupation (For adults): _____
10. Where do you go to school (For school going children): _____
11. Have you or your child come in close contact with any of these animals?
    - Poultry
      - Geese Ducks Chickens Other birds: _____
    - Mammals

- Goats Pigs Cows Donkeys Other Animals:_____

12. CURRENT ILLNESS

Why did you (your child) come to the hospital?

_____

Did you (your child) have any of the following signs and symptoms? (Tick where appropriate)

Headache Yes_____ No_____
Chills Yes_____ No_____
Cough Yes_____ No_____
Difficulty in breathing Yes_____ No_____
Sore throat Yes_____ No_____
Running nose Yes_____ No_____
Painful eyes Yes_____ No_____
Seizures Yes_____ No_____
Tick bite Yes_____ No_____
Abdominal pain Yes_____ No_____
Vomiting Yes_____ No_____
Diarrhea Yes_____ No_____
Blood in stool Yes_____ No_____
Bleeding Yes_____ No_____
Bruising Yes_____ No_____
Body rash Yes_____ No_____
Joint pain Yes_____ No_____
Muscle pain Yes_____ No_____
Dark urine Yes_____ No_____
Jaundice Yes_____ No_____

13. Have you or your child have other signs and symptoms that we have not mentioned? _____ If any bleeding, where is or was the bleeding?: (N/A, Gums, Nose, Injection Sites, mouth) _____
14. For how many days have you or your child been sick? _____
    - When did the sickness start? _____
15. How many days of work have you missed or how many school days have your child missed:_____
16. Is there anyone else you know with similar illness, signs or symptoms? Yes No
    - Who (husband, sister schoolmate, etc.):_____ when did they become ill_____

- Who (husband, sister schoolmate, etc.):_____ when did they become ill_____
- Who (husband, sister schoolmate, etc.):_____ when did they become ill_____

17. If taken what was your temperature when you came to hospital? _____^{o}C

18. Interviewer to record diagnosis by the MOH provider:

    _____

    _____

19. Interviewer to record treatment prescribed by MOH provider:

    _____

    _____

7.6 Appendix 6 : Turnitin Anti-plagiarism report

Tick-Borne Pathogens as Etiologies of Undifferentiated Acute Febrile Illness in Kenya: Geographical Distribution, Risk Factors and The Tick Bacteriome as a Bio-Indicator of Tick - Borne Zoonosis

ORIGINALITY REPORT

13%	9%	8%	3%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	academic.oup.com Internet Source	1%
2	html.scirp.org Internet Source	1%
3	www.ncbi.nlm.nih.gov Internet Source	<1%
4	clearafred.wits.ac.za Internet Source	<1%
5	www.frontiersin.org Internet Source	<1%
6	repository.sustech.edu Internet Source	<1%
7	kipdf.com Internet Source	<1%
8	M.J. Maze, Q. Bassat, N.A. Feasey, I. Mandomando, P. Musicha, J.A. Crump. "The	<1%

	epidemiology of febrile illness in sub-Saharan Africa: implications for diagnosis and management", Clinical Microbiology and Infection, 2018 Publication	
9	Submitted to Universiti Teknologi MARA Student Paper	<1%
10	P. Steinmann, B. Bonfoh, O. Peter, E. Schelling, M. Traore, J. Zinsstag. "Seroprevalence of Q-fever in febrile individuals in Mali", Tropical Medicine and International Health, 2005 Publication	<1%
11	repository.up.ac.za Internet Source	<1%
12	www.parasitetesting.com Internet Source	<1%
13	emedicine.medscape.com Internet Source	<1%
14	journals.plos.org Internet Source	<1%
15	Submitted to Universiti Sains Malaysia Student Paper	<1%
16	Submitted to University of Surrey Student Paper	<1%

