Bacterial microbiome of *Rhipicephalus sanguineus* ticks collected from dogs in the Mnisi community, South Africa

A dissertation submitted in fulfilment of the requirements for the degree Master of Science Veterinary Science Tropical Diseases in the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria

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DECLARATION

I hereby declare that this dissertation which I submit for the degree Magister Scientiae in the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, South Africa is my original research work and has not been submitted by me to any institution or University for the conferment of an award.

RAckermann

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Date

DEDICATION

This thesis is dedicated to my parents, Erica Ackermann and Ernst Ackermann, whom without I would not have made it this far. Somehow in an endless universe we found each other and I am so grateful to be able to call you my parents. Your support, dedication, sacrifices and love throughout my life has allowed me to flourish into the young woman I am today. Thank you for always believing in me and teaching me that no dream is ever too big.

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LIST OF ABBREVIATIONS

16S rDNA	16S ribosomal Deoxyribonucleic Acid
16S rRNA	16S ribosomal Ribonucleic acid
AIC	Akaike Information Criteria
BLASTn	Basic local alignment search tool nucleotide
bp	basepair
CCS	Circular Consensus Sequencing
CI	Confidence Interval
CLE	*Coxiella*-like Endosymbiont
CME	Canine Monocytic Ehrlichiosis
°C	Degrees Celsius
CO1	Cytochrome c oxidase 1
DBTK	DNeasy Blood and Tissue Kit
DNA	Deoxyribonucleic acid
g	Gram
ng	Nanogram
pg	Pictogram
HGA	Human Granulocytic Anaplasmosis
HHWRS	Hans Hoheisen Wildlife Research Station
Kb	Kilobase
MEGA	Molecular evolutionary genetics analysis
ml`	Millilitre
µl	Microliter
µM	Micromolar
mm	Millimetre
min	Minute
MSF	Mediterranean Spotted Fever
MPEE	Modified PureGene Ethanol Extraction
nMDS	Non-metric Multidimensional Scaling
OR	Odds Ratio
OTU	Operational Taxonomic Units
PCA	Principal Component Analysis
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction

r	Correlation Coefficient
RDP	Ribosomal Database Project
SAT	Saliva Assisted Transmission
TBD	Tick-borne Disease
TBP	Tick-borne Pathogen
USA	United States of America

Bacterial microbiome of *Rhipicephalus sanguineus* ticks collected from dogs in the Mnisi community, South Africa

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DISSERTATION SUMMARY

In Mnisi, a rural community in South Africa, *Rhipicephalus sanguineus* is one of the most prevalent ticks found on dogs. The community lies at the wildlife/livestock/human interface where humans are at risk of tick-borne diseases. The aim of this study was to investigate the diversity of the bacterial microbiome in *R. sanguineus* that may impact human health. Over a 12-month period, *R. sanguineus* (n=1,788), *Rhipicephalus simus* (n=61), *Rhipicephalus turanicus* (n=73), *Amblyomma hebraeum* (n=68), *Haemaphysalis leachi* (n=219) and *Hyalomma truncatum* (n=1) ticks were collected from 64 dogs. Genomic DNA was extracted from salivary gland and midgut tissues of 62 *R. sanguineus* tick pools (1 pool = 10 ticks); identifications were confirmed using Cytochrome c oxidase I barcoding. The 16S rRNA gene was amplified using barcoded primers and sent for Pacific Bioscience's circular consensus sequencing. Characterisation of the bacterial microbiome of midgut and salivary gland pools revealed a total of 260,312 sequences with Proteobacteria (85.44%) being the most prevalent phylum found; with *Anaplasma* (21.69%), *Coxiella* (12.12%) and *Ehrlichia* (19.94%) species dominating the microbiome. Further classification of *Ehrlichia* revealed 95.46% *Ehrlichia canis* and 4.54% *Ehrlichia* species *Anaplasma* consisted of 15.36% *Anaplasma centrale*, 75.82% *Anaplasma platys* and 8.82% *Anaplasma* species Phylogenetic analysis indicated that the *A. centrale* and *A. platys* clustered with various other published *A. centrale* and *A. platys* sequences, respectively. It also confirmed that all *Ehrlichia* species sequences detected in this study are *E. canis* sequences (94.46%). Furthermore, we determined that the *Coxiella* sequences detected in the study belong to the *R. sanguineus Coxiella*-like endosymbionts group. Assessment of risk factors for *R. sanguineus* infestation indicates that higher average monthly temperatures have a significant association with an increased risk of *R. sanguineus* tick infestations on dogs. Additionally, rearing chickens at the household was significantly associated with a decreased risk of *R. sanguineus* tick infestations on dogs. Our study indicated that *R. sanguineus* could be a potential reservoir for important bacterial pathogens of zoonotic importance.

CHAPTER 1

GENERAL INTRODUCTION

Tick-borne diseases (TBDs) have a major economical and health impact in South Africa due to the high biodiversity of pathogens infecting wild and domestic animals. This poses a critical threat especially to rural communities that depend on livestock as a source of income, labour and food (Sansoucy *et al.* 1995). The effect of tick-borne pathogens (TBPs) on livestock animals has been widely studied, however its impact on human health, particularly in rural communities is not well known (Hotez and Kamath 2009). Further research on the bacterial microbiome of ticks is required to characterize the diversity and prevalence of TBPs in the rural communities of South Africa.

The Mnisi community is a rural community located northeast of the Bushbuckridge Municipal area in Mpumalanga Province, South Africa. Mnisi, which is situated on the land of the Mnisi Traditional Authority, is located at a wildlife/livestock/human interface, with several game reserves surrounding the area. The main source of subsistence is agricultural and livestock farming, with cattle being the most important source. Goats and chickens are also plentiful with a small presence of donkeys and pigs. Ownership of domestic dogs has also become more common in the community. This creates an environment where pathogens are easily transmitted between wildlife reservoirs, livestock and domesticate dogs in the community.

There are various factors in a village or at a household that can influence tick abundance in the area and on domestic animals. These factors are classified as host factors, tick factors and environmental factors. Host factors include, but are not limited to availability and composition of the hosts (Cobbold *et al.* 2015), sex of the host, reproductive state of the hosts (Pollock *et al.* 2012) and behaviour of the hosts. Tick factors can include sex, reproductive state, host preference and host-questing behaviour (Süss *et al.* 2008). Environmental factors can cover a wide range of aspects such as temperature, pressure, moisture, seasonality and habitat (Debeila 2012). Not only do these factors affect tick infestations, but they can also influence the microbiome of ticks (Narasimhan and Fikrig 2015; Van Treuren *et al.* 2015) .

AIM OF THE STUDY

The aim of this study was to characterize the bacterial microbiome of *Rhipicephalus sanguineus* ticks collected from dogs in the Mnisi community, South Africa using a next-generation sequencing approach. Also, a questionnaire was conducted to determine possible factors that could have an influence on the tick abundance in the area and on domestic animals.

OBJECTIVES

- Determine the composition and diversity of the bacterial microbiome of *R. sanguineus* ticks collected from domestic dogs in Clare A, Mnisi using a microbiome sequencing approach.
- Determine the phylogenetic relatedness of *Anaplasma*, *Ehrlichia* and *Coxiella* spp. found to closely related species using 16S rDNA phylogenetic analyses.
- Determine if potential exposure factors at households in Clare A, Mnisi affected *R. sanguineus* populations on domestic dogs by conducting a questionnaire.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

Zoonotic pathogens are capable of infecting both animals and humans, and are therefore notably important (Taylor *et al.* 2001). Many emerging communicable diseases in humans can be ascribed to zoonotic pathogens arising from wildlife or domestic animal reservoirs (Taylor *et al.* 2001; Dantas-Torres *et al.* 2012). This poses a significant problem to rural communities in South Africa, as many of these communities lie at the livestock/human interface, with some in close contact with wildlife (Simpson *et al.* 2018). This wildlife/livestock/human interface facilitates the spread of zoonotic pathogens (Berggoetz *et al.* 2014), especially in areas with a lack of medical infrastructure.

The main pathway of transmission of zoonotic pathogens are arthropod vectors (Jones *et al.* 2010). Ticks constitute the majority of vectors carrying animal pathogens, and comes in second after mosquitoes for human vector-borne diseases (de la Fuente *et al.* 2008). Ticks are hematophagous (Parola and Raoult 2001; Kazimírová and Štibrániová 2013; Suppan *et al.* 2018) and acquire pathogens from an infected host during a blood meal (Kazimírová and Štibrániová 2013). The pathogens are taken up with the blood and will progress through the tick's body until it finally reaches the salivary glands where it can then infect a new host (Bowman and Sauer 2004; Šimo *et al.* 2017). Certain pathogens are also capable of replicating in different tick tissues, while it progresses through the body.

Tick-associated micro-organisms are capable of greatly influencing the colonization of pathogens in the tick body (Ross *et al.* 2018). Studies have shown that endosymbionts in the tick microbiome can have diverse interactions with their host as well as with other potentially pathogenic microbes (Clay and Fuqua 2010). The microbiome community and their interactions play a significant role in the tick's survival, physiology and pathogen acquisition (Clay and Fuqua 2010). These interactions create a dynamic microbiome community.

Characterizing the diversity and prevalence of zoonotic pathogens and their interaction with the tick microbiome is thus pertinent to understanding their epidemiology. This is of importance in the rural communities of South Africa where communities rely on livestock for various resources; with limited available medical resources.

2.2 TICKS AND PATHOGEN TRANSMISSION

Ticks consist of two families known as Ixodidae and Argasidae (Black and Piesmant 1994). Tick species in the Ixodidae family are also known as ixodid or hard ticks. The name is derived from their hard sclerotized scutum (dorsal plate), whereas soft ticks lack a scutum (Estrada-Peña *et al.* 2004). Soft ticks also have ventral capituli (mouth parts), meaning their mouth parts are located under their body. Conversely, hard ticks have anterior capituli, and their mouth parts can be seen from above as they are not hidden beneath the body (Estrada-Peña *et al.* 2004). Soft ticks penetrate deep into the host skin, are able to feed at high speed, and will feed repeatedly. This can cause substantial damage to the host. In contrast with soft ticks, hard ticks do not penetrate the host epidermis as deeply, and only feed once for a prolonged period of time during each developmental stage. The ixodid ticks include many important ticks, such as *Amblyomma*, *Ixodes*, *Dermacentor*, *Haemaphysalis* and *Rhipicephalus* (Barker and Murrell 2004).

Although only a small fraction of these ticks are known to transmit disease-causing pathogens (Jongejan and Uilenberg 2004), ticks still constitute a major threat to animal and human health. Tick species that are capable vectors can carry and transmit certain pathogens without any adverse effects, while some do experience a decrease or increase in fitness (de la Fuente *et al.* 2016). Nevertheless, ticks are ideal vectors due to their hematophagous feeding habits, which causes direct damage to the host and puts them in close proximity to veterinary and medically important pathogens (de la Fuente *et al.* 2008). While some ticks have a high vector competence, (a high aptitude to obtain and transmit a disease agent microbe from a reservoir host), and can acquire various pathogens, other ticks are closely associated with certain pathogens (de la Fuente *et al.* 2017). Many of these ticks also maintain a widespread distribution which is increasing due to climate change, as it allows them to adapt quicker to regions once limited by cold winters (Gray *et al.* 2009). Ixodid ticks also have certain properties that aid them in pathogen transmission. These properties include extended feeding periods, wide host variation, painless bites to avoid detection, as well as increased feeding on humans in certain species (Parola and Raoult 2001).

2.3 ATTACHMENT AND BLOOD-FEEDING

To be able to get to the blood vessels during feeding, ticks have to insert their hypostome through the skin of the host (Dantas-Torres 2008; Richter *et al.* 2013; Štibrániová *et al.* 2013; Suppan *et al.* 2018). In order to then anchor itself more securely to the host, the tick will secrete a type of cement that is produced in the salivary glands (Dantas-Torres 2008; Suppan *et al.* 2018). The cement consists of a mixture of mainly proteins, lipids and glycoproteins (Suppan *et al.* 2018). Other than anchorage, the cement has another role; that of filling in any gaps between the mouthparts inserted into the host and the host's skin. This prevents any loss of fluids and increases blood uptake (Suppan *et al.* 2018).

During penetration and feeding, ticks can damage many blood vessels, creating a large haemorrhagic pool in the dermis of the host (Kazimírová and Štibrániová 2013; Štibrániová *et al.* 2013). Normally, this would cause the host to react by forming a haemostatic plug and activating vasoconstriction and immune responses, which in turn would lead to tissue remodelling and healing of the wound (Kazimírová and Štibrániová 2013; Štibrániová *et al.* 2013). This process would disrupt feeding and eventually eliminate the tick. However, due to a long period of host-parasite co-evolution, ticks have evolved counter measures, allowing them to complete the feeding process. They manage this by using their salivary glands to produce various biologically active compounds that inhibit coagulation, platelet aggregation, inflammation and immunity of their hosts (Mans 2011; Kazimírová and Štibrániová 2013; Štibrániová *et al.* 2013; Valdés 2014; Kotál *et al.* 2015). When the tick is fully engorged, it can detach and drop off in order to completely digest its blood meal in a more sheltered area.

2.3.1 TICK MIDGUT

The tick midgut is the first barrier encountered by microbes that have been taken up during a blood meal. During feeding the midgut can go through some dramatic changes to prepare for the digestion of the blood meal (Agyei and Runham 1995). Different microbes take up during the blood meal will proceed through the tick in different ways - they can either be stored in the midgut and undergo rounds of development and replication until the next feeding sessions, or continue to the salivary glands, where they can then undergo development and replication (Lejal *et al.* 2019). Ross *et al.* (2018) suggested that bacterial growth could be inhibited in the midgut of hard ticks, as it is an ill-suited environment. For example, the midgut of *Ixodes scapularis* has very low levels of a thiamin, which is a vital nutrient for bacterial growth. Certain bacteria such as *Borrelia burgdirferi* are able to live under these conditions due to the evolution of distinctive metabolic strategies, allowing them to survive in low-thiamin environments. Other bacteria that lack these metabolic strategies might not be able to survive in this environment (Zhang *et al.* 2016).

2.3.2 TICK SALIVARY GLANDS

Tick salivary glands are very intricate, multifunctional glands that bear a resemblance to grapes. Each single structural unit is called an acinus. The salivary glands consist of many acini that are divided into four different types (I, II, III, and IV). Salivary glands are involved in various responsibilities, such as the acquisition and transmission of pathogens (Bowman and Sauer 2004), osmoregulation (which in ticks are not under the control of the Malpighian tubules) (Bowman and Sauer 2004; Dantas-Torres 2008) and the excretion of ions and water into the bite site (Suppan *et al.* 2018). During feeding the salivary glands of female ixodid ticks can enlarge by a factor of 25, not only in mass but also in protein content (Bowman

and Sauer 2004). This phenomenon also occurs in male ixodid ticks, but to a lesser extent (Bior *et al.* 2002).

Tick saliva produced by the salivary glands is very complex and contains large amounts of proteins and non-protein molecules, and it is the transmission route of pathogens to a new host. Pathogens are able to exploit molecules in the tick saliva to advance their survival, replication, development and transmission. This process is termed saliva-assisted transmission (SAT) (Liu and Bonnet 2014). Ticks have a remarkable ability to survive for extended periods in the environment without feeding. The salivary glands are able to absorb water from unsaturated air in the surrounding environment, which aids their ability to survive without a blood meal (Kazimírová and Štibrániová 2013).

2.3.3 THE PROCESS OF PATHOGEN ACQUISITION AND TRANSMISSION

For the majority of tick-borne pathogens (TBPs), acquisition happens during a blood meal (Bowman and Sauer 2004; Kazimírová and Štibrániová 2013). As stated previously, ticks alternate between regurgitating saliva and sucking up blood, which contains pathogens. The blood and pathogens will move into the midgut (Šimo *et al.* 2017) where the blood is digested (Figure 2 - 1). The midgut is an important defence barrier for pathogen infection in the tick itself. During pathogen acquisition and subsequent transmission, pathogens need to be able to overcome heterophagic digestion and then colonize or pass through cells of the epithelia surrounding the digestive tract (Liu and Bonnet 2014). Eventually they must then migrate into the haemocoel (Šimo *et al.* 2017). Here the pathogens can go through rounds of development and replication, after which they move into the salivary glands (Bowman and Sauer 2004; Šimo *et al.* 2017). Some pathogens are very specific and will only replicate and develop in the salivary glands, such as *Theileria annulata,* which has a high specificity for the type III acini (Bowman and Sauer 2004). Other pathogens such as certain viruses do not have cell specificity, and will replicate in either the salivary glands or the tick's body (Bowman and Sauer 2004). During the next blood meal on a healthy host, saliva containing the acquired TBPs will be injected into the haemorrhagic pool and infect the host (Šimo *et al.* 2017).

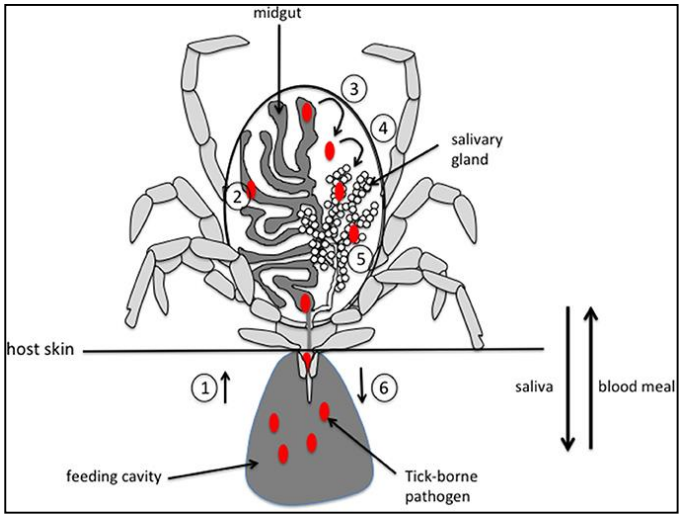

Figure 2 - 1: Diagram indicating the process of a blood meal, during which pathogens can be acquired and transferred by the tick. (1) During feeding, pathogens are taken up and ingested along with the blood. (2) The pathogens then enter the midgut, where they can cross the digestive tract epithelium. (3) The pathogens then invade the body of the tick, (4) and eventually cross over the salivary gland epithelium (5) to invade the acini. (6) During a next blood meal, pathogens are introduced into the new uninfected host along with the saliva. Modified from Šimo *et al.* (2017)

2.4 TICK MICROBIOME

Microbiome studies have become an important part of research focused on human pathogenic diseases. Given the immense role ticks play in the transmission of zoonotic diseases such as Lyme disease and Q-fever, the tick microbiome is of great significance for further research. Research conducted on tick microbiomes used to be limited, mostly focusing on whole ticks, rather than specific tissues, (Ponnusamy *et al.* 2014; Van Treuren *et al.* 2015) and only included a few life stages. Recent studies have focused on using different tissues of the tick, such as the midgut and the salivary gland (Gall *et al.* 2016)(Gall *et al.* 2016), as well as using different life stages (Zolnik *et al.* 2016).

It is possible for a tick to be co-infected with multiple pathogens at once, however, they are also able to host various endosymbiotic bacteria. These tick-borne endosymbionts have evolved to provide a range of functions, such as dietary supplementation, immune responses and survival for the host (Scarborough *et al.* 2005; Clay and Fuqua 2010; Ahantarig *et al.* 2013). Most importantly, they influence pathogen transmission and acquisition (Clay and Fuqua 2010).

Literature defines two types of endosymbionts, namely primary and secondary. Primary endosymbionts are obligate microbes that are vital for their host's fitness and survival. Secondary endosymbionts are not entirely essential to their host's fitness, as they do not likely provide any essential benefits. Furthermore, primary endosymbionts are usually vertically transmitted (from mother to offspring) (Zhong *et al.* 2007; Brouqui 2011; Ahantarig *et al.* 2013). If strictly vertically transmitted endosymbionts are beneficial in any way for host reproductive fitness, it will allow the endosymbiont to enhance their own transmission, and

they will increase in frequency (Ahantarig *et al.* 2013). Bacterial symbionts that are able to transmit vertically in ticks include *Rickettsia*-, *Coxiella*-, and *Francisella*-like organisms (Noda *et al.* 1997). These endosymbionts not only influence the colonisation and transmission of pathogenic TBPs, (Macaluso *et al.* 2002) but in humans, they could cause mild symptoms (Apperson *et al.* 2008). This can be seen in *Dermacentor variabilis*, where a rickettsial infection can hinder the transovarial transmission of a different *Rickettsia* species (Macaluso *et al.* 2002).

Most of these endosymbionts are thought to be non-pathogenic to mammals (Beard *et al.* 1998; Ahantarig *et al.* 2013). The reason as to why a symbiont that is closely related to a pathogenic bacterium remains non-pathogenic, is still unclear. There are a few opposing theories as to the evolution of these symbionts. Weller *et al.* (1998) thought that more pathogenic bacteria were derived from symbionts that were vertically transmitted, whereas Noda *et al.* (1997) theorised that symbionts are derived from closely related, more pathogenic bacteria present in the host. A recent study by Duron *et al.* (2015a) has provided further evidence that pathogenic bacteria were derived from symbionts in ticks. They found four distinct tick-borne *Coxiella*-like clades that were not identical to *Coxiella burnetii*. They demonstrated that these tick-borne *Coxiella* strains were maternally transmitted from the cytoplasm to the egg, indicating that it was primarily an endosymbiont. Given that the tick-borne *Coxiella* strains showed a high genetic diversity compared to *C. burnetii* and that *C. burnetii* was similar to one of the *Coxiella*-like clades, they theorized that the pathogenic *C. burnetti* was derived from tick-borne bacteria that evolved to infect vertebrate cells

2.4.1 TICK-BORNE PATHOGENS

Ticks are capable of transmitting various pathogens, including bacterial pathogens (*Rickettsia, Anaplasma, and Ehrlichia)*, viral pathogens (*Coltivirus, Flavivirus*) and parasites (*Babesia*) (Dantas-Torres *et al.* 2012). Many of these pathogens are zoonotic and can infect both animals and humans.

The emergence of Lyme disease (caused by *Borrelia burgdirferi*) put a large focus on the global importance of ticks and TBPs on human public health (Wormser 2006). Various other TBPs such as babesiosis, ehrlichiosis and anaplasmosis are gaining more interest among veterinarians and physicians (Dantas-Torres *et al.* 2012).

2.4.1.1 *Ehrlichia* species

Ehrlichia canis is a bacterial pathogen that causes canine monocytic ehrlichiosis (CME) (Harrus and Waner 2011). *Ehrlichia canis* is a widespread pathogen, but information regarding its distribution in Africa is sparse. *Ehrlichia canis* was thought to only infect canine species, but recently it was isolated from human

samples (Perez *et al.* 2006; Harrus and Waner 2011; Bouza-Mora *et al.* 2017), were it presents clinical symptoms compatible with ehrlichiosis.

2.4.1.2 *Rickettsia* species

Rickettsia species are gram-negative aerobic bacteria that parasitize host cells (Raoult and Roux 1997). About 15 emerging tick-borne rickettsioses have been documented (Parola *et al.* 2005), including *R. conorii* (Socolovschi *et al.* 2009b), *R. rickettsii* (Bratton and Corey 2005), *R. massiliae* (García-García *et al.* 2010) and *R. africae* (Parola and Raoult 2001).

2.4.1.3 *Anaplasma* species

Anaplasmosis is a disease caused by various species of the *Anaplasma* bacteria. *A. marginale* and *A. centrale* are two species that infect cattle (Rymaszewska and Grenda 2008). Dogs can be infected by *A. platys* that causes canine infectious cyclic thrombocytopenia. It does not have a high pathogenicity and infected dogs do not usually show clinical signs. However, dogs that have been co-infected with *E. canis* and *A. platys* have presented with severe canine ehrlichiosis (Hua *et al.* 2000). It has a world-wide distribution and is thought to be transmitted by *R. sanguineus* ticks (Sanogo *et al.* 2003). The only *Anaplasma* species that is pathogenic towards humans is *A. phagocytophilum*. This pathogen causes Human Granulocytic Anaplasmosis (HGA), which causes mild symptoms in most cases (Rymaszewska and Grenda 2008).

2.4.1.4 *Babesia* species

Babesia species, which cause Babesiosis, are able to parasitize host erythrocytes. *Babesia* species are able to infect a large variety of vertebrates and can be transmitted by various hard tick species (M'ghirbi and Bouattour 2008). The main causative agents of babesiosis in canines are *B. canis vogeli*, *B. canis canis* and *B. canis rossi. B. canis canis* is mainly transmitted by *Dermacentor reticulates*. In tropical and subtropical regions, *R. sanguineus* transmits *B. Canis vogeli*. The third *Babesia* species, *B. canis rossi,* is spread throughout South Africa by *H. Leachi* (Uilenberg *et al.* 1989; Uilenberg 2006). Clinical symptoms of canine babesiosis (biliary fever) include anaemia, influenza like symptoms, fever and renal failure (Bratton and Corey 2005).

2.4.2 TICK BACTERIAL ENDOSYMBIONTS

2.4.2.1 *Rickettsia*-like symbionts

Rickettsia species are intracellular, gram negative bacteria, and many species of ticks have been associated with *Rickettsia* endosymbionts (Ahantarig *et al.* 2013). *Rickettsia* species are globally distributed and are the causative agent of various diseases in invertebrate and vertebrate hosts, including humans (Raoult and Roux 1997). The pathogenicity of any of the *Rickettsia* endosymbionts are unknown,

however, some have very little or no pathogenicity. Goddard (2009) found that *Rickettsia parkeri* which was thought to be non-pathogenic, actually cause spotted-fever-like symptoms in humans. Although some of these endosymbionts have little pathogenicity, they are able to influence the physiology of ticks, especially the transmission and acquisition of other microbes.

2.4.2.2 *Coxiella*-like symbionts

Ticks such as *Rhipicephalus*, *Haemaphysalis* and *Ixodes* have been found to harbour endosymbionts that are related to *Coxiella burnetii,* however, these *Coxiella*-like endosymbionts (CLEs) were only identified in small numbers in the *Rhipicephalus* species (Bernasconi *et al.* 2002). The pathogenic *C. burnetii* causes acute Q fever, a zoonotic disease of humans and ruminants, and chronic endocarditis (Jasinskas *et al.* 2007). Q-fever is endemic in humans in South Africa with 42% prevalence in susceptible community areas. It is likely that the existence of these CLEs in these ticks can be attributed to the uptake of *C. burnetii* from an infected host, and its subsequent evolution within the tick. CLEs have a reduced genome and lack certain proteins that the pathogenic *Coxiella* have. Thus it was thought that *C. burnetii* was the only pathogenic organism in the genus, and the *Coxiella*-like microbes were thought to be non-pathogenic (Jasinskas *et al.* 2007). A study done by Jourdain *et al.* (2015) showed that four of the five most common *C. burnetii* detection methods cross-react with CLEs, causing a possible overestimation of *C. burnetii* infection. Recently, various CLEs were found infecting ruminants, birds and humans, causing mild clinical symptoms almost similar to Q-fever (Duron *et al.* 2014, 2015b). This could mean that CLEs in South Africa are capable of causing Q-fever. Additionally, these CLEs were found in high numbers in the salivary glands of *A. americanum*, signifying that they possibly influence pathogen transmission or acquisition of other pathogens (Klyachko *et al.* 2007).

2.4.2.3 *Francisella*-like symbionts

Francisella species are gram-negative bacteria and include species such as *F. philomiragia, F. noatunensis, F. tularensis* (the causative agent of tularemia) and *Francisella*-like bacteria. It can be transmitted through various mechanisms, including tics, infected animals (Petersen *et al.* 2009), and environmental contact (Barns *et al.* 2005). Hard ticks and soft ticks have recently been found harbouring *Francisella*-like endosymbionts that are closely related to *F. tularensis* (Michelet *et al.* 2013; Kaufman *et al.* 2018). Unfortunately, these *Francisella*-like endosymbionts can cross-react with *F. tularensis* in the available detection methods. This causes misidentification of the *F. Tularensis* infection (Kugeler *et al.* 2005).

