Detection of pathogens in ticks collected from Tanzania using specific Polymerase Chain Reaction and Next-Generation Sequencing

Ajanaw Yizengaw, Baye

Graduate School of Public Health

Yonsei University,

Department of Global Health Security,

Division of Global Health Security Response Program

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Directed by Professor : Tai-Soon Yong
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Ajanaw Yizengaw, Baye

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This certifies that the Master`s Thesis
of Baye Ajanaw Yizengaw is approved.

Tai-Soon Yong

Thesis Committee Member: Professor Tai-Soon Yong

Thesis Committee Member: Professor Myung Ken Lee

Myeong Heon Shin

Thesis Committee Member: Professor Myeong Heon Shin

Graduate School of Public Health
Yonsei University
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List of Abbreviation

BLAST:	Basic Local Alignment Search Tool
EtOH:	Ethnanol
MSF:	Meditranian Spotted Fevere
NCBI:	National Center for Biotechnology Institute
OTUs:	Operational taxnomic Units
PCoA:	Prinicipal coordinate analysis
PCR:	polymerase chain reaction
PCs:	principal coordinates
RPM:	Revolution Per Minute
SFGs:	Spoted Fevere Gruops
spp:	**species**
TAE:	Tris-Acetate Buffer
UPGMA:	Unweighted pair group method with arithmetic mean
V1-V4:	hyper Variable region 1-9

Abstract

Background: Ticks are blood sucking arthropods that play a vital role in the transmission of a variety of pathogens to humans and animals. This tick also carries several other pathogens that cause human disease, including agents of *Anaplasmosis, Babesiosis, Borreliosis, Rickettsia, Coxiella* spp and others. Thus, tick borne diseases detection approaches using next-generation sequencing (NGS) of 16S rRNA gene amplicon enhance the efficiency of diagnosis and control strategies. In this study, detection of bacteria and protozoa pathogen was performed in ticks collected from wild animals from Serengeti National Park, Tanzania.

Methods: Total 136 hard ticks were collected from wild animals (wildebeest, buffalo, zebra, and lion) in Tanzania in 2014-2016. Hard (I*xodidae*) tick's DNA was extracted from the ticks and pathogen-specific PCR was performed. In addition, microbiome study using NGS on of 16S rRNA gene amplification was performed.

Result: In this pathogen specific analysis, 72 out of total 136 tick samples were positive for any potential pathogens and the detection rate 52.94%. The detection rate of pathogen in ticks from wildebeest, buffalo, zebra, lion were 64.7%, 60.6%, 54.24%, and 33.33%, respectively. The commonly detected potential pathogen was *Coxiella* spp. (38.24%), followed by *Rickettsia* spp. (13.24%), and *Theileria* spp. (0.74%). While *Anaplasma* spp, *Bartonella* spp, and *Borrelia* spp were not detected in ticks.

Microbiome study was performed on 16 tick samples. The number of bacterial species identified in ticks ranged from 70 to 122 among samples. The number of identified bacterial species and bacterial composition were not different between groups. The average relative abundances of Coxiella spp. in wildebeest, buffalo, and zebra were found as 0.01%, 24.33% and 26.93% respectively. The average relative abundances of *Rickettsia* spp. in wildebeest, buffalo, and zebra were found to be 0%, 0.28%, and 0.76%. Other potential pathogens were detected. All tick samples positive by NGS approach were found to be positive in pathogen-specific PCR approach in this study.

Conclusion: Detection and analysis of ticks collected from wild animals demonstrated that Rickettsia and Coxiella pathogen detection rate were high in this study ,among the targeted pathogens.

The microbiomes of bacterial composition varied between tick's host animals, and the most occurrence microbiota from the members of Coxiellaceae, Francisellaceae, and Rickettsiaceae families (Phylum: Proteobacteria) were the most abundant. Therefore, Potential pathogens were detected in tick samples collected from wild animals in Tanzania using specific PCR and NGS approaches. In the future, NGS application for detection of pathogens could be considered since it is accurate and time saving.

Key words: Tick, PCR, 16S rRNA, NGS, Microbiomes.

I. Introduction

1.1. Research background:

Ticks are the blood sucking arthropods that play vital role in the transmission of a varies of pathogens to humans and animals through long term contact with the host body and are some of the most relevant disease vectors for human and other animals [1].The most predominantly know tick families are hard(*Ixodidae*) and soft (*Argasidae*) ticks and they are able to transmit wide range of pathogens with 700 and 200 species in the world respectively [2].Now the vector carried by a day`s disease has re-emerging and spread at accelerated level ,causing substantial morbidity and mortality worldwide. The newly emerging tick borne related microorganism and causing disease agents have been studied, but the well organized and multi-sectorial response to wards lacking mitigation strategies for novel tick borne diseases[3].The most common tick borne bacterial in most developed countries, such as Europe and America, has been identified in ticks (i.e. tick-borne) related to animals, humans and others. Among them, the most commonly detected bacteria pathogens are *Borrelioses, Ehrlichiosis, Anaplasmosis,* and tick-borne *Rickettsia* diseases are some of the emerging diseases which have been described throughout the world in recent years[4]. However, studies with advanced techniques in east Africa area are minimal and need additional finding. So far east Africa countries have been reporting tick borne pathogens from livestock, wild animals, and their environment. To this end, a promising avenue of disease control involves targeting the vector microbiome, the community of microbes inhabiting the vector. The vector microbiome plays a pivotal role in pathogen dynamics, and manipulations of the microbiome have led to reduced vector abundance or pathogen transmission for a handful of vector-borne diseases.

In Tanzania and infection rate 62.4% for *Theileria* spp., 17.6% for *Babesia bigemina,*and, 15.9% for *Anaplasma marginale*, 7.4% for *Ehrlichia ruminantium* and 4.5% for *Babesia bovis* [5] while another finding on comparing specific area stated that the detection rate of tick borne pathogen of the two region(Masawa and Iringa) in Tanzania among 300 ticks was reported 77.5% and 60.7% respectively [6].The overall prevalence tick borne pathogen

in Ethiopia 96.9% including *Theileria* spp, *Anaplasma* spp, *Babesia bigemina*,and *Ehrlichia spp* among 392 sample collected from cattle[7]. The study conducted in Kenya has identified *Ehrichiosis* (12.48%), *anaplasmosis* (6.32%), *Rickettsiosis* (6.15%), *Theileria* spp.0.51% and *Babesia* 1.37% among pooled tick pools[8].

As we see the above information, tick borne disease pathogens occurrence and distribution within similar geographical regions, will have transmission impact across the region. Acknowledging the works, we planned to study molecular detection and their characteristics of tick-borne pathogens by using PCR and next generation sequencing (NGS) among samples collected from wild animals from Tanzania. The study aims to identify and determine their diversity in microbiomes of bacterial pathogen among ticks collected wild animals from Serengeti National Park, Tanzania.

II. Literature review

2.1. preface

Tanzania is the most large and diverse country in east Africa. Among the unique resources, endowed with large and valuable forest resources. But the country facing serious environmental problems of degradations such as forest which need immediate consequence of the biodiversity[9]. Tanzania has about 33.5-million-hectare forest and woodlands. Due to the biodiversity abundance the country classified as mega biodiversity along with Brazil, democratic Congo, and Indonesia.

Tanzania is noted for both the sheer number of wildlife available throughout the country and for the variety on offer. There are more than four million wild animals in Tanzania representing 430 different species and subspecies. The country houses some 20% of Africa's large mammal population. Zebras, giraffes, elephants, wildebeest, buffaloes, hippos, antelopes, and gazelles are common animals. Larger predatory animals like lions, cheetahs and leopards are also found. Along with the familiar African mammals are approximately 60,000 insect species, 25 types of reptiles and amphibians, around 100 species of snakes and many fish species.

Two of the most popular areas to visit in Tanzania are Serengeti National Park and Ngorongoro Conservation Area. Serengeti National Park is the oldest national park in Tanzania and covers 14,763 square kilometers (5,700 square miles). It is home to the magnificent and world-famous wildebeest migration. A million wildebeest spend three weeks mating and giving birth to around 8,000 calves a day, then make the journey north across the rivers. The wildebeest migration happens at the same time as approximately 200,000 zebras and 300,000 Thomson's gazelles go searching for grazing pastures. Along with wildebeest, zebras and gazelles are a further 500 birds including ostrich and secretary birds, as well as buffalo, elephants, giraffes, impalas, lion, leopards, hyena and many more.