17	Curtis L. Fritz. "Emerging Tick-borne Diseases", Veterinary Clinics of North America: Small Animal Practice, 2009 Publication	<1 %
18	www.mdpi.com Internet Source	<1 %
19	ThankGod E. Onyiche, Cristian Răileanu, Oliver Tauchmann, Susanne Fischer et al. "Prevalence and molecular characterization of ticks and tick-borne pathogens of one-humped camels (Camelus dromedarius) in Nigeria", Parasites & Vectors, 2020 Publication	<1 %
20	link.springer.com Internet Source	<1 %
21	Submitted to Higher Education Commission Pakistan Student Paper	<1 %
22	www.science.gov Internet Source	<1 %
23	Borna A. Nyaoke, Marianne W. Mureithi, Caryl Beynon. "Factors associated with treatment type of non-malarial febrile illnesses in under-fives at Kenyatta National Hospital in Nairobi, Kenya", PLOS ONE, 2019 Publication	<1 %

168	William L. Nicholson, Daniel E. Sonenshine, Bruce H. Noden, Richard N. Brown. "Ticks (Ixodida)", Elsevier BV, 2019 Publication	<1 %
169	Ilias Chaligiannis, Isabel G. Fernández de Mera, Anna Papa, Smaragda Sotiraki, José de la Fuente. "Molecular identification of tick-borne pathogens in ticks collected from dogs and small ruminants from Greece", Experimental and Applied Acarology, 2018 Publication	<1 %

Exclude quotes	On	Exclude matches	Off
Exclude bibliography	On		

Supervisor: Dr. Kariuki Njaanake

Sign:

Date: 31st March 2022

Tick-Borne Pathogens as Etiologies of Undifferentiated Acute Febrile Illness in Kenya: Geographical Distribution, Risk Factors and The Tick Bacteriome as a Bio-Indicator of Tick - Borne Zoonosis

GRADEMARK REPORT

FINAL GRADE

/0

GENERAL COMMENTS

Supervisor: Dr. Kariuki Njenga
Sign:
Date: 31st March 2022

Instructor

PAGE 1

PAGE 2

PAGE 3

PAGE 4

PAGE 5

PAGE 6

PAGE 7

PAGE 8

PAGE 9

PAGE 10

PAGE 11

PAGE 12

PAGE 13

PAGE 14

PAGE 15

PAGE 16

PAGE 17

PAGE 18

Distribution and Risk Factors of Eight Tick-Borne Bacterial Pathogens as Etiologies of Undifferentiated Acute Febrile Illness and Bacteriome in Ticks Collected from Domestic Livestock from Different G

ORIGINALITY REPORT

15%	13%	5%	3%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	mafiadoc.com Internet Source	<1%
2	www.astmh.org Internet Source	<1%
3	academic.oup.com Internet Source	<1%
4	erepository.uonbi.ac.ke Internet Source	<1%
5	ir.jkuat.ac.ke Internet Source	<1%
6	usir.salford.ac.uk Internet Source	<1%
7	ugspace.ug.edu.gh Internet Source	<1%
8	www.mdpi.com Internet Source	<1%

Dr. H. Kariuki Njaanake
Supervisor
Signed
Date 7/01/22

7.7 Appendix 7 Manuscripts

A Duo 4-Plex Real Time PCR for Detection of Eight Tick-Borne Zoonoses in Kenya

Beth Mutai[1,2], Kariuki Njaanake[2], Kimita Gathii[1], Benson B. Estambale[2,3], John N. Waitumbi[1]

[1]US Army Medical Research Directorate-Kenya, Kenya Medical Research Institute, Kisumu, Kenya
[2]Department of Medical Microbiology, University of Nairobi, Nairobi, Kenya
[3]Jaramogi Oginga Odinga University of Science and Technology, Kisumu, Kenya
Email: john.waitumbi@usamru-k.org

How to cite this paper: Mutai, B., Njaanake, K., Gathii, K., Estambale, B.B. and Waitumbi, J.N. (2019) A Duo 4-Plex Real Time PCR for Detection of Eight Tick-Borne Zoonoses in Kenya. *Open Journal of Clinical Diagnostics*, **9**, 1-15.
https://doi.org/10.4236/ojcd.2019.91001