2.4.3 STABILITY OF THE TICK MICROBIOME

The composition of the bacterial microbiome in ticks can change remarkably over space and time (Jones *et al.* 2010; Gall *et al.* 2016). These changes can in part be due to vector-, host- and environmental-characteristics (Hawlena *et al.* 2013). In the midgut, the bacterial species that are acquired from a host

can be considerably altered, especially by competition between bacterial pathogens and endosymbionts (Hawlena *et al.* 2013). A study done by Ross *et al.* (2018) found that wild, unfed hard ticks did not have a diverse microbiome, but rather a limited core microbiome, which was in contrast to various other studies previously done on hard ticks (Andreotti *et al.* 2011; Narasimhan and Fikrig 2015; Abraham *et al.* 2017; Budachetri *et al.* 2017). A limited microbiome could be due to various factors that inhibit bacterial growth, such as the lack of required nutrients, conserved immunity elements that target bacteria, (Chou *et al.* 2015) as well as extended periods between feeding that could lead to desiccation of the tick.

Ticks acquire a portion of their microbiome during a blood meal from their hosts. Certain host characteristics could potentially influence the pathogens acquired during this blood meal, such as age, gender and roaming habits of the host (Hawlena *et al.* 2013). A study done by Hawlena *et al.* (2013) showed that host-factors had little influence on the tick microbiome. They theorized that this was because the majority of endosymbionts were vertically transmitted and would remain stable between generations of ticks.

Ticks may spend considerable time feeding on their hosts, but most of their time is spent in the environment during their free-living stages (during moulting and host-questing periods). This puts the ticks in direct contact with environmental contaminants that could potentially alter their microbiome. Differences in the environment, such as temperature or habitat type, could influence the microbiome of ticks (Hawlena *et al.* 2013; Gall *et al.* 2017). Some studies found that there were no differences in the microbiome between populations in different environments, while other studies found large differences (Jones *et al.* 2010; Menchaca *et al.* 2013; Clayton *et al.* 2015; Van Treuren *et al.* 2015).

2.5 TICK CONTROL METHODS

Ticks feed on a host for a certain period of time, depending on the species, and the rest of their life cycle is spent in the environment while they lay eggs or undergo moulting. For effective tick control methods, an integrated strategy would have to be undertaken to target ticks on animals as well as ticks in the environment, as the majority of ticks are found in the environment.

2.5.1 CHEMICAL CONTROL

Various chemical methods of tick control for dogs are available such as shampoos, sprays, spot-on formulations, dips, powders and collars (Jongejan and Uilenberg 1994). Acaricides are very efficient at eradicating infestations and preventing any re-infestations within a certain time frame. Methods for environmental tick control are limited and will only be effective if a confined area is treated. Various factors can influence environmental tick control, such as the severity of the infestation, the presence of infestations in surrounding areas, as well as environmental conditions. The use of acaricides as a tick

control method should be well managed, as the overuse of acaricides can lead to environmental and health pollution as well as acaricide resistance (Malan 2016).

2.5.2 NON-CHEMICAL CONTROL

To avoid overuse of acaricides and to improve the effectiveness, non-chemical control methods should be used in conjunction with chemical control methods. Non-chemical methods include reducing favourable habitats by sealing any cracks or crevices as well as removing or cutting all grass and reeds around homes (Dantas-Torres 2008). Manual removal of ticks, such as removing ticks from dogs or other animals, and removing ticks found close to a house could also be implemented. Another method that could be implemented is biological control. It has been demonstrated that a formulation of entomopathogenic fungi can be harmful to ticks (Felix *et al.* 2009).

2.6 TICKS AND TICK-BORNE PATHOGENS IN SOUTH AFRICA

During the 20th century, TBPs were managed effectively through vector control programmes. However, since the 21st century, accompanied by global interconnectedness, TBPs have become a rising threat to human health (Chitanga *et al.* 2014). Several other aspects have also contributed to the increased threat, such as the expanding distribution of tick populations as a result of climate change, and the increased interaction between humans, livestock, and wildlife (Gray *et al.* 2009).

South Africa's climate is becoming increasingly warmer, which can have a big impact on the expansion and distribution of tick populations. The impact of a changing environment and climate has been a controversial topic in recent literature (Süss *et al.* 2008; Caminade *et al.* 2019). It is thought that global warming will cause the distribution of tick species to the north. Recent studies have demonstrated that *Rhipicephalus sanguineus* ticks exposed to higher temperatures were more aggressive and had a higher tendency to bite humans (Parola *et al.* 2008; Socolovschi *et al.* 2009a), therefore elucidating the notion that a warmer climate will increase the risk of human parasitism in South Africa (Parola *et al.* 2008).

2.6.1 EFFECT OF TICK-BORNE DISEASES ON RURAL COMMUNITIES

Due to the high biodiversity of wild and domesticated animals that act as hosts for ticks and reservoirs of TBPs in South Africa, they are crucial economical and health threats in the country. Specifically, to the rural communities of South Africa, where many people practice small-scale or traditional farming, not only as a source of income, but also for various other reasons (Sansoucy *et al.* 1995). Small-scale farming includes agriculture and animal keeping at a small scale, instead of at commercial scale. Cattle are the most common animals used for traditional farming, while goats and chickens are also utilized quite often. Pigs and donkeys are less common in these areas. These livestock animals are hosts to many insect

vectors, including ticks, and the economic losses due to reduced milk yield and cattle mortality caused by TBPs have a negative economic impact on the communities (Makala *et al.* 2003).

Furthermore, TBPs have an impact on rural communities when domesticated dogs become infected (Dantas-Torres 2008). Dogs are very competent hosts for various tick species, such as *R. sanguineus* that is well adapted to survive in kennels and areas close to dogs. This causes an increase in tick survival and tick density close to homes (Dantas-Torres 2008; Dantas-Torres *et al.* 2012), further facilitating the spread of TBPs to humans. In South Africa, TBPs in livestock has been well studied, as it has a major economic impact on rural communities as well as on commercial farming. The impact of TBPs on humans has not been as widely studied (Hotez and Kamath 2009), creating a knowledge gap that makes it difficult to analyse the importance of TBPs in South Africa.

Various communities have implemented certain control measures to reduce the tick density within the communities and on their livestock. These programs employ weekly cattle dips into acaricides as well as veterinary assistance to ensure healthier livestock and domesticated dogs in the communities (Choopa 2015). Unfortunately, the overuse of chemical control methods have led to acaricide-resistant tick populations, thus further research is necessary to identify alternate effective tick control prevention methods (Walker *et al.* 2014).

2.7 RHIPICEPHALUS SANGUINEUS

The *Rhipicephalus genus* consists of about 79 species (Barker and Murrell 2004). The species in this genus are characterised as being inornate, small and showing only slight sexual dimorphism. *Rhipicephalus sanguineus*, otherwise known as the brown dog tick is a three-host tick that completes its life cycle primarily on dogs, but it can also be found on various domestic and wild animals as well as on humans (Estrada-Peña and Jongejan 1999). This makes it a very important vector of zoonotic pathogens (Milhano *et al.* 2015). It has a wide distribution, but is mainly found between latitudes 50°N and 35°S. A study conducted by Bryson *et al.* (2000) on the ectoparasites of dogs in rural resource-poor communities located in the North West Province of South Africa, identified several tick species, including *H. leachi, A. hebraeum*, *R. appendiculatus, R. evertsi evertsi*, *R. simus* and *R. sanguineus.* The study revealed that *R. sanguineous* was the most predominant tick species in rural-resource-poor communities compared to more affluent areas.

2.7.1 TAXONOMY AND IDENTIFICATION

Rhipicephalus sanguineus falls in the subfamily Rhipicephalinae which belongs to the Metastriata lineage within the Ixodidae family. Latreille first described *R. sanguineus* in 1806. Even though the type specimen is absent, it remains the type species of the *Rhipicephalus* genus (Pegram *et al.* 1987b). There are various

theories as to the origin of *R. sanguineus*. One theory postulates that *R. sanguineus* is an African tick species that was distributed globally by dogs (Hoogstraal 1956), whereas another theory believes that *R. sanguineus* is a Mediterranean species (Morel and Vassiliades 1962).

The exact taxonomic classification of *R. sanguineus* is still unclear (Pegram *et al.* 1987b). *Rhipicephalus sanguineus* is a species complex which consists of 12 tick species being grouped under the same name (Table 2 - 1) (Pegram *et al.* 1987b). A species complex is delineated as a group of different species that are closely related and thus share some common phenotypic characters (Coimbra-Dores *et al.* 2016). This leads to difficulty in differentiating between species in the complex.

Table 2 - 1: *Rhipicephalus sanguineus* species complex. Data modified from Nava *et al.* (2015).

Species	Reference	Date
Rhipicephalus sanguineus	Latreille	1806
Rhipicephalus sulcatus	Neumann	1908
Rhipicephalus rossicus	Yakimov and Kohl-Yakimov	1911
Rhipicephalus schulzei	Olenev	1929
Rhipicephalus pumilio	Schulze	1935
Rhipicephalus pusillus	Gil Collado	1936
Rhipicephalus turanicus	Pomerantzev	1940
Rhipicephalus leporis	Pomerantzev	1946
Rhipicephalus guilhoni	Morel and Vassiliades	1963
Rhipicephalus moucheti	Morel	1965
Rhipicephalus bergeoni	Morel and Balis	1976
Rhipicephalus camicasi	Morel, Mouchet and Rodhain	1976

Rhipicephalus sanguineus is a small, elongated tick ranging from medium brown to yellow in colour (Figure 2 - 2). They are inornate and show very slight sexual dimorphism. They have short palps and the presence of eyes and festoons (Dantas-Torres 2008). They have hexagonal basis capituli which is an identifying character. However, it is not easily distinguishable from other species in the *Rhipicephalus* genus, such as *R. turanicus* (Estrada-Peña *et al.* 2004) (Figure 2 - 3). With morphological identification, the problem arises where different populations of one species display small morphological differences and will be assigned as two distinct species, while two separate species that share morphological traits may be assigned as one species (Pegram *et al.* 1987b). Thus identification is only possible through microscopy or DNA (Deoxyribonucleic acid) bar-coding (Dantas-Torres 2008). Although these species are morphologically similar, they do differ in various other ways, especially in regards to behaviour, ecology and vector capacity characteristics, which can have a large impact on the bacterial pathogens they harbour (Estrada-Peña *et al.* 2004).

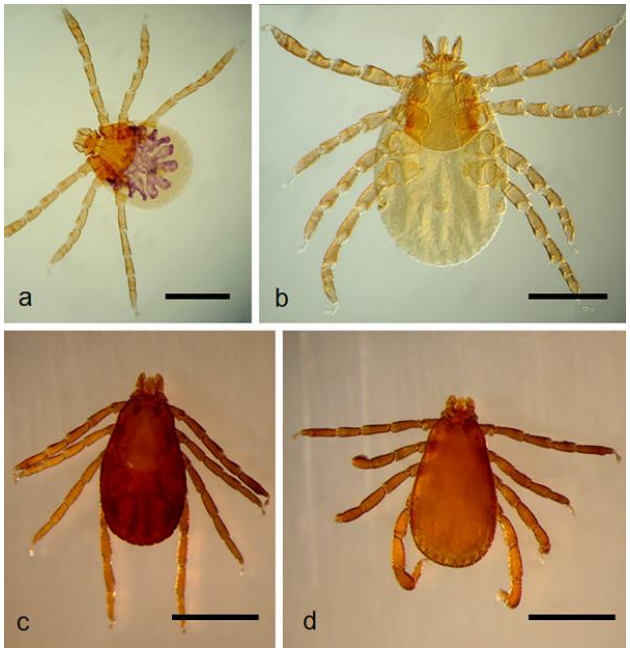

Figure 2 - 2: All life stages of *Rhipicephalus sanguineus*. (A) Larva (B) Nymph (C) Adult female (D) Adult Male. Image modified from Dantas-Torres (2010).

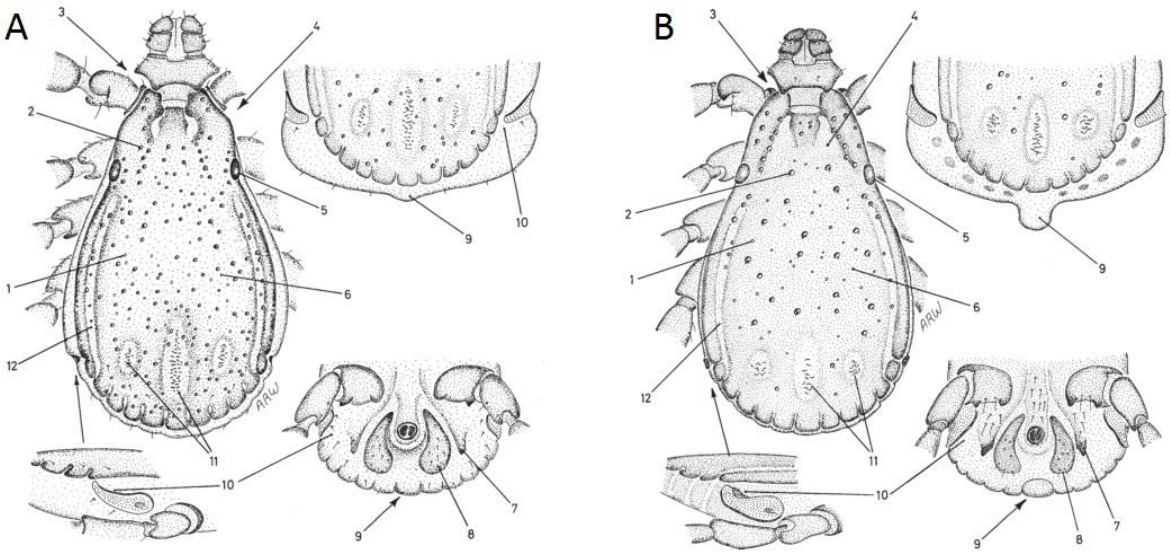

Figure 2 - 3: (A) *Rhipicephalus sanguineus*. (B) *Rhipicephalus turanicus*. (A1-B1) Sparsely distributed, small to medium interstitial punctuations. (A2) Indistinct setiferous punctations. (B2) Distinct setiferous punctations. (A3-B3) Anterior spurs on coxae 1 not dorsally visible. (A4) Cervical field depressions are not evident and has no wrinkles. (B4) Cervical field depressions are evident with no wrinkles. (A5) Eyes are slightly convex. (B5) Flat eyes. (A6) Pale conscutum. (B6) Dark conscutum. (A7-B7) Large accessory adenal plates. (A10) Narrow spiracle plate tails. (A8-B8) adenal plates are narrow and trapezoid in shape. (A9-B9) Broad caudal appendage in males that have fed. (B10) Broad spiracle plate tails. (A11-B11) Distinct posterior grooves with wrinkles. (A12) Distinct lateral groove with smooth texture. (B12) Distinct lateral groove with punctuate texture in South Africa. Image modified from Estrada-Peña *et al.* (2004)

2.7.2 BIOLOGY AND ECOLOGY

2.7.2.1 Developmental stages and life cycle

Rhipicephalus sanguineus ticks go through four developmental stages, which includes the egg, larva, nymph and adult stages (Figure 2 - 4). *Rhipicephalus sanguineus* is a three-host tick, meaning that all three active developmental phases (larva, nymph and adult) will feed just once over a certain period of time. The process of casting off the outer cuticle (ecdysis) then occurs in the environment and not on the host (Dantas-Torres 2008).

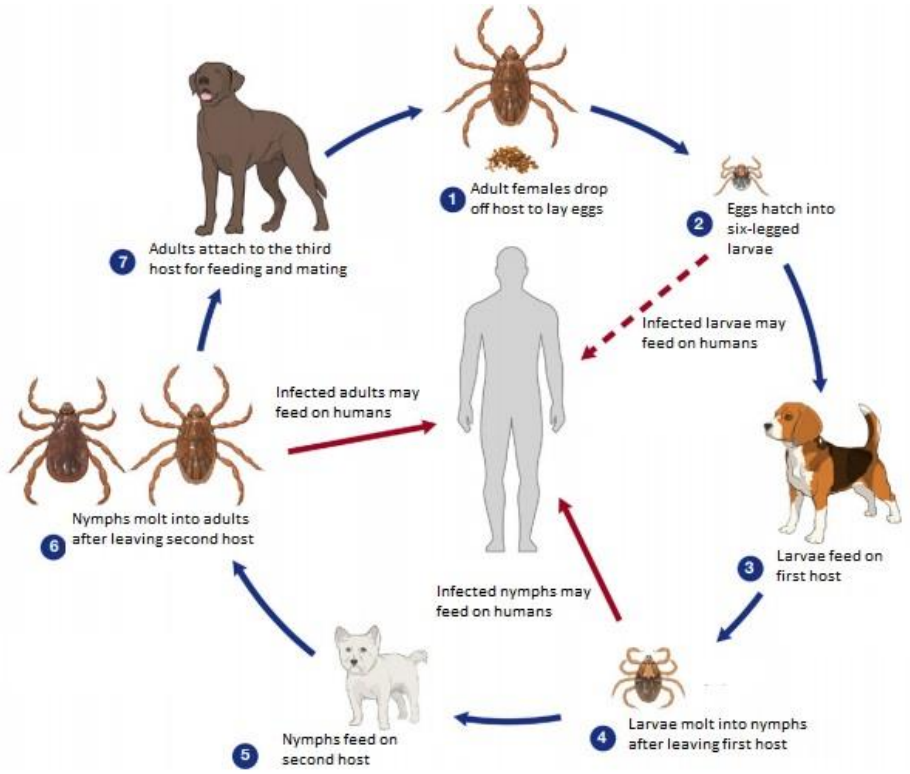

Figure 2 - 4: A diagram depicting the life cycle of a three host-tick. Image modified from Centres for Disease Control and Prevention (2015).

The life cycle begins when an engorged female drops off from the host and lays eggs that will hatch into larvae. The incubation period for *R. sanguineus* eggs are 6 to 23 days (Pegram *et al.* 1987a). *Rhipicephalus sanguineus* eggs are small, dark brown and spherical (Dantas-Torres 2008). When the larvae hatch they have only three legs and are small, with an average width of 0.39 mm and length of 0.54 mm) (Cooley 1946). The larvae have to find a host to feed on for 10 to 13 days before they fall off and moult into the nymph stage (Pegram *et al.* 1987a). The larval moulting phase can last for five to 15 days (Pegram *et al.* 1987a). If larvae are unable to feed, they can survive in the environment without a host for eight months (Goddard 1987).

Nymphs look like the adults and also have four legs. Nymphs are however sexually immature (no genital aperture present) and smaller than the adults with an average length ranging from 1.14 mm to 1.3 mm and an average width ranging between 0.57 mm to 0.66 mm (Cooley 1946). These nymphs then feed on a new host for three to 11 days, fall off and moult into adults (which can take from nine to 47 days) (Pegram *et al.* 1987a; Estrada-Peña *et al.* 2004). Unfed adults and nymphs are able to survive in the environment without a host for 19 months and six months, respectively.

Adults are sexually mature, larger than the nymphs, and also have four legs. Males can range in length from 2.28 mm to 3.18 mm, and width from 1.11 mm to 1.68 mm (Cooley 1946). The males are flat and their scutum is covered in small pits. Before females feed and become engorged, they can resemble males in length (ranges between 2.4 mm and 2.7 mm), width (ranges between 1.44 mm and 1.68 mm), colour and shape (Cooley 1946). However, the scutum of the female does not cover the entire back allowing her to become engorged. Adult females feed for 21 days on the host (Pegram *et al.* 1987a). When the female becomes engorged her body turns grey-blue and her length can increase to 11.5 mm and her width to 7.5 mm (Cooley 1946).

Engorged females will detach in order to process the blood meal and subsequently lay eggs in a protected place during oviposition, which can range from 16-18 days (Koch 1982). *Rhipicephalus sanguineus* will lay her eggs in cracks and crevices that are close to the dog's resting place, making it easier for larvae to locate their host. During the oviposition, an engorged female can lay an average of 4000 eggs, with a maximum of 7273 eggs (Koch 1982). After oviposition, the female dies (Jacobs *et al.* 2004). The ideal temperature for oviposition in *R. sanguineus* ranges from 20°C to 30°C (Sweatman 1967). Under ideal environmental conditions, the entire life cycle can be completed within 10 weeks (Estrada-Peña *et al.* 2004).

The parameters of moulting periods, oviposition and other biological factors vary significantly under laboratory conditions when *R. sanguineus* ticks are exposed to variable temperatures, relative humidity and hosts. Factors such as moulting and engorgement periods also vary greatly under field conditions and are affected by host availability and temperature (Mumcuogliu *et al.* 1993). It has been established that *R. sanguineus* ticks are not extremely dependent on moisture in their environment and are thus capable of inhabiting drier regions (Yoder *et al.* 2006).

2.7.2.2 Primary host and secondary hosts

Rhipicephalus sanguineus can parasitize almost all vertebrates, but their primary host during their adult and immature life-stages are almost exclusively dogs (Jacobs *et al.* 2004). It is likely that they evolved alongside burrowing carnivores. As dogs became domesticated, contact between these carnivores and

humans increased, as their dwellings were in close proximity. These ticks subsequently evolved to parasitize both domesticated dogs and humans (Gray *et al.* 2009). Although they are a very widespread species, the presence of domesticated dogs are necessary to maintain large tick populations. Even though dogs are their main hosts, *R. sanguineus* ticks show an opportunistic host selection under certain circumstances, such as high environmental tick infestation and a low host prevalence. Rodents and other small mammals can be hosts to immature stages, while larger mammals are parasitized by the adults (Estrada-Peña and Jongejan 1999). It has not been very common for *R. sanguineus* ticks to bite humans, however in the literature there has been an increase in cases reporting humans being parasitized by *R. sanguineus* ticks (Goddard 1989; Estrada-Peña and Jongejan 1999; Demma *et al.* 2005).

2.7.2.3 Off-host ecology

Since three-host hard ticks feed once during each life stage and moult in the environment, they spend an average of 94 - 97% of their life without a host, in the environment (Needham and Teel 1991). Many ixodid ticks are found outside in the open air environment when they are not on a host (exophilic behaviour), however *R. sanguineus* can usually be found indoors (endophilic behaviour), climbing up walls and structures, such as furniture (Demma *et al.* 2005). In rural community areas where there is a large host population, *R. sanguineus* populations are able to thrive, as it is highly adaptable to enclosed spaces, such as dog kennels or areas around human homes. Females may lay their eggs near the dog kennel, in the dog bedding or in small cracks around the house, causing heavy infestations (Bryson *et al.* 2000). In South Africa, all stages of *R. sanguineus* are most prevalent between October and May, and are less prevalent during the winter months (Estrada-Peña *et al.* 2004).

2.7.2.4 Host seeking behaviour

Larvae, nymphs and adults require stimuli to indicate that a host is close, so that they can attach and feed. These stimuli include chemical, temperature and mechanical signals. Ticks are very sensitive to these different signals. Chemical stimuli include carbon dioxide and ammonia emitted by the host, temperature stimuli include the body temperature of the host, while mechanical stimuli include airborne vibrations produced by the host (Parola and Raoult 2001). Host-seeking behaviour and strategies in Ixodid ticks can vary greatly. Some Ixodid ticks deploy a method whereby they wait on vegetation with their front legs extended and then attach to hosts as they walk past. Other methods include ticks running toward a host after emergence, or remaining in the nest of the host. Certain species have a very distinct method while other ticks such as *R. sanguineus* can use several methods (Parola and Raoult 2001).

2.7.2.5 Attachment and blood-feeding

As discussed previously, ticks need to insert their hypostome into the skin of their hosts to be able to feed. This process can differ between ticks. Primitive ticks such as the *Amblyomma* species need to insert

and attach very deeply. In contrast, more advanced ticks such as the *Rhipicephalus* species and *Dermacentor* species have mouthparts that do not penetrate the dermis and they attach only superficially (Bowman *et al.* 1997). During a blood meal, *R. sanguineus* shows an initial period of slow feeding during which blood is continuously digested in the midgut. This initial period is followed by a subsequent period of reduced digestion, leading to rapid engorgement (Parola and Raoult 2001).

2.7.2.6 Veterinary and medical importance

Rhipicephalus sanguineus has a large vector capacity and can transmit pathogens that cause various diseases in vertebrate animals. Even though *R. sanguineus* is able to transmit *B. canis* in certain regions of the world, a recent study has proposed that it is unable to transmit it in South Africa (Uilenberg *et al.* 1989). It is thus not a very important vector of *B. canis* in South Africa. *Rhipicephalus. sanguineus* is a capable vector of *E. canis* and is able to transmit it transovarially and transstadially.

Although humans are not primary hosts of *R. sanguineus*, its role in transmission of *Rickettsia* to people has been studied and documented (Palmas *et al.* 2001). *Rhipicephalus. sanguineus* is able to transmit three species of *Rickettsia*: (1) *R. conorii*, which causes Mediterranean spotted fever (MSF) (Socolovschi *et al.* 2009b). *R. conorii* is a widespread pathogen and is present throughout Africa including South Africa (Rovery and Raoult 2008). Research conducted by Levin *et al.* (2012) indicated that domestic dogs were capable of being a reservoir for *R. conorii*, thus playing an important role in the spread of MSF. (2) *R. rickettsii* the causative agent of Rocky Mountain spotted fever (Bratton and Corey 2005) a potentially fatal disease in humans (Parola *et al.* 2008). (3) *R. massiliae*, an emerging pathogen that causes spotted fever (García-García *et al.* 2010).

It has also been hypothesized that *R. sanguineus* could be a possible vector of *Anaplasma platys* (Simpson *et al.* 1991). *Rhipicephalus. sanguineus* is also able to transmit *Hepatozoon canis*, however not through usual methods. Infection occurs when infected *R. sanguineus* ticks are ingested by the host (Giannelli *et al.* 2013).

Apart from being a vector, *R. sanguineus* can also act as a reservoir for pathogens that are able to transmit transovarially. This keeps the pathogen circulating in nature across numerous generations (Bremer *et al.* 2005). The medical and veterinary importance of these ticks is not only due to their vector capacity, but also because they can cause severe clinical illness due to their serious infestations of their hosts. This can lead to anaemia, tick paralysis, and skin abscesses (Otranto *et al.* 2012).

2.7.2.7 Susceptibility of dogs to *Rhipicephalus sanguineus* infestations and tick saliva

It has been shown that *R. sanguineus* salivary glands can block the action of histamine (Chinery and Aitey-Amith 1977). The saliva of *R. sanguineus* is also able to impair T-cell proliferation, and it impairs macrophages from performing microbicidal activities (Bowman *et al.* 1997). This allows it to survive during the initial infection period. Different hosts have shown variable levels of resistance. Guinea pigs have displayed resistance to succeeding infestations of *R. sanguineus* ticks, and are used as model hosts. Dogs and mice exposed to consecutive *R. sanguineus* infestations have failed to develop a delayed-type hypersensitivity (DTH) response (Ferreira *et al.* 2003; Cavassani *et al.* 2005). *Rhipicephalus sanguineus* ticks have evolved alongside dogs and have evolved immunodulatory factors to control the immune response in dogs, allowing dogs to become re-infested.

2.7.3 CONTROL OF *RHIPICEPHALUS SANGUINEUS* TICKS

The most frequently used chemical control methods for *R. sanguineus* ticks are acaricides (Dantas-Torres 2008). However, acaricide resistance is increasing and this method is not as effective as it once was. *Rhipicephalus sanguineus* has been shown to be very resistant to dichlorodiphenyltrichloroethane permethrin and coumaphos, as well as slightly resistant to amitraz (Miller *et al.* 2001; Rodriguez-Vivas *et al.* 2017).