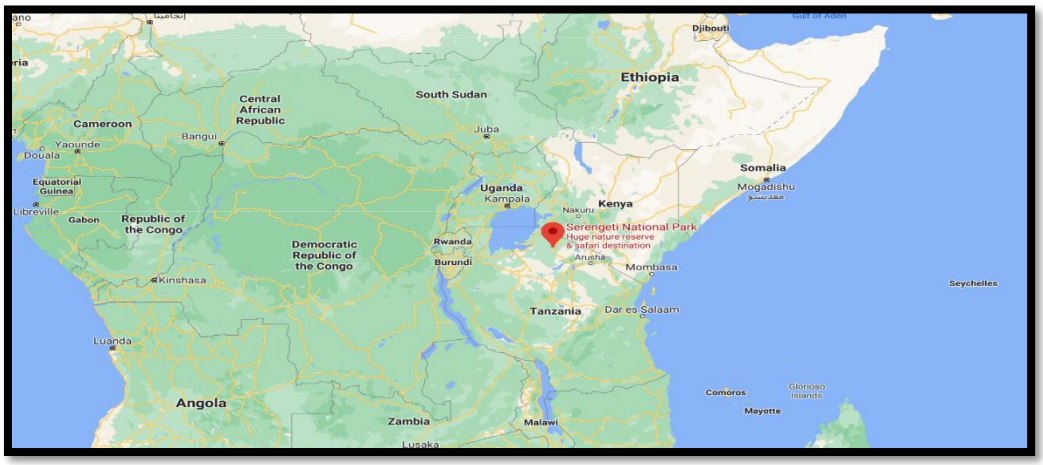

Figure 1 Serengeti National Park sample and animal conservation site, Tanzania
sources: https://www.google.com/maps/place/Serengeti,+Tanzania/

2.2. *Borrelia* spp

Borrelia is a genus of spirochete phylum bacteria. It causes Lyme disease, also known as Lyme borreliosis, a zoonotic, vector-borne disease mainly transmitted by ticks and lice, depending on the bacterial species[10][11].Among currently known 52 species of *Borrelia*, 21 are belongs to the Lyme disease group while 29 belong to the relapsing fever group, and two are members of a genetically distinct third group typically found in reptiles[12]. It is transmitted to humans through the bite of infected blacklegged ticks. Typical symptoms include fever, headache, fatigue, and a characteristic skin rash called erythema migran. If left untreated, infection can spread to joints, the heart, and the nervous system. Lyme disease is diagnosed based on symptoms, physical findings (e.g., rash), and the possibility of exposure to infected ticks.

2.3. Piroplasmidae

Piroplasm's are tick borne parasite which found int the throughout the world[13]. Its associate in nursing order of the parasite within the phylum Apicomplexa and that they are divided by the binary fission as protozoan parasites. It possesses sexual and asexual phase, and they include the tick parasite babesia and Theileria.

The family Piroplasmidae includes the genera *Babesia* (or Piroplasma), *Babesiella* (or Microbabesia), and Nuttallia. Representatives cause serious diseases in animals, along with *piroplasmosis, babesiosis* and *nuttaliases* [14][15].

The genera Babesia and Theileria (phylum Apicomplexa, order Piroplasmidae) are protozoan parasites that infect erythrocytes of a variety of vertebrate hosts, including domestic and wild animals, with some *Babesia* spp. also infecting humans[16].

Babesiosis is a rare and life-threatening infection of the red blood cells that is usually spread by ticks. It is caused by tiny parasites called Babesia. The kind that most often affects humans is called *Babesia microti* where Theileria species infect a good vary of each domestic and wild animals and area unit transmitted by tick ticks of the genera *Amblyomma, Hyalomma* and *Rhipicephalus* [17].

DNA of piroplasm's was detected in 162 of 548 (29.5%) blood samples and 9 out of 97(9.3%) ticks. the very best prevalence in blood samples was ascertained in Chad in 2016 with 72.9% positivism rate[13].Similar study indicated thar Piroplasmidae were appeared to be mixed infection and the detection rate in tick from wild animals were two fold lower than cattle[6].generally most study in Africa has been done in domestic animals than wild animals.

2.4. Rickettsiaceae

Rickettsiae are a complex of compulsively intracellular Gram-negative microorganism found in ticks, lice, fleas, mites, chiggers, and mammals. these consist of the *Rickettsiae, Ehrlichia*, *Orientia*, and *Coxiella* genera. those zoonotic pathogens reason infections that spread to several organs in the blood[18].The Ixodidae tick family are main sources of biological vectors of bacteria labeled in the order Rickettsiae and incorporate tick-borne intracellular bacterial organisms that are pathogenic to humans and domestic animals among the households that make up the *Rickettsia* [19] Ixodid ticks worldwide (e.g., *Haemaphysalis H. longicornis, flava, Ixodes persulcatus, and I. nipponensis Rhipicephalus sanguineus,* are the main Vectors / reservoirs with a broad spectrum among the species[20].Human and animals risk having rickettsia infections while contacting to tick populated areas.

Transmission and duration take place to 5-14 days incubation for maximum rickettsiae diseases.

2.5. *Coxiella* spp

The obligate intracellular gram negative, gamma proteobacteria which infect cause the animals and Q fever humans in the worldwide and its Coxiella burnttii which is types of vector borne pathogenic bacteria which causes diseases to human, animals, and others. All *Coxiella burnttii* tick borne spp are not pathogenic like Coxiella-like endosymbionts Which was found to have a high prevalence of 57 percent and 46 percent of these were also present in confirmed C ticks. Isolates of *burnttii* [21]

Ticks act as reservoirs and are responsible for the transmission of the pathogen to animals via bite fecal contamination and Tick transmitted animal *Coxiella* has been identified with about 40 different tick species[22].

For instance , a recent study in Tanzania found that bacterial zoonosis caused 26 per cent of acute fever cases, of which 20 per cent were Q fever, caused by Coxiella burnttii, and 30 per cent were Rickettsiosis, caused by *Rickettsia,* a spotted fever community[23]. Similar research in Tanzania on the interplay of effects of wildlife loss and environment on ticks and tick-borne diseases, the prevalence of *C.burntti* isolates were screened at 43 per cent (1⁄4 58 out of 136)[21].

2.6. Morphology and Taxonomy of ticks

According to WHO,2017 report Ticks are among the most important vectors of human and animal diseases caused by protozoa rickettsiae, bacteria, viruses, and some helminths. They rank second only to mosquitoes as vectors of life threatening or debilitating human and animal diseases. The tick's form consists of a capitulum (head) and a flattened, oval-shaped body called the idiosoma. Adult ticks and nymphs have eight legs, though larvae emerge from the egg with only six. Hard ticks have a hardened plate on the dorsal surface called a scutum. On females, this scutum takes up approximately 1/3 of the dorsal surface and can be useful in differentiating tick species. On males, the scutum covers the entire dorsal surface and limits their feeding ability. The tick's mouthparts are located on the capitulum and

are made up of the chelicerae and hypostome, which are used to penetrate and secure the tick to its host. During feeding, ticks secrete substances that help anchor it to the host, act as an anesthetic to mask the pain from the bite and prevent blood from coagulating. Since ticks are efficient feeders and tenacious once attached, there is potential for transmitting disease. Mainers should be in the habit of performing tick checks after frequenting tick habitat.

Both soft (*Argasidae*) and hard (*Ixodidae*) ticks are members of the following taxonomic groups: Phylum: Arthropoda Subphylum: Chelicerata Class: Arachnida Subclass: Acari Order: Parasitiformes Suborder: Ixodidae (Metastigmata) and the family Ixodidae. Ticks also incorporate different types microbiome composition at phylum, genus, or species level. The commonly taxa includes Proteobacteria ,Acinetobacter

Bacteroides ,Firmicutes and others. The relative abundance across base line in male ticks (range = 54.7%–83.4%, mean = 67.4%), followed by Actinobacteria (4.3%–41.4%, 16.6%), Bacteroidetes (1.6%–13.2%, 9.3%), and Firmicutes (1.7%–17.8%, 6.7%)[24].A more complex microbiome comprised of *Mycobacterium* (mean = 23 percent), Acinetobacter, was found to be similar in male tick (n = 13) taxes.(22 percent), *Methylobacterium* (4 percent), Sphingomonas (5 percent),Corynebacterium (2%), *Staphylococcus* (2%), *Escherichia* (2%), *Rickettsia* (2%), *Sphingobium* (2%), *Rhizobium* (1%), *Pseudomonas* (1%), and other[25].

2.7. Bacterial diversity

A lot of studies have been studied and stated that the diversity of microbiomes can be surveyed through measures of alpha and beta diversities. Alpha diversities quantities measures the number of the species in a test and their extent (species richness), whereas beta diversity qualities measures the divergence between tests (genetic relatedness)[26]. In addition the Diversity metrics depend on the taxonomic resolution of sequences and sequencing depth, however 16S NGS of one to three hypervariable regions results in read lengths of ~200–500 bp, which is a sufficient length for the taxonomic resolution of many, but not all, bacterial species [26]. In spite of the fact that all region V1-V4 (hyper variable Region 3&4)

have been most commonly focused on in tick microbiomes considers, a later study about that compared the bacterial diversity obtained from sequencing region V1-V9 on the Ion Torrent found the region V2,V3,V4,V6,V7,V8 and V9 gave the most compressive estimates of the bacterial families and the V4 region resulted in with the highest estimated diversity[27].In addition, within a few bacterial genera, the hypervariable districts of 16S are profound moderated between species, which limits species-level distinguishing proof. Moreover, the choice of the similitudes cut off and clustering calculation utilization to choose operational taxonomic units(OTUs) and sequencing blunders rate can too influence taxonomic resolution[26]. This study aims to identify the diversity of the bacteria using V3-V4 (hypervariable region) of 16s rRNA.

2.8. Emerging Disease bacterial disease in east Africa

Major tick-borne disease transmitted by hard ticks *(Ixodidae*) and includes Anaplasma phagocytophilum, Borrelia burgdorferi sensu lato, Rickettsia spp. and Babesia spp. These pathogens cause the most prevalent tick-borne diseases such as human granulocytic anaplasmosis (A. phagocytophilum), Lyme diseases (*B. burgdorferi*), spotted fever (*Rickettsia* spp.) and *babesiosis* (*Babesia* Spp)[28].

Other major human pathogens may occasionally be transmitted by ticks, including *Francisella* tularemia and *Coxiella Brunetti*. Notably, the distribution of Natural tick-borne pathogens include wildlife and livestock Which pose double animal and human health risks Which pose double animal and human health risks([28].

The relapsing fever group comprises diverse zoonotic agents transmitted through the bite of soft ticks of the genus Ornithodoros, which are responsible for recurrent fevers associated.