Received: December 20, 2018
Accepted: March 3, 2019
Published: March 6, 2019

Abstract

Ticks harbor multiple pathogens, most of which can be transmitted to humans. The ensuing zoonoses display non-specific symptoms that make definitive diagnosis difficult. We report here the development and evaluation of multiplex real time polymerase chain reaction (qPCR) assays for eight tick-borne zoonoses (TBZ). The assays were organized in duo formats of 4-plex each. Format 1 was optimized for *Anaplasma phagocytophilum*, *Coxiella burnetii*, *Borrelia burgdoferi and Ehrlichia chaffeensis.* Format 2 was optimized for *Rickettsia* species (spp.), *Bartonella* spp., *Borrelia* spp. other than *B. burgdoferi* and *Babesia* spp. Synthetic plasmids were used to show that the assays can specifically detect all target sequences in the same reaction tube. Assays were assayed eight times to determine assay performance and the limit of detection was determined as the lowest plasmid concentration that was amplified for all the targets. Standard curves of threshold cycle (Ct) versus copy numbers were generated and used to determine linearity and efficiency of the assays. Pairwise comparison of singleplex and multiplex assays was done using Bland-Altman plots. Prevalence was calculated as overall percentage of positive patients to each

Bacteriome in Ticks Collected from Domestic Livestock in Kenya

Beth Mutai[1,2], Kariuki Njaanake[2], Kimita Gathii[1], Benson B. Estambale[2,3], John N. Waitumbi[1*]

[1]US Army Medical Research Directorate—Africa/Kenya Medical Research Institute, Kisumu, Kenya
[2]Department of Medical Microbiology, University of Nairobi, Nairobi, Kenya
[3]Jaramogi Oginga Odinga University of Science and Technology, Bondo, Kenya
Email: *john.waitumbi@usamru-k.org

How to cite this paper: Mutai, B., Njaanake, K., Gathii, K., Estambale, B.B. and Waitumbi, J.N. (2022) Bacteriome in Ticks Collected from Domestic Livestock in Kenya. *Advances in Microbiology*, **12**, 67-82.
https://doi.org/10.4236/aim.2022.122006

Received: December 2, 2021
Accepted: February 15, 2022
Published: February 18, 2022

Abstract

Background: Metagenomics approaches are increasingly being utilized as "dipstick" for microbial carriage. In this study, 16S rRNA metagenomics was used to probe for microbial community that resides in the ticks, those they pick from the environment, wildlife and livestock and to identify potential tick borne zoonoses. **Methods:** Tick DNA from 463 tick pools collected from domestic animals between 2007 and 2008 were amplified with primers that target the 16S rRNA V3-V4 domain and then sequenced on Illumina Miseq platform using 300 cycles version 3 kits. Ticks were pooled according to species and animal from which they were collected. A non-target control was used to track laboratory contaminants. Sequence data were analyzed using Mothur v1.3 pipeline and R v3.3.1 software and taxonomy determined using SILVA rRNA database. Shannon diversity index was used to compute bacterial diversity in each tick species before computing the means. **Results:** A total of 645 bacteria genera grouped into 27 phyla were identified. Four phyla contributed 97.4% of the 36,973,934 total sequences. Proteobacteria contri-

Vector-Borne and Zoonotic Diseases, VOL. 20, NO. 5 | Original Articles

normal

Serological Evidence of Yersiniosis, Tick-Borne Encephalitis, West Nile, Hepatitis E, Crimean-Congo Hemorrhagic Fever, Lyme Borreliosis, and Brucellosis in Febrile Patients Presenting at Diverse Hospitals in Kenya

Josphat Nyataya, Moureen Maraka, Allan Lemtudo, Clement Masakhwe, Beth Mutai, Kariuki Njaanake, Benson B. Estambale, Nancy Nyakoe, Joram Siangla, and John Njenga Waitumbi

Published Online: 28 Apr 2020 | https://doi.org/10.1089/vbz.2019.2484

Tools Share

Abstract

Data on pathogen prevalence is crucial for informing exposure and disease risk. We evaluated serological evidence of tick-borne encephalitis (TBE), West Nile (WN), Hepatitis E virus (HEV), Crimean-Congo Hemorrhagic Fever (CCHF), Yersiniosis, Lyme Disease (LD), and brucellosis in 1033 patients presenting with acute febrile illness at 9 health care facilities from