Ticks also spend the majority of their lives in the environment, and acaricide treatment of the environment will only be effective if it is used in restricted areas. Thus non-chemical control methods should also be implemented for *R. sanguineus* ticks. As *R. sanguineus* ticks are well adapted to live close to their hosts and in and around homes, all cracks or gaps should be closed up. All grass or weeds around homes should be kept short or removed. Dogs should also be periodically checked for ticks, and the ticks should be subsequently removed and destroyed by putting it in alcohol.

2.8 METHODS FOR NEXT GENERATION SEQUENCING

2.8.1 USE OF 16S RDNA PRIMERS TO TARGET BACTERIA

The 16S rDNA gene has been widely used in the characterisation of bacterial populations. The 16S rDNA gene contains highly conserved regions and highly variable regions (Figure 2 - 5). The highly conserved regions act as fixed points that are used during primer design, whereas the highly variable regions are used during sequencing to distinguish between different bacteria (Tringe and Hugenholtz 2008). These primers are “universal”, meaning they can amplify the 16S rDNA gene of the majority of bacterial species. A full length 16S rDNA gene is made up if nine variable regions which are then divided by nine highly conserved regions (Baker *et al.* 2003).

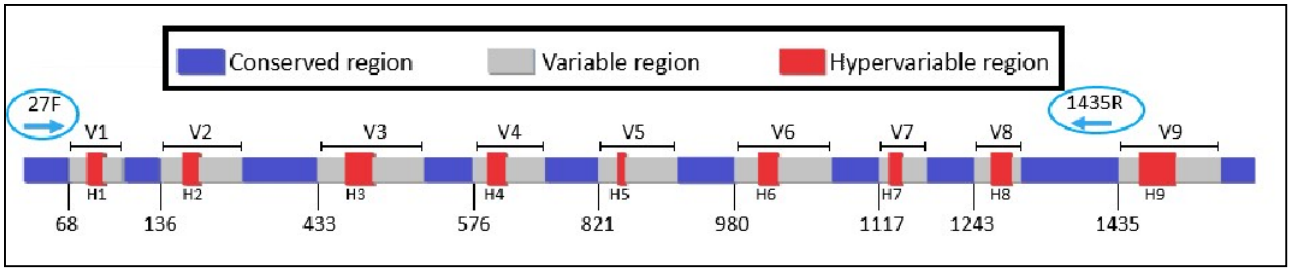

Figure 2 - 5: A diagram indicating the 16S rDNA gene that consists of highly conserved (blue), highly variable (grey) and hypervariable regions (red).

2.8.2 PacBio Circular Consensus Sequencing

The template that is used for PacBio sequencing is created by ligating a hairpin adapter sequence to each end of the double stranded DNA template (Figure 2 - 6). This creates a single-stranded circular molecule containing the template DNA. The hairpin adaptors contain a priming site where the DNA polymerase will bind and start the sequencing process of the template DNA. The enzyme will continue sequencing the single-stranded circular molecule, and depending on the length of the template DNA, it could possibly sequence the entire molecule multiple times. This creates long read lengths with high accuracy (99.85). The enzyme will eventually fall off, or be killed, due to fluorescent excitation. PacBio CCS (Circular Consensus Sequencing) sequencing is capable of sequencing DNA templates up to 3 (kilobase) kb. PacBio has a high error rate (10-15%), however these errors are randomly incorporated and will differ every time the template is sequenced. It can thus easily be detected.

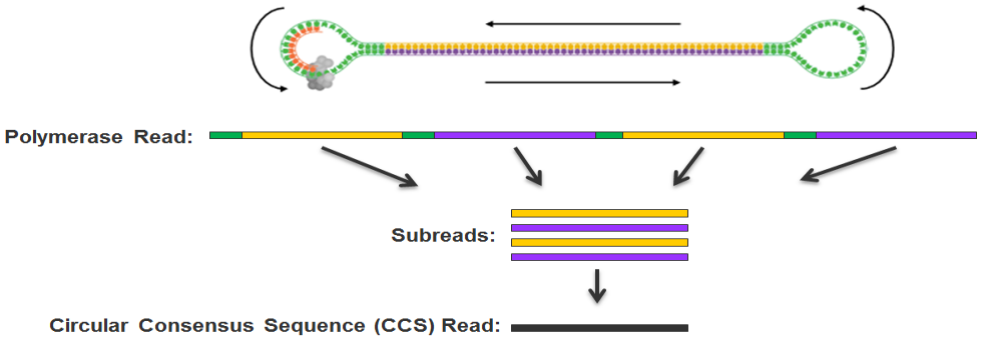

Figure 2 - 6: Illustration of the PacBio template used for circular consensus sequencing. The green sequences are hairpin adaptors that have been ligated to the double-stranded DNA template (indicated in yellow and purple). A polymerase then binds to the hairpin adapters and sequences the template DNA, producing a single-stranded polymerase read which consist of the hairpin adaptors and the DNA template. These reads are then merged to form a circular consensus sequence read. Image was taken from Pacific Biosciences Terminology.

CHAPTER 3

MATERIALS AND METHODS

3.1 ETHICAL APPROVALS

This project was approved by the following committees: Faculty of Veterinary Science Research Ethics Committee (REC010-18) and Animal Ethics Committee (V012-18); Faculty of Humanities Research Ethics Committee (GW20180719HS). Permission was obtained to conduct the research, in terms of Section 20 of the Animal Diseases Act of 1984, from the Department of Agriculture, Forestry and Fisheries (DAFF), South Africa, with reference number 12/11/1/1/8.

3.2 STUDY AREA AND SAMPLE COLLECTION

The Mnisi community is a rural community situated at the wildlife/livestock/human interface in the northeastern corner of the Bushbuckridge Municipality in Mpumalanga, South Africa (Figure 3 - 1). It consists of approximately 29,500 ha of communal land, of which >75% borders private and provincial conservation areas. The total population in the study area is estimated at 40,000–50,000 individuals in ~8,500 households, spread over 12 villages (Statistics South Africa 2012). Smallholder agro-pastoralist farming (~1,300 farming households) is the primary subsistence activity, and ownership of domestic animals is common.

Sampling was done in the Clare A village of the Mnisi community (Figure 3 - 1). The study was originally designed to be conducted in Clare A, Eglington and Clare B, as various other studies have been done in these areas before. Unfortunately, ethical approval was not received for Eglington and Clare B, thus sampling and questionnaire interviews could only be conducted Clare A. Questionnaires were conducted at households with dogs; these dogs were then examined for ticks which were subsequently collected. If no ticks were visible on the dogs, only the questionnaire were conducted. Ticks and blood samples were collected from six dogs per month over a 12-month period (July 2018 – July 2019).

The minimum number of samples (dogs) required was calculated to be 79, with a confidence interval of 95%, and assuming that the pathogen of interest (i. e. *Anaplasma*) Is present in the Clare A tick population at greater than 3% prevalence, (http://epitools.ausvet.com.au/). Only dogs older than six weeks or weighing more than 500 g were included in the study. The dogs that showed any clinical symptoms of disease or other serious injuries were not included. Each dog was restrained by the owner and checked for the presence of ticks. If ticks resembling the *Rhipicephalus* genus were present, they were collected by grasping them with a pair of blunt forceps and gently pulling them away from the host's skin. Any other

species of ticks present on the dog were also collected. Live ticks were placed in a specimen bottle, and stored in a cool box until transported back to Hans Hoheisen Wildlife Research Station (HHWRS) for identification.

Whole blood samples were also collected from dogs where ticks were collected, depending on the temperament of the dogs, the permission of the owner, as well as the difficulty of collecting blood. Blood was collected into EDTA vacutainer tubes (Becton, Dickinson and Company, New Jersey, United States) by cephalic vein puncture using standard protocols. The EDTA tubes were then stored in a cool box until transported back to HHWRS, where they were aliquoted into microcentrifuge tubes for storage at -80°C for future projects.

Figure 3 - 1: A map indicating the Mnisi Community, flanked by the Kruger National Park, Manyeleti Game reserve, Sabi Sands and Andover. The sample area (Clare A) is indicated with a red star ("Mnisi Community Programme").

3.3 TICK IDENTIFICATION AND DISSECTIONS

After collection, the collection tubes containing all the ticks were placed into a temperature and humidity controlled chamber for a two-day period to allow for the digestion of their blood meal. Since microbes contained in the blood meal move from the mouthparts to the midgut and finally to the salivary gland over time, this ensured that any microbes detected had been assimilated by the tick, and were not picked up from the blood meal. The average minimum temperature of the tick chamber for the duration of the study was 21.48°C (std = 0.54) (Table A 1 in the Appendix), while the average maximum temperature was 26.28°C (std = 1.77) (Table A 2). The average humidity was 56.85 (std = 5.54) (Table A 3).

All ticks were then identified morphologically according to Estrada-Peña *et al.* (2004). The minimum ticks per dog needed for further microbiome analysis was 10 *R.* sanguineus adult male ticks. If a sample from a dog had 10 male adult *R. sanguineus* ticks present, the 10 ticks were pooled (biological replicate) and the sample was used for further molecular analysis. All remaining ticks, including any male *R. sanguineus* ticks beyond the 10 males needed, were stored in 70% ethanol (Merck and Company, New Jersey, United States) for future studies.

Of the 1,180 male *R. sanguineus* ticks collected, 62 tick pools (10 ticks per pool) were selected for dissections, DNA extractions and microbiome sequence analysis (Figure 3 - 1). Since it is technically difficult to morphologically distinguish between *R. sanguineus* and *R. turanicus* ticks (Walker 2000), total genomic DNA of these 24 morphologically selected *R. sanguineus* ticks was sent to GeneLethu Laboratories (Johannesburg, South Africa) for cytochrome c oxidase I (COI) barcoding to confirm the species identity.

It has previously been shown that *R. sanguineus* ticks contain pores on their scutum that trap environmental contaminants (Soares *et al.* 2013). To prevent contamination in downstream processing, all adult male *R. sanguineus* ticks were surface sterilized, using a triple tick rinse (Scoles *et al.* 2005). Ticks were vigorously washed in a 0.25% Corox (Ecolab, Minnesota, United States) and 0.25% ethanol water solution, to remove any environmental contamination. Thereafter, samples were washed twice with sterile water to remove any remaining environmental contaminants and excess tick wash. The triple rinse process was performed twice in order to ensure complete removal of external contaminants.

Prior to dissection, baseplate wax sheets (Keystone industries, Pennsylvania, United States) were cut up into small strips, placed in ethanol and air-dried. This was used instead of a petri dish to prevent the scalpel from slipping during dissection. Three small drops of Hyclone HBSS/Modified buffer (GE Healthcare Life Sciences, Utah, United States) was placed onto a strip of the baseplate wax. One male tick was then placed into one of the drops of Hyclone HBSS/Modified buffer. A number 5 tweezer was used to hold the tick in place and a Hyde single edged razor blade (Hyde, Massachusetts, United States) was used for dissection. All dissection tools were surface sterilized between pooled samples to minimize contamination (Scoles *et al.* 2005; Gall 2016; Gall *et al.* 2016, 2017). To remove the scutum, the tick was cut along all four sides, (Figure 3 - 2A). The dorsal and ventral sides were then pulled apart to reveal the tick tissues (Figure 3 - 2B). The salivary gland tissues were then carefully removed and placed into one of the clean drops of Hyclone HBSS/Modified buffer to wash the tissues. The midgut was then also carefully removed and placed into the second clean drops of Hyclone HBSS/Modified buffer to wash the tissues. The salivary gland and midgut tissues were then respectively placed into a storage solution. During the initial stages of the study, the tissues of three adult male *R. sanguineus* ticks per dog were pooled into

one sample, due to a limited availability of *R. sanguineus* ticks on the dogs. As the population of *R. sanguineus* ticks increased during the study period the tissues of 10 adult male *R. sanguineus* ticks per dog were pooled.

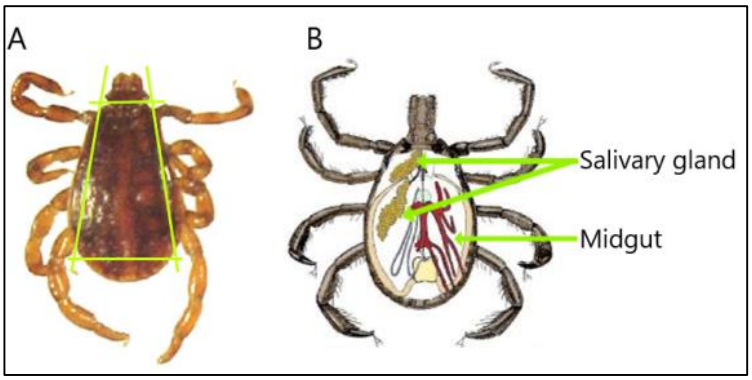

Figure 3 - 2: Illustration of the dissection process. (A) A razor blade was used to cut along the green line, allowing the separation of the dorsal and ventral parts, exposing the tick tissues. (B) A diagram indicating the salivary glands and the midgut tissues in a tick. Image modified from Edwards *et al.* (2009).

3.4 GENOMIC DNA EXTRACTION

Two DNA extraction methods were used to extract total genomic DNA from the midgut (n=62) and salivary gland (n=62) pools after dissection. DNA from a total of 56 midgut and salivary gland pools (each pool containing 3-10 ticks from one dog) was extracted using a modified PureGene Ethanol Extraction (MPEE) method (Scoles *et al.* 2005). A further six midgut and salivary gland pools (each pool containing 10 ticks from one dog) were extracted using a DNeasy Blood and Tissue kit (DBTK) method (Qiagen, Hilden, Germany).

For the MPEE method, all salivary gland and midgut tissues were stored in a storage solution of 490 µl cell lysis (Merck and Company, New Jersey, United States) and 10 µl proteinase K (Thermo Fisher Scientific, Massachusetts, United States) after dissection. All samples were incubated at 56°C at 300 rpm on a thermomixer R (Eppendorf, New York, United States) for 12 hours before DNA extraction. A mastermix of glycogen (Thermo Fisher Scientific, Massachusetts, United States) and cell lysis solution with a final concentration of 2 µl/mL was prepared and 400 µl of this mastermix was added to all samples after incubation. Samples were mixed with a vortex (Scientific Industries Inc., New York, United States) for 3 seconds. Thereafter 200 µl protein precipitate (Qiagen, Hilden, Germany) was added and samples were vortexed for an additional 10 seconds. The samples were then centrifuged on an Eppendorf 5415R centrifuge (Eppendorf, Hamburg, Germany) at 4°C for 10 min at 16 000 g after which 800 µl of the supernatant was transferred to a clean 1.5 mL Eppendorf tube (Eppendorf, Hamburg, Germany) and 500 µl isopropanol (Merck and Company, New Jersey, United States) was added. The samples were then inverted 30 times and centrifuged at 4°C for 15 min at 16 000x g. The 100% isopropanol was then removed

with a pipette and 500 µl 70% ethanol was added to the pellet. The samples were then inverted 5 times and centrifuged at 4°C for 10 min at 16 000 x g. The 70% ethanol was removed with a pipette and the microcentrifuge tube containing the pellet was air-dried for eight hours. After the ethanol evaporated, 50 µl DNA hydration (Qiagen, Hilden, Germany) was added and the samples were incubated at room temperature for 15 min (minutes) after which they were stored at -20°C until further processing.

For the DBTK method, all salivary gland and midgut tissues were stored in 180 µl ATL buffer and 20 µl proteinase K. The samples were then vortexed and incubated at 56°C at 300 rpm for 12 hours. After the incubation, 200 µl 96% ethanol was added and the samples were mixed by pulse-vortexing for 15 seconds. The samples were then added to the spin column in a 2 ml collection tube and centrifuged at ≥6000 x g for 1 min. All centrifugation steps were performed at room temperature (15°C-25°C). The collection tubes were then replaced by new 2 ml collection tubes and 500 µl AW1 buffer was added to the spin columns. The samples were then centrifuged at ≥6000 x g for 1min. The collection tubes were replaced by new 2 ml collection tubes and 500 µl AW2 buffer was added to the spin columns. The samples were centrifuged at full speed for 3 min. The collection tubes were then replaced by new 1.5 ml microcentrifuge tubes and 200 µl buffer AE was added to the spin columns. The samples were incubated at room temperature for 5 min and centrifuged at ≥6000 x g for 1 min. The flow-through in the 1.5 ml microcentrifuge tubes was then stored at -20°C until further processing.

The quantity and absorbance at 260 AU and 280 AU was measured for 2 µl DNA of each sample using the spectrophotometer. The elution buffer of either of the two DNA extraction methods was used as a blank for the corresponding samples. The purity of the DNA samples was checked using the 260/280 ratio obtained. We expected the ratio values for our samples to be approximately 1.8, as this is the optimal value accepted as good quality DNA (Kalb and Bernlohr 1977; Turashvili *et al.* 2012). To observe the integrity of the genomic DNA, a 5:2 ratio of DNA template and gel loading dye orange x6 (New England Biolabs, Massachusetts, United States) was examined by gel electrophoresis on a 1.5% (w/v) agarose gel stained with 2% ethidium bromide in a 1x TAE buffer (0.04 M Tris Acetate pH 8, 0.002 M EDTA). A 1,000 basepair (bp) ladder was used as a size reference. The gel was run for 30 min at 120 V and visualised with the Bio-Rad Molecular Imager, ChemiDoc XRS+ system (Bio-Rad Laboratories, California, United States).

3.5 16S rDNA PCR AMPLIFICATION AND SEQUENCING

The 16S rDNA was amplified in triplicate from 24 samples the same barcoded primers to allow samples to be pooled (Table 3 - 1). The 16S rDNA gene region (variable region V1-V8) was amplified using universal barcoded 16S rDNA primers; 27F (5'-AGA GTT TGA TCM TGG CTC AGA ACG-3') and 1435R (5'-CGA TTA CTA GCG ATT CCR RCT TCA-3') (Turner *et al.* 1999; Gall *et al.* 2016) (Table 3 - 2). Amplification was performed in a 20 µl reaction, consisting of 1X Phusion Flash High-Fidelity PCR (Polymerase chain

reaction) Master Mix (consisting of Phusion Flash II DNA Polymerase, dNTPs, reaction buffer, and $MgCl_2$) (Thermo Fisher Scientific, Massachusetts, United States), 0.15 µM of the forward (27F) and reverse primer (1435R), and a final concentration of ~ 9ng/µl of DNA template. According to the guidelines of the Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific, Massachusetts, United States), for a 20 µl reaction the quantity of DNA should range between 1 pg-10ng. The reaction was made up to 20 µl with double distilled H_2O. *Anaplasma centrale* vaccine strain (Onderstepoort Biological Products) was used as the positive control while PCR grade water was used as a no template negative control.

Amplification was done using a PCR thermocycler 2720 (Applied Biosystems, California, United States) with the following cycle conditions: Initial denaturation at 98°C for 30 seconds, followed by 40 cycles of 10 seconds at 98°C for denaturation, 30 seconds at 60°C for annealing, and 30 seconds at 72°C for extension. A final extension period followed this at 72°C for 10 min, and a final hold period at 4°C. The triplicate replicates of one sample were then added together. The purity and quantity of 2 µl of each PCR product was checked on a spectrophotometer. The blank for the spectrophotometer consisted of ddH_2O. The integrity of the PCR products was then examined by running a 5:2 ratio of PCR product and gel loading dye orange x6 on a 1.5% (w/v) agarose gel stained with 2% ethidium bromide in a 1x TAE buffer (0.04M Tris Acetate pH 8, 0.002 M EDTA). A 1,000 bp ladder was used as a size reference. The gel was run for 30 min at 120 V and visualised with the Bio-Rad Molecular Imager, ChemiDoc XRS+ system (Bio-Rad Laboratories, California, United States). The amplified 16S rDNA samples were submitted to the Washington State University's Sequencing Core for Pacific Bioscience (PacBio, Menlo Park, CA) Circular Consensus Sequencing (CCS).

Table 3 - 1: Sample and primer information for the library prepared for CCS.

Origin of samples	DNA ID	Host animal	Barcoded primer ID*
Clare A	1M	Dog	F1R2
Clare A	1S	Dog	F1R3
Clare A	2M	Dog	F1R4
Clare A	2S	Dog	F1R5
Clare A	3M	Dog	F2R2
Clare A	4M	Dog	F2R1
Clare A	5M	Dog	F2R3
Clare A	5S	Dog	F1R1
Clare A	6M	Dog	F2R4
Clare A	6S	Dog	F2R5
Clare A	7M	Dog	F3R1
Clare A	7S	Dog	F3R2
Clare A	8M	Dog	F4R3
Clare A	8S	Dog	F4R5
Clare A	9M	Dog	F5R2
Clare A	9S	Dog	F5R3
Clare A	11M	Dog	F3R3
Clare A	11S	Dog	F3R4
Clare A	12M	Dog	F4R1
Clare A	12S	Dog	F3R5

* See Table 3-2 for primer information

Table 3 - 2: 16S rDNA universal, sample-specific bar-coded primers

Name	Code	*Multiplex identifier (MID) sequence	Primer	Length (bp)
MID1-27F	F1	CGT ATC GCC TCC CTC GCG CCA TCAG ACG AGT GCGT	27F†	1,275
MID2-27F	F2	CGT ATC GCC TCC CTC GCG CCA TCAG ACG CTC GACA	27F†	1,275
MID3-27F	F3	CGT ATC GCC TCC CTC GCG CCA TCAG AGA CGC ACTC	27F†	1,275
MID4-27F	F4	CGT ATC GCC TCC CTC GCG CCA TCAG AGC ACT GTAG	27F†	1,275
MID5-27F	F5	CGT ATC GCC TCC CTC GCG CCA TCAG TCA GAC ACGA	27F†	1,275
MID1-1435R	R1	CTA TGC GCC TTG CCA GCC CGC TCAG ACG AGT GCGT	1435R♦	1,275
MID2-1435R	R2	CTA TGC GCC TTG CCA GCC CGC TCAG ACG CTC GACA	1435R♦	1,275
MID3-1435R	R3	CTA TGC GCC TTG CCA GCC CGC TCAG AGA CGC ACTC	1435R♦	1,275
MID4-1435R	R4	CTA TGC GCC TTG CCA GCC CGC TCAG AGC ACT GTAG	1435R♦	1,275
MID5-1435R	R5	CTA TGC GCC TTG CCA GCC CGC TCAG ATC AGA CACG	1435R♦	1,275

*red font= Adaptor sequence, blue font= Key sequence, green font= MID sequence.
†27F primer sequence = 5'-AGA GTT TGA TCM TGG CTC AGA ACG-3'
♦1435R primer sequence = 5'-CGA TTA CTA GCG ATT CCR RCT TCA-3'

3.6 MICROBIOME SEQUENCE ANALYSIS

Binning, trimming and filtering of sequence data obtained was conducted using the Pacific Biosciences software according to the set sequence size range and 99% precision. The format of the raw sequence reads for the 24 samples obtained from PacBio, was changed from Fastq to Fasta, using CLC Genomics workbench version 7 (developed by CLC Bio. http://www.clcbio.com). The sequences were then compared against a 16S rDNA database and an in-house developed *Anaplasma* database, using the BLASTn (Basic local alignment search tool nucleotide) on NCBI BLAST 2.9.0+ command-line application (Camacho *et al.* 2009). This provided a list of sequences with reference numbers corresponding to different bacteria. These sequences were then filtered to 98% identity, and classified to species level. All sequences corresponding to a specific species were grouped together and classified as Operational Taxonomic Units (OTUs). Additionally, all sequence reads were analysed on the Ribosomal Database Project (RDP) (Cole *et al.* 2009; Gall *et al.* 2016, 2017) and sequence reads that had less than 98% identity were classified to genus level (Jones *et al.* 2010; Bonnet *et al.* 2014). The sequences that corresponded to a specific genus, were grouped together and classified as OTU. All OTUs in a sample that represented less than 1% of the total OTU count of that sample, were classified as rare.

3.7 STATISTICAL ANALYSIS FOR MICROBIOME SEQUENCE DATA

The composition of the microbial community in the midgut and salivary gland pools from *R. sanguineus* ticks were analysed using the community ecology package, vegan version 2.5-6 in R Studio version 2.1 (R Core Team, 2017). Alpha diversity rarefaction curves were plotted to calculate the average species diversity of the bacteria in the midgut and salivary gland pools.

The Beta diversity (an indication of the extent with which samples differ from one another) between the MPEE and DBTK methods as well as between the midgut and salivary gland tissues was compared with a permanova test using a 1000 permutations, and the Bray-Curtis dissimilarity metric using the community ecology package, vegan version 2.5-6 in R Studio version 2.1 (R Core Team, 2017). The Beta diversity was visualized with a non-metric multidimensional scale (nMDS) plot. This was followed by testing if the assumption of homogeneity of multivariate dispersion was met for the permanova test, using a betadisper test. Clustering of samples indicated a similar bacterial microbiome, while samples that were dispersed indicated variability.

Principal component analysis (PCA) was performed to quantify the similarities and dissimilarities of the bacterial communities in the midgut and salivary gland pools. Dimensions were created from the linear combinations of the variables. This analysis was done on the FactoMineR package on R Studio version 2.1 (R Core Team, 2017). Individuals that clustered together in the PCA plot were considered to share similar

bacterial profiles, while samples that were dispersed showed variability. The position of the variables on plane of the plot indicated positive correlation, while variables positioned on the other plane of the plot indicated negative correlation. The correlation coefficient (r) was extracted from the PCA and indicated the correlation between the variables and the dimensions. The correlation coefficient was considered significant if the p-value was lower than 0.05.

3.8 SEQUENCE AND PHYLOGENETIC ANALYSIS

Consensus sequences of organisms of interest (i.e. *Anaplasma*, *Ehrlichia* and *Coxiella*) were extracted in CLC genomics workbench version 7 (http://www.clcbio.com). The sequences were saved individually and changed to Fasta format and imported to CLC Main Workbench 8 (http://www.clcbio.com). Alignments were created for *Anaplasma*, *Ehrlichia* and *Coxiella* species, respectively using CLC Main Workbench 8 (http://www.clcbio.com). Homologous sequences from GenBank were also included in the alignments. The alignments were then run through MAFFT online version 7 (http://mafft.cbrc.jp/alingments/server/index.html) with default parameters, to determine if all sequences were in the correct orientation and of the correct length. The aligned matrix was then examined and edited manually on CLC Main Workbench 8 (http://www.clcbio.com). The aligned matrix was exported as a Fasta alignment and uploaded onto Mega (Molecular evolutionary genetics analysis) version 7.0.26 (Kumar *et al.* 2016). Maximum likelihood and Neighbour Joining trees where constructed using Mega version 7.0.26 (Kumar *et al.* 2016). The best model for each tree was determined with the model test on Mega version 7.0.26 (Kumar *et al.* 2016). The best model for *Anaplasma* species was determined as the Kimura 2-paramter model with a discrete gamma distribution (G) and evolutionary invariable sites (I). The best model for *Ehrlichia* and *Coxiella* species was determined as the Kimura 2-paramter model with a discrete gamma distribution (G). Support values calculated was based on a 1000 bootstrap replicates. The accession numbers of the references sequences are available in Table A 4 of appendix A.