The relapsing fever crew comprises various zoonotic agents transmitted through the bite of soft ticks of the genus Ornithodoros, which are accountable for recurrent fever related with spirochetemia. Recently, new relapsing fever, tick-borne borrelia were discovered, namely

B. miyamotoi, alongside with various new uncultured borrelia such as *Candidiatus Borrelia* Algeria, unrecognized *Borrelia* spp. A*mblyomma* and *Rhipicephalus* ticks in Ethiopia and an unnamed new species in *Ornithodoro porcinus* ticks in Tanzania[2].

2.9. Geographical distribution.

For some species of ticks there are numerous distribute records of the geological destination in which they have been found. These records can be changed over into maps which provide a common sign of where a species is likely to be found since of where it has been found recently. If a species has solely ever been recorder north of the Sahara, then it is unlikely to be found to south. However, this essential resource in identification has various complications. For example, the kind of habitat in which the species is discovered is possibly to be a whole lot extra broadly allotted than the current geographical range of the tick[29].

Some of the current examples are the introduction of cattle from Tanzania onto Grand Comoros resulting in East Coast fever outbreaks in 2003-2004, transmitted by way of Rhipicephalus Appendiculatus to local cattle, and the introduction of Rhipicephalus microplus into West Africa as a result of the importation of Girolando cattle from Brazil between 2000 and 2009[30].

Thus, a tick which is situated in a similar habitat however a faraway geographical place from its standard distribution should have developed to become imported recently. Likewise these maps rely on records from tick surveys which differ in their extent from country to country, so gaps in distribution on a map may additionally mean that no person has regarded for them there and mostly distributed region in abundance from east to west sub-Sahara and central Africa.[31].

III. Hypothesis, question, and purpose

3.1. Study hypothesis

Aiming that using the PCR for pathogen specific detection and NGS technique which would help to identify the bacterial abundance on the selected samples.

Since ticks are blood feeding insects and they bite all animals, vertebrates and are responsible for victor borne disease carriers and they are the second victor borne disease sources and burden of disease in the world.

3.2. Study questions

The study question deal with to determining the prevalence of the bacterial pathogen and what kind of disease-causing bacteria and types of bacteria would be detected?

3.3. Study purpose and significance.

The purpose of the study to identify human diseases causing pathogens from ticks and determine their molecular characteristics. The study would help to indicate source of emerging tick borne bacterial pathogens and could be important information regarding one health approach emerging disease prevention and control strategies.it would be an important information for health educators , travels and other societies who have close relation with wild animals and the environments.

It also will indicate the characteristics of the pathogen and their diversity among the selected study sources and their hosts under their environment as well as provide important information regarding tick borne disease which emanated from wild animal habitat for community health prevention.

3.4. The detailed objectives

The study aims to detected and molecular characteristics of tick-borne bacterial pathogens by using PCR and NGS technique.

3.4.1 Specific objective

- Determine the prevalence of the disease-causing pathogens using PCR and to see co-infection patterns of targeted pathogens among where ticks were collected.
- Determine the molecular characterization of the bacterial pathogens and their microbiome abundance among selected samples.

IV. Materials and Methods

4.1 Materials

4.1.1. Materials and Apparatus

For this study materials and equipment's up to final completion includes, micropipette tips , sample extraction and PCR amplification reagents, agarose, TAE buffer(0.5X) , DNA ladders and loading and gel stain, gel electrophoresis, Polymerases chain reaction(PCR),and Next generation sequencer machine would have used for each laboratory activities.

4.1.2. Study Period and Design

The cross-sectional retrospective design was applied, and the study was conducted from June 2020 to December 2020 Yonsei University, South Korea. This study would target 16S rRNA gene of the tick sample to identify bacterial pathogen under controlled laboratory conditions. Samples were collected from Tanzania in 2014-2016.

4.1.3. Sample Collection

All the ticks selected for this study was randomly selected from stored tick biobank in Department of Environmental Medical Biology and Institute of Tropical Medicine, Yonsei University College of Medicine. Original the ticks were collected from Lion, Buffalo, zebra,

and wildebeest would include in as a host. All selected tick was at adult stages and includes both male and females.

Ticks collected from the dead and official hunting wild animals (buffalo, lion, zebra, and wildebeest) from Naabi, EBU-2436, and CTA-2436, SCA and DBL-2454 area, from January 2014 to March 09, 2016 in Tanzania. Stored ticks were fixed with EtOH and transferred from Tanzania to Yonsei University and well preserved under controlled laboratory conditions at Yonsei University.

4.2. Methods

4.2.1. DNA Extraction

DNA was extracted from clean tick samples using a commercial kit for insect genomic DNA protocol (Nucleospin® DNA insect) procedures. Mainly the process aiming to preparing sample for lyse tick body, binding the DNA and eluting the final DNA. The procedures as follow.

The first, the tick put into bead tube by adding 100µl elution buffers (EB), 40µl buffer MG and 10µl liquid proteinase k and the Agitate on swing mill for 20 minutes and centrifuge for 1 minute at 11,000RPM.

The second, was adjusting the binding condition by adding 600µl MG buffer following centrifuging for 1 minute at 11,000RPM. The third, binding DNA by loading 600µl supernatant into Nucleospin® DNA insect column and centrifuge for 1 minute at 11,000RPM.

The fourth, washing silica membrane by adding 500µl BW buffer and B5 buffers and the centrifuge for 1 minute at 11,000 RPM, respectively. Finally drying, transferring to the new spin column into 1.5 ml nuclease free tube and then adding 30µl elution buffers following 1-minute incubation and centrifuge for 2 minutes at 11.000RPM and stored the extract at -20^{o}c for further use. Each step will be done on the clean bench and all materials were autoclaved to remove contamination. DNA concentration quantified was checked by using Nano drop (Thermo ND-1000, USA).

4.2.2. PCR amplification and Detection

Amplification and the detection of the DNA of the tick have done through pathogen specific primers (table 1). Total of 7 pathogen specific primers were used for detection disease causing bacterial and protozoa. Each reaction contains 16.9µl double distilled water, 2.5µl deoxy nucleotides and 10x reaction buffer, 0.1µl Taq DNA polymerase enzymes and 1 µl template with total 25µl. All used primers run on PCR according to the manufacturer instructions with a little a bit optimization in the laboratory and all PCR programs has annexed (annex 1). The Amplified PCR products were visualized using gel electrophoresis on 1 % agarose with gel stain for checking successful amplification as well as detection of pathogen by observing the presents of highly quality band with the specified DNA ladder as reference and then the gel captured a for documentation using gel documentation system with canon camera.

The Amplified DNA products were Sequenced, cleaning and alignment were conducted manually using Bioedit (version 7.0.5.2) [33]. Sequences were then identified with the use of the Basic Local Alignment Search Tool (BLAST), (NCBI BLAST). Selected sequences amongst those obtained were deposited in GenBank and the pathogen selected with highest similarity percentage among the reference data.

4.2.3. High throughput sequencing of the 16S rRNA gene amplicon

The V_3–V_4 region of 16S rRNA was amplified by PCR using previously described protocol and the amplification done using the bacterial universal primers pair
5'- TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCW-3`.
5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGG-TATCTAATCC-3') [32].PCR amplification for region of was performed using 5µl DNA with total 25 µl and following the denaturation at 95^{o}C for 3 minute and again for 30 seconds ,55^{o}C for 30 seconds for annealing ,72^{o}C for 30 second and for elongation at 72^{o} C for 5 minutes with 25 cycles by using Applied Biosystems Veriti Thermal Cycler.

A limited-cycle amplification step was performed to add multiplexing indices and Illumina sequencing adapters. The libraries were normalized, pooled, and sequenced using the MiSeq platform (Illumina MiSeq V3 cartridge [600 cycles]; Illumina, San Diego, CA, USA) according to the manufacturer's instructions.

Table 1. List of primer used for detection of bacterial Pathogen

Pathogens	Primer sequence	Product size (bp)e	Reference
Anaplasmataceae	EHR1GAACGAACGCTGGCGGCAAGC	610	[24]
	newEHR2b -CACGCTTTCGCACCTCAGTGTC		
	EHR3 -TGCRTAGGAATCTRCCTAGTAG	524	
	newEHR2b-CACGCTTTCGCACCTCAGTGTC		
Rickettsia spp.	Rr17k.1p- TTTACAAAATTCTAAAAACCAT	539	[33]
	Rr17k.539n- TCAATTCACAACTTGCCATT		
	Rr17k.90p - GCTCTTGCAACTTCTATGTT	450	
	Rr17k.539n- TCAATTCACAACTTGCCATT		
Borellia spp	NewLDfb-GTAAACGATGCACACTTGGTG	524	[24]
	newLDrbTCCGRCTTATCACCGGCAGTCT	487	
	Outer1 - AARGAATTGGCAGTTCAATC		
	Outer2-GCATTTTCWATTTTAGCAAGTG ATG		
	Inner2ACATATTCAGATGCAGA-CAGAGGTTCTA	389	
	Inner2-GAAGGTGCTGTAGCAGGTGCTG GCTGT		
Coxiella spp	Q5- GCGGGTGATGGTACCACAACA	501	
	Q3- GGCAATCACCAATAAGGGCCG		
	Q6- TTGCTGGAATGAACCCCA	325	

	Q4- TCAAGCTCCGCACTCATG		
Piroplasmidae	BJ1- GTCTTGTAATTGGAATGATGG	476-520	
	BN2- TAGTTTATGGTTAGGACTACG	735	
Bartonella spp	QHVFE1TTCAGATGTGATGTGATCCCAAGC		[34]
	QHVFE3- AACATGTCTGAATATATCTTC		
	QHVEF12GCAGCTAAGAATCTTCCG-CAATGG	484-569	
	QHVEF14CAACCATGCAGCACCTGTATAT		
Babesia spp.	BabGF2- GYYTTGTAATTGGAATGATGG	359	[35]
	BabGR2- CCAAAGACTTTGATTTCTCTC		
gltA	gltAF-5`GGCTAATGAAGCGGTAATAA AT-ATGCTT3 `	341	[36]
	gltAR5`TTTGCGACGGTATACCCATAGC-3		

4.3.Statically Analysis

The detection rate of each bacterial species was analyzed according to relationship host animals, and the abundance of the tick-borne pathogens from the tick and the relation to pathogens to the tick's species types. simple's mathematical procedure was used on Microsoft Excel using on current version 2019 16.0.6742.2048.

4.3.1. Phylogenetic Analysis

A phylogenetic tree will be constructed using MEGA version 7.0 program [37] with DNA sequences obtained from this study and those from the same pathogens already available in the Gen Bank. Unweighted pair group method with arithmetic mean (UPGMA) clustering was used for phylogenetic tree analysis. all pathogen specific primers result will be analyzed for the relationship and their lineages.

4.3.2. Bioinformatics and Its Analysis.

The data analysis for the identification of microbiome of microbial species from tick samples among the host animals.

Bioinformatic analyses were performed as follows.i Raw reads were processed through a quality check, and low-quality (< Q25) reads were filtered using Trimmomatic 0.32[33]. Paired-end sequence data were then merged using PandaSeq[38]. Primers were trimmed using the ChunLab in-house program (ChunLab, Inc., Seoul, Korea), applying a similarity cut-off of 0.8. Sequences were denoised using the Mothur pre-clustering program, which merges sequences and extracts unique sequences, allowing up to two differences between sequences[39]. The EzBioCloud database[40] was used for taxonomic assignment using BLAST 2.2.22[41], and pairwise alignments were generated to calculate similarity [42] The UCHIME algorithm and non-chimeric 16S rRNA database from EzBioCloud were used to detect chimeric sequences for reads with a best hit similarity rate of < 97%[43]. Sequence data were then clustered using CD-Hit and UCLUST[43][44]. All the described analyses were performed with EzBioCloud, a commercially available ChunLab bioinformatics cloud platform for microbiome research (https://www.ezbiocloud.net/). The reads were normalized to 12,000 to perform the analyses. We computed the Shannon index[45], unweighted pair group method with arithmetic mean (UPGMA) clustering[46], principal coordinates analysis (PCoA)[47],and permutational multivariate analysis of variance (PERMANOVA)[48] based on the generalized UniFrac distance[49].We used the Wilcoxon rank-sum test to test for differences in the number of operational taxonomic units (OTU) and used the Shannon index to compare microbiome diversity between the two groups.

V. Result

5.1. Detection rate among the selected wild animal ticks

In the pathogen specific PCR, 72 out of total 136 tick samples were positive for any potential pathogens (52.94%). The detection rate of pathogen in ticks from Wildebeest, Buffalo, Zebra, Lion were 64.7%, 60.6%, 54.24 %, and 33.33%, respectively. The most detected potential pathogen was *Coxiella* spp. (38.24%), followed by *Rickettsia* spp. (13.24%), and *Theileria* spp and *Babesia* spp were (0.74%) respectively. While *Anaplasma* spp, *Bartonella* spp, and *Borrelia* spp w ere not detected in ticks (Table 2).

Since ,the detection rate for disease causing pathogens increase , the animals are vulnerable for the above pathogens. In a manner that is consistent with the animals (host) Wildebeest (64.7%)), Buffalo (60.6%) Zebra (54.24% and Lion (33.33%) have infected with bacterial tick-borne pathogen (table 3). the Naabi and Ebu-2426 samples and host living locations were free of the targeted pathogens (Table 2).

Table.2. Location , numbers of wild animals and feeding ticks, and detection rate of pathogens

		no of tick	sex		Number of infected ticks							
Location	Sample sources		M	F	*Baronella*	*Borrelia*	Anaplasma	*Rickettsia* spp	*Coxiella* spp	*Theileria* spp	*Babesia* spp	Total (%)
naabi-	Lion	17	13	4	0	0	0	0	0	0	0	0
naabi-plain	Lion	10	6	4	0	0	0	0	9	0	0	9(90)
Ebu-2467	Zebra	17	12	5	0	0	0	2	14	0	0	16(94.11)
2B-4475	Zebra	12	8	4	0	0	0	5	11	0	0	16(22.22)
Ebu-2426	Zebra	30	26	4	0	0	0	0	0	0	0	0
SCA-2438	Buffalo	16	11	5	0	0	0	3	15	0	0	18(25)
SCA-	Buffalo	17	16	1	0	0	0	0	2	0	0	3(4.16)
CTA-2436	wildebeest	4	2	2	0	0	0	1	0	0	0	1(1.38)
CTA-2458	wildebeest	7	5	2	0	0	0	4	1	0	0	5(6.94)
Dbl-2454	wildebeest	4	2	2	0	0	0	3	0	0	0	3(4.16)
CTA-2455	wildebeest	2	1	1	0	0	0	0	0	1	1	2(2.78)
	Total (%)	136	102	34	0	0	0	18(13.24)	52(38.24)	1(0.74)	1(0.74)	72(52.94)

Table 3. Detected pathogens compared with sample sources(wild animals)

tick sources (animals)	Number of ticks	*Rickettsia* spp	*Coxiella spp*	*Theileria spp*	*Babesia spp*	Total (%)
Lion	27	0	9	0	0	9(33.3)
Buffalo	33	3	17	0	0	20(60.6)
Zebra	59	7	25	0	0	32(54.24)
Wilde-beest	17	8	1	1	1	11(64.7)
Total (%)	136	18(13.24)	52(38.24)	1(0.74)	1(0.74)	72(52.94)

5.2. Percentage of detected bacterial pathogen among tick species

In this study, to determine the targeted bacterial pathogen from ticks which have been collected from wild animals, total of 7 disease specific primers has been used (table 1).Among the ticks species, *Rhipicephalus pulchellus, Rhipicephalus evertsi*, and *Rhipicephalus Appendiculatus* were the most detected ticks' species as shown (table.4). whereas any pathogens were not detected in *Rhipicephalus Muhsamae* and *Rhipicephalus leachi* species. The frequently detected pathogens were occurred *47(58.02%),*12(54.54,9(50%) *Rhipicephalus pulchellus, Rhipicephalus evertsi*, *Rhipicephalus Appendiculatus respectively*(table 4).

Table 4. Detected bacterial spp and selected tick species type.

Tick species type		Bacterial pathogen		Protozoal pathogens		
	total	*Rickettsia* spp	*Coxiella* spp	*Theileria* spp	*Babesia* spp	total (%)
Hyalomma Tranctum	1	1	0	0	0	1(00)
Amblyomma Lepidum	2	2	0	0	0	2(100)
Amblyomma Gemma	2	1	0	0	0	1(50)
Rhipicephalus Evertsi	22	2	10	0	0	12(54.54)
Rhipicephalus Muhsamae	6	0	0	0	0	0
Rhipicephalus Appendiculate	18	0	9	0	0	9(50)
Rhipicephalus Pulchellus	81	12	33	1	1	47(58.02)
Rhipicephalus Marginatum	1	0	0	0		0
Rhipicephalus Leachi	3	0	0	0	0	0
total (%)	136	18(13.24)	52(38.4)	1(0.74)	1(0.74)	72(53))

5.3.Co infection

Out of 72 positive ticks, 10 ticks (7.35%) were found co-infected with two pathogens. One co-infection detected was *Coxiella Burnetti* with *Rickettsia* spp in two tick species such *Rhipicephalus pulchellus, Rhipicephalus evertsi* tick collected from zebra (n=6),buffalo(n=3) and wildebeest (n=1) (Table 5).

Table 5. Identified tick species from wild animals and their co infection

Pathogen	No of positive tick from wild animals				Ttal(n=136)
single infection	Lion (N =27)	Zebra (N=59	Bufalo(N=33)	Wildebeest (N=17)	%
Bartonella spp	0	0	0	0	0
Borrelia spp	0	0	0	0	0
Anaplasma spp.	0	0	0	0	0
Rickettsia spp	0	7	3	8	18
Coxiella spp	9	25	17	1	52
Theileria spp	0	0	0	1	1
Babesia spp	0	0	0	1	1
subtotal	9	32	20	11	72(52.29)*
Mixed infections					
Rickettsia spp.+ *Coxiella spp.*	0	6	3	1	10(90)
Subtotal	0	6	3	1	10(7.35)*
Total	9	38	23	14	84

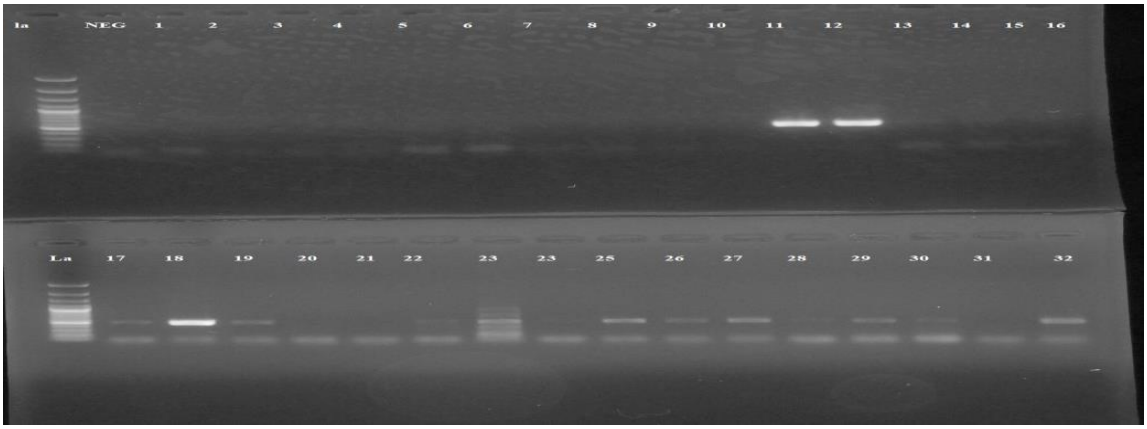

Figure 2 .Gel electrophoresis of Coxiella burnttii polymerase chain reaction.
Amplified polymerase chain reaction product with amplicon size of 501 bp.(La: reference ladder ,Neg; negative control and the numbers are sample ID).

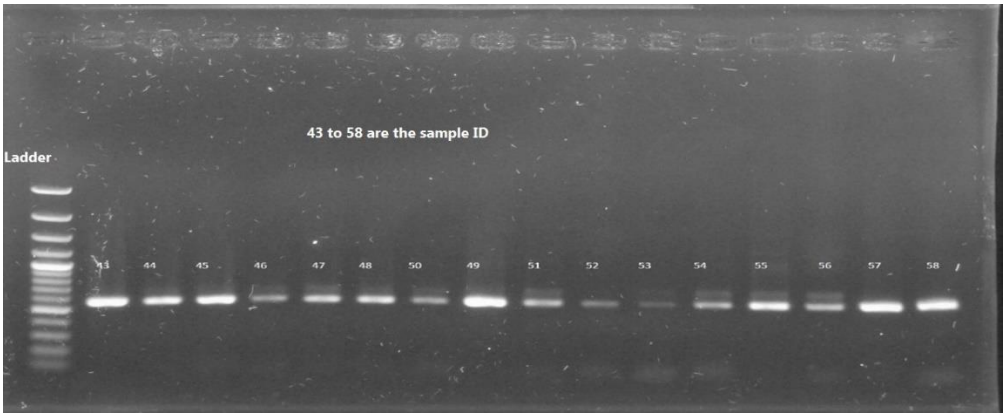

Figure 3. Gel electrophoresis of Rickettsia species polymerase chain reaction. Amplified polymerase chain reaction product with amplicon size of 450 bp.

5.4. Species Identification

The PCRs were performed in an Applied Biosystems Veriti Thermal Cycler (Applied Biosystems, Foster City, CA) with the following conditions: denaturation at 95^{0}C for 5 min, and then 40 cycles of denaturation at 95^{o}C for 30 s, annealing at 52^{o}C for 1 min, and elongation at 72^{o}C for 30 sec and 70^{o}C for 5 minutes for gltA and ompA genes, respectively.

One Sequence data for tick-extracted DNA samples that had been identified as *Rickettsia* using pathogen specific PCR and primer which comparison to 341 bp citrate synthase (gltA) gene, were obtained from NCBI's data base from a previous study[50]. The sample is closely related with *Rickettsia Aeschlimannii* strain *and Rickettsia Raoultii* strain Khabarovsk as figures 4.

Rickettsia Aeschlimannii, which was first isolated and documented from *Hyalomma marginatum* ticks collected in Morocco in 1997 and , has also been found in Zimbabwe, Niger, and Mali[51]. Whereas R.Raoultii also identified as a novel species of SFG rickettsiae through genotypic and phenotypic analysis and named Rickettsia Raoultii in 2008[52].Its pathogenic and cause for SFGs such as Tick-borne lymphadenopathy(TBOLA), and MSF and distributed in different contents.

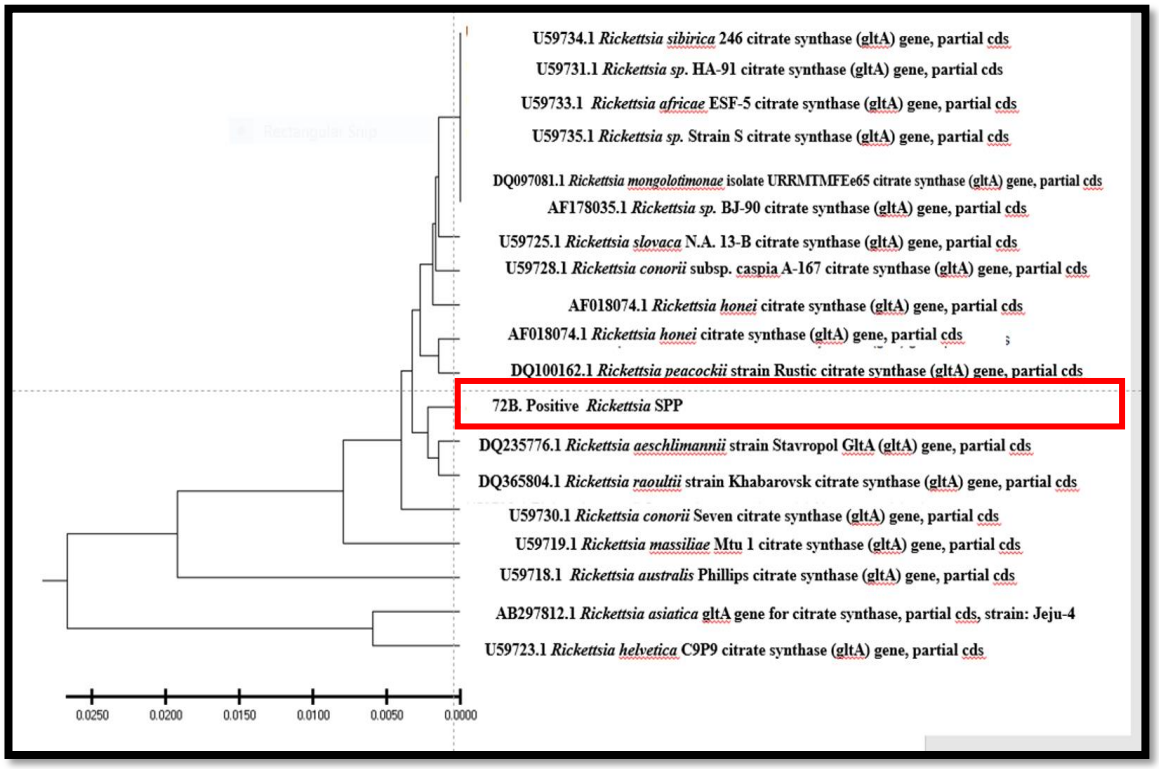

Figure 4. Phylogenetic tree of gltA gene sequence of Rickettsia phylotype Ixodidae ticks

5.5. Microbiome Result

5.5.1 Alpha diversity of tick's microbiome between different host species.

The ticks sample hosts were wild animals such as zebras, buffalo wildebeests and total of 16 ticks DNA(Wildebeest (n=3), Buffalo (n=6), and Zebra (n=7)) were used for microbiome analysis(16S rRNA). The average reads for the selected bacteria were 49,733 reads assigned to 360 species (OTU), 47,373 reads assigned to 200 species, 61,076 reads assigned to 490 species for each, respectively. The number of identified operational taxonomic unit (OTU) abundance, highest median observation in Zebra (122) and lowest in buffalo (70) but no significance difference among the groups (figure 4).

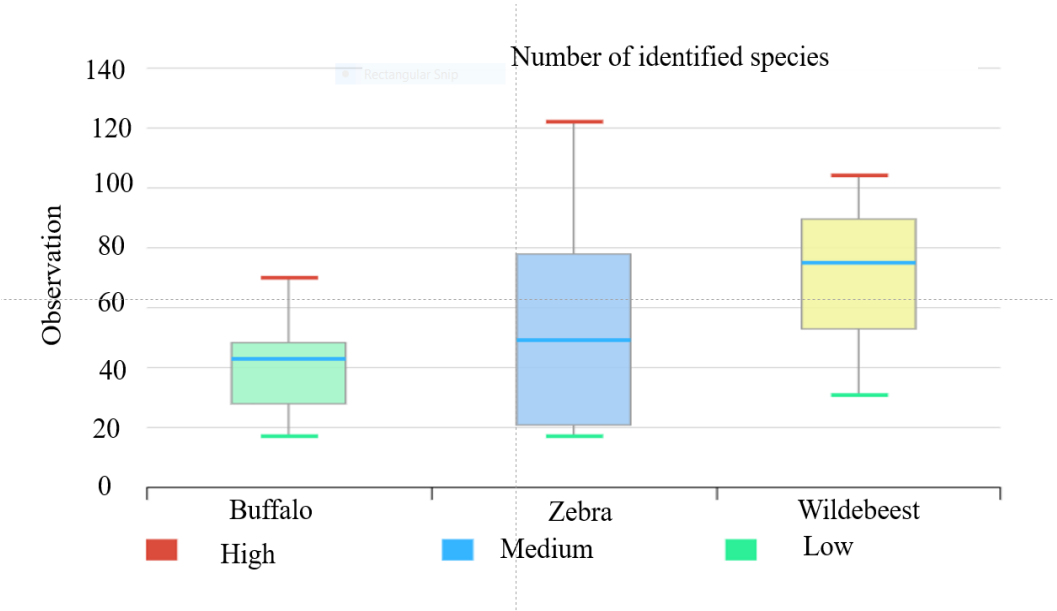

Figure 5. Alpha diversities of the tick's microbiome collected from wild animals(Number of identified species).

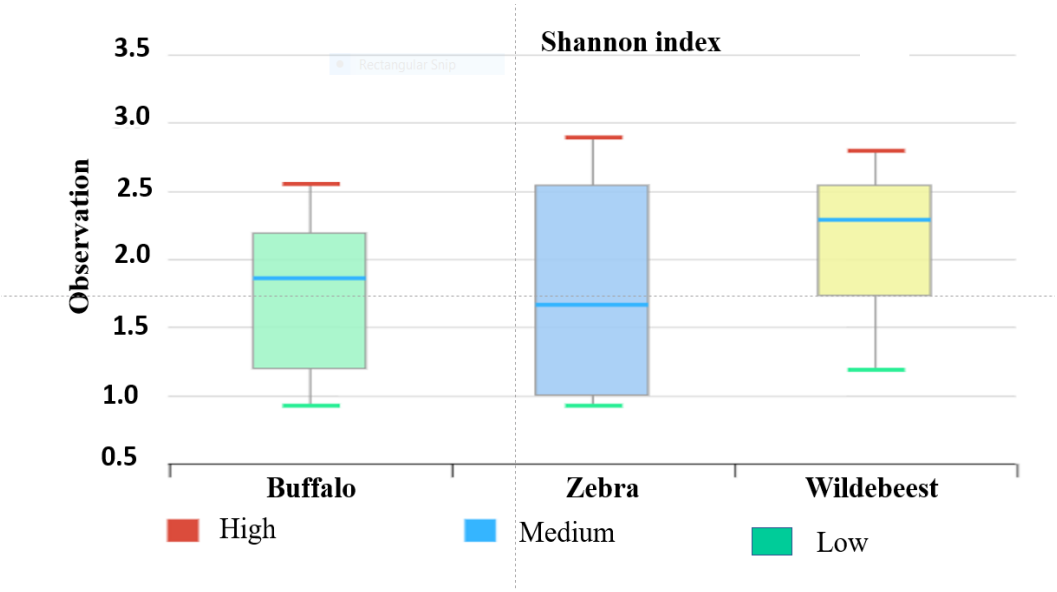

Figure 6 Shannon index of microbiomes of ticks collected from wild animals

As shown (figure 6) results indicated the Shannon index (ecology of microbiomes), among the three groups, species diversely distributed as well as richness and the evenness of the species community increase. The median observation in wildebeest (2.37) were higher than Zebra (1.87 and Buffalo (1.76) recorded.

5.5.2. Beta diversity of tick's microbiomes between different host species.

Beta diversity examination incorporating abundance and taxonomic relatedness, showed evidence of different bacterial communities among tick species host animals.

The principal-coordinate analysis indicates that ,the differences in taxonomic compositions of bacterial communities among the tick samples groups were evenly distributed (figure 7).the most diversified from the groups ,ticks from Buffalo bacteria and better distribution in terms of the environmental relationships might be contribution.

The principal coordinates (PCs), each of which indicates a certain species variability ,diversity and distantly distributed among the groups.

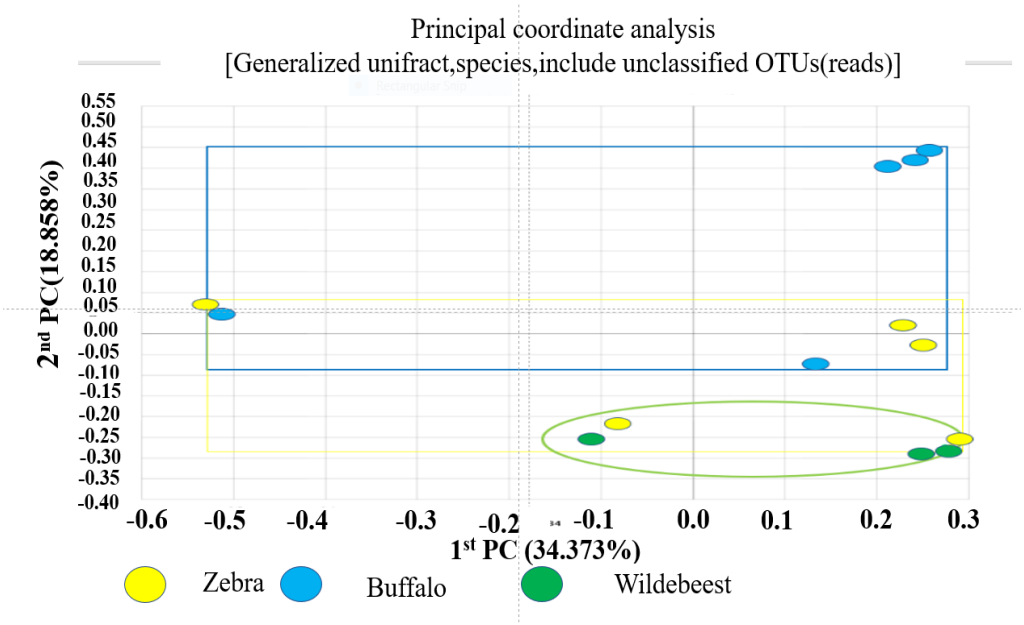

Figure 7. Principal Coordinate analysis (PCoA) of beta diversity of bacterial groups.

The UPGMA clustering showed that the samples were organized according to group; tick samples microbiomes were mainly clustered as shown figure.8.The most similar clustering indicating that samples in the microbiomes group shared relatively similar in bacterial composition distribution and their frequency in tick samples.

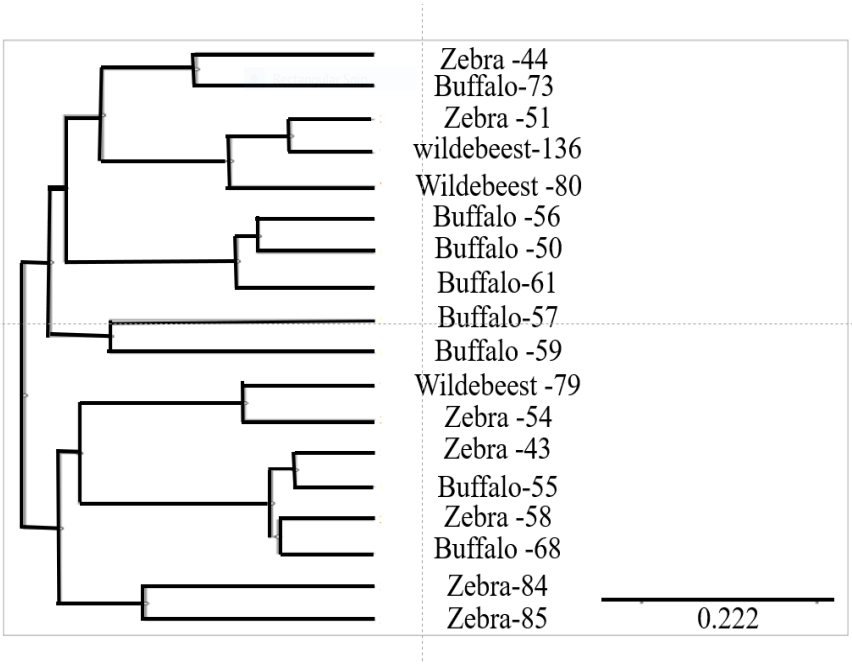

Figure 8 .Unweighted pair group method with arithmetic mean (UPGMA) clustering analysis of microbial communities of the ticks collected from wild animals.

5.5.3. Relative abundances of the microbiome

The abundance microbiome of the of bacterial taxa at the species level in tick samples collected from Zebra, Buffalo, and wildebeest. Each bar describes the average relative abundance value of independent animal. Species containing greater than already calculated and less than 1% reads shown(figure 8).

The bacterial taxa abundance among the selected ticks samples , *Acinetobacter guillouiae* groups(25.90%),*Finegoldia Magna* (22.33%),under 1% coverages (17.08%),and *Escherichia coli* groups in wildebeest ticks, Coxiella groups (24.33%),*Corynebacterium falsenii* (14.77%), C*utibacterium acnes* groups (13.75%), and under 1% coverage (11.47%)
in Buffalo ticks and *Coxiella* groups (26.93%),under 1% coverages (14.47%),*Corynebacterium xerosis* group (12.24%) were in zebra tick were the highest observed taxonomic coverage and would have a possibility in be a potential source of infectious bacteria to animals and humas.

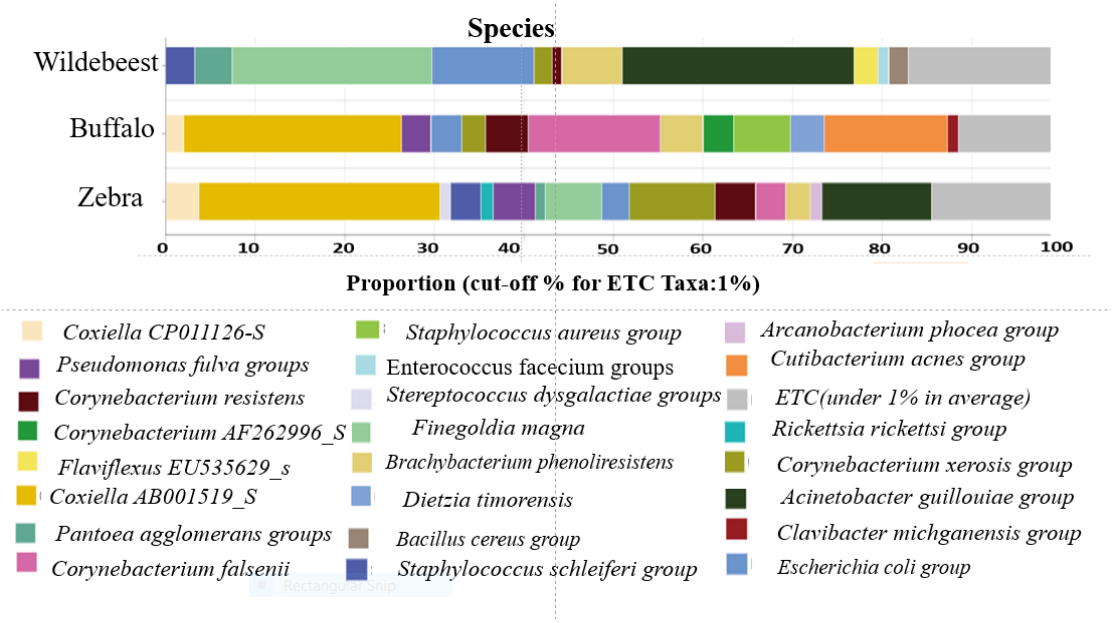

Figure 9 Average Bacterial taxa composition at the specie. Level.

Table 6:Tick average bacterial composition coverage in Percentage.

Taxa Name	Wildebeest tick(%)	Bufallo ick(%)	Zebra Tick(%)
Finegoldia magna	0.221	0	0.062
Bacillus cereus group	0.021	0	0
Corynebacterium resistens	0.010	0.047	0.045
Unclassified In higher Taxonomic Rank	0.005	0.001	0.004
Escherichia coli group	0.113	0.034	0.030
Enterococcus faecium group	0.011	0	0
Pantoea agglomerans group	0.041	0	0.011
Corynebacterium xerosis group	0.020	0.026	0.095
Flaviflexus EU535629_S	0.0267	0	0
Acinetobacter guillouiae group	0.257	0	0.121
Staphylococcus schleiferi group	0.0323	0	0.033
Brachybacterium phenoliresistens	0.0670	0.047	0.027
ETC(Under 1% In average)	0.1697	0.114	0.143
Coxiella group	0	0.242	0.267
Cutibacterium acnes group	0	0.137	0
Coxiella CP011126 group	0	0.020	0.037
Staphylococcus aureus group	0	0.063	0
Dietzia timorensis	0	0.0375	0
Clavibacter michiganensis group	0	0.012	0
Corynebacterium AF262996_S	0	0.0338	0
Corynebacterium falsenii	0	0.147	0.032
Pseudomonas fulva group	0	0.032	0.046
Arcanobacterium Phocae group	0	0	0.012
Streptococcus dysgalactiae group	0	0	0.011
Rickettsia rickettsii group	0	0	0.013

Bacterial families identified in tick species, represented as relative number of sequences in Figure 9, show 24 dominant taxa. While bacterial composition varied between tick's species, sequences from the members of Coxiellaceae, Francisellaceae, and Rickettsiaceae families (Phylum: Proteobacteria) were the most abundant.

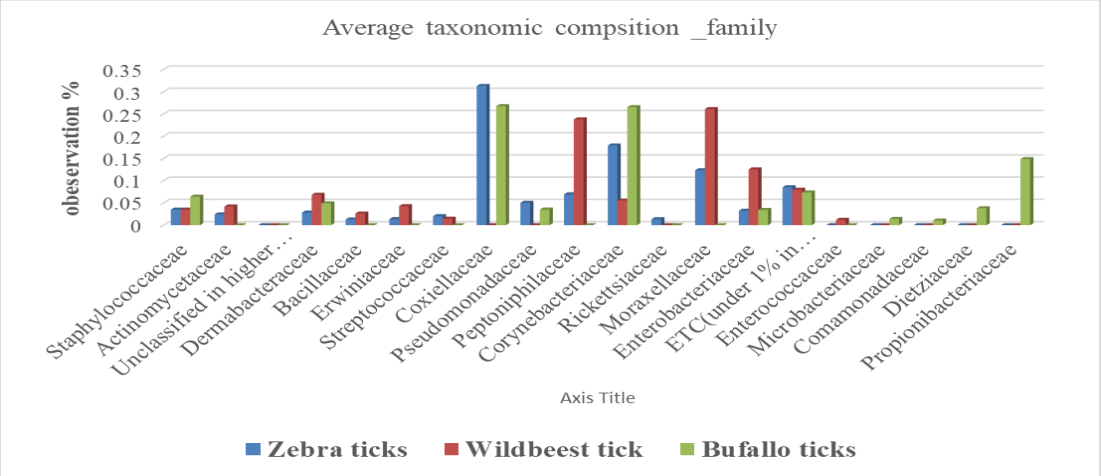

Figure 10 Average taxonomic composition at family level for the three groups.

Contemplating a large number of bacteria was categorized into the phylum Proteobacteria both in all three set of tick samples, we therefore defined phylum with relative abundance ≥0.1% as predominant phylum (Figure 10).Relative abundance show that bacteria belonging to the Proteobacteria phylum were the most abundant and diverse taxa classified, followed by Actinobacteria and Firmicutes. The highest composition coverage observed in the ticks proteobacteria in zebra and wildebeest ticks while the actinobacteria composition higher in Buffalo ticks followed by zebra and wildebeest respectively(figure. 12).

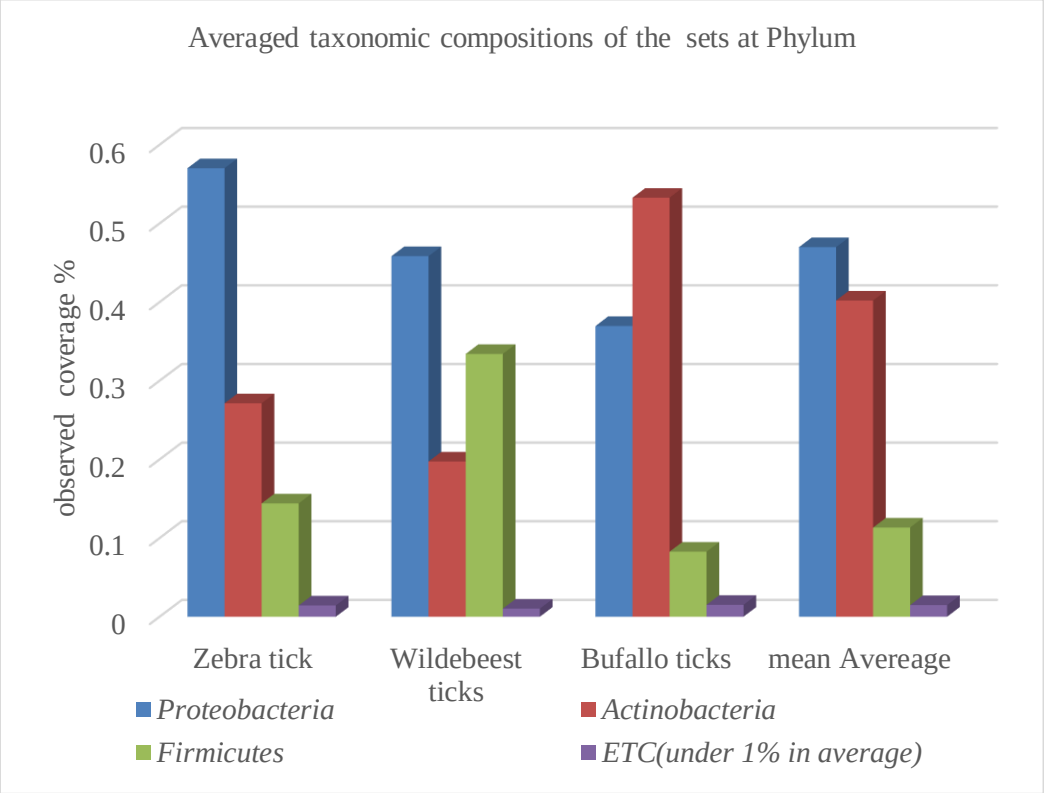

Figure 11 Average taxonomic composition of the groups Phylum.

VI. Discussion

Special emphasis would be important for wild animals, their environment and mutual relationship which contribute for vector borne disease such as tick-borne disease. The tick-associated area and the distribution area of each vector are observed worldwide. Around 20 among 21 species are transmitted by ticks, serving as both the vector and the main reservoir of the spotted fever population[53].

Studying the tickborne pathogens in eastern Africa regions about their prevalence, diversity and composition would have a great importance, since the area diversified and rich in wild and livestock's, animals, and other anthropoids. Since wild animals share common living area and mutually benefited to each other's which lead them for sharing disease vectors(tick) and pathogens. These and other information help to minimize the disease transmission from human to animals and vies versal. Different Studies have been conducted tick borne disease in livestock (cattle), vertebrate while in wild animals were tiny efforts had been done. The ultimate effort was to identify the human diseases causing pathogens in tick species collected from wild animals such as Lion, Buffalo, Zebra and Wildebeest in Serengeti National Park, Tanzania, and PCR and 16S rRNA NGS was applied. The detection rate of the pathogen and a variety of pathogenic bacteria and protozoa were identified ,whereas tick borne viral disease were not included in this study due to long time storage for the samples issue. In this finding, the common disease -causing pathogen detected were , *Coxiella burnetti, candidiatus rickettsia, Theileria luwenshuni* and *Babesia* spp while the *Anaplasma* spp, *Bartonella* spp and *Borrelia* bacterial pathogens were negative(table 2) and in addition, the overall detection rate 52.94% among 136 tick samples collected from wild animals.

The most positive pathogen among tick species were, *Hyalomma Tranctum, Amblyomma Lepidum, Rhipicephalus Evertsi Rhipicephalus Appendiculate* and *Rhipicephalus Pulchellus*. Overall, we have identified 4 pathogens from different tick species collected from wild animals.

The co-infections were observed between *candidiatus rickettsia* and *C. burnttii* in this study (table 5).

further investigations into their potential pathogenicity interaction with surrounding environment and routine surveillance would be important for disease transition mitigation in all communities. For instance, in this study, detection rate of 52 ticks were positive for *Candidiatus rickettsia* and *Coxiella* collected from buffalo and zebra, which is line with a previous report that indicated the detection rate of *Coxiella* spp and *Rickettsia* spp. were 43% and 5% in 136 ticks collected in Kenya [23].

In this study, the detection of *Anaplasma, Bartonella, Borrelia* pathogens were all negative. However, the possibility of diseases caused by these genera remains high in Tanzania, 15.9% for *Anaplasma*[5], in Ethiopia, the detection rate by RLB, including *A. marginale* (14.5%) and Anaplasma sp. 'Omatjenne' (25.5%)[7] , in Kenya anaplasmosis (6.32% *Anaplasma* ovis,14.36% *Anaplasma platys*, and 3.08% *Anaplasma bovis*,)[8], and the prevalence of *Anaplasma marginale* found in Mozambique study was than the 5.4 % (27/500)[54]. All these information's is like this finding which indicates, the eastern Africa region ,the tick-borne disease being prevalent and its persistence. Comparable study conducted in northern Tanzania ,nearest place for the study site, the most frequent detected pathogen was *Rickettsia*ceae with 65.5%(131/200) in wild animals and 16 % in cattle in Masawa and Iringa region[6] while in this study was 13.34% coverage.

Total of 16 hard (Ixodidae) and all belongs to *Rhipicephalus Pulchellus* tick samples were used for NGS to observe the abundance of the bacterial diversity and looks for pathogenic bacteria in taxa. From total reads (158,182) assigned 1,050 OTUs were identified from ticks 16S RNA. The highest average species observation of alpha diversity recoded in wildebeest (2.16), zebra (1.87) and buffalo (1.76) respectively.

The bacterial tax abundance among the selected wild animals, *Acinetobacter guillouiae* groups (25.90%) in wildebeest, *Coxiella* (24.33%) in buffalo and 26.93% in zebra were the highest abundance where as in similar study among 28 phyla of which Chlamydiae, Proteobacteria, Actinobacteria, and Firmicutes were the four most dominant phyla, accounting for >90% of the bacteria present in the tick samples[55].Proteobacteria coverage in wildebeest(45.84%) and zebra(57.02%) ticks show increment. The variation observed in the

bacterial community might be due to the blood meal, feeding had little or no impact on the microbial diversity associated with Ixodes ticks. Member of phyla proteobacteria and other members of the general are wide spread in the environments and some of them are pathogenic to human , animals and can induce clinically relevant opportunistic infection[56]. The principal coordinate analysis (generalized Unirac), we also could be able to see the variation in bacterial composition and difference between ticks collected from wild animals(figure 6).

Overabundance of extra bacteria have been detected in all ticks, which may be acquired either by means of the host (from the host's skin or ingested by way of ticks during host[57]. However, among the target for this study, we can be able to identify the mostly common disease rickettsia, and *Coxiella* bacteria species in tick collected from zebra and buffalo, and other infectious bacterial taxa (fig 4).

Medically important related tick-borne bacteria which includes *Anaplasma* spp., *Borrelia* spp., *Coxiella* Sp., *Ehrlichia* spp., *Francisella* spp. and *Rickettsia* spp. Grouped as alphaproteobacteria family and has *Rickettsia* spp. is one of them[58]. All the varied environmental microbiota that may gain access into the tick, solely many become genuine members of the tick microbiome. as a result of diet plays a central role in shaping the composition of the class microbiome, it's possible that hematophagy may choose surely microorganism genera[59].

Even if, we have a concern for sample size and representativeness, the result shown that wild animals are highly infected with tick borne disease and the prevalence also high. Endosymbionts relationships of ticks allow them to vertical pathogen transmission, such as *Coxiella* and *Rickettsia* while the tick while feeding on an infected host[60]. In this investigation, both pathogen examined are universally important Coxiella burnttii , the causative agent of Q fever is known to be an developing zoonotic illness, whereas rickettsia pathogen are mindful for a number of spotted fever counting Africa tick

bite fever (caused by Rickettsia Africae) in our study site. Both are extremely severe and potentially fatal disease in humans and animals.

VII. Conclusion

- Detection and analysis of ticks collected from wild animals demonstrated that Rickettsia and Coxiella pathogen detection rate were high in this study ,among the targeted pathogens.
- The microbiomes of bacterial composition varied between tick's host animals, and the most occurrence microbiota from the members of Coxiellaceae, Francisellaceae, and Rickettsiaceae families (Phylum: Proteobacteria) were the most abundant.
- Some target pathogens were detected using specific PCR and NGS approaches, therefore ,it's important for detail information and easy decision making.
- In the future, NGS application for detection of pathogens could be considered since it is accurate and time saving regardless of its cost and infrastructure.
- Beside site specific study, further national level study incorporated all tick-borne disease in wildlife, livestock and environmental sources for humans and zoonotic disease under one health perspectives necessary important.

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IX. Annex-1

	First PCR, Anaplasma-taceae				Second PCR, Anaplasmataceae				
	Vol-ume	Tem pera-ture	Time	cy cle		Vol-ume	Tem pera-ture	Time	cy cle
EHR1 F	1µl	94°c	3m		EHR3 F	1µl	94°c	3 m`t	
newEHR2 b	1µl	94°c	0.30s e		newEHR2 b	1µl	94°c	0.30s e	35
dntp mix	2.5µl	63°c	0.30s e	35	dntp mix	2.5µl	52°c	0.30s e	
10taq mix buffer	2.5µl	72°c	0.30s e		10 taq mix buffer	2.5µl	72°c	0.30s e	
Taq	0.1µl	72°c	10 m		Taq	0.1µl	72°c	10m	
Dw	16.9µ l	4°c	∞		Dw	16.9µ l	4°c	∞	
Extract DNA	1µl				Extract DNA	1µl			
Total	25µl				Total	25µl			

Bartonella spp PCR Condition									
first PCR					Second PCR				
	1x		Time	Cy-cle		1x		Time	Cy-cle
QHVE1 F	1 ul	94°c	3 minutes		QHVE12 F	1ul	94°c	3 minutes	
QHVE3 R	1 ul	94°c	30 sec		QHVE 14 R	1ul	94°c	30 sec	
d NTP mix	2.5 ul	55°c	30 sec	40	D TNP mix	2.5 ul	55°c	30sec	40
10 x Taq buffer	2.5 ul	72°c	30 sec		10 x Taq buffer	2,5 ul	72°c	30 sec	
Taq	0.1 ul	72°c	5 minutes		Taq	0,1 ul	72°c	5 minutes	
DW	16.9ul	4°c	∞		DW	16.9ul	4°c	∞	

Tem-plate	0.5				Template	o.5			

Babesia spp PCR Condition				
	1x	Temperature	Time	Cycle
Bab GF2	1ul	94c	3min	
BabGR2	1ul	94c	30 Sec	
d NTP mix	2.5ul	55c	30 sec	40 cycles
10x Taq buffer	2.5ul	72c	0.30 sec	
Taq	0.1ul	72c	5min	
DW	16.9 ul	4c		
Template	0.5ul			
Total	25			

	First PCR, Borrelia species			
	Volume	Temperature	Time	cycle
New LDfb	1µl	94oc	3m	
new LDrb	1µl	94oc	0.30se	
dntp mix	2.5µl	61oc	0.30se	40
10 Taq mix buffer	2.5µl	72oc	0.30se	
Taq	0.1µl	72oc	10 m	
Dw	16.9µl	4oc	∞	
Extract DNA	1µl			
Total	25µl			

	First PCR, Rickettsia spp				Second PCR, Rickettsia spp				
	Vol-ume	Tem pera-ture	Time	cy cle		Vol-ume	Tem pera-ture	Time	cy cle
Rr17k.1p	1µl	94°c	3m		Rr17k.P90	1µl	94°c	3m	
Rr17k.539 n	1µl	94°c	0.30s e		Rr17k.539 n	1µl	94°c	0.30s e	35
dntp mix	2.5µl	55°c	1m	35	dntp mix	2.5µl	57°c	1m	
10 taq mix buffer	2.5µl	72°c	1m		10 taq mix buffer	2.5µl	72°c	1m	
Taq	0.1µl	72°c	7m		Taq	0.1µl	72°c	7m	
D.w	16.9µ l	4°c	∞		D.w	16.9µ l	4°c	∞	
Extract DNA	1µl				Extract DNA	1µl			
Total	25µl				Total	25µl			

	First PCR Coxiella				Second PCR, Coxiella				
	Vol-ume	Tem-pera-ture	Time	cy-cle		Vol-ume	Tem-pera-ture	Time	cy-cle
Q3 R	1µl	94°c	3m		Q4 R	1µl	94°c	3m	
Q5 F	1µl	94°c	0.30se		Q6 F	1µl	94°c	0.30se	35
dntp mix	2.5µl	56°c	0.30se	35	dntp mix	2.5µl	52°c	0.30se	
10 taq mix buffer	2.5µl	72°c	0.30se		10 taq mix buffer	2.5µl	72°c	0.30se	
Taq	0.1µl	72°c	5m		Taq	0.1µl	72°c	5m	
D.W	15.9µl	4°c	∞		D.W	15.9µl	4°c	∞	
Ex-tract DNA	2µl				Ex-tract DNA	2µl			
Total	25µl				Total	25µl			

Piroplasmide PCR Condition				
	1x	Temperature	Time	Cycle
BJ1 F	1ul	94c	5min	
BN2 R	1ul	94c	1min	
d NTP mix	2.5ul	55c	1min	40 cycles
10x taq buffer	2.5ul	72c	1min	
Taq	0.1ul	72c	5min	
DW	16.9 ul	4c		
Template	0.5ul			
Total	25			