3.9 ASSESSMENT OF RISK FACTORS FOR *RHIPICEPHALUS SANGUINEUS* INFESTATIONS

3.9.1 STUDY DESIGN AND SELECTION OF HOUSEHOLDS

A questionnaire study was conducted (Appendix B) to determine if host and environmental factors, as well as management practices influenced infestation of domestic dogs with *R. sanguineus* ticks in households in the Mnisi community, Mpumalanga Province, South Africa. The questionnaire interviews were conducted at 150 households that owned domestic dogs in the Clare A area, after the owner gave written informed consent. The sample size for the questionnaire interviews was determined using Cochran's equation with a correction for small populations (Cochran 1963; Israel 1992). As the Mnisi area has an average of 8500 households divided into 12 villages, we extrapolated that Clare A has an average

of 700 households, with 25% of these households having dogs. Following Cochran's equations, and assuming that Clare A has a 175 households with dogs, the minimum sample size required for the questionnaire interviews was calculated to be 120.

3.9.2 HOUSEHOLD INTERVIEWS AND ENVIRONMENTAL DATA

In each household, the interviews were performed in the owner's native language, a facilitator who translated and guided the interview. An animal handler was also present who helped secure the animals to keep them calm during tick collection. The questionnaire was pre-tested in one household and amended to ensure that participants understood the questions and that there was good flow of the questions and the responses. The interviews were conducted between 08:00 and 13:00 and each interview took about 10 min. The interviews comprised a list of open- and close-ended questions to collect both qualitative and quantitative data that were regarded as potential exposure factors to *R. sanguineus* infestation amongst the dogs. The collected data included: household characteristics, demographics of the dogs (e.g. number and age), tick control practices (hand picking of ticks, dipping), animal health care (veterinary assistance), dog activities and raising method (e.g. hunting, herding with cattle, confinement), and presence of other animal species in the household (cattle, goats, pigs, chicken) as shown in Table 6. In each household, dogs were examined for ticks, and these were collected and their number and species recorded. Only *R. sanguineus* infestation was used in the analysis for risk factors. The monthly temperature and rainfall for the study area was recorded at the weather station at Hluvukani, Mnisi, which is the closest weather station to the sampling area.

3.9.3 DATA ANALYSIS

The data collected from household interviews and environmental measurements were entered into Microsoft Excel sheets, and quantitative data was standardised by conversion into categories. The number of dogs were categorised as ≤ 2 or ≥ 3 dogs, while the age of dogs was sub-grouped as ≤2 years or ≥3 years. The number of chickens in the household were categorised as ≤10 chicken or >10 chicken. We hypothesize that chickens could scavenges on the ticks present in the surrounding area, and therefore their numbers in household may affect tick infestations in dogs. The environmental measurements were categorised as 0 to 19°C or ≥20°C for temperature and 0 to 40 mm or ≥41 mm for rainfall.

Univariate analysis of associations was performed considering the *R. sanguineus* infestation as a binary outcome (low *R. sanguineus* infestation [0 to 10 ticks] or high infestation [≥11]. Each household characteristic (potential exposure factor) was tested for significant association with level of tick infestation using the chi-square test. Prevalence ratios and odds ratios (OR) of dogs with high tick infestation (≥11 ticks) with respect to the household exposure factors were obtained as shown in Table

6. Thirteen variables in the univariate analysis with a p-value ≤0.5 were included in an initial multivariable logistic regression model, in which tick infestation was a binary outcome (low or high infestation). A Generalised Linear Model function was performed, considering a binomial distribution, and employing a stepwise elimination procedure in order to arrive at the minimal adequate model that minimised the Akaike Information Criteria (AIC). Hosmer-Lemeshow (χ^2) was used as a goodness of fit test. Statistical analysis was carried out using R Console version 3.2.1 (R Core Team, 2017) at 5% level of significance.

CHAPTER 4

RESULTS

4.1 TICK IDENTIFICATION

During the 12-month collection period, a total of 2,685 ticks were collected from 64 dogs in the Mnisi community. This consisted of 2,212 (82.38%) adult ticks morphologically identified to belong to seven species (Figure 4 - 1), 429 (15.98%) immatures and 44 (1.64%) unidentified ticks. Overall, 1,788 (80.83%) adult *R. sanguineus* ticks were collected (of which 1,180 were males). From the 429 immature ticks collected, 94 (21.91%) were identified as *Rhipicephalus* and 335 (78.09%) were identified as *Amblyomma*.

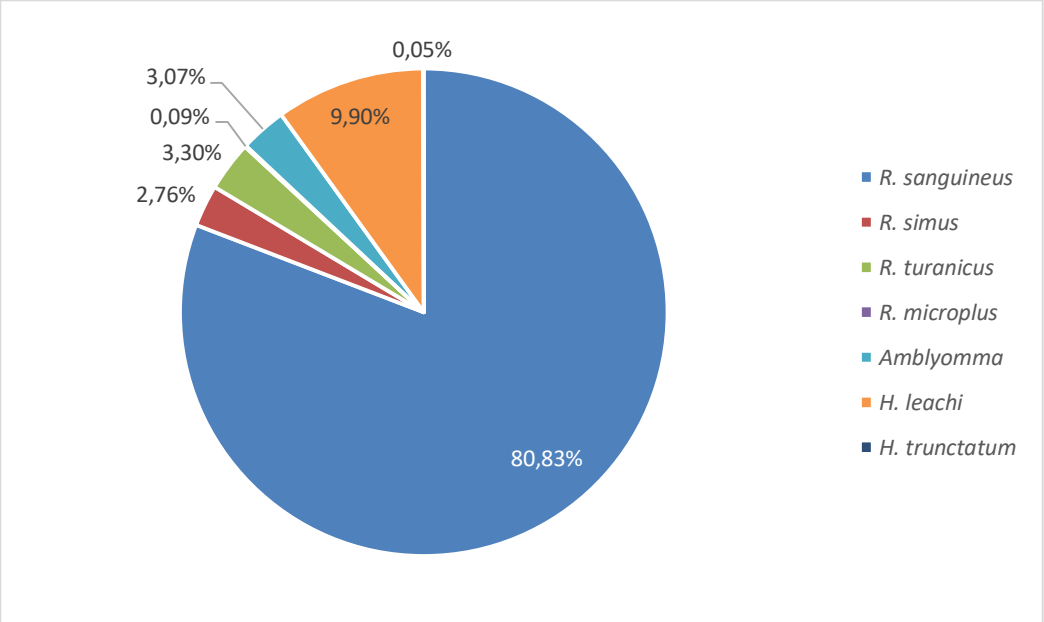

Figure 4 - 1: A chart indicating the identity and abundance of adult ticks collected from dogs in 64 households in the Mnisi community in Mpumalanga Province, South Africa from June 2018 to June 2019.

Cytochrome c oxidase 1 (CO1) barcoding was subsequently used to confirm the species identification of 24 morphologically selected *R. sanguineus* ticks. CO1 results identified 13 of the 24 ticks as *R. leporis* and 7 as *R. sanguineus* (Table 4 - 1). Four of the ticks did not yield results. All samples were subsequently considered to be *R. sanguineus*, as the distribution of *R. leporis* does not extend to South Africa.

Table 4 - 1: COI barcoding results: Species identity and the corresponding percent identity of ticks in each sample (excluding samples 3S, 4S, 10M and 10S).

Sample ID	Species Identification	Percentage Identity
1M	*Rhipicephalus leporis*	99.83%
1S	*Rhipicephalus leporis*	99.65%
2M	*Rhipicephalus sanguineus*	99.83%
2S	*Rhipicephalus sanguineus*	100%
3M	*Rhipicephalus sanguineus*	99.84%
4M	*Rhipicephalus leporis*	99.83%
5M	*Rhipicephalus leporis*	99.44%
5S	*Rhipicephalus leporis*	99.67%
6M	*Rhipicephalus sanguineus*	99.61%
6S	*Rhipicephalus sanguineus*	99.84%
7M	*Rhipicephalus leporis*	99.62%
7S	*Rhipicephalus leporis*	99.33%
8M	*Rhipicephalus leporis*	99.45%
8S	*Rhipicephalus leporis*	99.64%
9M	*Rhipicephalus leporis*	99.45%
9S	*Rhipicephalus leporis*	799.20%
11M	*Rhipicephalus sanguineus*	99.82%
11S	*Rhipicephalus sanguineus*	99.64%
12M	*Rhipicephalus leporis*	99.82%
12S	*Rhipicephalus leporis*	99.67%

4.2 GENOMIC DNA EXTRACTION AND 16S RDNA AMPLIFICATION

From the 56 midgut and 56 salivary gland pools extracted using the modified PureGene Ethanol Extraction (MPEE) method, the 16S rDNA could only be PCR amplified from six midgut pools and six salivary gland pools (Table 4 - 2). From the six midgut and six salivary gland pools extracted using the DNeasy Blood and Tissue Kit (DBTK) method, the 16S rDNA could be PCR amplified from all pools (Table 4 - 3). Each sample was amplified in triplicate using the barcoded 16S rDNA primers, and the triplicates were combined to create one sample. The integrity of the PCR products were examined by gel electrophoresis (Figure 4 - 2 and Figure 4 - 3).

Table 4 - 2: Summarized sample data indicating the sampling date, number of ticks per pool and the DNA quantity and quality, of 6 midgut and 6 salivary gland pools from *Rhipicephalus sanguineus* ticks, extracted using the modified PureGene ethanol extraction method

	Sampling Date (2019)	Sample ID	Amount of ticks per pool	Tick tissue	Concentration (ng/µl)	260/280
Modified PureGene ethanol extraction (MPEE)	February	1M	10	Midgut	56.16	1.9
	February	1S	10	Salivary Gland	157.05	1.9
	February	2M	10	Midgut	27.69	2.1
	February	2S	10	Salivary Gland	100.84	2.0
	February	3M	10	Midgut	33.35	2.1
	February	3S	10	Salivary Gland	70.04	2.0
	February	4M	10	Midgut	40.34	2.0
	February	4S	10	Salivary Gland	58.63	2.0
	March	5M	10	Midgut	11.47	1.3
	March	5S	10	Salivary Gland	41.61	1.1
	March	6M	10	Midgut	38.27	1.3
	March	6S	10	Salivary Gland	76.73	1.3

Table 4 - 3: Summarized sample data indicating the sampling date, number of ticks per pool and the DNA quantity and quality, of 6 midgut and 6 salivary gland pools from *Rhipicephalus sanguineus* ticks, extracted using DNeasy Blood and Tissue kit method

	Sampling Date (2019)	Sample ID	Amount of ticks per pool	Tick tissue	Concentration (ng/µl)	260/280
DNeasy Blood and Tissue Kit (DBTK)	June	7M	10	Midgut	10.93	2.0
	June	7S	10	Salivary Gland	21.56	2.0
	June	8M	10	Midgut	10.39	2.0
	June	8S	10	Salivary Gland	16.51	2.0
	July	9M	10	Midgut	6.85	2.2
	July	9S	10	Salivary Gland	29.63	2.0
	July	10M	10	Midgut	22.37	2.2
	July	10S	10	Salivary Gland	43.56	2.1
	July	11M	10	Midgut	12.00	2.1
	July	11S	10	Salivary Gland	31.15	2.0
	July	12M	10	Midgut	16.34	1.9
	July	12S	10	Salivary Gland	41.59	2.0

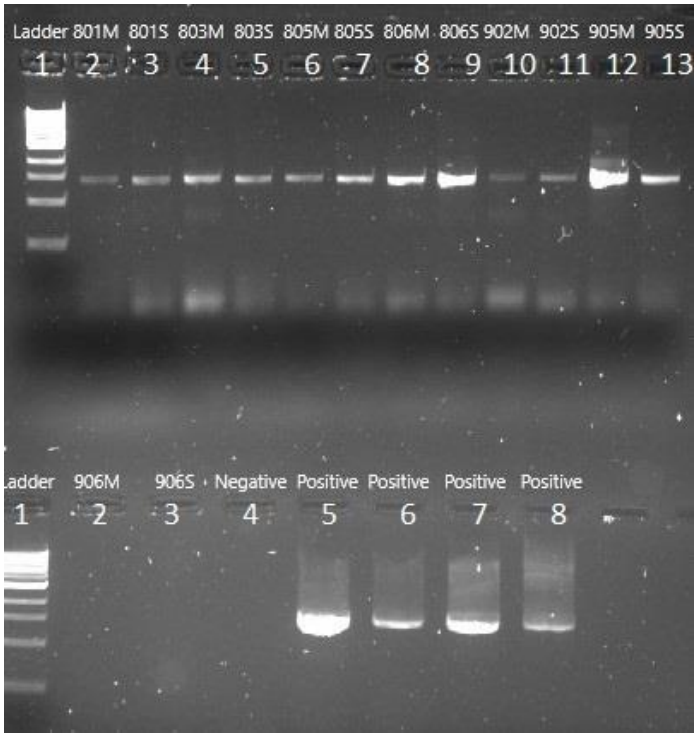

Figure 4 - 2: Visualization of an agarose gel of genomic DNA extracted using the modified PureGene ethanol extraction method. Top row: Lane 1 indicates a 1 kb (kilobase) ladder. Lane 2-13 contains samples. Bottom row: Lane 1 contains a 1 kb ladder. Lane 2-3 contains samples. Lane 4 contains a negative control. Lane 5-8 contains positive controls.

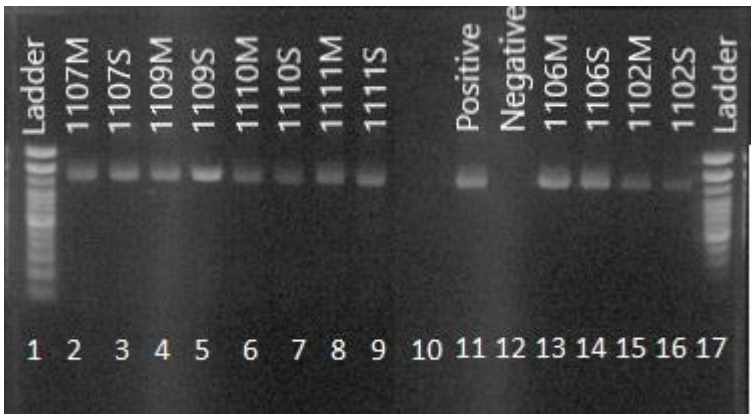

Figure 4 - 3: Visualization of an agarose gel of genomic DNA extracted using the DNeasy extraction kit. Lanes 1 and 17 contains a 100 bp (basepair) ladder. Lanes 2-9 and lanes 13-16 contains samples. Lane 10 is empty. Lane 11 contains a positive control and lane 12 contains a negative control.

4.3 MICROBIOME SEQUENCING AND STATISTICAL ANALYSIS

PacBio Circular Consensus Sequencing (CCS) of all 12 midgut and 12 salivary gland samples generated a total of 26,0312 bacterial 16S rDNA sequences. One of the midgut samples dehydrated during transportation to the sequencing facility and only provided 267 sequences and was subsequently removed from the analysis. A rarefaction curve using the mean species diversity of the bacterial

populations in the tick tissues indicated that all samples approached the saturation plateau, showing that there was enough sequence coverage to correctly represent all the bacterial populations (Figure 4 - 4).

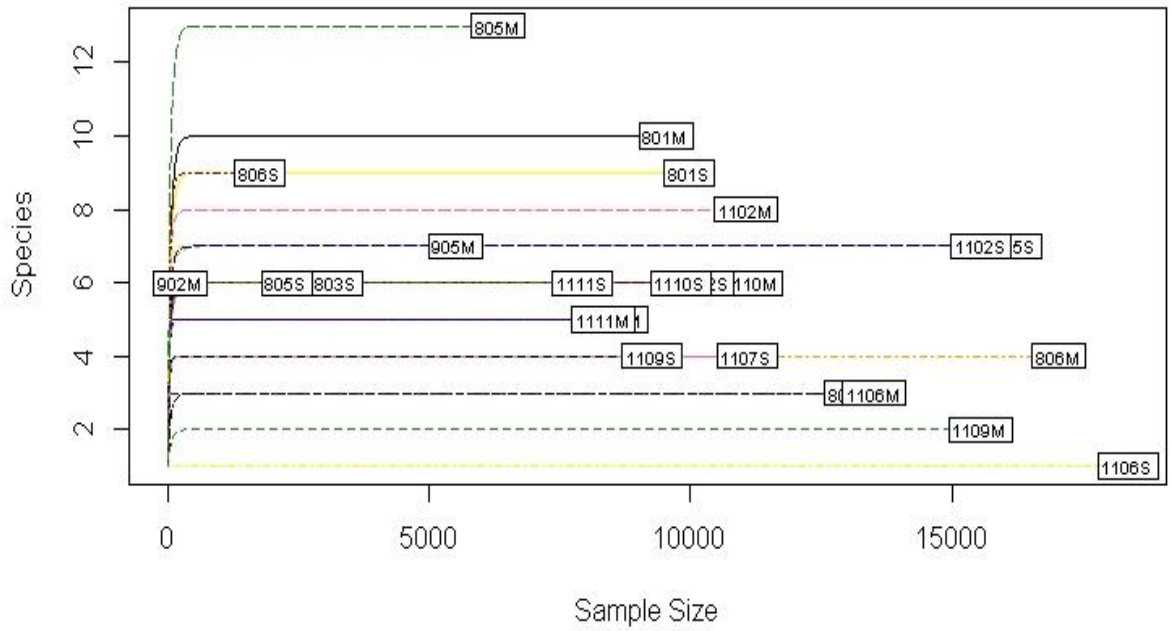

Figure 4 - 4: Rarefaction curves of tick tissue samples from Clare A in the Mnisi community. The read depth was plotted against the mean species diversity of the bacterial populations in the tick tissue samples.

Further analysis revealed 39 species within 34 genera belonging to seven phyla, excluding all bacteria with a frequency lower than 1%, which was classified as 'rare' or 'unclassified bacteria' (Figure 4 - 5). Actinobacteria (Figure 4 - 6) and Proteobacteria (Figure 4 - 7) were the most prevalent phyla found during the study and made up 15.52% and 85.44% of the total bacteria, respectively. The remaining bacteria consisted of Bacteroidetes (0.09%) (Figure 4 - 8), Firmicutes (0.76%) (Figure 4 - 9), Euryarchaeota (0.06%), Planctomycetes (0.02%), Verrucomicrobia (0.01%), rare bacteria (8.05%) and unclassified bacteria (0.05%). The Euryarchaeota, Planctomycetes and Verrucomicrobia were present in very low quantities in only a few samples. The Actinobacteria further consisted of *Propionibacterium* (48.1%), *Cutibacterium* (46.74%), *Brevibacterium* (0.3%), *Microbacterium* (4.7%) and *Pseudonocardia* (0.16%). The Firmicutes phyla was comprised of *Bacillus* (13.45%), *Lactobacillus* (0.40%), *Lysinibacillus* (9.77%), *Staphylococcus* (31.79%) and *Streptococcus* (44.58%) while the Bacteroidetes phylum consisted of *Hydrotalea* (86.46%) and *Haliscomenobacter* (13.54%). Proteobacteria consisted of 18 genera: *Actinobacter* (0.6%), *Anaplasma* (21.69%), *Aureimonas* (5.78%), *Azospirillum* (0.05%), *Campylobacter* (0.07%), *Coxiella* (12.12%), *Ehrlichia* (19.94%), *Kosakonia* (1.46%), *Limnobacter* (0.01%), *Methyloversatilis* (0.02%), *Microvirga* (3.24%), *Paracoccus* (0.43%), *Pseudomonas* (13.4%), *Ralstonia* (0.08%), *Roseomonas* (0.04%), *Snodgrassella* (0.48%), *Sphingomonas* (20.96), and *Stenotrophomonas* (0.06%).

For further analysis, we only focussed on the *Anaplasma, Ehrlichia* and *Coxiella* sequences found.

Further examination of the *Anaplasma* genus using only the Ribosomal Database Project (RDP) database, indicated that the *Anaplasma* species consisted of 68.36% *A. phagocytophilum* and 31.64% *Anaplasma* species After further classification with the in-house developed *Anaplasma* database, we detected 15.36% *A. centrale* with 100% sequence identity to *A. centrale* Israel strain (CP001759), 75.82% *A. platys* with a 100% identity to *A. platys* isolate D25 (MK81441) and 8.82% *Anaplasma* species We did not detect any *A. phagocytophilum* with the in-house *Anaplasma* database. The *A. centrale* was distributed across most of the MPEE method samples, while the DBTK samples did not contain any *A. centrale*. Sample 8M and 8S from the DBTK method were the only two samples that contained *A. platys*.

The *Ehrlichia* genus consisted of 95.46% *E. canis* with 100% sequence identity to the *E. canis* isolate TrKysEcan3 (KJ513197) and 4.54% *Ehrlichia* species The *E. canis* was very prevalent in four of the DBTK samples (9M, 9S, 10M and 10S), and present in trace amounts in only one MPEE method sample. *Coxiella* species *w*as detected (8.86%) in most of the MPEE samples, and was only present in small amounts in two of the DBTK samples.

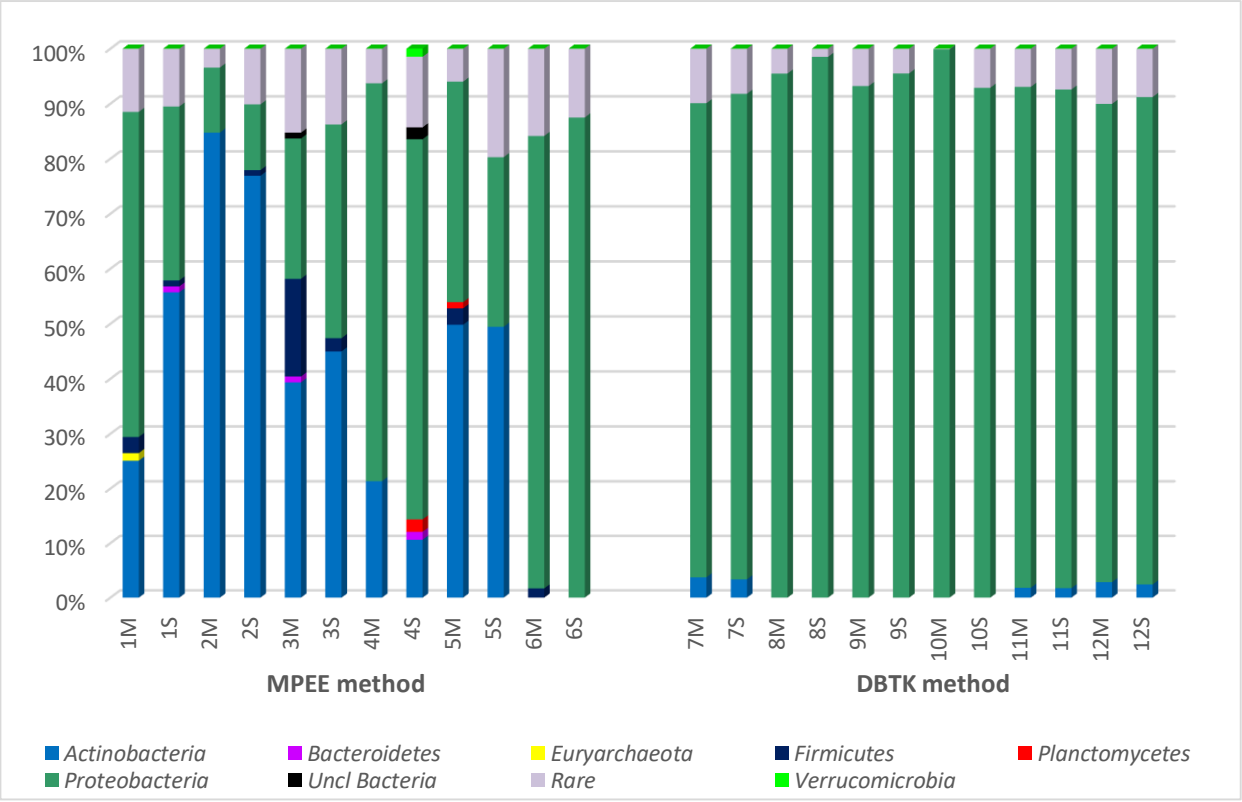

Figure 4 - 5: A stacked bar chart indicating the prevalence and diversity of the bacterial phyla of midgut (M) and salivary gland (S) pools, extracted using two different extraction methods (MPEE and DBTK) from *Rhipicephalus sanguineus* ticks collected from Clare A in the Mnisi community, Mpumalanga, South Africa

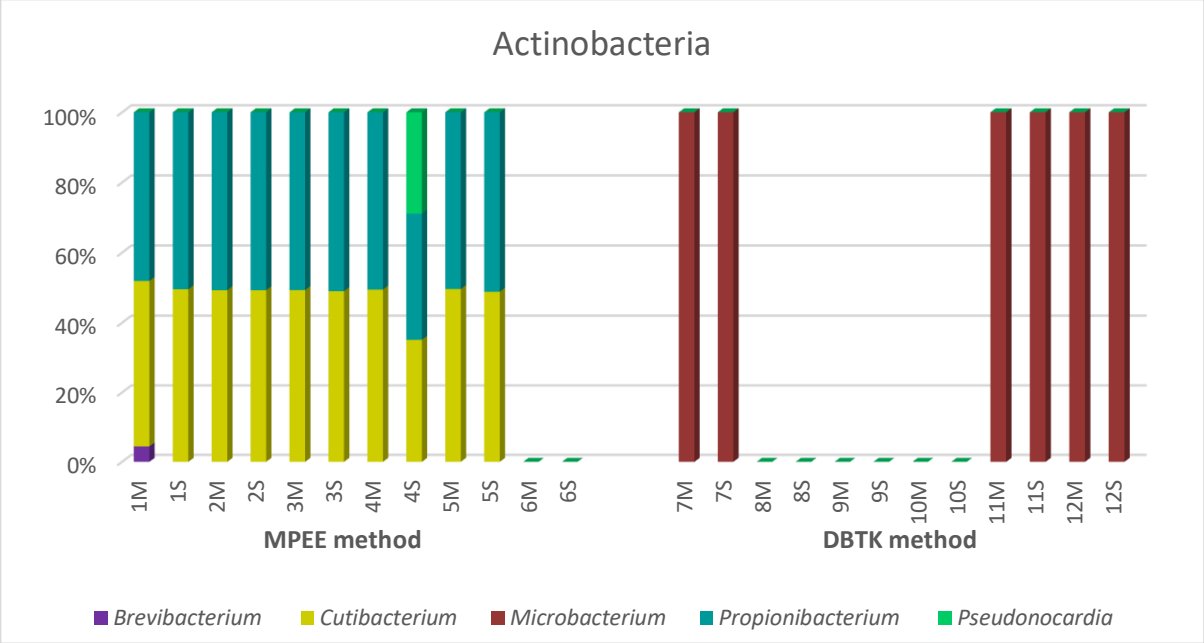

Figure 4 - 6: A stacked bar chart indicating the prevalence and diversity of the Actinobacteria phylum of midgut (M) and salivary gland (S) pools, extracted using two different extraction methods (MPEE and DBTK) from *Rhipicephalus sanguineus* ticks collected from Clare A in the Mnisi community, Mpumalanga, South Africa

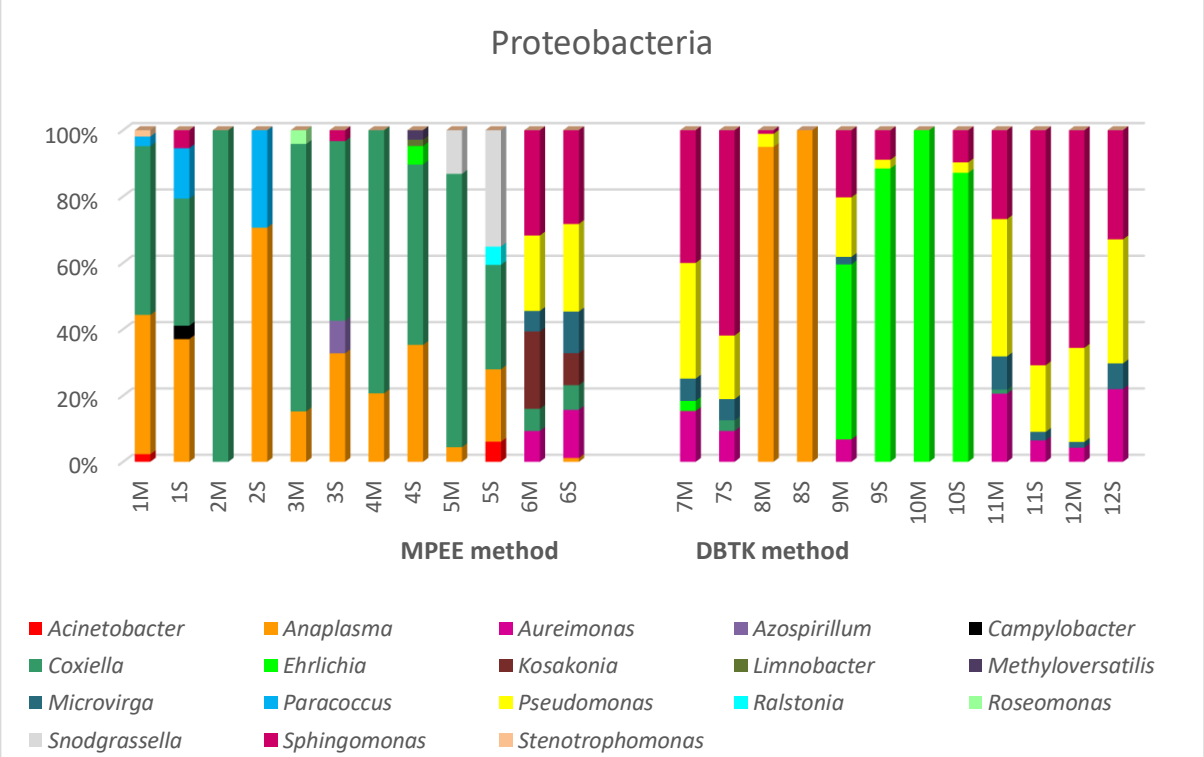

Figure 4 - 7: A stacked bar chart indicating the prevalence and diversity of the Proteobacteria phylum of midgut (M) and salivary gland (S) pools, for the MPEE and DBTK extraction methods from *Rhipicephalus sanguineus* ticks collected from Clare A in the Mnisi community, Mpumalanga, South Africa

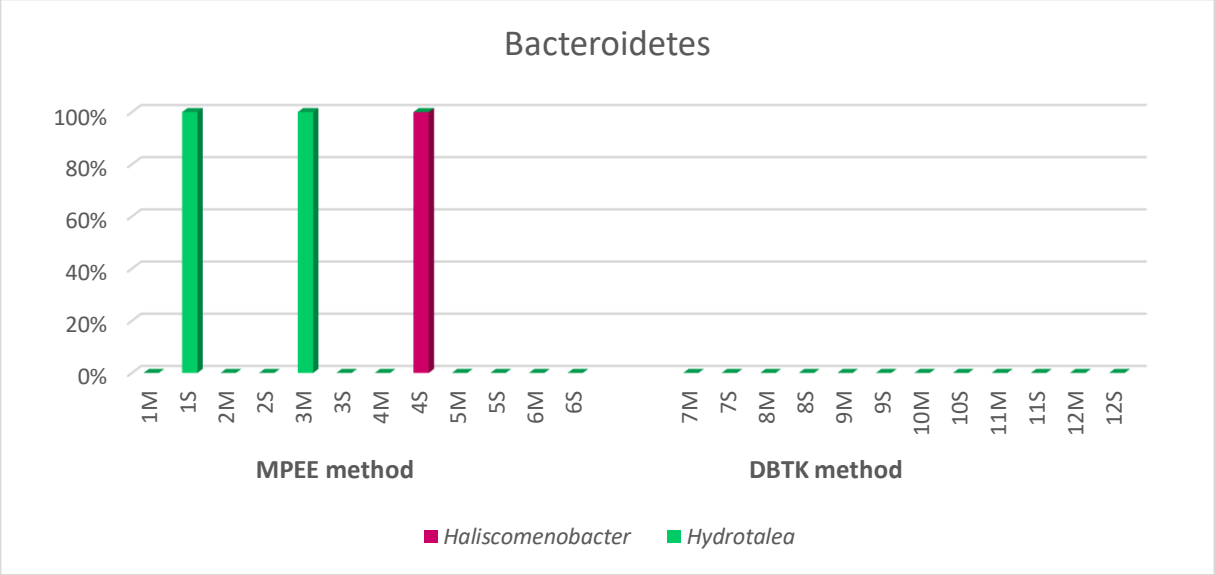

Figure 4 - 8: A stacked bar chart indicating the prevalence and diversity of the Bacteroidetes phylum of midgut (M) and salivary gland (S) pools, for two extraction methods (MPEE and DBTK) from *Rhipicephalus sanguineus* ticks collected from Clare A in the Mnisi community, Mpumalanga, South Africa

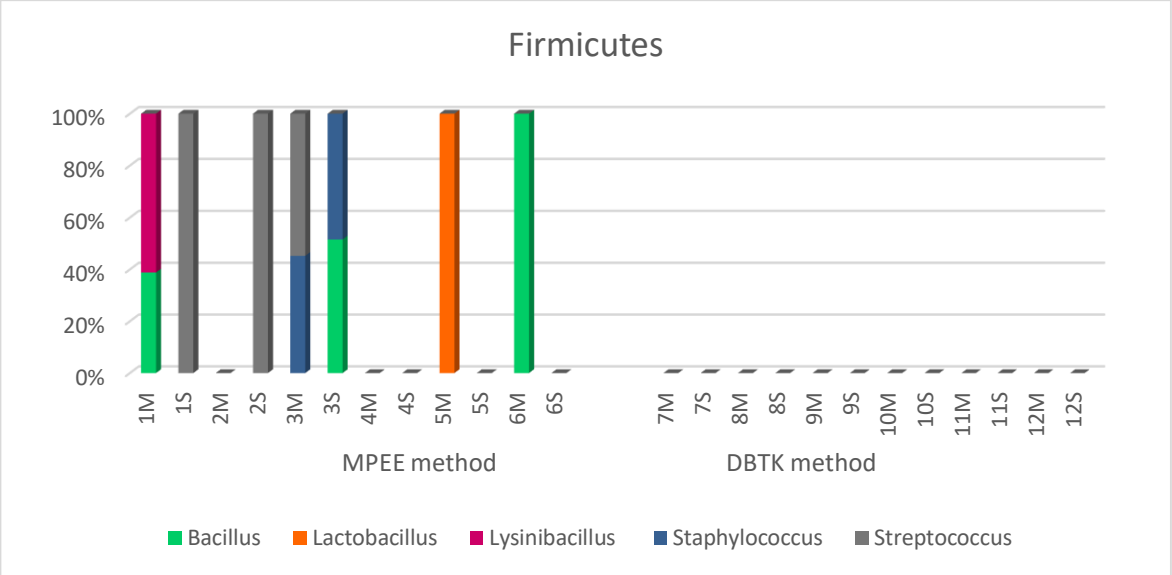

Figure 4 - 9: A stacked bar chart indicating the prevalence and diversity of the Firmicutes phylum of midgut (M) and salivary gland pools (S), for two extraction methods (MPEE and DBTK) from *Rhipicephalus sanguineus* ticks collected from Clare A in the Mnisi community, Mpumalanga, South Africa

The Beta diversity of the bacterial community between the MPEE and DBTK extraction methods was visualised using both an nMDS (Non-metric multidimensional scaling) (Figure 4 - 10) and PCA plot. The nMDS plot was visualized in two dimensions (stress = 0.12) and showed that ten of the MPEE samples clustered together, while two of the MPEE samples clustered around the DBTK extracted samples. The DBTK extracted samples also clustered together, with two outliers clustering far away from all the samples. A permanova test indicated that there were statistical differences in the beta diversity of the

bacteria between the MPEE and the DBTK extraction methods (p = 0.001). A betadisper test showed that the assumption of homogeneity of multivariate dispersion was met (p = 0.89).

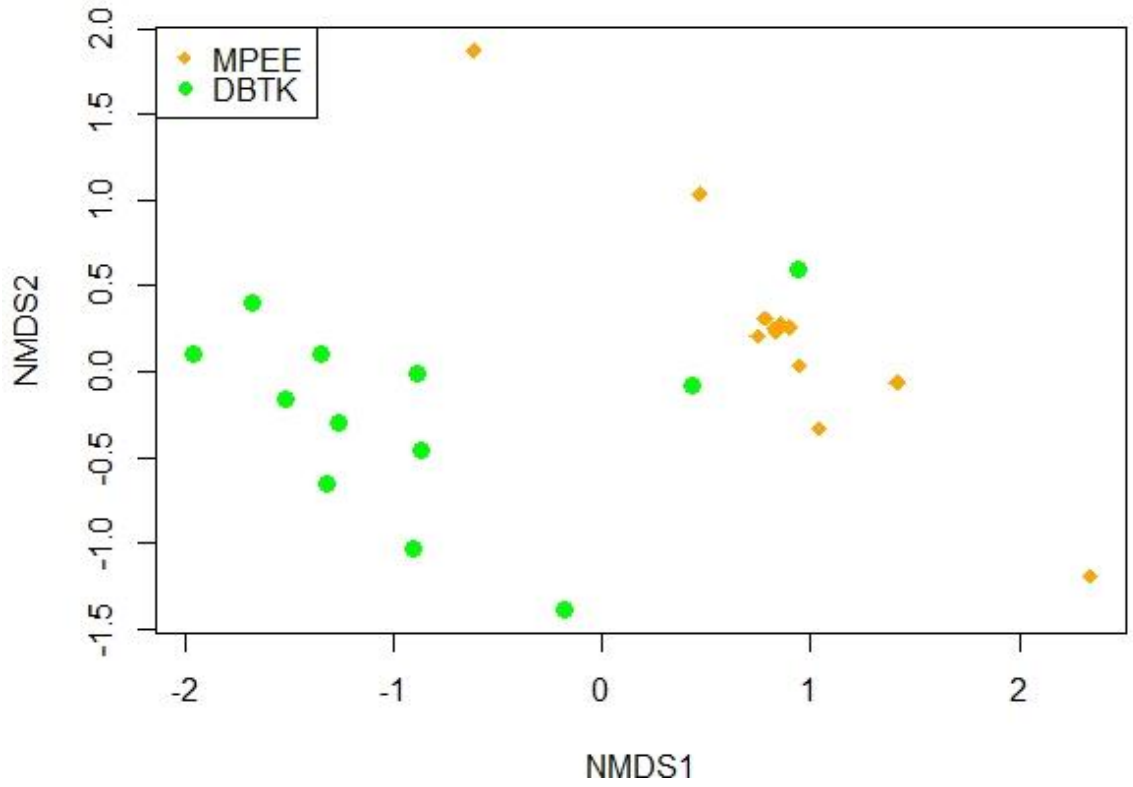

Figure 4 - 10: Non-metric multidimensional scaling (nMDS) ordination of the bacterial microbiome communities of *Rhipicephalus sanguineus* midgut and salivary gland pools, comparing the MPEE and DBTK extraction methods, using the Bray-Curtis similarity coefficient of the square-root transformed (Operational Taxonomic Units) OTU relative abundances. The symbols signify the bacterial microbiome of individual samples, and the distances between each symbol signify the similarities between those samples (symbols that are closer together are more similar than distant symbols). Samples extracted by MPEE method are indicated in yellow and samples extracted by the DBTK method are indicated in green.

The Beta diversity of the bacterial community between the two tissue types (midgut and salivary gland) were visualised using both an nMDS (Non-metric multidimensional scaling) (Figure 4 - 11) and PCA plot. The nMDS plot was visualized in two dimensions (stress = 0.12) and showed no distinct clustering between the two groups. There were two outliers, one midgut sample and one salivary gland sample. A permanova test indicated that there were no statistical differences in the beta diversity of the bacteria between the midgut and salivary gland tissues (p = 0.97). A betadisper test showed that the assumption of homogeneity of multivariate dispersion was met (p = 0.9).

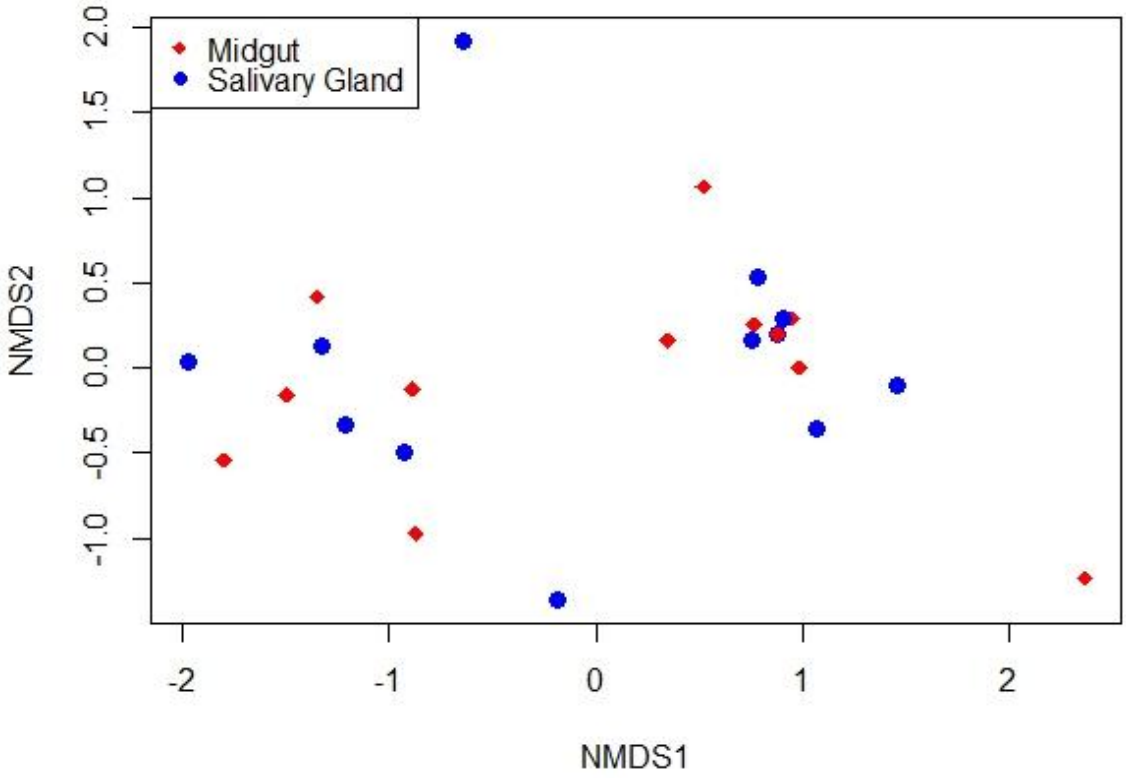

Figure 4 - 11: Non-metric multidimensional scaling (nMDS) ordination of the bacterial microbiome communities of *Rhipicephalus sanguineus* comparing the midgut and salivary gland TISSUES using the Bray-Curtis similarity coefficient of the square-root transformed (Operational Taxonomic Units) OTU relative abundances. The symbols signify the bacterial microbiome of individual samples, and the distances between each symbol signify the similarities between those samples (symbols that are closer together are more similar than distant symbols). The midgut samples are indicated by red and the salivary gland samples by blue.

PCA of the individuals factor map indicated that samples 6M, 6S, 7M, 7S, 10S, 11M, 11S, 12M, 12S shared some similarities owing to their position on the bottom left quarter of the PCA plot (Figure 4 - 12). Salivary gland pools 1S and 5S shared similarities as they were both in the bottom right quarter. Sample 1M was also in the bottom right quarter, however it was positioned distant from samples 1S and 5S. Samples 2M, 2S, 3M, 3S, 4M, 8M and 10M were all found in the top right quarter indicating that they likely shared some similarities. Sample 4M was also found in the top right quarter, but was positioned distant from the other samples in that quarter. Samples 5M, 8S, 9S and 9M also shared some similarities and grouped together in the top left quarter.

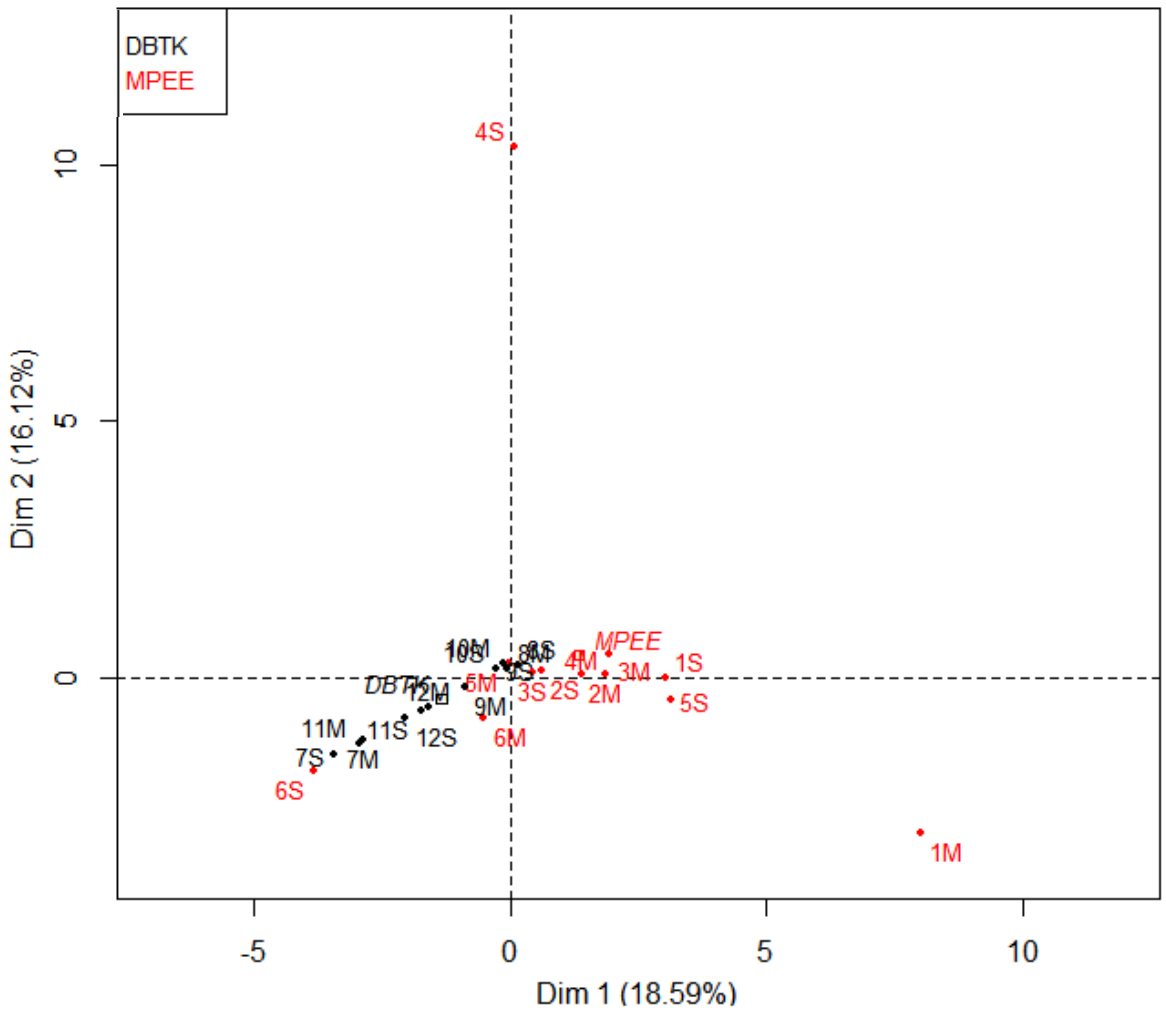

Figure 4 - 12: PCA plot of individuals factor map of the midgut and salivary gland samples from *Rhipicephalus sanguineus* ticks from dogs in Clare A, Mnisi community, South Africa. Dim 1 and Dim 2 are dimensions/principal components produced from linear combinations of variables. On each axis the percentage of variability for each dimension is given in brackets.

The variables factor map PCA plot (Figure 4 - 13) revealed that *Brevibacterium*, Gp6, *Stenotrophomonas* and *Lysinibacillus*, expressed by the correlation coefficient (R = 0.7) were significantly and positively associated with dimension 1, and significantly and negatively correlated to dimension 2 (R = - 0.3). *Acinetobacter* (R = 0.7), *Cutibacterium* (0.6), *Propionibacterium* (R = 0.6), *Bacillus* (R = 0.5). *Paracoccus* (R = 0.5), *Coxiella* (R = 0.3) and *Hydrotalea* (R = 0.3) were all significantly and positively correlated to dimension 1. Conversely, *Kosakonia* (R = -0.3) and *Microbacterium* (R = -0.5) were negatively associated with dimension 1. *Pseudonocardia*, *Methyloversatilis*, *Prosthecobacter*, *Haliscomenobacter* and *Limnobacter* were all positively correlated with dimension 2, expressed by the correlation coefficient (R = 0.9). *Microvirga* (R = -0.6), *Sphingomonas* (R = -0.7), *Aureimonas* (R = -0.7) and *Pseudomonas* (R = -0.7) were all significantly and negatively associated with dimension 1. Additionally, *Microvirga*, *Sphingomonas*, *Aureimonas* and *Pseudomonas*, expressed by the correlation coefficient (R = 0.3), were all significantly and negatively associated with dimension 2.

When connecting the PCA plot of the variables factor map to the individuals' factor mapped, it shows that samples 1M, 1S and 5S were associated with the variables, *Brevibacterium*, Gp6, *Stenotrophomonas* and *Lysinibacillus* which were the main contributors in the bottom right quarter. Samples 6M, 6S, 7M, 7S, 10S, 11M, 11S, 12M and 12S were linked to the variables, *Microvirga, Sphingomonas, Aureimonas*, *Pseudomonas* and *Lysinibacillus*, which were the main contributors in the bottom left quarter. Samples 15M, 8M, 9M and 9S were linked to the variables *Kosakonia*, *Pseudonocardia*, *Methyloversatilis*, *Prosthecobacter*, *Haliscomenobacter* and *Limnobacter*. Samples 2M, 2S, 3M, 3S, 4M, 4S, 8M and 10M were linked to a combination of *Pseudonocardia*, *Methyloversatilis*, *Prosthecobacter*, *Haliscomenobacter*, Limnobacter, *Actinobacter*, *Cutibacterium*, *Propionibacterium*, *Bacillus*, *Paracoccus*, *Coxiella* and *Hydrotalea*.

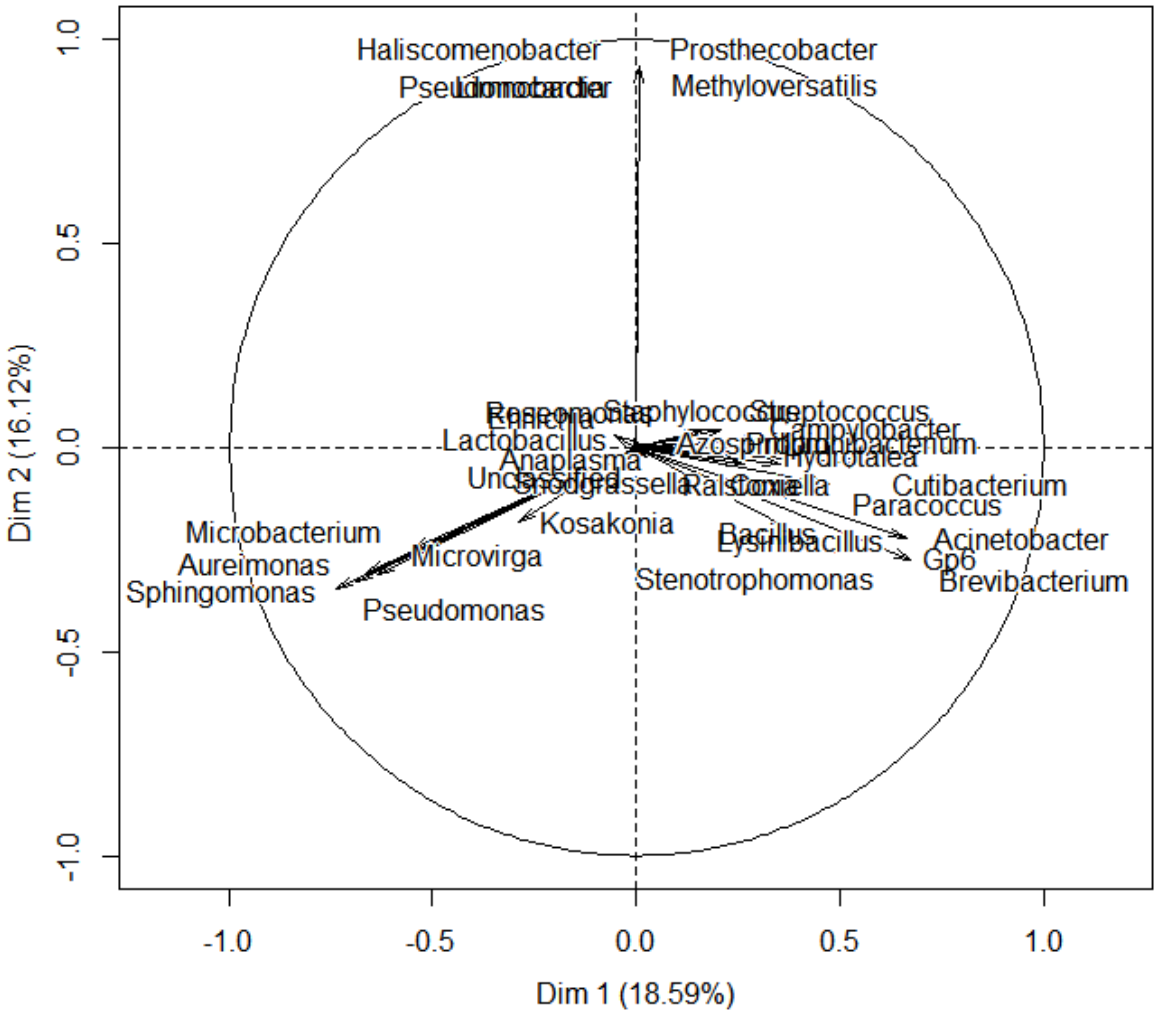

Figure 4 - 13: A PCA plot of the variables factor indicating the bacterial genera found within the bacterial microbiome of the midgut and salivary gland pools of *Rhipicephalus sanguineus* tick collected from dogs in Clare A, in the Mnisi community, South Africa. Dim 1 and Dim 2 are the dimensions/principal components produced from the linear combinations of the variables. The percentage variability for each dimension is indicated in brackets on each axis.

4.4 SEQUENCE AND PHYLOGENETIC ANALYSIS

Five *Anaplasma*, four *Ehrlichia* and four *Coxiella* 16S rDNA sequence variants were extracted from the microbiome sequence dataset and used in subsequent phylogenetic analyses.

Basic local alignment search tool nucleotide (BLASTn) homology searches showed that four of the obtained *Anaplasma* sequences (Ac 1, Ac 2, Ac3, Ac 4) had 99.5-100% sequence identity to the published sequences of *A. centrale* (Israel vaccine strain) (AF309869), an *A. centrale* previously described from *R. simus* ticks in South Africa (AF414869), and *A. centrale* sequences more recently described from cattle in Uganda (KU686783, KU686784). One sequence (Ap 1) had 100% sequence identity to various published *A. platys* sequences; with 99.66% sequence identity to the *A. platys* type strain (M82801). It furthermore had 99.9% sequence identity to *Anaplasma* species Mymensingh (MK814448, MK814449, MF576175) and 99.83% to *Anaplasma* species Omatjenne (KC189853).

A comparison of estimated evolutionary divergence between the observed *A. centrale* gene sequences and those of closely related *Anaplasma* sequences was subsequently compared by determining the number of base differences per partial 16S rRNA gene sequence [1,190 bp]. One sequence (Ac 1) were identical over a 1 191 bp region of the 16S rRNA gene to that of the *A. centrale* Israel vaccine strain and the *A. centrale* previously described from *R. simus* ticks; the remaining three sequences differed by 2-6 bp from these published *A. centrale* gene sequences.

The observed sequence similarities were confirmed by phylogenetic analyses. Maximum likelihood and neighbour-joining techniques were used to reveal the phylogenetic relationships between the partial 16S rDNA sequences obtained to related *Anaplasma* species previously deposited in GenBank. A representative tree obtained by the maximum likelihood method is shown in Figure 4 - 14. The neighbour joining phylogenetic tree can be found in appendix A (Figure A 1).

The maximum likelihood phylogenetic tree of *Anaplasma* species 16S rDNA sequences indicated that the four *A. centrale* sequence variants obtained in this study formed a monophyletic group with *A. centrale* (Israel strain) and the *A. centrale* previously described from *R. simus* ticks in South Africa (Figure 4 - 14) with moderate bootstrap support (62). The *A. platys* sequence obtained in this study grouped with the *A. platys* reference sequences, however, with low bootstrap support (58) (Figure 4 - 14). There was good bootstrap support (98) for the grouping of the *A. platys* group with *Anaplasma* species Omatjenne (U54806), *Anaplasma* species Mymensingh (MF576175) and *Anaplasma* species dog9 (KP006405).

The BLASTn homology searches showed that four *Ehrlichia* sequences obtained (Eh 1, Eh 2, Eh 3, Eh 4) had 99.76-100% sequence identity to previously published sequences of *Ehrlichia canis* described from

dogs in Thailand (EF139458) and Israel (U26740) and an *Ehrlichia* species Described from a sheep in South Africa (U54805).

An evaluation of the estimated evolutionary divergence between the obtained *Ehrlichia* sequences and closely related *Ehrlichia* sequences were then compared by looking at the amount of base differences over the partial 16S rRNA gene sequence (1,263 bp). Three of the sequences (Eh 1, Eh 3 and Eh 4) was identical over the entire 16S rRNA gene region to the *E. canis* sequence described from a dog in Thailand (EF139458) and the *E. canis* sequence described from a sheep in South Africa (U54805). These three sequences also had one basepair difference over the entire region to an *E. canis* sequence described from a dog in Israel (U26740). The remaining sequence (Eh 2) had one basepair difference over the entire region with all three described *E. canis* sequences.

Phylogenetic analysis was used to confirm the sequence comparisons. Maximum likelihood and neighbour-joining methods were applied to show the relationship between the obtained partial 16S rRNA gene sequences and *Ehrlichia* sequences from GenBank. A representative maximum likelihood tree is shown in Figure 4 - 15, while a representative neighbour-joining tree is shown in appendix A (Figure A 2). The maximum likelihood phylogenetic analysis supported the relationship of the four *Ehrlichia* variants from this study, to other *Ehrlichia canis* sequences described from dogs in Thailand (EF139458) and Israel (U26740) and from a Sheep in South Africa (U54805), with good bootstrap support (99).

BLASTn homology searches indicated that the four *Coxiella* sequence variants obtained (Co 1, Co 2, Co 3, Co 4) had 98.74-99.46% sequence identity to *Coxiella*-like endosymbionts (CLEs) described from *R. sanguineus* ticks (KP994843, D84559), and a *R. turanicus* tick (KP994845).

Assessment of the estimated evolutionary divergence between the *Coxiella* sequences obtained in this study and other closely related *Coxiella* sequences were compared to determine the number of base differences over a 1,114 bp region of the 16S rRNA gene. All four sequences (Co 1, Co 2, Co 3 and Co 4) differed from the CLEs described from two *R. sanguineus* ticks (KP994843, D84559), by 2-3 bp. They also differed from the CLEs described from a *R. turanicus* (KP994845) and *R. microplus* (KP994839) ticks by 2-6 bp.

Further phylogenetic analysis using maximum likelihood and neighbour-joining methods were used to indicate the relationship between the 16S rRNA gene region of the *Coxiella* sequences obtained during this study and other *Coxiella* sequences from GenBank. A representative maximum likelihood tree (Figure 4 - 16) showed that the *Coxiella* sequences formed a monophyletic group with other CLEs described from two *R. sanguineus* ticks (KP994843, D84559) and an *R. turanicus* tick (KP994845), with good bootstrap support (89). A representative neighbour-joining tree is shown in appendix A (Figure A 3).

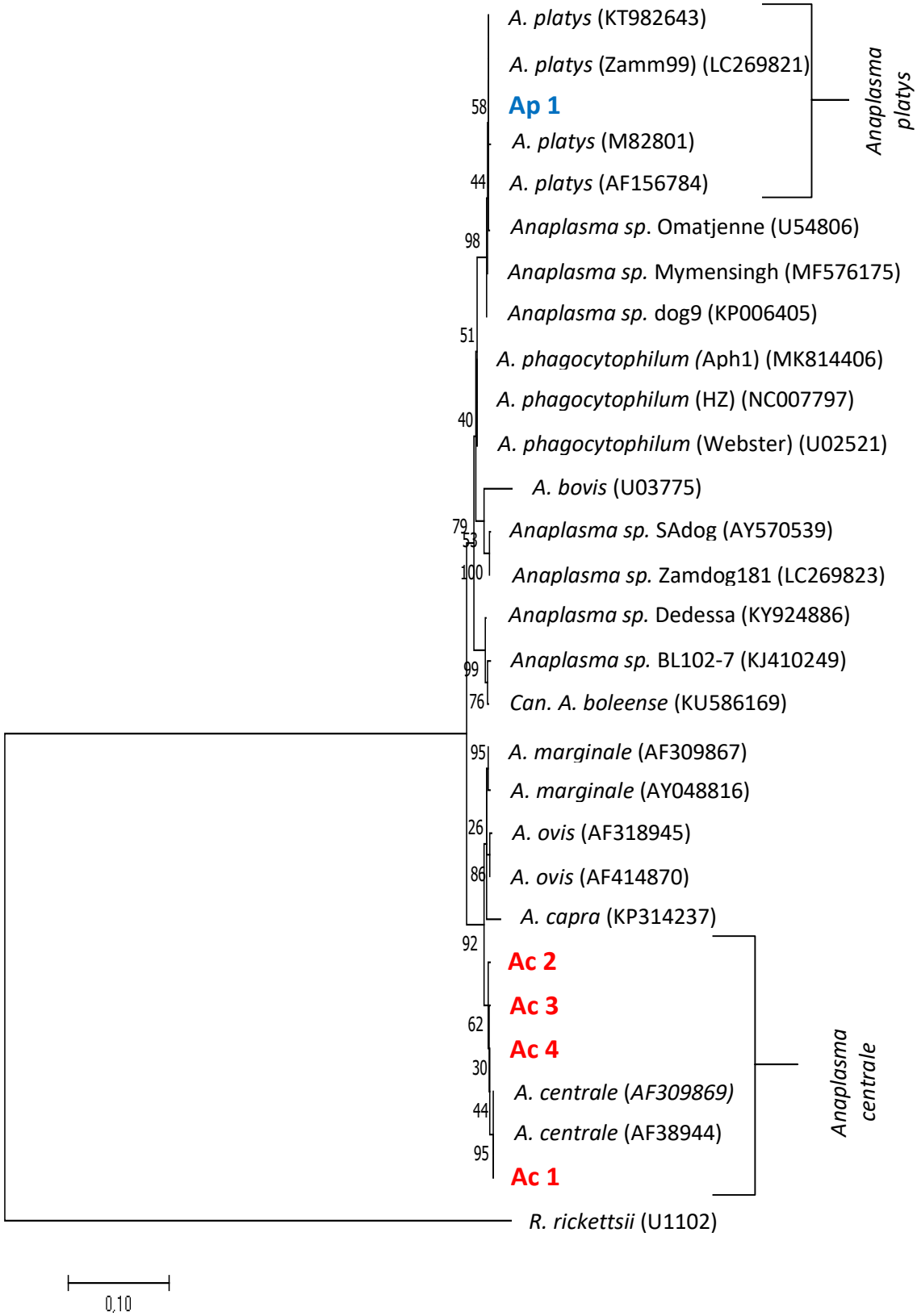

Figure 4 - 14: The evolutionary history of *Anaplasma* species was inferred using the Maximum Likelihood method based on the Kimura 2-paramter model with a discrete gamma distribution (G) and evolutionary invariable sites (I). The bootstrap value (1000 bootstraps) is indicated at each node. Representative *A. centrale* and *A. platys* sequences from *Rhipicephalus sanguineus* from this study are indicated in red and blue, respectively. Evolutionary analyses were conducted in MEGA7 7.0.26 (Kumar et al. 2016).

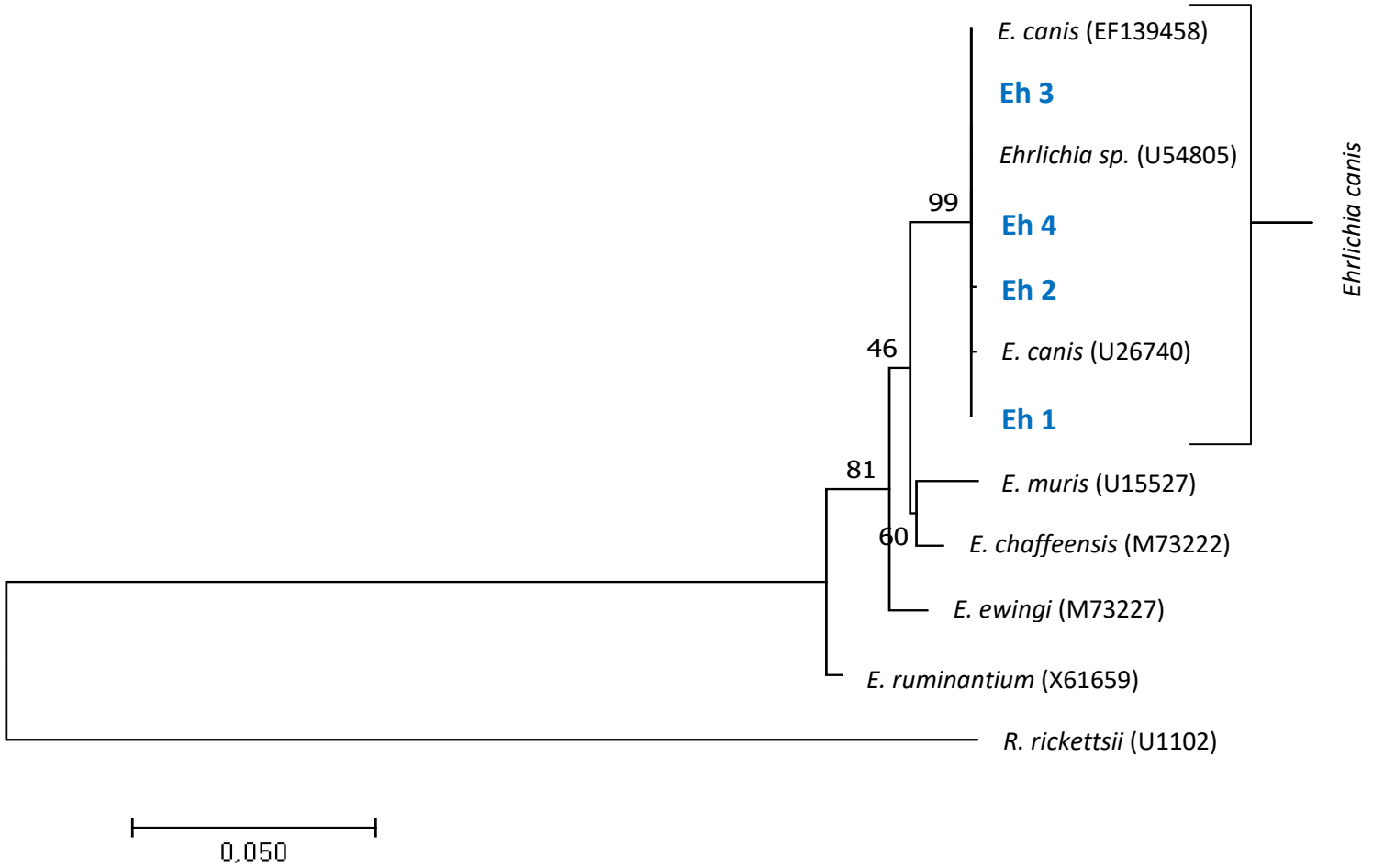

Figure 4 - 15: The evolutionary history of *Ehrlichia* species was inferred using the Maximum Likelihood method based on the Kimura 2-paramter model with a discrete gamma distribution (G). The bootstrap value (1000 bootstraps) is indicated at each node. Representative *Ehrlichia canis* sequences from *Rhipicephalus sanguineus* from this study are indicated in blue. Evolutionary analyses were conducted in MEGA7 7.0.26 (Kumar et al. 2016).

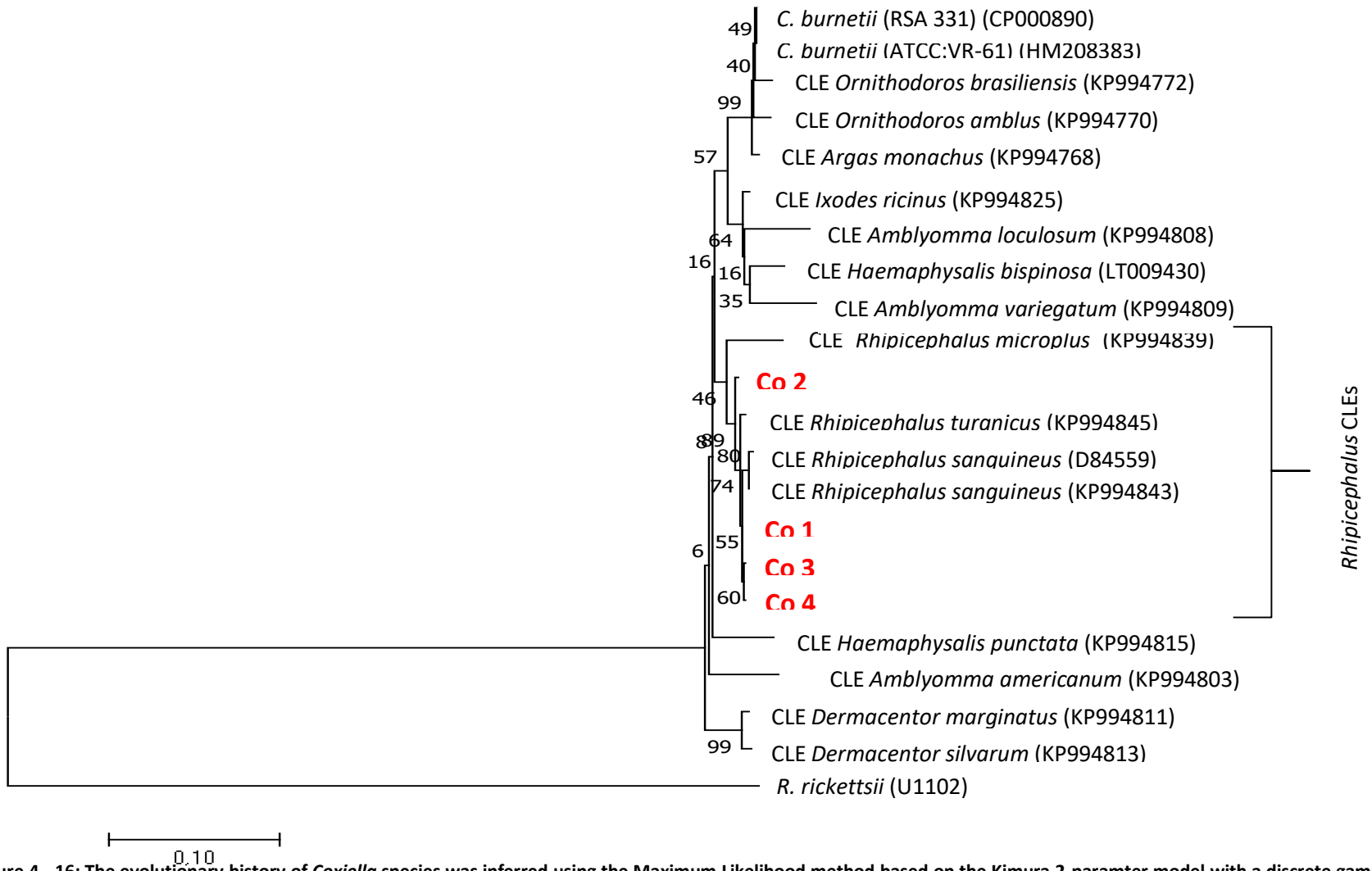

Figure 4 - 16: The evolutionary history of *Coxiella* species was inferred using the Maximum Likelihood method based on the Kimura 2-paramter model with a discrete gamma distribution (G). The bootstrap value (1000 bootstraps) is indicated at each node. Representative *Coxiella* species sequences from *Rhipicephalus sanguineus* from this study are indicated in red (Co 1, Co 2, Co 3 and Co 4). Evolutionary analyses were conducted in MEGA7 7.0.26 (Kumar et al. 2016). CLE = *Coxiella*-like endosymbiont.

4.5 ASSESSMENT OF RISK FACTORS FOR *RHIPICEPHALUS SANGUINEUS* INFESTATIONS

4.5.1 UNIVARIATE ANALYSIS FOR *RHIPICEPHALUS SANGUINEUS* TICK INFESTATION

Out of the 16 variables studied in the univariate analysis (Table 4 - 4), only four (herding of cattle with dogs, having cattle or chicken in the household and monthly temperature) showed a statistically significant association with *R. sanguineus* infestation ($p<0.05$). While a higher monthly temperature (≥20°C) was associated with increased odds of high tick infestation [OR (Odds Ratio) =2.21, *p* = 0.02), having cattle (OR = 0.48, p = 0.04) or chicken (OR = 0.46, p = 0.03) in the household and the herding of cattle with dogs (OR = 0.39, p = 0.03) were each associated with reduced odds of high tick infestation. Other factors that were associated with an increased risk of high infestation with ticks (OR≥1.5) were older dogs (OR = 1.52), hand picking of ticks (OR = 1.50) and use of dogs for hunting (O R= 1.82), although the associations were not statistically significant ($p>0.05$).

Table 4 - 4: Descriptive statistics and univariate associations between potential household-level exposure factors and *Rhipicephalus sanguineus* tick infestations amongst dogs in the Mnisi community area, in Mpumalanga Province, South Africa

Characteristic	Category	Number of dogs with a high tick infestation (≥11 ticks) /total (%)	Odds ratio [95% CI (Confidence Interval)]	*p*
No. of dogs	≤2	38/102 (37.3)		
	≥3	17/48 (35.4)	0.92 (0.45, 0.89)	0.83
Age of dog	≤2 years	21/67 (31.3)		
	≥3 years	34/83 (41.0)	1.52 (0.77, 2.99)	0.22
Hand picking of ticks	No	30/91 (36.7)		
	Yes	25/59 (42.4)	1.50 (0.76, 2.94)	0.24
Dip tank	No	42/111 (37.8)		
	Yes	13/39 (33.3)	0.82 (0.38, 1.77)	0.62
Vet assistance	No	49/134 (36.6)		
	Yes	6/16 (37.5)	1.04 (0.36, 3.04)	0.94
Hunting	No	49/138 (35.5)		
	Yes	6/12 (50.0)	1.82, (0.56, 5.94)	0.32
Herding	No	47/113 (41.6)		
	Yes	8/37 (21.6)	0.39 (0.16, 0.92)	0.03
Roam freely	No	21/66 (31.8)		
	Yes	34/84 (40.5)	1.46 (0.74, 2.89)	0.28
Constrained	No	45/127 (35.4)		
	Yes	10/23 (43.5)	1.40 (0.57, 3.45)	0.46
Cattle	No	39/90 (43.3)		
	Yes	16/60 (26.7)	0.48 (0.23, 0.97)	0.04
Goats	No	45/109 (41.3)		
	Yes	10/41 (24.4)	0.46 (0.20, 1.03)	0.06
Pigs	No	48/127 (37.8)		
	Yes	7/23 (30.4)	0.72 (0.28, 1.88)	0.50
Chickens	No	24/49 (49.0)		
	Yes	31/101 (30.7)	0.46 (0.23, 0.93)	0.03
Number of Chickens	≤10	33/82 (40.2)		
	≥11	22/68 (32.4)	0.71 (0.36, 1.39)	0.32
Monthly temp.	≤19°C	24/84 (28.6)		
	≥20°C	31/66 (47.0)	2.21 (1.13, 4.36)	0.02
Monthly rainfall	≤40mm	30/76 (39.5)		
	≥41mm	25/74 (33.8)	0.78 (0.40, 1.52)	0.47

Results from questionnaire interviews involving 150 households and tick collection from 150 dogs carried out from June 2018 through March 2019. Infestation was categorised as low or none (0-10) and high (>11 ticks)

4.5.2 MULTIVARIATE ANALYSIS FOR *RHIPICEPHALUS SANGUINEUS* TICK INFESTATION

The 13 variables that had $p \leq 0.5$ from univariate analysis were included in an initial model for multivariable logistic regression analysis. The most adequate logistic model comprised five variables (Using the dog for hunting, total monthly rainfall, having chickens in the household, allowing the dog to roam freely and average monthly temperature), based on AIC (Akaike Information Criteria) values (Table 4 - 5). Higher monthly temperatures (≥20°C) were significantly associated with increased odds of a tick infestation amongst dogs (OR = 2.89, p = 0.009). The practices of taking dogs out for hunting (OR = 2.38) and allowing dogs to roam freely (OR = 1.72) were also associated with increased odds of tick infestation, although the associations were not statistically significant (p>0.05) (Table 4 - 5). On the other hand, rearing of chicken in the household and higher monthly rainfall (≥41 mm) were each associated with decreased odds of high infestation with *R. sanguineus*, and the former showed a statistically significant association (p = 0.02). The Hosmer and Lemeshow goodness of fit test was not statistically significant ($\chi^2 = 6.96$, p = 0.54) showing that the model fitted the data well, with the observed data matching the values expected in theory.

Table 4 - 5: Results of a multivariable logistic regression on the level of *Rhipicephalus sanguineus* tick infestation among dogs from households in Mpumalanga Province in South Africa

Characteristic	Category	Number of dogs with a high tick infestation (≥11 ticks) /total (%)	Odds ratio (95% CI)	*p*
Hunting	No	49/138 (35.5)		
	Yes	6/12 (50)	2.38 (0.27, 1.39)	0.20
Monthly Rainfall	≤40mm	30/76 (39.5)		
	≥41mm	25/74 (33.8)	0.49 (0.22, 1.09)	0.08
Chickens	No	24/49 (49.0)		
	Yes	31/101 (30.7)	0.41 (0.19, 0.85)	0.02
Roam	No	21/66 (31.8)		
	Yes	34/84 (40.5)	1.72 (0.79, 3.76)	0.17
Monthly Temp	≤19°C	24/84 (28.6)		
	≥20°C	31/66 (47)	2.89 (1.30, 6.45)	0.009

150 dogs sampled for ticks and questionnaire interviews done in 150 household units from June 2018 to March 2019.
Tick infestation level was considered as a binary outcome (Low or none, 0-10 and high, >11 ticks)
CI, confidence interval
***Hosmer and Lemeshow $\chi^2 = 6.9618$, df = 8, p-value = 0.5408**

CHAPTER 5

DISCUSSION

Horak *et al*. (2002) recorded the identities of ixodid ticks feeding on humans in South Africa; 20 ixodid tick species representing six genera were identified, with species of the genus *Rhipicephalus* the most numerous. Most bites were inflicted by *Amblyomma hebraeum, Haemaphysalis leachi, Hyalomma marginatum, H. rufipes, H. truncatum, R. appendiculatus, R. gertrudae* and *R. simus*. *Rhipicephalus sanguineus,* the brown dog tick is almost an exclusive parasite of domestic dogs and is well adapted to living in kennels or human dwellings with its canine host (Walker et al., 2000), where it may also bite people in the safety of their own homes (Horak et al., 2002). It is known to transmit *Ehrlichia canis*, *Babesia canis, Rickettsia* species and *Anaplasma platys.* The aim of this study was to characterize the bacterial microbiome of *R. sanguineus* ticks collected from dogs in the Mnisi community, and to determine its potential impact on human and animal health in the community. The study suggested that the bacterial microbiome of *R. sanguineus* ticks in the community is diverse, and possibly influenced by external factors.

5.1 BACTERIAL MICROBIOME OF *RHIPICEPHALUS SANGUINEUS* TICKS

In our study, we collected *R. sanguineus* (80.83%), *R. simus* (2.76%), *R. turanicus* (3.30%), *A. hebraeum* (3.07%), *H. leachi* (9.90%), *H. truncatum* (0.05%) and *R. microplus* (0.09%) ticks from a total of 64 dogs over a 12-month period. Since it is often difficult to identify ticks belonging to the *Rhipicephalus* genus as they share various morphological characteristics, we sequenced the cytochrome c oxidase 1 (CO1) gene of 24 morphologically identified *R. sanguineus* ticks to confirm the species identity on a molecular level. Using the COI species database, the selected ticks were identified as *R. sanguineus* and *R. leporis* with a sequence identity of more than 99%. A study done by Hornok *et al.* (2017) showed that the COI haplotypes of *R. sanguineus* and *R. leporis* collected from the same country only differed by about 10 bp, indicating that they are genetically very similar. When looking at the 16S rRNA gene sequence, they found even less variation (Hornok *et al.* 2017). To date, *R. leporis* has not been identified within South Africa, and has only been collected from the Ivory Coast and Kenya (Hornok *et al.* 2017). The fact that *R. leporis* has not been identified within South Africa and due to the known genetic similarity between *R. leporis* and *R. sanguineus*, we assumed that the *R. leporis* species identified in our study, was in fact *R. sanguineus*. Thus, all ticks dissected during this study were classified to be *R. sanguineus*.

Two genomic DNA extractions were used to extract DNA from the tick salivary gland and midgut tissues. It has been shown that commercially available DNA extraction kits harbour possible bacterial

contaminants that negatively influence downstream microbiome sequencing data analysis (Salter *et al.* 2014). We, therefore, initially used the PureGene Ethanol Extraction Method (MPEE) in an attempt to minimize possible bacterial contaminant DNA in our DNA extracts. During the first few months of our study, we struggled to find sufficient numbers of *R. sanguineus* ticks per dog (on average three instead of the planned 10/dog). Using the MPEE extraction method on three ticks per pool resulted in low DNA quantities and a subsequent low 16S rDNA PCR amplification (10.7%) success rate. In later months, we succeeded in collecting 10 ticks per dog; we also switched over to a column-based DNeasy Blood and Tissue Kit (DBTK). We successfully amplified the 16S rDNA from the genomic DNA extracted from all 24 tick tissue pools using the DBTK extraction kit. We subsequently found that there were significant differences in the bacterial microbiome found using the two different extraction methods.

Analysis of the microbiome composition of the midgut and salivary gland tissue pools from *R. sanguineus* ticks revealed the presence of seven phyla, comprising 34 genera and 39 species. However, no significant difference in the bacterial diversity described from the midgut and salivary gland tissues could be found. This is in contrast with other studies that found tissue-specific differences in the bacterial microbiome (Gall 2016; Gall *et al.* 2017). Of the phyla found, the Proteobacteria (85.44%) was the most prevalent phylum found; with *Anaplasma* (21.69%), *Ehrlichia* (19.94%) and *Coxiella* (12.12%) species dominating the microbiome.

Anaplasma sequences were detected from 12 of the 24 tick pool samples (50%). Operational Taxonomic Units (OTU) clustering classified *A. platys* (75.82%) and *A. centrale* (15.36%). *Anaplasma platys* sequences were detected from one tick pool; both from the midgut (86.1%) and salivary gland (96.6%) tissues. One *A. platys* 16S rDNA consensus sequence was subsequently extracted from the microbiome sequence dataset and phylogenetically shown to cluster with previously described *A. platys* sequences.

Anaplasma platys, which causes infectious canine thrombocytopenia (Harvey *et al.* 1978) has been recognised as an emerging zoonotic pathogen (Maggi *et al.* 2013; Arraga-Alvarado *et al.* 2014). It has been detected in some African countries. In sub-Sahara Africa it has been identified in dogs and ticks (*R.* sanguineus, *R*. *turanicus* and *Haemaphysalis leachi*) from Nigeria (Kamani *et al.* 2013), and in *R. sanguineus* ticks collected from dogs in the Congo (Sanogo *et al.* 2003) as well as in dogs and two tick species (*R. sanguineus* and *R. camicasi*) in Kenya (Matei *et al.* 2016). In the southern Africa region, it has been identified in *R. evertsi evertsi* ticks from wild and domestic ruminants in South Africa (Berggoetz *et al.* 2014) as well as in Zambia from domestic dogs (Vlahakis *et al.* 2018). It is thought that in South Africa, *R. sanguineus* plays a role in *A. platys* transmission and may be the reservoir host. *Rhipicephalus sanguineus* is one of the most predominant tick species on the dogs of the Mnisi community (Mulder 2014). They are also known to occasionally bite and feed on humans, thus our findings are of great

significance and highlights the possible risks associated with the presence of *R. sanguineus* ticks close to dogs and humans and the possible transmission of *A. platys*.

An unexpected finding was the detection of *A. centrale* sequences from 10 tick pools; both from the midgut (40%) and salivary gland (60%) tissues. Four 16S rDNA sequence variants were extracted from the microbiome sequence dataset and phylogenetically shown to be *A. centrale*. *Anaplasma centrale* was confirmed as a separate species from *A. marginale* (Khumalo *et al.* 2018), and is the cause of a less virulent form of bovine anaplasmosis (Carelli *et al.* 2008). It has been detected in several African countries; and shown to be freely circulating in cattle and wildlife species in South Africa (Khumalo *et al.* 2018). Dogs are not known to be a reservoir host of *A. centrale* but it has been shown that *R. simus* and *Dermacentor andersoni* are capable of transmitting *A. centrale* (Ueti *et al.* 2009).

In a recent study by Kolo (PhD thesis, 2019), *A. centrale* sequences were detected from two dog blood samples in the Mnisi community using a microbiome sequencing approach. The author speculated that the detection of *A. centrale* in dogs was likely an incidental finding due to the deep sequencing approach and the fact that ticks feeding on these dogs carry these pathogens rather than these pathogens truly infecting the dogs. We subsequently tested the blood samples collected from the dogs (that the *R. sanguineus* ticks were collected form) using a duplex quantitative polymerase chain reaction (qPCR) for the specific detection of *A. marginale* and *A. centrale* DNA (Makgabo 2018). All blood samples tested negative.

In the study by Kolo (2019), *Anaplasma* species ZAM dog (14.8%) and *A. phagocytophilum* (0.3%) sequences were also obtained from dog blood samples collected in the Mnisi community using a microbiome sequencing approach. No *A. platys* could, however, be detected in any of the *R. sanguineus* ticks sampled in the Kolo study. The authors speculated that *R. sanguineus* should be considered as a possible vector for *Anaplasma* species southern Africa dog (the name they suggested for the previously described *Anaplasma* species ZAM dog and *Anaplasma* species SA dog) in South Africa; however, we could not detect this species in our study.

Ehrlichia sequences were identified in six of the 24 samples (25%); OTU clustering identified *E. canis* (95.46%). Four *E. canis* sequences from the microbiome dataset were extracted and phylogenetically clustered with other previously described E. *canis* sequences.

Ehrlichia canis is the disease causing agent of canine monocytic ehrlichiosis (CME) which infects dogs globally, except in New Zealand and Australia (Kelly 2000). Molecular evidence of the occurrence *E. canis* in humans has been reported in Venezuela (Perez *et al.* 1996, 2006) and Costa Rica (Bouza-Mora *et al.* 2017), suggesting the zoonotic potential of this agent. *Rhipicephalus sanguineus* and *Dermacentor*

variabilis (Johnson *et al.* 1998) are the recognized vectors for *E. canis. Ehrlichia canis* has been detected throughout southern Africa in countries such as Namibia, where it was serologically identified in 53.8% of dogs screened (Manyarara *et al.* 2015). In South Africa it was molecularly identified in 3% of dogs (Matjila *et al.* 2008) and 19% of ticks collected from dogs and cats (Mtshali *et al.* 2017).

A study done by Kolo (2014) detected *Ehrlichia* species in *R. sanguineus* ticks that were collected from dogs in the Mnisi area. Another study by Kolo (2019) detected *E. canis* in 60% of the dogs sampled in the Mnisi area using a microbiome approach. The authors indicated that these results were from dogs that did not show any symptoms, and speculated that these dogs had overcome the acute phase and had a sub-clinical infection. During our study, ticks were only collected from dogs that did not show any clinical symptoms of disease, which could validate the findings of the study done by Kolo (2019). As *R. sanguineus* is a competent vector of *E. canis,* it can be assumed that the dogs were infected with *E. canis*, however further investigation will be required.

In the Kolo study they detected a significant negative correlation between *E. canis* and *Anaplasma* species infection in dogs; dogs that had high burdens of *E. canis* infection in their blood had lower burdens of *Anaplasma* species and vice versa. This phenomenon of bacterial interference between rickettsial species has been previously described in *Dermacentor* ticks (Macaluso *et al.* 2002; Gall *et al.* 2016). We also observed this phenomenon in this study, however, it was not significantly supported by the Principal Component Analysis (PCA); possibly due to our small sample size.

Coxiella burnetii causes Q fever, a zoonotic infection that presents as an acute to chronic fever and pneumonia in humans, as well as causing abortion in livestock (Vanderburg *et al.* 2014). Q fever is endemic in South Africa with up to 59% prevalence in vulnerable communities (Quan *et al.* 2014). Recent sero-prevalence study carried out on veterinary staff, cattle farmers and herders in the Mnisi community found 60.9% of individuals positive for anti-*Coxiella* antibodies (Simpson *et al.* 2018). In our study, *Coxiella* sequences were detected from 13/24 (54%) tick pool samples. Further OTU clustering of the sequences could not identify the *Coxiella* species. Phylogenetic analysis of four *Coxiella* species sequences extracted from the microbiome dataset, indicted that they clustered with other previously described *Coxiella*-like endosymbionts (CLE) and not with *C. burnetti;* this is as expected as *R. sanguineus* is not a known vector of *C. burnetii* (Lalzar *et al.* 2012). Phylogenetic analysis also showed a clear distinction between the CLEs described from different tick genera. The *Coxiella* sequences obtained from this study grouped together with CLEs from other *R. sanguineus* ticks as well as a CLE from an *R. turanicus* tick.

Coxiella-like endosymbionts (CLEs) are bacteria that are globally present in various tick species and in the spleen of some wild mammals (Brinkmann *et al.* 2019). These CLEs have differentiated from the closely

related C. *burnetii,* which is a pathogenic species. CLEs act as symbionts to their hosts, inferring certain fitness advantages such as defence, immunity, nutrition as well as pathogen acquisition and transmission (Duron *et al.* 2015a). Some CLEs have been shown to be pathogenic and have been identified infecting animals and humans, causing symptoms similar to Q-fever (Jourdain *et al.* 2015). It has also been discovered that 4/5 current molecular methods used to detect *C. burnetii*, cross-react with other CLEs. This leads to a possible overestimation of *C. burnetii* detection (Jourdain *et al.* 2015). It is highly possible that Q-fever infections in South Africa is not only due to *C. burnetii* but also due to CLEs.

In our study, we detected *Coxiella* species in all samples that contained *A. centrale*, indicating a possible positive correlation between these two species; unfortunately, this was not statistically significant. Tick symbionts such as *Coxiella* species, do not only have an effect on tick survival, but can also effect pathogen survival and transmission (Moutailler *et al.* 2016). It is thus possible that *Coxiella* species have a positive effect on *A. centrale*, increasing the acquisition of this pathogen, or its survival within the tick.

Furthermore, a prospective negative correlation was seen between *Coxiella* species and *E. canis*. These correlations were also not statistically supported by the PCA. This negative correlation could be due to resource competition between *Coxiella* and *Ehrlichia* species within the tick, where the one outcompetes the other. It is also possible that *Coxiella* decreases the pathogen's acquisition, survival and transmission. It has previously been shown that a *Coxiella*-like endosymbiont of *Amblyomma* ticks can weaken the transmission of *E. chaffeensis* (Klyachko *et al.* 2007). We also detected quite low amounts of *Coxiella*, compared to a pilot study (unpublished) conducted in Clare B and Eglington in the Mnisi community on the bacterial microbiome of *R. sanguineus*, which found an average of 96.5% *Coxiella*. This is expected, as *Coxiella* is a known endosymbiont of *R. sanguineus* ticks, and is thought to provide a fitness advantage to the tick (Duron *et al.* 2015a).

We then also looked at the possibility of bacterial contaminants present in the microbiome datasets generated; either due to the preparation of the tick tissues or during the DNA extraction process. Some of the bacteria detected have shown no previous associations with tick microbiomes, and we can assume that these could be possible contaminants. These bacteria include: *Azospirillum*, *Campylobacter*, *Cutibacterium*, *Domibacillus*, *Gp6*, *Hydrotalea*, *Methyloversatilis*, *Microvirga*, *Corynebacterium* and *Prosthecobacter*. For future tick microbiome studies, we would recommend that: (i) During the tick wash steps, DNA be extracted from the triple tick rinse wash solution to act as negative control and be subjected to 16S rDNA microbiome analysis. This will provide a bacterial profile of possible environmental contaminants that could then be subtracted and excluded from the tick microbiome dataset, in order to provide a better representation of the tick microbiome. (ii) Care be taken when dissecting out the midgut and salivary gland tissues to avoid any possible cross-contamination between tissue types. Tissues should

be washed in buffer solution before further processing. (iii) A negative water control should be included if a kit-based DNA extraction method is used, as it has been shown that these extraction kits contain bacterial contaminants (Salter *et al.* 2014). Once again, this will provide a bacterial profile of possible environmental contaminants that could then be subtracted/excluded from the tick microbiome dataset.

5.2 ASSESSMENT OF RISK FACTORS FOR *RHIPICEPHALUS SANGUINEUS* INFESTATION

We then also looked at data concerning household, environmental and management characteristics in relation to infestation of dogs with *R. sanguineus* ticks. This part of our study focussed only on dogs that were infested with ticks of the *Rhipicephalus* genus, whereas other dogs that visibly had only other species, such as *Amblyomma*, were not sampled. Most of the ticks collected were *R. sanguineus*, although a few ticks of other species were also collected.

Multivariable analysis showed that high monthly temperature was significantly associated with increased odds of high infestation with *R. sanguineus* ticks. *Rhipicephalus sanguineus* has been shown to be dependent on temperature during different stages such as moulting and feeding (Mumcuogliu *et al.* 1993; Parola *et al.* 2008; Socolovschi *et al.* 2009a). During the initial collection months (July to September 2018), we found very low quantities of *R. sanguineus* ticks on the dogs (average n=5). The tick count started increasing during November and December, when temperatures started rising above 20°C, and reached a maximum during January (average n=17). This is likely because the ideal temperature for oviposition in *R. sanguineus* ticks is between 20°C to 30°C (Sweatman 1967). The life cycle of *R. sanguineus* ticks from oviposition to the adult phase can take an average of 30 days to complete, which would result in a high number of ticks during January and February on the dogs, which corresponds to our data. This increased risk of tick infestation with an increase in temperature could be very detrimental to animal and human health in South Africa, as well as globally. Due to a changing climate, South Africa is becoming progressively warmer, which could lead to much larger *R. sanguineus* populations and infestations on animals as well as humans. A study done by Parola *et al.* (2008) showed that higher temperatures influenced *R. sanguineus* ticks, causing them to have a higher tendency towards attachment and feeding on humans. This occurrence could also cause an increase in the distribution of *R. sanguineus* into areas that were previously too cold to colonize (Süss *et al.* 2008; Caminade *et al.* 2019).

Higher rainfall was associated with decreased odds of *R. sanguineus* infestations amongst the dogs. This is expected, as it has been shown that *R. sanguineus* do not particularly depend on moisture in their environment for survival, and can inhabit drier regions (Yoder *et al.* 2006). Rearing chicken in the household was also associated with decreased risk of a high *R. sanguineus* infestation from multivariable analysis. It is thought that chickens at a household scavenge on the ticks when they are present in the

environment, thus lowering the tick population. The practices of using dogs for hunting and free roaming led to increased risk of tick infestation. Dogs that are taken out for hunting or roam in the surrounding environment are exposed to different habitat types and consequently high exposure to ticks in the environment.

5.3 CONCLUSION

In conclusion, this study reveals a highly diverse bacterial microbiome of *R. sanguineus* ticks collected from dogs in Clare A of the Mnisi community in South Africa; indicating that *R. sanguineus* could be a potential reservoir for important bacterial pathogens of zoonotic importance. It indicates that *R. sanguineus* ticks are vectors of *A. platys* and *E. canis* in the community. It also shows that *R. sanguineus* is capable of carrying *A. centrale,* however further research will be needed to determine if *R. sanguineus* is a possible vector of *A. centrale* or if this is a novel strain of *A. centrale*. It also upholds the theory that *R. sanguineus* ticks harbour a *R. sanguineus* specific *Coxiella*-like endosymbiont within its microbiome. Tick symbionts such as *Coxiella*, require additional research as they play such a large role in the survival of ticks and the transmission of pathogens.

The *A. platys, E. canis* and the CLEs described all have previously been shown to have zoonotic potential and highlight the potential zoonotic risk of *R. sanguineus* ticks to animal and human health in the Mnisi community.

Moreover, this study determined that high temperatures increases the *R. sanguineus* tick population on dogs at the household level, while rainfall did not affect the population. With a warming climate in South Africa, this could pose a high risk to the community as populations of *R. sanguineus* will likely increase, which could lead to a surge in zoonotic tick-borne diseases. We also found that having livestock in contact with dogs can decrease the infestation of *R. sanguineus* ticks on the dogs. However, this almost certainly does not decrease the overall *R. sanguineus* population in the area. It is possible that having chickens in contact with the dogs could decrease the tick populations, as we theorize that the chickens eat the ticks in their immediate environment.

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APPENDIX A

Table A 1: The minimum temperature for the temperature and humidity controlled chamber for August 2018 to April 2019.

Date	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
August	20,2	20,2	20,2			20,2	20,2	20,2	20,2	20,2			20,2	20,2	20,2	20,2	20,2			20,2	20,2	20,2	20,2	20,2			20,2	20,2	20,2	19,4	19,3
September			19,3	19,3	19,3	19,3	19,3				19,3	19,3	19,3	19,3			19,3	19,3	19,3	19,3	19,3			19,3							
October	19,3	19,3	19,3	19,3	19,3			19,3	19,3	19,3		19,3			19,3		19,3	19,3					22	20,2	19,1	19			18,8	18,8	18,8
November	18,8	18,8			18,8	18,8	18,8	18,8	18,8			18,8	22,2	22,5	22	22			21,6	21,6	21,6		21,6	21,6		21,5	21,5	21,5		21,3	
December	21,3		21,3	21,3	21,3	21,3	21,3	21,3	21,3	21,3	21,3	21,3	22,6	22,6				22,4	22,3	22,3	22,8			22,6			22,5	22,3			22,8
January		22,7	23	22,8			22,7		22,8	22,8	22,5			22,3		22,3	22,3	22,3			22,3		22,1	22,1	22,1			22,3	23	23,6	22,4
February	22,3			22,4	22,4	22,4	22,4	22,4	22,4	23,2	23	22,6	22,6	24,9	23,8	23,8		23,3	23,3	23,3	23,3				22,6	22,6	23,3	23,3	23,3		
March		23,3	23,8	23,8	23,4	23,8	23,8	23,7			23,5	24,7		23,8	23,8	23,7	23,5	23,8	23,4	23,4	23,4	23,4			23,7	23,4					
April	23,4	23,4	22,9	22,9	22,9			22,6	22,9	23,4	23	23,1			21,9	22	23,3	23,1	22,8			22,8	22,8	22,5	23,2	23,2			22,6	22	

Table A 2: The maximum temperature for the temperature and humidity controlled chamber for August 2018 to April 2019.

Date	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
August	22,9	23,4	23,4			23,4	23,4	23,4	23,4	23,4			23,4	23,4	23,4	23,4	23,4			23,4	23,6	23,6	23,6	23,6			28	28	28	28	28
September			28	28	28	28	28			28	28	28	28	28			28	28	28	28	28			28								
October	28	28	28	28	29,2			29,2	29,2	29,4		29,4			29,4		29,4	29,4					34,8	34,8	34,8	34,8			34,8	34,8	34,8
November	4,8	34,8			34,8	34,8	34,8	34,8	34,8			34,8	24,3	24,6	24,6	24,6			24,6	24,6	24,6		24,6	24,6		24,6	24,6	24,6		24,6	
December	24,6		24,6	24,6	24,6	24,6	24,6	24,6	24,6	24,6	24,6	23,6	24,2	24,2				24,2	24,1	24	24			24,2			24,5	23,9			24,3
January		24,2	23,9	24,1			24,3		24,2	24,3	24,4			24,4		24,4	24,4	24,4			24,2		24	26,7	27,3			24,3	23,4	25,2	24,2
February	24			27	27	27	27	27	27	25	25	25	25	25	25,2	25,5		26	26	26	26				25	25	24,7	24,9	24,9			
March		26,1	25	25	25	25	25,5	25,8			29,6	25,3		28,5	28,5	28,5	25,9	26	26	26	26	26			25,8	25					
April	25,4	25,4	24,9	24,9	24,9			25	25,2	25,6	25,1	24,9			26,1	26	25,1	25,4	25,1			25,1	24,6	24,9	25	25,1			26,1	25,5	

Table A 3: The humidity for the temperature and humidity controlled chamber for August 2018 to April 2019.

Date	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
August	33,6	32,7	32,7			32,7	32,7	32,7	32,7	32,7			26,3	26,3	26,3	26,3	26,3			26,3	26,3	26,3	26,3	26,3			26,3	26,3	26,3	26,3	26,3
September			26,3	26,3	26,3	26,3	26,3			23	22,7	22,7	22,7	22,7			23,3	23,08	23,13	24,4	23,4			23,4								
October	22,7	22,7	22,7	22,7	22,7			22,7	22,7	22,7		22,7			22,7		22,7	22,7					58,2	58,2	58,2	58,7			59,2	59,8	64,6
November	64	61,3			69,1	69,1	69,1	69,1	69,1			69,1	48,1	52,6	53,2	55,4			61,9	61,9	61,9		61,9	61,9		61,9	61,9	65,9		74,5	
December	74,5		74,5	74,5	74,5	74,5	74,5	74,5	76,6	76,6	76,6	75,6	63,6	68,22				69,2	65,2	66,9	69			67,1			71,8	66,7			71,2
January		73,3	76,9	69,4			82,7		76	78,6	80,4			80,4		80,4	80,4	80,4			77,6		62,5	64,8	68,3			72,2	68,2	70,9	70,5
February	72			74	74	74	74	74	74	69,3	69,5	69,5	69,5	72,1	74,1	74,1		77,9	77,9	77,9	77,9				76,6	76,6	74,5	77,5	77,8			
March		77,8	78,1	75,4	77,7	74,3	76,7	74,9			74,9	71,1		73,9	73,9	73,9	73,5	74,2	74,2	74,2	74,2	74,2			60,1	62,1					
April	68,3	68,3	65,4	65,4	65,4			66,1	67,8	67,5	66	64,7			68,7	68,7	68,7	65,9	62,8			63,8	61,7	63,3	64,9	65			68,9	59,2	

Table A 4: Genbank accession numbers of the sequences used in the phylogenetic analysis of the *Anaplasma*, *Ehrlichia* and *Coxiella* species

Accession number	Organism	Strain/Country	Host
Ehrlichia			
U26740	*E. canis*	Israel	Dog
EF139458	*E. canis*	Thailand	Dog
M73222	*E. chaffeensi*	USA*	Human
M73227	*E. ewingi*	USA*	Dog
U15527	*E. muris*	Tokyo	Mouse
X61659	*E. ruminantium*	USA*	Cattle
U54805	*E. spp*	Strain Germishuys	Sheep
Anaplasma			
U03775	*A. bovis*	South Africa	Cattle
KP314237	*A. capra*	China	Tick
AF309869	*A. centrale*	Strain Israel	Cattle
AF318944	*A. centrale*	South Africa	Cattle
AF309867	*A. marginale*	Florida	-
AY048816	*A. marginale*	USA*	Cattle
AF318945	*A. ovis*	Mozambique	Sheep
AF414870	*A. ovis*	South Africa	Sheep
NC007797	*A. phagocytophilum*	USA	Human
U02521	*A. phagocytophilum*	USA	Human
MK814406	*A. phagocytophilum* Aph1	South Africa	Dog
AF156784	*A. platys*	China	-
KT982643	*A. platys*	India	Dog
M82801	*A. platys*	USA*	Dog
LC269821	*A. platys* ZAM 99	Zambia	dog
KJ410249	*Anaplasma* sp. BL102-7	China	*Hyalomma asiaticum*
KY924886	*Anaplasma* sp. Dedessa	Ethiopia	Cattle
KP006405	*Anaplasma* sp. Dog9	Philippines	Dog
MF576175	*Anaplasma* sp. Mymensingh	Bangladesh	Cattle
U54806	*Anaplasma* sp. Omatjenne	Namibia	*Hyalomma truncatum*
AY570539	*Anaplasma* sp. SA dog	South Africa	Dog
LC269823	*Anaplasma* sp. ZAM dog	Zambia	Dog
KU586169	*Candidatus Anaplasma* boleense WHANSA	China	*Anopheles sinensis*
Coxiella			
HM2088383	*Coxiella burnetii*	Strain ATCC:VR-615	-
CP000890	*Coxiella burnetii*	Strain RSA 331	-
NR104916	*Coxiella burnetii*	-	-
KP994803	*Coxiella*-like endosymbiont	USA*	*Amblyomma americanum*
KP994815	*Coxiella*-like endosymbiont	England	*Haemaphysalis punctata*
KP994811	*Coxiella*-like endosymbiont	France	*Dermacentor marginatus*
KP994813	*Coxiella*-like endosymbiont	China	*Dermacentor silvarum*
KP994808	*Coxiella*-like endosymbiont	France	*Amblyomma loculosum*

KP994809	*Coxiella*-like endosymbiont	France	*Amblyomma variegatum*
LT009430	*Coxiella*-like endosymbiont	Malaysia	*Haemaphysalis bispinosa*
KP994825	*Coxiella*-like endosymbiont	-	*Ixodes ricinus*
KP994772	*Coxiella*-like endosymbiont	Brazil	*Ornithodoros brasiliensis*
KP994770	*Coxiella*-like endosymbiont	Peru	*Ornithodoros amblus*
KP994768	*Coxiella*-like endosymbiont	Argentina	*Argas monachus*
KP994839	*Coxiella*-like endosymbiont	-	*Rhipicephalus microplus*
KP994845	*Coxiella*-like endosymbiont	-	*Rhipicephalus turanicus*
D84559	*Coxiella*-like endosymbiont	USA*	*Rhipicephalus sanguineus*
KP994843	*Coxiella*-like endosymbiont	Brazil	*Rhipicephalus sanguineus*
Outgroup			
U11021	*Rickettsia rickettsii*	Ohio	-

*** USA = United States**

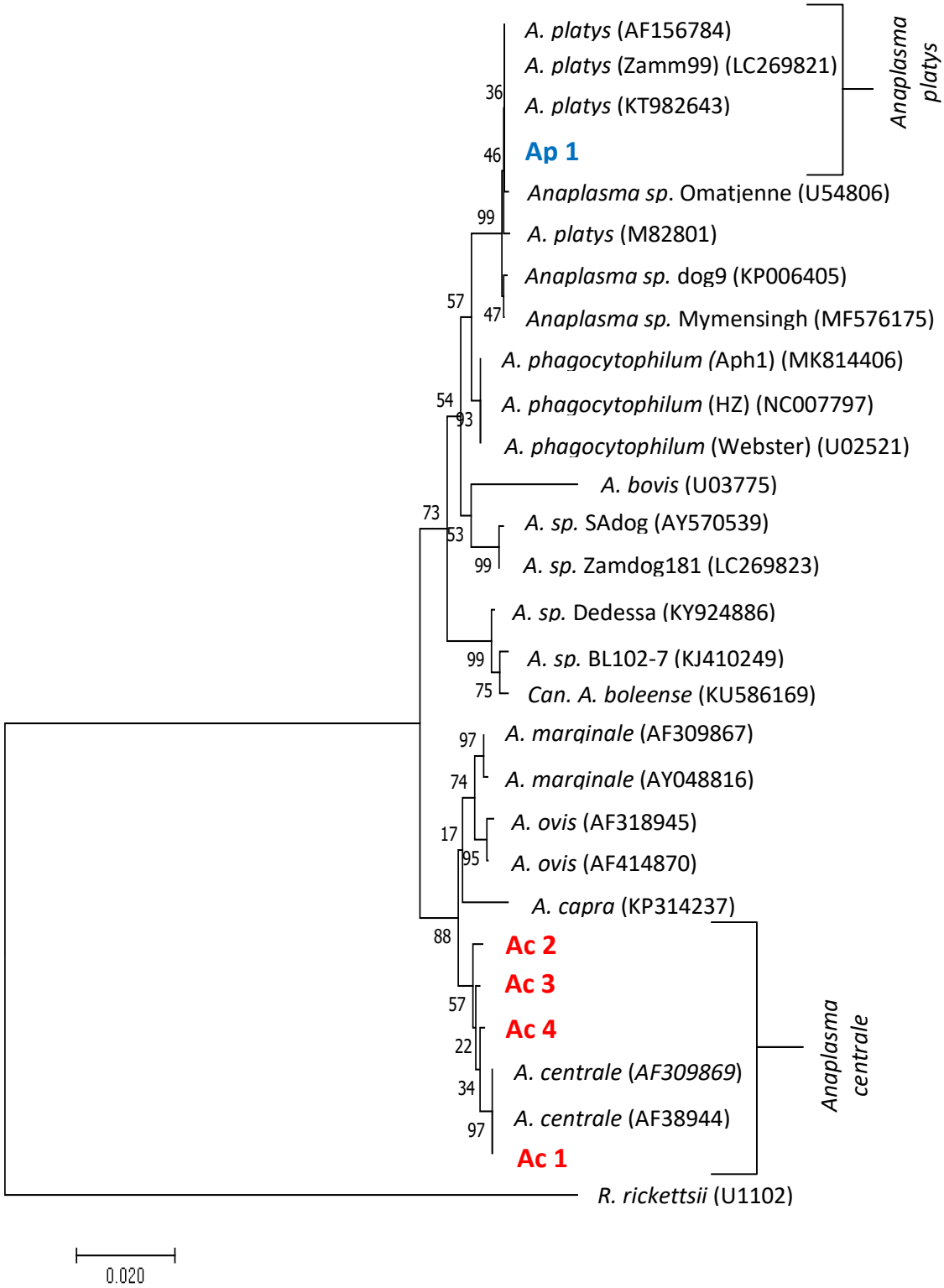


Figure A 1: The evolutionary history of *Anaplasma* species was inferred using the Neighbour Joining method based on the Kimura 2-paramter model with a discrete gamma distribution (G). The bootstrap value (1000 bootstraps) is indicated at each node. Representative *Anaplasma centrale* and *Anaplasma platys* sequences from *Rhipicephalus sanguineus* from this study are indicated in red and blue respectively. Evolutionary analyses were conducted in MEGA7 7.0.26 (Kumar et al. 2016).

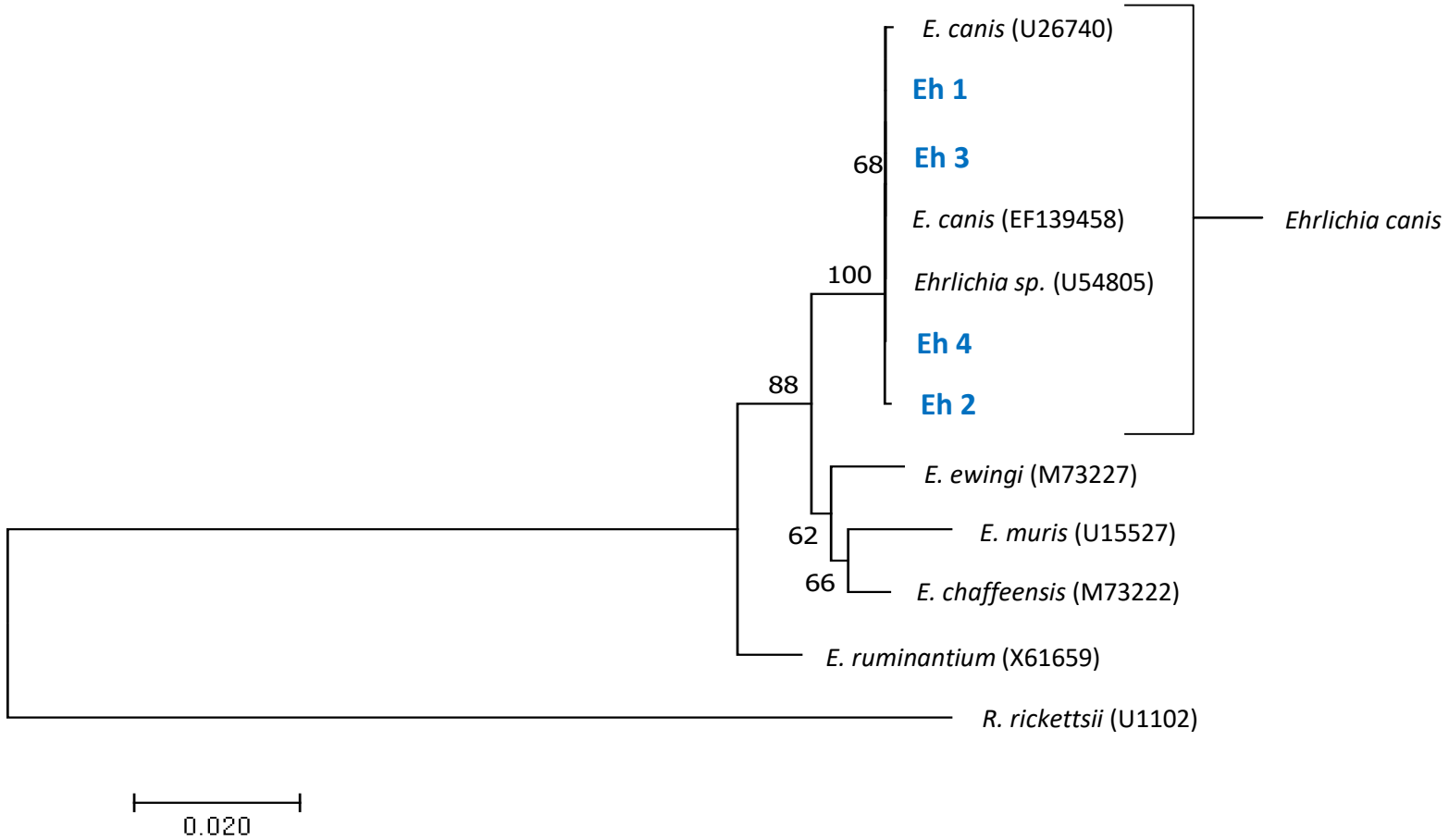


Figure A 2: The evolutionary history of *Ehrlichia* species was inferred using the Neighbour Joining method based on the Kimura 2-paramter model with a discrete gamma distribution (G). The bootstrap value (1000 bootstraps) is indicated at each node. Representative *Ehrlichia canis* sequences from *Rhipicephalus sanguineus* from this study are indicated in blue. Evolutionary analyses were conducted in MEGA7 7.0.26 (Kumar et al. 2016).

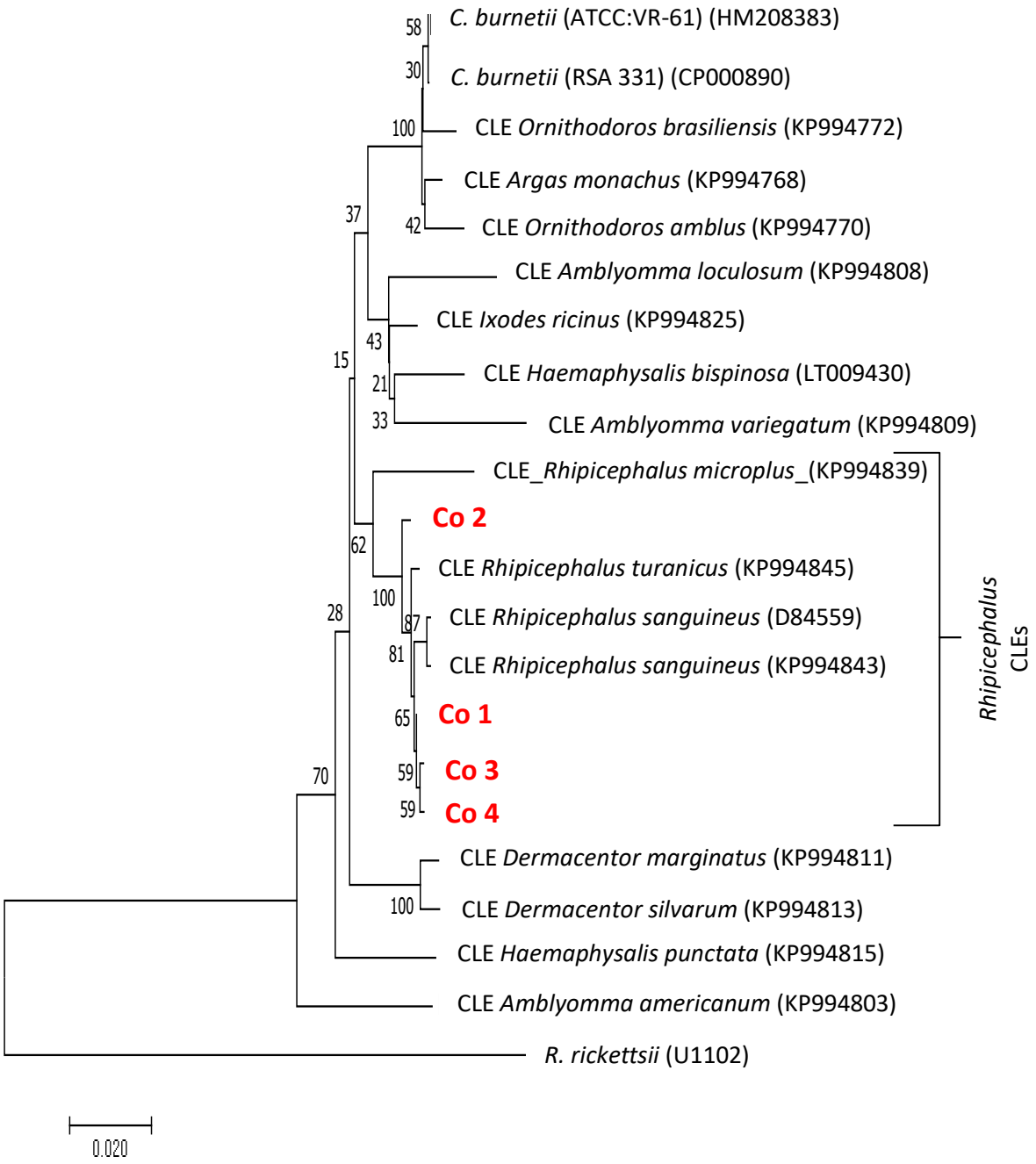
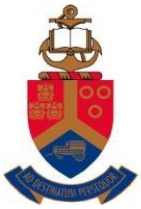


Figure A 3: The evolutionary history of *Coxiella* species was inferred using the Neighbour Joining method based on the Kimura 2-paramter model with a discrete gamma distribution (G). The bootstrap value (1000 bootstraps) is indicated at each node. Representative *Coxiella* species sequences from *Rhipicephalus sanguineus* from this study are indicated in red (Co 1, Co 2, Co 3 and Co 4). Evolutionary analyses were conducted in MEGA7 7.0.26 (Kumar et al. 2016). CLE = *Coxiella*-like endosymbiont.

APPENDIX B

QUESTIONNAIRE



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The bacterial microbiome of *Rhipicephalus sanguineus* ticks collected from domestic dogs in the Mnisi communal area, South Africa

Researcher	Rebecca Ackermann
Student number	14170508
Department	Tropical and Veterinary Science, University of Pretoria

Dear Participant

I am an **MSc** student at the **University of Pretoria** in the Department of **Tropical and Veterinary Science.** You are invited to volunteer to participate in our research project on **The bacterial microbiome of *Rhipicephalus sanguineus* ticks collected from domestic dogs in the Mnisi communal area, South Africa**.

This letter gives information to help you to decide if you want to take part in this study. Before you agree you should fully understand what is involved. If you do not understand the information or have any other questions, do not hesitate to ask us. Your participation in this study is voluntary. You can refuse to participate or stop at any time without giving any reason. We would like you to complete a questionnaire. This may take about 10 min. Your answers and information will be kept in a safe place to ensure confidentiality. We will be available to help you with the questionnaire or to fill it in on your behalf. By completing the questionnaire and signing the informed consent you cannot recall your consent. We will not be able to trace your information. Therefore, you will also not be identified as a participant in any publication that comes from this study.

Purpose of the study:

The purpose of the study is to determine what diseases are in the Mnisi community that can affect human health. We believe that ticks from the dogs in the Mnisi community, carry diseases that can affect both animals (livestock and dogs) and humans. To see which diseases these ticks have, we need to collect ticks and blood from your dog. We also need to take a photograph of the dog, and a questionnaire will be given to you to fill in.

Tick Control Information

Ticks can be controlled by removing them manually from dogs and other domestic animals around your house. You can also ask for veterinary assistance or purchase tick repellent at the Hluvukani Animal Health Clinic. Dogs can also be dipped along with the cattle at the weekly dip tanks. If a tick is found on you or your family, remove it using tweezers by grasping the tick and pulling gently backward. Do not squeeze or twist the tick. Monitor the tick bite for signs of a rash and any symptoms such as fever, headache, joint pain or flu-symptoms. If any symptoms arise, visit a health clinic.

The team involved in the study:

- Principle investigator: Rebecca Ackermann (Tick collection and photograph)
- Professional veterinarian: Ilana van Wyk (Blood collection)
- Hluvukani Animal Health workers: Dog handling, questionnaire

Research signature	**Participant signature**	**Date**
_____	_____	_____

Questions

1) Village
   a) Clare A
   b) Clare B
   c) Eglington

2) House number: _____

3) Number of dogs at household:
   a) 0
   b) 1
   c) 2
   d) 3
   e) ≥4

4) Age of dog being sampled: _____ (Please specify months or years)

5) Microchip number of dog being sampled (if applicable): _____

6) May a photo be taken of your dog:
   a) Yes
   b) No

7) Do you remove any ticks from your dogs:
   a) Yes
   b) No

8) If yes which method do you use?
   a) Manually remove ticks from the dog
   b) Dip dogs at the cattle dip
   c) Assistance from veterinarian staff
   d) Other (please specify): _____

9) Do you take your dogs hunting or herding or are they allowed to roam freely?
   a) Hunting
   b) Herding
   c) Walk around freely
   d) Kept at the house (not allowed out)

10) Have you or a family member ever found a tick on your body?
   a) Yes
   b) No

11) Have you or a family member ever been bitten by a tick?
   a) Yes
   b) No

12) Have you or a family member ever had signs of fever
   a) Yes
   b) No

13) Types of other animals present at the household
   a) Cattle
   b) Goats
   c) Pigs
   d) Chickens
   e) Other (please specify): _____

14) Number of chickens at the household:
   a) 0
   b) 1-3
   c) 4-7
   d) 8-10
   e) ≥10

APPENDIX C

ETHICAL APPROVALS

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Research Ethics Committee

PROJECT TITLE	**Tick-borne disease dynamics at the human/wildlife/livestock interface in the Mnisi community area of South Africa**
PROJECT NUMBER	**REC010-18**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Rebecca Ackermann**

DISSERTATION/THESIS SUBMITTED FOR	**MSc**

SUPERVISOR	**Prof Marinda Oosthuizen**

APPROVED	Date	**11 September 2018**
CHAIRMAN: UP Research Ethics Committee	Signature	N.M. Duca

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Tick-borne disease dynamics at the human/wildlife/livestock interface in the Mnisi community area of South Africa**
PROJECT NUMBER	**V012-18**
RESEARCHER/PRINCIPAL INVESTIGATOR	**R Ackermann**

STUDENT NUMBER (where applicable)	**U_14170508**
DISSERTATION/THESIS SUBMITTED FOR	**MSc**

ANIMAL SPECIES	**Adult Canine**
NUMBER OF ANIMALS	**79**
Approval period to use animals for research/testing purposes	**March 2018 – March 2019**
SUPERVISOR	**Prof. M Oosthuizen**

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED (*With conditions)	Date 26 March 2018
CHAIRMAN: UP Animal Ethics Committee	Signature

Conditions: Only 79 animals may be used;
Animals have to be more than 500g and more than 6 weeks old
Advise owners regarding tick control
Diseased animals have to be excluded

S4285-15

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

Extension No. 1

PROJECT TITLE	**Tick-borne disease dynamics at the human/wildlife/livestock interface in the Mnisi community area of South Africa**
PROJECT NUMBER	**V012-18 (Not including Amendment 1)**
RESEARCHER/PRINCIPAL INVESTIGATOR	**R Ackermann**

STUDENT NUMBER (where applicable)	**U_14170508**
DISSERTATION/THESIS SUBMITTED FOR	**MSc**

ANIMAL SPECIES	**Adult Canine**
NUMBER OF ANIMALS	**51**
Approval period to use animals for research/testing purposes	**March 2019 – March 2020**
SUPERVISOR	**Prof. M Oosthuizen**

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED (*With conditions)	Date 1 March 2019
CHAIRMAN: UP Animal Ethics Committee	Signature

Conditions:
Only 79 animals may be used;
Animals have to be more than 500g and more than 6 weeks old
Advise owners regarding tick control
Diseased animals have to be excluded

S4285-15

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Humanities
Research Ethics Committee

6 September 2018

Dear Ms Ackermann

Project:	**The bacterial microbiome of Rhipicephalus sanguineus ticks collected from domestic dogs in the Mnisi communal area, South Africa**
Researcher:	**R Ackermann**
Supervisor:	**Prof MC Oosthuizen**
Department:	**Veterinary Tropical Diseases**
Reference number:	**14170508 (GW20180719HS)**

Thank you for your response to the Committee's correspondence.

I have pleasure in informing you that the Research Ethics Committee formally **approved** the above study at an *ad hoc* meeting held on 6 September 2018. Data collection may therefore commence.

Please note that this approval is based on the assumption that the research will be carried out along the lines laid out in the proposal. Should your actual research depart significantly from the proposed research, it will be necessary to apply for a new research approval and ethical clearance.

We wish you success with the project.

Sincerely

Prof Maxi Schoeman
Deputy Dean: Postgraduate and Research Ethics
Faculty of Humanities
UNIVERSITY OF PRETORIA
e-mail: PGHumanities@up.ac.za

cc: Prof MC Oosthuizen (Supervisor)

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
Reference: 12/11/1/1/8

Prof Marinda Oosthuizen
Department of Veterinary Tropical Diseases,
Faculty of veterinary Sciences
University of Pretoria, Onderstepoort Campus
0110.
South Africa
Tel: 012 529 8390
E-mail: Marinda.oosthuizen@up.ac.za

RE: Permission to do research in terms of section 20 of the ANIMAL DISEASES ACT, 1984 (ACT NO 35 of 1984)

Dear Prof Oosthuizen

Your application, requesting for permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers.

I am pleased to inform you that permission is hereby granted to perform the following study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. All tick and blood samples collected from dogs in the Mnisi community should be send to Hans Hoheisen research facility in the Kruger National Park for processing and storage, and a veterinary permit should be issued prior to their movement;
3. Only extracted DNA should be send together with a veterinary permit to the DVTD lab at the University of Pretoria for further molecular analysis;

4. All potentially infectious material utilised or generated during or by the study is to be destroyed at completion of the study;
5. Only a registered waste disposal company may be used for the removal of waste generated by or during the study;
6. Records must be kept for five years for auditing purposes;
7. If required, an application for an extension must be made by the responsible researcher at least one month prior to the expiry of this Section 20 permit. Please apply in writing to HerryG@daff.gov.za;
8. The study is approved as per the application form sent with an email dated 02 May 2017 and the correspondence thereafter. Written permission from the Director: Animal Health must be obtained prior to any deviation from the conditions approved for this study under this Section 20 permit. Please apply in writing to HerryG@daff.gov.za.

Title of research/study: Tick-borne disease dynamics at the human/wildlife/livestock interface in the Mnisi community area of South Africa.

Researcher: Prof Marinda Oosthuizen
Institution: University of Pretoria
Permit Expiry date: 31 December 2019
Our ref Number: 12/11/1/1/8

Kind regards,

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date: 2018 -07- 0 6

SUBJECT: *PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO. 35 OF 1984)*

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
Reference: 12/11/1/1/8

Prof Marinda Oosthuizen
Department of Veterinary Tropical Diseases (DVTD)
Faculty of veterinary Sciences
University of Pretoria, Onderstepoort Campus
0110.
South Africa
Tel: 012 529 8390
E-mail: Marinda.oosthuizen@up.ac.za

RE: DISPENSATION ON SECTION 20 APPROVAL IN TERMS OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984) FOR: *"Tick-borne disease dynamics at the human/wildlife/livestock interface in the Mnisi community area of South Africa."*

A dispensation is hereby granted on Point 4 of the Section 20 approval that was issued for the above mentioned study (attached):

i) DNA samples should be stored at DVTD under strict access control;

ii) Blood samples collected from dogs in Mnisi community should be kept at Hans Hoheisen research facility under strict access control;

iii) Stored samples may not be outsourced or used for further research without prior written approval from Director: Animal Health.

Kind regards,

DR. MPHO MAJA
DIRECTOR: ANIMAL HEALTH
Date: 2018 -07- 0 6

Permit in	Permit out

DEPARTMENT OF AGRICULTURE

MPUMALANGA

Permit number: 20190130ORK-UMMP1

DEPT. LANDBOU

LITIKO LETEKULIMA

MASTER

VETERINARY PERMIT TO MOVE ANIMALS / ANIMAL PRODUCTS
VEEARTSENYPERMIT VIR VERVOER VAN DIERE / DIEREPRODUKTE
IMVUMO YEKUHAMBISA TILWANE / TINTFO LETIHABULISAKO TETILWANE

In terms of the Animal Diseases Act No. 35 of 1984 permission is hereby granted to:
Kragtens die Wet op Dieresiektes Nr. 35 van 1984 word toestemming hiermee verleen aan:
Ngeligunya leMtsetfo weTifo teTilwane No. 35 wa 1984:

Name: / Naam: / Libito:	REBECCA ACKERMANN	ID No.:	9402240384080
Address: / Adres: / Likheli:	UNIVERSITY OF PRETORIA		
to move with: / om te beweeg met: / uvunyelwa yekuhambisa:	AMOUNT IN WORDS: ONE HUNDRED	SPECIES: CANINE + TICKS	LIVE TICKS / LIVE / PRODUCT: BLOOD
Identified as follows: / geïdentifiseer as volg: / letikhonjiswe nga:	EDTA BLOOD SAMPLES LIVE TICKS		
from the farm/diptank area/place: / vanaf die plaas/dipbakgebied/plek: / kusuka epulazini/edibhini/endzaweni:	CLARE A VILLAGE, BBR EAST	STOCK CARD NUMBER	in the district of / in die distrik / esigodzini sase: BOHLABELA
to the farm/diptank area/abattoir/place: / na die plaas/dipbakgebied/abattoir/plek: / kuya epulazini/edibhini/emadzeleni/endzaweni:	HANS HOHEISEN W.R.S		in the district of / in die distrik / esigodzini sase: BOHLABELA

On the following conditions:

1. This permit:
   (a) is valid for 10 days from date of issue and for one consignment only
   (b) must accompany the animals / products mentioned above;
   (c) must be produced for inspection on demand by any land or animal owner or police or veterinary official;
   (d) must be kept at a destination until collected by a veterinary official.
2. For game / game products a nature conservation permit is also needed.
3. Animals and products must be visibly free from external parasites.
4. Animals are to be transported in accordance with animal welfare guidelines.
5. Other conditions:

Op die volgende voorwaardes:

1. Hierdie permit:
   (a) is geldig vir 10 dae vanaf datum van uitreiking en slegs vir een besending;
   (b) moet die diere / produkte hierbo vermeld vergesel;
   (c) moet vir inspeksie getoon word op aanvraag van enige grond- of dier-eienaar of polisie- of veeartsenybeampte;
   (d) moet by bestemming gehou word totdat dit deur 'n veeartsenybeampte geneem word.
2. Vir wild / wildprodukte word 'n natuurbewaringspermit ook benodig.
3. Diere en produkte moet sigbaar vry van uitwendige parasiete wees.
4. Diere moet volgens dierewelsynsriglyne vervoer word.
5. Ander voorwaardes:

Ngaphansi kwemibandela lelandzelako ngaphansi:

1. Lemvumo:
   (a) [illegible] 10 [illegible]
   (b) [illegible]
   (c) [illegible]
   (d) [illegible]
2. [illegible]
3. [illegible]
4. [illegible]
5. [illegible]

* SAMPLE LIST TO BE CAPTURED AND ACCOMPANY RESULT

6. Retention for / Quarantine for / Direct slaughter within: days after arrival at destination

Seal no/s	N/A	—	Place / Plek / Indzawo:	HANS HOHEISEN
Vehicle reg. no/s	DY77 UHGP	—	Date / Datum / Lusuku:	2019-01.30

I hereby certify that all applicable control measures for issuing of this permit have been complied with.

PRINT NAME: DR. HKM RAMUDI
Issuing Veterinary Official:

SIGNATURE
State Veterinarian / Staatsveearts / Dokotela weTilwane

IMPORTANT NOTE / BELANGRIKE NOTA / SATISO LESIBALULEKILE
Permit holder who does not comply with this permit or any condition thereof is guilty of an offence.
Permithouer wat in stryd met hierdie permit of enige voorwaarde daarvan optree, is skuldig aan 'n misdryf.
Lonalemvumo longalandzeli lemvumo kumbe nguphi umbandzela wayo uphula umtsetfo.

DATE STAMP 0009
2019-01.30

DISTRIBUTION:

Permit in	Permit out

Permit number: 2019080UOKK-OMMPT

DEPARTMENT OF AGRICULTURE

MPUMALANGA

DEPT. LANDBOU

LITIKO LETEKULIMA

MASTCK

VETERINARY PERMIT TO MOVE ANIMALS / ANIMAL PRODUCTS
VEEARTSENYPERMIT VIR VERVOER VAN DIERE / DIEREPRODUKTE
IMVUMO YEKUHAMBISA TILWANE / TINTFO LETIHABULISAKO TETILWANE

In terms of the Animal Diseases Act No. 35 of 1984 permission is hereby granted to:
Kragtens die Wet op Dieresiektes Nr. 35 van 1984 word toestemming hiermee verleen aan:
Ngeligunya leMtsetfo weTifo teTilwane No. 35 wa 1984:

Name: / Naam: / Libito:	REBECCA ACKERMANN	ID No.:	9 4 0 2 2 4 0 3 8 4 0 8 0
Address: / Adres: / Likheli:	UNIVERSITY OF PRETORIA		

to move with: / om te beweeg met: / uvunyelwa yekuhambisa:	ONE HUNDRED (AMOUNT IN WORDS)	CANINE + TICK (SPECIES)	LIVE / PRODUCT: BIOLOGICAL SAMPLES
Identified as follows: / geïdentifiseer as volg: / letikhonjiswe nga:	CANINE BLOOD SAMPLES + TICKS		

from the farm/diptank area/place: / vanaf die plaas/dipbakgebied/plek: / kusuka epulazini/edibhini/endzaweni:	CLARE A (H.A.C)	STOCK CARD NUMBER	in the district of / in die distrik / esigodzini sase	BOHLABELA
to the farm/diptank area/abattoir/place: / na die plaas/dipbakgebied/abattoir/plek: / kuya epulazini/edibhini/emadzeleni/endzaweni:	HANS HOHEISEN WRS		in the district of / in die distrik / esigodzini sase	BOHLABELA

On the following conditions:
1. This permit: 30 DAYS
(a) Is valid for ~~10 days~~ from date of issue and for one consignment only
(b) must accompany the animals / products mentioned above;
(c) must be produced for inspection on demand by any land or animal owner or police or veterinary official;
(d) must be kept at a destination until collected by a veterinary official.
2. For game / game products a nature conservation permit is also needed.
3. Animals and products must be visibly free from external parasites.
4. Animals are to be transported in accordance with animal welfare guidelines.
5. Other conditions:

Op die volgende voorwaardes:
1. Hierdie permit:
(a) is geldig vir 10 dae vanaf datum van uitreiking en slegs vir een besending;
(b) moet die diere / produkte hierbo vermeld vergesel;
(c) moet vir inspeksie getoon word op aanvraag van enige grond- of dieresienaar of polisie- of veeartsenybeampte;
(d) moet by bestemming gehou word totdat dit deur 'n veeartsenybeampte ingesamel word.
2. Vir wild / wildprodukte word 'n natuurbewaringspermit ook benodig.
3. Diere en produkte moet sigbaar vry van uitwendige parasiete wees.
4. Diere moet volgens dierewelsynsriglyne vervoer word.
5. Ander voorwaardes:

[illegible]:
1. [illegible]
(a) [illegible] 10 [illegible]
(b) [illegible]
(c) [illegible]
(d) [illegible]
2. [illegible]
3. [illegible]
4. [illegible]
5. [illegible]

* SAMPLE DETAILS TO BE CAPTURED ON ACCOMPANYING LIST PLEASE.

6. Retention for / Quarantine for / Direct slaughter within: .... days after arrival at destination

Seal no/s	N/A	/	Place / Plek / Indzawo:	HANS HOHEISEN
Vehicle reg. no/s	DY 77 VHGP	/	Date / Datum / Lusuku:	2019-03-04

I hereby certify that all applicable control measures for issuing of this permit have been complied with.

PRINT NAME: DR NKM RAMPE	SIGNATURE
Issuing Veterinary Official:	State Veterinarian / Staatsveearts / Dokotela weTilwane

IMPORTANT NOTE / BELANGRIKE NOTA / SATISO LESIBALULEKILE

Permit holder who does not comply with this permit or any condition thereof is guilty of an offence.
Permithouer wat in stryd met hierdie permit of enige voorwaarde daarvan optree, is skuldig aan 'n misdryf.
Lonalemvumo longalandzeli lemvumo lomba [illegible] wayo uphula umtsetfo.

DATE STAMP 0009
2019.03.04

DISTRIBUTION: