

Exploration of tick-borne pathogens and microbiota of dog ticks collected at Potchefstroom Animal Welfare Society

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DEDICATION

This thesis is dedicated to the late Nettie Coetzee. For her inspiration and lessons to overcome any obstacle that life may present.

God called home another angel we all love and miss you.

“We are the scientists, trying to make sense of the stars inside us.”

-Christopher Poindexter

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ABBREVIATIONS

ADP	Adenosine diphosphate
ARC	Agricultural Research Council
ATP	Adenosine triphosphate
BIC	Bayesian Information Criterion
BLAST	Basic Local Alignment Search Tool
CFT	Complement-fixation test
CI	Confidence interval
CO1	Cytochrome oxidase subunit 1
CO ₂	Carbon dioxide
DE	Germany
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
<i>gltA</i>	Citrate synthase encoding gene
IF	Immunofluorescence
IgG	Immunoglobulin G
ITS	Internal transcribed spacer
MEGA	Molecular Evolutionary Genetics Analysis
ML	Maximum likelihood
MRS	MiSeq Reporter Software
NaCl	Sodium chloride

NaOH	Sodium hydroxide
NCBI	National Center for Biotechnical Information
NGS	Next-generation sequencing
NTR	General Time Reversible
OTU	Operational taxonomic unit
OVAH	Onderstepoort Veterinary Academic Hospital
PANDAsseq	PAired-end Assembler for Illumina sequences
PAWS	Potchefstroom Animal Welfare Society
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pH	Potential of Hydrogen
QIIME	Quantitative Insights Into Microbial Ecology
rRNA	Ribosomal ribonucleic acid
TEM	Transmission electron microscopy
Tris-HCl	Trisaminomethane- Hydrochloric acid
USA	United States of America
w/v	Weight per volume
ZA	South Africa

RESEARCH OUTPUTS

Conference papers

van Wyk C, Taioe MO, Mtshali MO, Mienie CMS, La Grange DC, Mert MJ, Terera S. Thekisoie OMM. Dog ticks and their associated pathogens in the JB Marks Local Municipality, South Africa. Oral presentation. **16-18 September 2018, 47th Annual PARSA conference, Tshipise Forever Resort, Limpopo, South Africa. Page 20.**

ABSTRACT

Ticks are blood-feeding pests of domestic and wild animals. Next to mosquitoes ticks are notorious for the transmission of pathogenic organisms to their mammalian hosts. Companion animals also suffer from tick infestations and this is problematic due to their close association with humans. In South Africa, there is a need for constant supply of up to date information of ticks infesting companion animals and their associated tick-borne pathogens using modern diagnostic techniques. Furthermore, some of the pathogens infecting companion animals are zoonotic resulting in diseases which are also of importance to human health. The current study was aimed at identifying tick species infesting urban dogs admitted at the Potchefstroom Animal Welfare Society (PAWS) and known pathogens of veterinary importance using microscopy and PCR. Moreover, the 16S metabarcoding of the tick gut, salivary gland, and egg microbiota was conducted using Illumina next-generation sequencing (NGS) platform. Different tick sterilization methods were also tested to determine their effectiveness to eliminate surface bacteria on the ticks.

During the current study, a total of 592 ticks were collected from 61 dogs admitted to PAWS, mainly originating from the JB Marks Local Municipality and a few dogs originating from Merafong City and Emfuleni Local Municipalities. Tick species were identified as *Haemaphysalis elliptica* (39%) and *Rhipicephalus sanguineus* (61%) by both morphological and molecular analysis. Phylogenetic analysis of the *Haemaphysalis* and *Rhipicephalus* genera, using the CO1 and ITS2 genes, supported the respective monophyly of these ticks compared to other ticks of the same genera on the NCBI database. Results of this study indicated ticks were most abundant the PAWS kennels, where companion animals are co-housed than in stray dogs from residential areas where grooming is rare.

Morphological detection of pathogens infecting *H. elliptica* indicated respective infestation rates of 29% (32/112) and 2% (2/112) with *Rickettsia* sp. and *Anaplasma* sp. Whilst in *R. sanguineus* there was respective infestation rates of 14% (13/92) and 1% (1/92) with *Rickettsia* sp. and *Babesia* sp. Molecular analysis detected respective infestation rates of 2% (1/49), 8.2% (4/49), 14.3% (7/49), 22.4% (11/49) of *Anaplasma phagocytophilum*, *Ehrlichia canis*, *Rickettsia conorii*, and *Coxiella burnetii* from *H. elliptica* DNA. Whilst 3.6% (2/55), 5.5% (3/55), and 5.5% (3/55) of *E. canis*, *R. conorii*, and *C. burnetii* was PCR positive from *R. sanguineus* DNA. In addition, *H. elliptica* eggs had an infestation rate of 10.5% (2/19) for *C. burnetii*. This is an indication that *C. burnetii* is possibly being transmitted both transstadially and transovarially in this tick species.

The metagenomic analysis of data generated by NGS from DNA of *H. elliptica* and *R. sanguineus* whole ticks, midguts, salivary glands, and eggs produced 1111515 assembled sequences and 404 Operational Taxonomic Units (OTUs) up to genus level. There was a total of 25 detected bacterial phyla. Dominating bacterial phyla detected were Proteobacteria (57.2%), Actinobacteria (17.4%), and Bacteroidetes (12%). This was followed by Firmicutes (7.8%) and Cyanobacteria (2.7%). Bacterial phyla that were unassigned had a total relative abundance of 14.2%. Bacterial genera of veterinary, ecological, and medical importance were also detected in lower relative abundances including *Bacillus*, *Staphylococcus*, *Streptococcus*, *Clostridium*, *Ehrlichia*, *Rickettsia*, *Wolbachia*, *Neisseria*, *Campylobacter*, *Proteus*, *Coxiella*, *Borrelia*, and *Pseudomonas*.

Several bacterial phyla detected during metagenomic diagnosis in this study were previously reportedly isolated from agricultural environments. This is in accordance with Ixodid ticks spending most of their life cycle off hosts. Due to this, the current study also evaluated several tick sterilization methods in order to determine the most effective method for removing external bacteria on tick surface. After washing ticks with 70% ethanol, PBS, 10% Bleach and 10% Tween 20, observations of this study revealed that a combination of washing first with 10% Tween 20 and then followed by 70% ethanol is effective for removal of external bacteria on tick surface without causing damage to the tick tissue and DNA.

This current study has documented that *H. elliptica* and *R. sanguineus* ticks are infesting urban dogs admitted at PAWS. Furthermore, this study has showed that these tick harbour several pathogens which are known for causing tick-borne disease, some of which are zoonotic. This data will be useful to veterinarians in municipalities where sampled dogs originated, as it will enable formulation of proper tick and tick-borne disease control approaches. Additional studies are required to determine the effect of these ticks, their associated tick-borne pathogens and their microbiota on other companion animals and humans.

Keywords: Dog ticks, *Haemaphysalis elliptica*, *Rhipicephalus sanguineus*, Tick-borne pathogens, *Anaplasma phagocytophilum*, *Coxiella burnetii*, *Ehrlichia canis*, *Babesia* sp., *Rickettsia* sp., Metagenomics, Tick microbiota.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Background

Ticks are blood-feeding pests of domestic and wild animals which are capable of transmitting various pathogenic organisms. Companion animals such as dogs and cats are also victims of tick infestations which results in a bigger problem as they are in close association with humans. This places ticks as one of the most significant vectors of diseases infecting livestock, companion animals, and humans. Management of ticks, treatment of tick-borne diseases, and losses due to livestock hide damage are of economic concern (Jongejan and Uilenberg, 2004). Ticks are also harbouring various bacterial communities which are referred to as the microbiome or microbiota. The microbiota of ticks may influence tick-host-pathogen interactions (de la Fuente, Villar, *et al.*, 2016). Use of culture-independent techniques and innovation in molecular techniques has made it possible to determine the microbiota of ticks and in turn may aid with the development of new techniques to control ticks and tick-borne pathogens (Narasimhan and Fikrig, 2015).

1.2 Ixodid ticks

1.2.1 Systematics

Ticks are grouped along with other members of mites under the subclass Acari, within the class Arachnida, under the suborder Ixodida, which forms part of the order Parasitiformes (Gray *et al.*, 2013; Muruthi, 2015). In general, there are four families under which the ticks are classified. These families include the Ixodidae, Argasidae, Nuttalliellidae and a newly described family, namely Deinocerotonidae (Machado-ferreira *et al.*, 2015; Muruthi, 2015; Dantas-torres, 2018). Ticks in the Ixodidae family, are commonly known as hard ticks (Figure 1-1) because they contain a sclerotized dorsal plate (Dantas-torres, 2008) whilst in the Argasidae family ticks are commonly referred to as the soft ticks (Figure 1-2), as they contain a flexible leathery cuticle (Parola and Raoult, 2001). Nuttalliellidae family consists of one tick species, *Nuttalliella namaqua*, that was documented in South Africa, Namibia and Tanzania (Mans *et al.*, 2011; Latif *et al.*, 2012; Machado-ferreira *et al.*, 2015; Pugh, 2018). Nuttalliellids are distinguished from the former two families by the absence of setae, the appearance of the fenestrated plates, the corrugated integument, as well as the stigmata position (Figure 1-3).

Family Deinocerotonidae currently consists of the species, *Deinoceroton draculi*, which is a newly described extinct tick species based on fossil material originating from Myanmar (Peñalver *et al.*, 2017; Dantas-torres, 2018). Deinocerotonidae family has similar morphological features to the Nuttalliellids with several differences (Figure 1-4). Some of these differences include smooth basis capitula, pitted pseudoscutum, the presence of cervical grooves, the absence of cornua, smooth genital area, proximal capsule forming part of Haller's organ is entirely open, to name a few (Peñalver *et al.*, 2017). Ixodidae consist of two main groups, namely Prostriata and Metastriata (Beati and Keirans, 2001; Little *et al.*, 2007). Argasidae is divided into two subfamilies, namely Argasinae and Ornithodorinae (Parola and Raoult, 2001). Currently, the Deinocerotonidae and Nuttalliellidae families do not have any subdivisions (Dantas-torres, 2018).

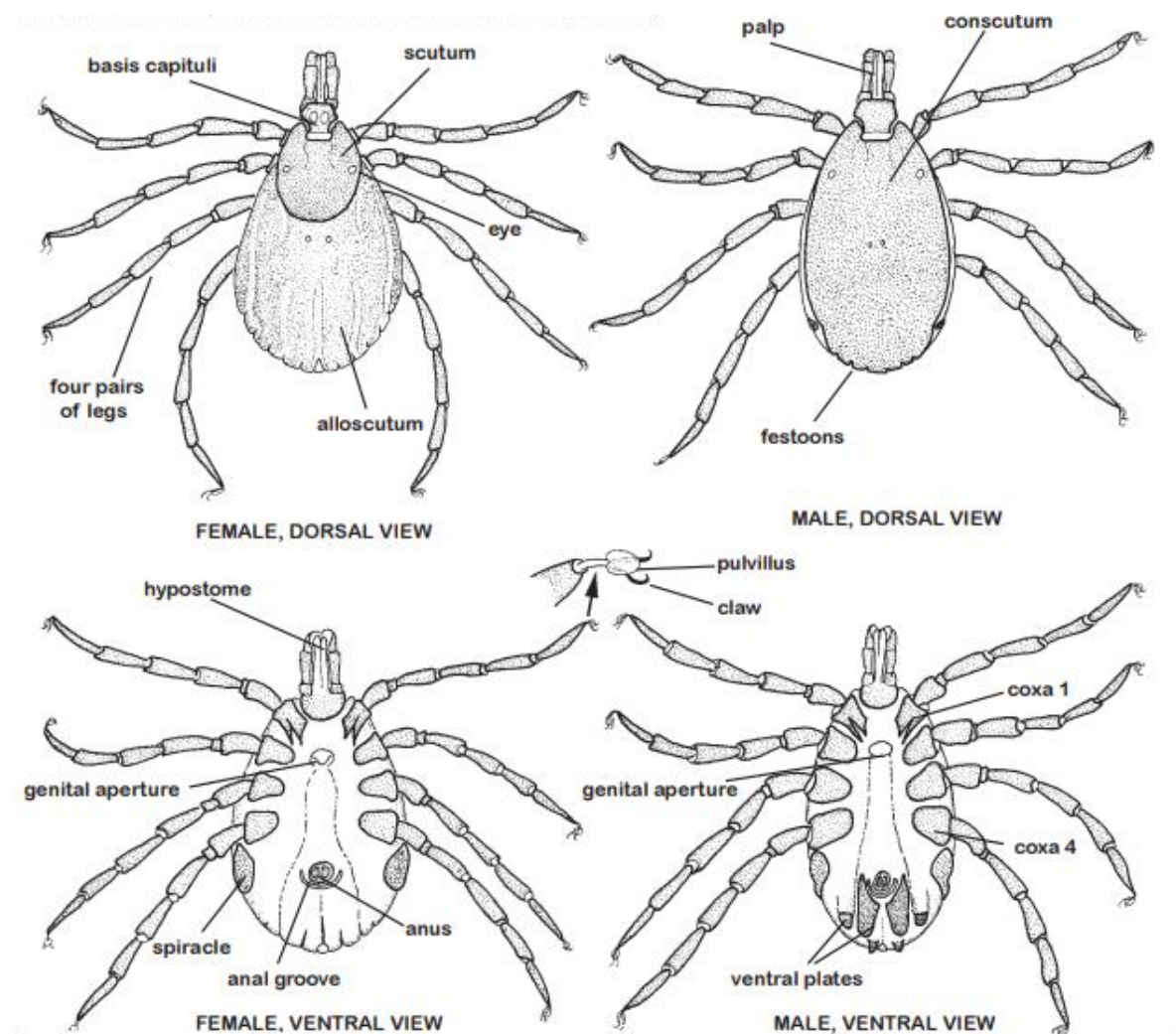


Figure 1-1: Dorsal and ventral view of an external structure of ticks of Ixodidae family (Walker *et al.*, 2014)

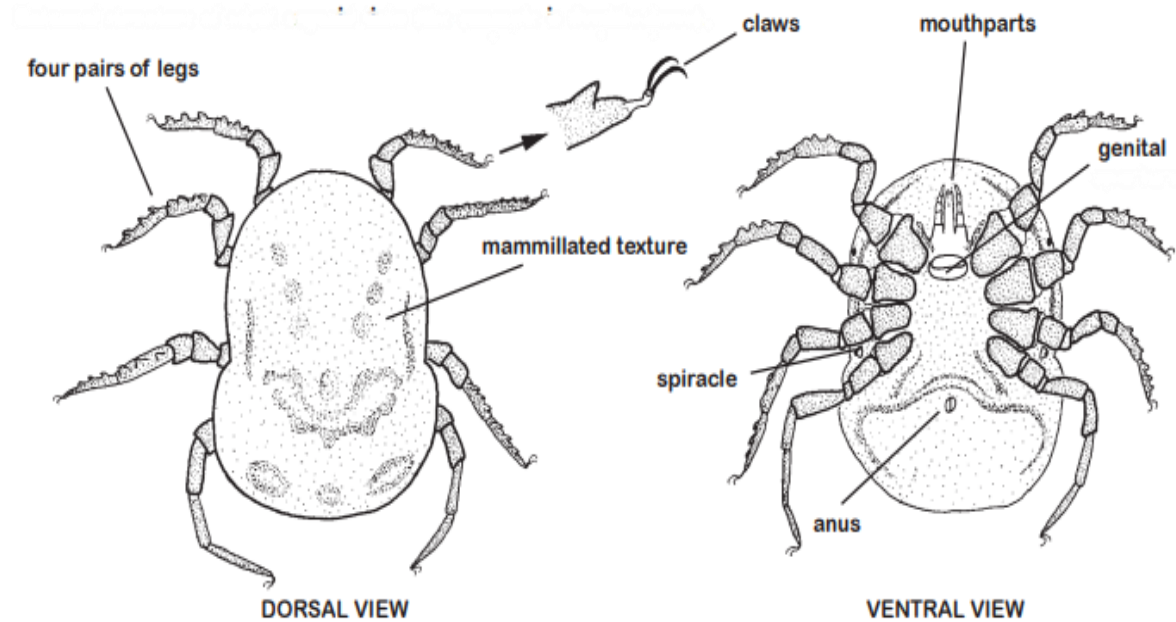


Figure 1-2: Dorsal and ventral view of an external structure of ticks of Argasidae family (Walker *et al.*, 2014)

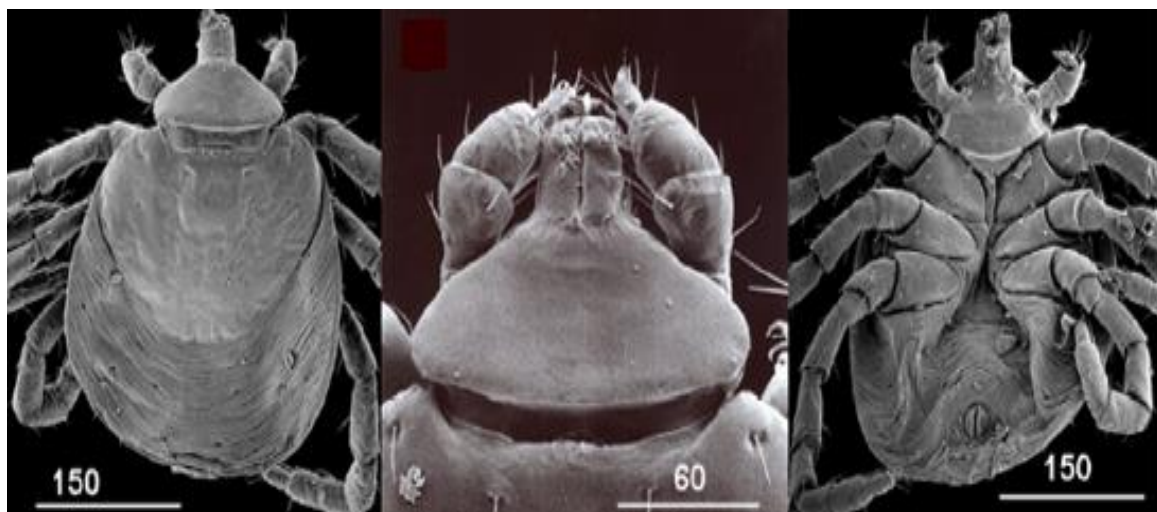


Figure 1-3: Dorsal and ventral view of an external structure of ticks of Nuttalliellidae family (Latif *et al.*, 2012)

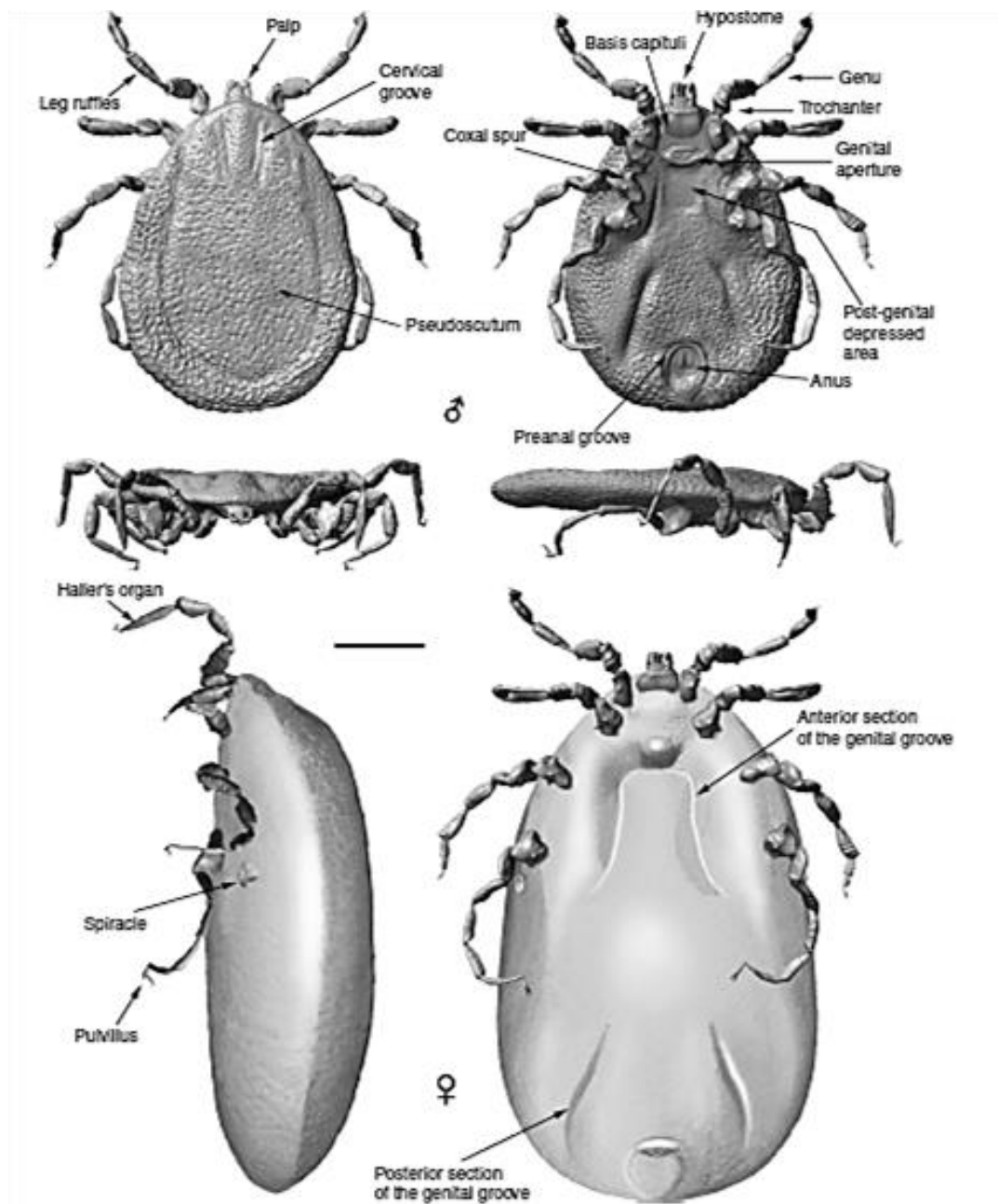


Figure 1-4: Dorsal and ventral view of an external structure of ticks of *Deinocerotonidae* family (Peñalver *et al.*, 2017)

1.2.2 Morphology

Ticks are acarines with body sizes ranging from 2 - 30 mm (Parola and Raoult, 2001). Larvae have three pairs of walking legs whereas nymphs and adults have four (Dantas-torres, 2008). In all the developmental stages antennae are absent. Bodies of ticks are not separated into a distinct thorax, head, and abdomen. Instead ticks consist of two body regions, namely the capitulum and the idiosoma (also known as the body region) (Parola and Raoult, 2001; Sonenshine and Roe, 2013).

The capitulum or the anterior part of the body contains the mouthparts, which include the cutting organ, sensory organs, and the hypostome. Hypostome is a median immobile organ containing several recurved teeth that aid to attach the tick to the skin of the host (Dantas-torres, 2008). In many tick species, eyes are absent. When eyes are present it doesn't aid ticks to have a thorough perception of the surrounding environment. Sensory organs are present to trace hosts and for communication purposes (Greay, 2014). Sensory organs include Haller's organ (sensory complex located on the dorsal surface of the tarsus on the first pair of legs consisting of a collection of gustatory and olfactory receptors) and hair-like structures located on the mouthparts, legs, and the body (Parola and Raoult, 2001).

Idiosoma is further divided into two parts, namely the anterior podosoma and posterior opisthosoma. Podosoma consists of the genital pore, present during the adult life stage, and the walking legs. In hard ticks. In the dorsal surface of Ixodid ticks, a sclerotized plate is present, known as the scutum in females and conscutum in males (Sonenshine and Roe, 2013). During a blood meal, the sclerotized plate remains unchanged whereas the rest of the body expands. Along the lateral margins, the scutum consists of lateral ridges and close to the midline cervical grooves are present. The surface of the scutum is covered with small pits and several setae. A genital groove is present on the ventral surface of the tick, situated medial to the coxae, expanding to the posterior body margin from the genital pore (Sonenshine and Roe, 2013).

On the posterior end of the scutum, the alloscutum are located. Alloscutum surface is distinguished by striations that signify shallow folds. Paired foveal pores are located posterior to the scutum. The anus and associated grooves, spiracles, plates, and several other

structures unique to different genera of ticks are also located on the opisthosoma. In several species, marginal grooves are present, located along the posterior and lateral body edges. Major body features located on the tick ventral surface include the anal aperture, spiracular plates, as well as the genital pore, during the adult life stage. Ticks of the Ixodidae family contain a spiracular plate housing the macula, functioning as the respiratory system opening. The whole body are coated by several pore-like sensilla and setae (Sonenshine and Roe, 2013). A circulatory system is present where all tissues and organs are covered by a circulating fluid, known as the haemolymph (Parola and Raoult, 2001).

1.2.3 Biology and ecology

1.2.3.1 Host finding strategies

The majority of Ixodid ticks are exophilic and remain separated from a host for more than 90% of the life cycle where the ticks are located in forests, meadows, or open environment (Dantas-torres, 2008). In general, ticks are seasonally active and search for hosts during favourable environmental conditions (Gray *et al.*, 2013). Changes that occur in chemical stimuli, such as carbon dioxide and ammonia, aromatic chemicals, humidity, phenols, body temperatures, and airborne vibrations associated with hosts act as stimuli for ticks (Dantas-torres, 2008).

Host-seeking strategies observed among exophilic ticks include the ambush strategy and hunter strategy. In the ambush strategy, used by *Rhipicephalus sanguineus*, ticks ascend vegetation, with the front legs extended forward while waiting for suitable hosts to attach to. When using the hunter strategy ticks leave their habitat and move towards and attacks a nearby host (Parola and Raoult, 2001; Dantas-torres, 2008, 2010). Certain exophilic tick species may use both strategies (Parola and Raoult, 2001). Endophilic tick species, such as those of the *Ixodes* genus make use of a third strategy, staying in the same environment as the host (Dantas-torres, 2008).

Certain tick species are host-specific and only attaches to a certain host when taking a blood meal, where other species have different hosts and host selectivity may vary during each developmental stage. Habitat distribution affects host selection since ticks are adapted to a specific environment (Dantas-torres, 2010). Ticks have different affiliations for humans and

certain Ixodid tick species, such as *Rhipicephalus sanguineus*, may feed on humans in the absence of suitable hosts (Dantas-torres, 2008, 2010).

1.2.3.2 Attachment and blood feeding

During attachment, the hypostome gets inserted into the host's skin. This is when the salivary glands create various substances that enter the host forming a feeding pool (Dantas-torres, 2008, 2010). Within the first twenty-four to thirty-six hours there is a limited intake of blood as attachment and penetration of the host are the main activities taking place (Parola and Raoult, 2001).

Ixodid ticks generate salivary secretions that aid with the successful uptake of blood and contain an anesthetic that makes Ixodid tick bites painless and go unnoticed by the host (Parola and Raoult, 2001). These salivary secretions contain a cement mix that secures the mouthparts of the tick to the host, as well as vasodilators, enzymes, antihemostatic, immunosuppressive, and anti-inflammatory substances (Parola and Raoult, 2001; Dantas-torres, 2008, 2010).

Ixodid ticks require extended periods for a successful blood meal to be taken. This period may last two to fifteen days that depends on the tick species, developmental stage, attachment site, and host type (Parola and Raoult, 2001). The first three to four days consist of an early slow feeding period followed by one to three days of rapid engorgement (Dantas-torres, 2008). During the engorgement period ticks may enhance their body mass by up to 120-fold (Parola and Raoult, 2001). During the feeding period, there is an interchange between taking a blood meal, salivation, and frequently regurgitation (Dantas-torres, 2008). Regurgitation especially occurs towards the end of tick engorgement. Early slow feeding period consists of constant consumption of the blood meal taking place in the midgut and occasional defecation. During the rapid engorgement period, there is limited consumption of the blood meal taking place (Parola and Raoult, 2001).

After completion of blood meal and separation from the host consumption of the blood meal continues. The blood meal is concentrated by the ticks, during transpiration, by the removal of

electrolytes and water in the salivary gland excretions and feces (Dantas-torres, 2008). Undigested remains coming from the midgut and waste produced by the excretory body are discarded by the anus (Parola and Raoult, 2001).

1.2.3.3 Developmental stages

There are four different developmental stages including eggs, larvae, nymphs, and adults (Dantas-torres, 2008; Gray *et al.*, 2013; Duan and Cheng, 2017) (Figure 1-5). In general, Ixodid ticks follow a three-host life cycle (Figure 1-6) where each developmental stage requires a single host (Dantas-torres, 2010). During each life stage, only one blood meal is required for the tick to molt and develop to the next life stage (Dantas-torres, 2008; Gray *et al.*, 2013). During each developmental stage, the tick locates, attaches, and takes a blood meal for numerous days on a suitable host (Parola and Raoult, 2001). After a blood meal is taken the tick detaches from the host, consumes the blood meal, and molt to enter the next developmental stage, or enter a state of postponed development and decreased metabolic activity, known as diapause (Belozarov *et al.*, 2002) (Figure 1-6). The duration of Ixodid ticks life cycle is normally two to three years. Environmental conditions such as photoperiod, temperature, and humidity may alter the life cycle duration to such an extent that it may take six months to six years (Parola and Raoult, 2001; Dantas-torres, 2008).

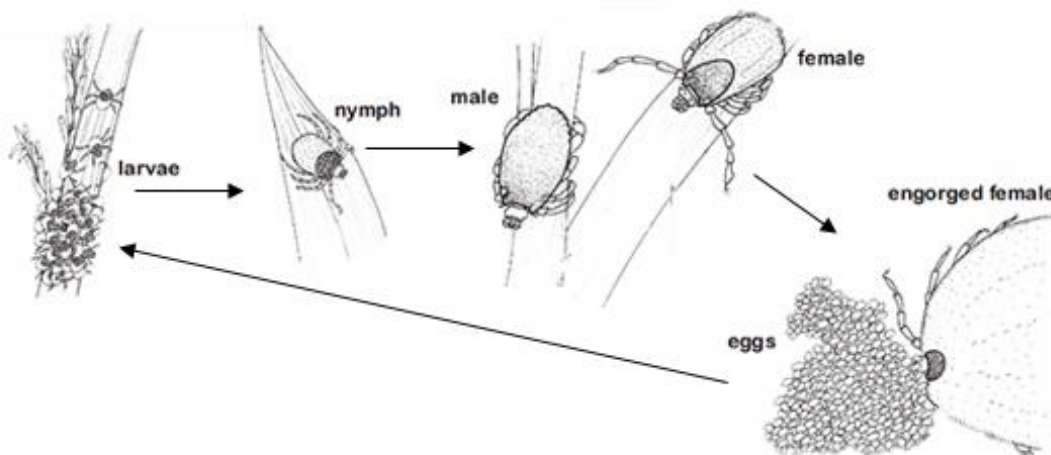


Figure 1-5: Basic life cycle of ticks (Walker *et al.*, 2014)

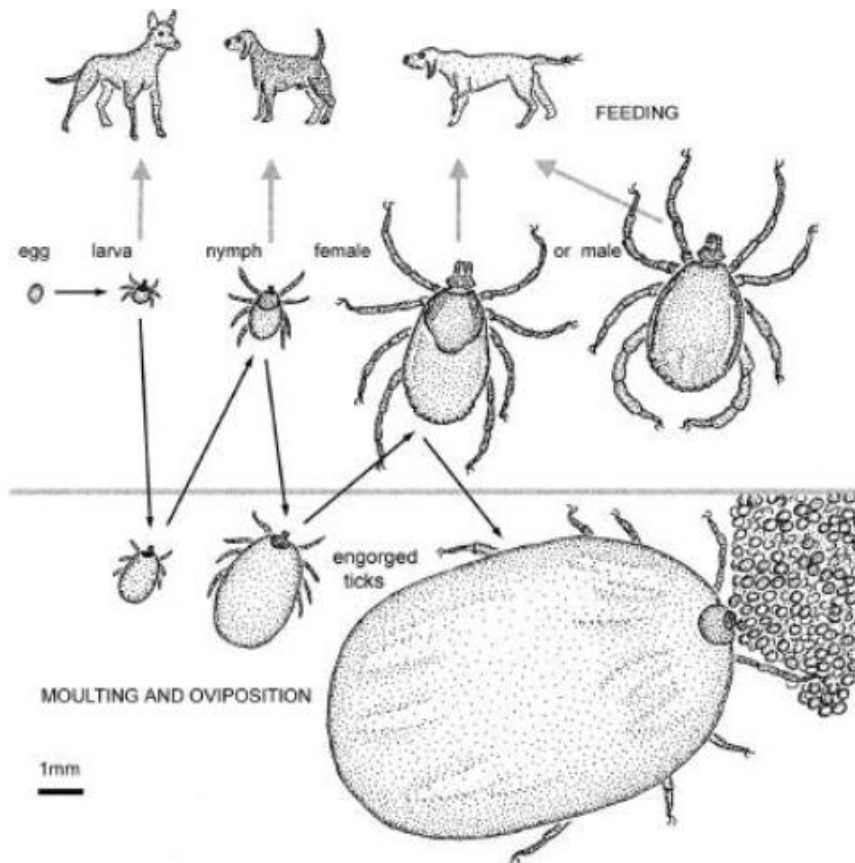


Figure 1-6: Three-host tick life cycle (Greay, 2014)

1.2.4 Geographical distribution

Ixodid ticks are susceptible to dehydration and are generally found in woodlands and grasslands (Parola and Raoult, 2001; Knight, 2016). Geographical distribution of every tick species is influenced by biotopes as well as optimal environmental conditions (Esteve-gassent *et al.*, 2016).

1.2.5 Tick control measures

Decrease and management of tick populations is a challenging task (Miller *et al.*, 2001). One strategy of tick control includes habitat modification by use of herbicides, vegetation control, as well as draining wet areas. These strategies are ineffective as they are short-term solutions and lead to ecological damage (Parola and Raoult, 2001; Kiss, Cadar and Spînu, 2012). Pyrethroids or organophosphates may be used with pheromones to control tick populations. This approach may pollute the environment and is lethal to humans and animals (Dantas-

torres, 2008; Kiss *et al.*, 2012). Acaricides may be used directly on hosts to interrupt the feeding process by eliminating attached ticks (Parola and Raoult, 2001; Davey *et al.*, 2006; Dantas-torres, 2008). Biological control methods such as the vaccination of tick hosts, sterilization of males by irradiation, and encouragement of natural predators, bacterial pathogens, as well as parasites of ticks may also be used (Parola and Raoult, 2001; Davey *et al.*, 2006; Dantas-torres, 2008; Kiss *et al.*, 2012; Šmit and Postma, 2017). Currently, tick management is established on the principle of integrated pest management. This principle is where various control methods are adjusted to a specific tick species or area by taking into consideration the environmental effects (Parola and Raoult, 2001; Demma *et al.*, 2005).

1.2.6 Molecular identification of tick species

Polymerase chain reaction (PCR) is still one of the most commonly used techniques for the identification of tick species. PCR is a primer-mediated enzymatic process utilized for the methodical amplification of genomic or complementary DNA sequences. This technique allows isolation and amplification of one sequence from a heterogeneous pool. PCR technique is based on the amplification of a specific gene sequence belonging to an individual organism (Petrulia and Conoci, 2017). Chain end complementary oligonucleotide primers consisting of a known synthetic DNA sequence are used for PCR amplification. PCR consists of various cycles, where each cycle is made up of three steps. Step one involves degradation of the template DNA. Second step involves the annealing of the synthetic oligonucleotide primers to the template DNA. Last step involves the addition of 4 kinds of dNTPs to the primers, with the aid of Taq DNA polymerase, in the 5' to 3' direction. After the completion of these steps, a new chain is generated that is similar to the original DNA template. After this step, a new cycle starts where the newly synthesized chain serves as the template DNA. Depending on the number of cycles the DNA products increase exponentially (Yu *et al.*, 2017). Commonly used genes for the identification of tick species include CO1, ITS2; 12S rRNA, 16S rRNA, and 18S rRNA (Barker, 1998; Beati and Keirans, 2001; Jizhou Lv *et al.*, 2014).

After amplification by using PCR the sequence of the DNA needs to be determined. Sanger sequencing, also known as the chain termination method of DNA sequencing, is commonly used. When using this method the DNA sample, one of the primers that were used for PCR, DNA polymerase, DNA nucleotides, and four dye-labeled chain-terminating dideoxynucleotides are combined. Reaction mixture is subsequently heated to allow

denaturation of the template DNA and cooled for the primer to bind to the single-stranded template. As the combination of the primer and single-stranded DNA is complete the temperature will be increased and new DNA will be generated starting from the primer with the aid of DNA polymerase. Nucleotides will continuously be added, by the DNA polymerase, until the chain terminating dideoxynucleotide is added. Similar to PCR this method also consists of various cycles. By the end of this process, the mixture consists of different lengths of fragments, where the final nucleotide is indicated at the end of these fragments by fluorescent dyes (Hindley, 2000; Liu *et al.*, 2016). These fragments are then subjected to a process known as capillary gel electrophoresis where the attached dye is detected and illumed by a laser. Colors of the dye are then recorded on a detector, therefore, revealing the original DNA sequence by the assembly of one nucleotide followed by the next nucleotide. Nucleotides are then resembled as a series of peaks by the detector and the DNA sequence is displayed as a series of peaks forming a chromatogram (Fu *et al.*, 1995; Chen *et al.*, 2017).

After sequencing of a specific gene is complete it allows the identification by combining the gene sequence to a similar known or previously identified sequence. Construction of phylogenetic trees where gene sequences of similar organisms are clustered together also show the similarities in gene sequences and is also widely used for evolutionary studies. Construction of phylogenetic trees relies on several construction methods. These methods include neighbour joining, maximum parsimony, minimum evolution, maximum likelihood, and minimum evolution, to name a few. Neighbour joining is an algorithm that clusters data for the rapid construction of phylogenetic trees, which are often unreliable when deeper divergence times are present. Maximum parsimony is a method that aims to shorten the length of tree branches by lowering the mutations. Like the maximum parsimony method, the minimum evolution method also aims to lower branch lengths by lowering the distance. Maximum likelihood method belongs to a strategy where several trees are examined and the appropriate tree is chosen based on a specific criterion (Saitou and Imanishi, 1989; Brooks *et al.*, 2007; Yang and Rannala, 2012).

1.2.7 Influence of ticks on humans, livestock and companion animals

Ticks are responsible for the transmission of a wide range of pathogenic organisms (Dantas-torres, 2008, 2010) including viruses, microorganisms, spirochetes, protozoa and rickettsiae (Jongejan and Uilenberg, 2004). This classifies ticks as significant vectors of illnesses concerning humans, livestock, and companion animals (Barker, 1998; Dantas-torres, 2010). In addition to the transmission of pathogens extended attachment periods of several tick species may cause host paralysis, irritation, allergy, and toxicosis (Felz *et al.*, 1996; Dantas-Torres *et al.*, 2006).

Impact of tick-borne pathogens on humans, companion animals, and livestock is considered in terms of disease and death (Dantas-torres, 2010; Berggoetz *et al.*, 2014b). In addition, tick-borne diseases also result in a decrease in livestock production (Spickett *et al.*, 2011; Berggoetz *et al.*, 2014b). Intensive application of acaricides or chemicals to control ticks are both expensive and lead to resistance to these chemicals (Davey *et al.*, 2006). Increased costs influence revenue of livestock owners (Schroder and Reilly, 2013; Esteve-gassent *et al.*, 2016). Tick-borne pathogens infecting companion animals amount to significant constraints to sporting events and international trade concerning these animals (Jongejan and Uilenberg, 2004).

1.2.8 Ticks infesting dogs in South Africa

1.2.8.1 *Haemaphysalis elliptica* Koch, 1844

The *H. elliptica*, or the yellow dog tick, is a three-host tick and is one of the two ticks that primarily feeds on domestic dogs (Walker *et al.*, 2014). This tick is also referred to as *Haemaphysalis leachi* as described by Audouin in 1826 (Guglielmone *et al.*, 2014; Horak *et al.*, 2018), however was renamed to *H. elliptica* after Apanaskevich *et al.* (2007) studied several tick specimens from Horak's collection originating from South African wild carnivores, domestic dogs, and vegetation previously identified as *H. leachi*. It was later determined that these ticks belonged to the *H. elliptica* or *H. colesbergensis* taxon. Apanaskevich *et al.* (2007) concluded that *H. elliptica* are distributed in South Africa extending north to Kenya and *H. leachi* is distributed in Egypt and Zimbabwe. Besides domestic dogs, other hosts include wild carnivores, like wild dogs, larger cats, jackals, and foxes. Larvae and nymphs may appear on a similar host as adults but would rather feed on murid rodents (Horak *et al.*, 2002).

Instances were documented where this tick feeds on cattle and other livestock (Walker *et al.*, 2014). These instances occur when livestock graze in areas where these ticks are present, and the ticks preferred hosts are unavailable, or when ticks detach from infested domestic animals and attach onto nearby livestock to take a blood meal (Baneth, 2014).

During the entire year adults of this tick species are present (Walker *et al.*, 2014). During spring to late summer or winter to early summer, these species numbers are at their highest (Horak *et al.*, 2002). This tick species is in an extensive range of climatic areas. Humid and warm conditions are preferred, although this species may occur where selected hosts are present (Walker *et al.*, 2014). Throughout sub-Saharan Africa where dogs are present this tick is prevalent, although they are scarce on dogs (Horak *et al.*, 2002).

1.2.8.2 *Rhipicephalus sanguineus* Latreille, 1806

The *R. sanguineus* is generally known as the pan-tropical dog tick, the kennel tick (Walker *et al.*, 2014), or the brown dog tick (Barker, 1998). This is a three-host tick where dogs are the preferred host for all the developmental stages. Other hosts include cattle and potentially humans. These hosts are generally parasitized to maintain tick populations when dogs are scarce (Little *et al.*, 2007; Dantas-torres, 2008; Dantas-torres *et al.*, 2013; Walker *et al.*, 2014).

This tick species is in all climatic areas of Africa (Walker *et al.*, 2014). The *R. sanguineus* are capable to survive in open environments and are adjusted to living in human homes (Horak *et al.*, 2002) and dog kennels (Walker *et al.*, 2014). Female ticks of this species produce eggs in gaps and cracks in the walls or under dogs' bedding (Dantas-torres, 2008; Walker *et al.*, 2014). Severe infestations are possible in kennels where the same dogs are present for extended time periods. Ticks may feed in the winter in artificially heated human homes (Miller *et al.*, 2001; Walker *et al.*, 2014).

1.3 Role of ticks as vectors of tick-borne diseases

Ticks are hematophagous and are capable of the transmission of disease-causing pathogens (Beati and Keirans, 2001; Shaw *et al.*, 2001; Berggoetz *et al.*, 2014a; de la Fuente, Waterhouse, *et al.*, 2016). After mosquitoes, ticks are the second global concern in their ability to transmit diseases (Shaw *et al.*, 2001; Dantas-torres, 2010; Merino *et al.*, 2013; Bonnet *et al.*, 2014).

1.3.1 Transmission of bacteria from ticks to hosts

Ticks become infected with bacteria by means of transstadial and transovarial transmission (Dantas-torres, 2008; Rynkiewicz *et al.*, 2015). Transstadial transmission requires the transmission of bacteria from one developmental stage to the next, from larvae followed by nymphs and adults. Transovarial transmission requires bacteria to be transmitted from one generation to the following by the female ovaries (Parola and Raoult, 2001).

For certain bacteria such as rickettsiae, all forms of transmission are possible. This is because members of the genus *Rickettsia* are present and multiply in nearly every organ and fluids of ticks, especially the ovaries and salivary glands. This permits the transmission of organisms both transovarially and transtadially, meaning the tick will also serve as a reservoir of the bacterial species. Not all tick species of a genus may transmit bacteria transstadially. Aforementioned is because certain bacterial species may be transmitted transovarially to the tick vector without infecting the salivary glands and in turn, does not infect the tick host (Parola and Raoult, 2001).

Since most Ixodidae ticks consist of a three-stage life cycle and a new host is required during each developmental stage pathogens may be spread amidst vertebrate species (Khoo *et al.*, 2016). This makes ticks important sources of zoonotic diseases. Bacteria may also be transmitted by co-feeding. This is when several ticks on the same host take a blood meal and bacteria may be transmitted to an uninfected tick from an infected tick (Kordick *et al.*, 1999; Kocan and de la Fuente, 2003).

1.3.2 Transmission of pathogens from ticks to humans

Zoonosis is presently recognized as tick-borne bacterial diseases of humans where the bacteria is sustained by ticks and wild or domestic hosts by means of natural cycles (Parola *et al.*, 2005). A single or several reservoirs and tick vectors may occur for every bacterial disease. Animal hosts are susceptible to disease-causing bacteria and to become reservoirs of infection bacteraemia must present for a prolonged period. Bacterial infections in ticks may rise as the duration of attachment on a reservoir host during the taking of a blood meal increases (Parola and Raoult, 2001).

Several tick species are capable of serving as vectors of pathogens and it is estimated that twenty-eight of these species may transmit pathogens of humans (Narasimhan and Fikrig, 2015). The *R. sanguineus* is an example of a tick that may infest humans (Dantas-torres *et al.*, 2013). This tick is host-specific and hardly feed on humans but is well adjusted to human urban environs (Dantas-torres, 2008, 2010). A number of pathogens transmitted by ticks to vertebrate hosts continue to increase, with fluctuations in climate conditions as one of the vital contributing factors (Bonnet *et al.*, 2014). Because of this, the development of new strategies is increasing for tick management, decreasing pathogens transmitted by ticks, and avoiding infection prevalence (Olwoch *et al.*, 2007; Narasimhan and Fikrig, 2015; Esteve-gassent *et al.*, 2016).

Disease-causing bacteria may be transmitted to humans when the attachment areas of ticks are infested with infected coxal fluid, salivary secretions, feces, or regurgitated midgut contents (Berggoetz *et al.*, 2014b). Transmission via indirect routes is also likely when scraped skin or eyes encounter contaminated fingers after the crushing of ticks. Bacteria are unlikely to be transmitted to humans during the taking of a blood meal if they are limited to ovarial tissue of arthropod hosts (Parola and Raoult, 2001).

Ticks may attach to humans at various sites but often occur around the neck, head, and the groin. The preferred site of attachment of *R. sanguineus* to humans includes the head area of minors and the entire body of adults (Dantas-torres, 2010). These preferred attachment sites may be due to host-seeking behaviour or the altitude of clumps created on vegetation by ticks (Parola and Raoult, 2001).

Ixodid ticks usually go unnoticed by humans because attachment and feeding are painless. Larvae and nymphs also go unnoticed because of their small body size (Parola and Raoult, 2001; Dantas-torres, 2010). Majority of tick bites are unreported and humans who have tick-borne diseases diagnosed do not report the history of the tick bite (Felz *et al.*, 1996; Demma *et al.*, 2005; Gray *et al.*, 2013). Rates of human infections with tick-borne bacterial diseases are influenced by tick- and human-related factors (Esteve-gassent *et al.*, 2016).

Certain ticks may be infected with and transmit several pathogenic bacteria. This results in a phenomenon known as co-infection. These ticks may attach, feed and transmit multiple organisms to humans. Multiple pathogenic organisms may lead to multiple infections and may cause unusual clinical appearances of tick-borne diseases (Parola and Raoult, 2001).

Tick-related factors include the prevalence of preferred hosts, willingness to feed on humans, and abundance of vector ticks and degree of infection (Esteve-gassent *et al.*, 2016). Human factors include the vulnerability to disease-causing bacteria, humans entering tick habitat, and the act of crushing attached ticks (Parola and Raoult, 2001).

1.3.3 Epidemiology of tick-borne pathogens

Source and distribution of tick-borne zoonosis have been the focal point of various hypotheses and consist of the conception of co-evolution between microorganisms, animal hosts, and ticks (Berggoetz *et al.*, 2014b). Tick-borne diseases are localized by geographical origin and are only present in areas with optimal conditions for animals and ticks involved in the circulation of the bacterial pathogens. Hosts and tick-vectors are then exposed to selective pressure because of this setting leading to the outcome of co-evolution (Parola and Raoult, 2001).

Spread and maintenance of infections to a new area requires reservoir hosts or vector ticks to locate ticks or hosts, respectively. These ticks or hosts needs to be susceptible to infections to be able to sustain pathogenic organisms (Parola and Raoult, 2001). Ticks may spread to new areas by walking but occurs only over brief distances that rarely surpass 50 m (Bonnet *et*

al., 2014). Ticks attached to hosts, especially to mammals or migrating birds (Budachetri *et al.*, 2017), traveling over long distances may also contribute to the spread of ticks (Bonnet *et al.*, 2014). Agricultural activities, a consignment of livestock over substantial distances, and altering of tick habitation also contribute to tick dispersal (de la Fuente, Waterhouse, *et al.*, 2016; Esteve-gassent *et al.*, 2016).

Epidemiology of tick-transmitted diseases is determined by the density of the host, degree of tick infestation, and infections of reservoir hosts. These rely on various ecological and physiological aspects including host immunity, host preference of the different tick developmental stages, host and bacteria compatibility, environmental conditions, host and tick seasonal activity, and intensity of tick-host contact (Parola and Raoult, 2001; Esteve-gassent *et al.*, 2016).

1.3.4 Pathogens transmitted by ticks infesting dogs in South Africa

Ability of dogs to transmit a wide variety of tick-borne pathogens is of veterinary and medical concern (Little *et al.*, 2007). Tick-borne diseases of dogs of most concern include babesiosis, hepatozoonosis, ehrlichiosis, and rickettsiosis (P. T. Matjila *et al.*, 2008) (Table 1-1).

Table 1-1: Common tick-transmitted diseases of dogs and humans of South Africa and other regions

Pathogen	Disease	Tick vectors	Distribution	Host	Reference
<i>Babesia canis</i>	Canine babesiosis	<i>R. sanguineus</i> , <i>Dermacentor marginatus</i> and <i>D. reticulatus</i>	Tropical and semitropical worldwide distribution	Dogs	Shaw <i>et al.</i> (2001); de la Fuente <i>et al.</i> (2008)
<i>Babesia vogeli</i>	Canine babesiosis	<i>R. sanguineus</i>	Tropical and semitropical worldwide distribution	Dogs	Shaw <i>et al.</i> (2001); de la Fuente <i>et al.</i> (2008)
<i>Babesia rossi</i>	Canine babesiosis	<i>H. elliptica</i>	Southern Africa	Dogs	Shaw <i>et al.</i> (2001); de la Fuente <i>et al.</i> (2008)
<i>Babesia gibsoni</i>	Canine babesiosis	<i>R. sanguineus</i> , <i>H. bispinosa</i> , and <i>H. longicornis</i>	Southern Europe, Asia, Africa, and the USA	Dogs	Shaw <i>et al.</i> (2001); de la Fuente <i>et al.</i> (2008)
<i>Hepatozoon canis</i>	Hepatozoonosis	<i>R. sanguineus</i> , <i>H. longicornis</i> , and <i>Amblyomma maculatum</i>	Africa, Southern Europe, Far East and the Middle East	Dogs	Shaw <i>et al.</i> (2001); de la Fuente <i>et al.</i> (2008)
<i>Ehrlichia canis</i>	Canine ehrlichiosis	<i>R. sanguineus</i>	Africa, Southern USA, Middle East, southern Europe, and eastern Asia	Dogs	Shaw <i>et al.</i> (2001); Dantas-torres (2008); de la Fuente <i>et al.</i> (2008)

Table 1-1 (continue): Common tick-transmitted diseases of dogs and humans of South Africa and other regions

Pathogen	Disease	Tick vectors	Distribution	Host	Reference
<i>Rickettsia conorii</i>	Mediterranean spotted fever	<i>R. sanguineus</i>	Africa, Europe, and Asia	Dogs and humans	Shaw <i>et al.</i> (2001); Horak <i>et al.</i> (2002); de la Fuente <i>et al.</i> (2008)
<i>Rickettsia conorii caspia</i>	Asrakhan fever	<i>R. sanguineus</i> , and <i>R. pumilio</i>	Africa, and Asia	Humans	de la Fuente <i>et al.</i> (2008)
<i>Anaplasma phagocytophilum</i>	Human Granulocytic Anaplasmosis and Canine Granulocytic Anaplasmosis	<i>R. sanguineus</i> , <i>Ixodes. persulcatus</i> group, <i>I. ricinus</i> , <i>I. scalpularis</i> , and <i>I. pacificus</i>	Europe and USA	Humans and various mammals	Taylor <i>et al.</i> (2007); de la Fuente <i>et al.</i> (2008); Mtshali <i>et al.</i> (2015)
<i>Coxiella burnetti</i>	Q fever	Various tick species	Worldwide distribution	Humans and various mammals	de la Fuente <i>et al.</i> (2008)

1.3.4.1 *Anaplasma phagocytophilum*

Anaplasma phagocytophilum is classified under the order Rickettsiales and under the family Anaplasmataceae (Woldehiwet, 2010). Natural infections have previously been documented in humans and a variability of wild and domestic animal species (Dumler *et al.*, 2005; Stuen *et al.*, 2013). Lethal cases were reported in humans, dogs, sheep, moose, cattle, deer, horses, roe, and reindeer and rodents (Taylor *et al.*, 2007; Woldehiwet, 2010; Stuen *et al.*, 2013). This pathogen is transmitted transstadially and is present in the tick for a part of its life cycle (Dumler *et al.*, 2005; Taylor *et al.*, 2007).

Pathogenesis and aetiology

The *A. phagocytophilum* has a preference towards phagocytic cells (Stuen *et al.*, 2013). This organism is one of the rare bacteria acknowledged to replicate and survive in neutrophil granulocytes (Dumler *et al.*, 2005; Stuen *et al.*, 2013). When the tick takes a blood meal, neutrophil-associated-inflammatory-responses are controlled by numerous stimuli utilized by tick sialome components (Carlyon and Fikrig, 2003; Stuen *et al.*, 2013). Orchestration of interactions between vectors and bacteria with the host defence mechanisms appear to encourage instead of managing transmission and infection. As a result, infected cells are more obtainable at the location of the tick bite and the circulating blood (Carlyon and Fikrig, 2003; Woldehiwet, 2010; Stuen *et al.*, 2013). A decreased quantity of circulating organisms present between periods of bacteremia may be due to immunologically altered periods in generations of antigenically diverse organisms, margination of infested granulocytes to an endothelial exterior, or momentary clearance of infected cells (Stuen *et al.*, 2013).

Previously it was documented that *A. phagocytophilum* may infect several cells and tissues (Woldehiwet, 2010; Stuen *et al.*, 2013). It was also demonstrated that intravascular myeloid cells tend to have a greater infestation rate than the cells sited within the bone marrow. This may signify that the myeloid cells precursor stages convey ligands varying from mature neutrophils, consequently being more intractable to binding and entering the organism (Dumler *et al.*, 2005; Stuen *et al.*, 2013).

By activating an anti-apoptosis cascade *A. phagocytophilum* are capable to postpone apoptosis of host cells (Dumler *et al.*, 2005; Stuen *et al.*, 2013). This is crucial to ensure

reproduction and intracellular survival of the pathogen in the neutrophil granulocytes, which is generally short-lived (Carlyon and Fikrig, 2003; Stuen, Granquist and Silaghi, 2013). Even though *A. phagocytophilum* is a Gram-negative bacterium this organism does not have peptidoglycans and lipopolysaccharides. Instead, they include cholesterol that makes up for the absence of membrane integrity (Woldehiwet, 2010; Stuen *et al.*, 2013). This enables Toll-Like Receptor and Nod-Like Receptor activation pathways to escape and infection of vertebrate immune cells (Dumler *et al.*, 2005; Stuen, Granquist and Silaghi, 2013).

Clinical signs

Reaction to fever may differ depending on factors such as host species, host immunological status, host age, and a variant of *A. phagocytophilum* (Stuen *et al.*, 2013). Various clinical symptoms are present in mammals, like anorexia, fever, apathy, depression, unwillingness to move, distal edema, and petechial bleedings. In dogs, the major clinical symptoms displayed include lameness, depression, as well as anorexia. In humans, clinical symptoms displayed vary from minor self-limiting febrile disease to deadly infections. Majority of human infections have no or minimal clinical manifestations. Human patients generally display unspecific influenza-like symptoms associated with malaise, fever, myalgia, and headache (Dumler *et al.*, 2005; Taylor *et al.*, 2007; Stuen *et al.*, 2013). Thrombocytopenia, anemia, leukopenia, and elevated alanine and aspartate aminotransferase activity within sera has been documented (Carlyon and Fikrig, 2003; Stuen *et al.*, 2013).

Diagnosis

Diagnosis of the disease is based on clinical symptoms; however, laboratory confirmation is needed to confirm the diagnosis (Carlyon and Fikrig, 2003; Stuen *et al.*, 2013). Observation of blood smears under a light microscope during the initial fever period (Taylor *et al.*, 2007; Stuen *et al.*, 2013). Blood smears are generally stained with MayGrünwald Giemsa where the pathogens appear as blue cytoplasmic presences, 1.5 to 5 µm in diameter, within granular leukocytes and monocytes (Hartelt *et al.*, 2004). Electron microscopy of blood and organ samples and immuno-histochemistry of tissue samples are also used (Stuen *et al.*, 2013). Molecular assays, including PCR of DNA from blood and tissue samples are generally used (Taylor *et al.*, 2007; Stuen *et al.*, 2013). The *A. phagocytophilum* cultivation within cell cultures used from isolated variants from human, sheep, dog, roe, deer, and horse patients was previously reported (Stuen *et al.*, 2013). Serological assays, including ELISA tests,

complement fixation tests, indirect immunofluorescent antibody tests, and counter-current immunoelectrophoresis tests are also used for detection of *A. phagocytophilum* infections (Taylor *et al.*, 2007).

Treatment

Tetracycline is the drug of choice (Carlyon and Fikrig, 2003; Stuen *et al.*, 2013). Use of 5 to 10 mg/kg doxycycline for a period of three weeks is commonly used for treatment of infection in dogs and cats (Taylor *et al.*, 2007). Successful use of doxycycline for human patients for seven to ten days have been documented (Carlyon and Fikrig, 2003; Stuen *et al.*, 2013). Rifampin therapy should be taken into consideration for patients risking unfavourable drug reactions (Woldehiwet, 2010; Stuen *et al.*, 2013).

1.3.4.2 *Ehrlichia canis*

Ehrlichia canis is classified under the order Rickettsiales, family Rickettsiaceae (Perez *et al.*, 1996; Taylor *et al.*, 2007). Ehrlichiae were identified as veterinary pathogens for a given time but is acknowledged as tick-borne pathogens of humans (Breitschwerdt *et al.*, 1998). First reported case of human monocytic ehrlichiosis was documented in 1987. *E. canis*, which is the cause of canine ehrlichiosis was the presumed infecting organism (Parola and Raoult, 2001).

The 16S rRNA gene sequence analysis and groESL heat-shock operon signify different genogroups of Ehrlichiae (Parola and Raoult, 2001). Genogroup three includes *E. canis* (Shaw *et al.*, 2001). Other species of the *E. canis* genogroup include *Ehrlichia chaffeensis* and *Ehrlichia ewingii* that have also been implicated in human diseases (Perez *et al.*, 1996; Breitschwerdt *et al.*, 1998). Only transstadial transmission of this pathogen is possible (Taylor *et al.*, 2007; Moraes-filho *et al.*, 2015).

Pathogenesis and aetiology

Symptoms of the illness affecting dogs caused by *E. canis* genogroup may be impossible to differentiate. Different strains may have variations in pathogenicity (Shaw *et al.*, 2001). Ehrlichiae, in vivo, generally infect cells occurring in membrane-bound vesicles, especially leukocytes, where bone marrow is the source of origin (Parola and Raoult, 2001). Intraphagosomal Ehrlichiae split by means of binary fission and generate a cluster of organisms known as a morula (Taylor *et al.*, 2007).

Incubation period lasts eight to twenty days, subsequently an acute, a subclinical, and a chronic phase follow. Acute phase lasts a period of two to four weeks (Taylor *et al.*, 2007; Waner, 2008). When infected with the acute phase of this disease immunological obliteration of the platelets take place (Shaw *et al.*, 2001; Waner, 2008). If the acute phase remains untreated the disease enters the subclinical phase. Dogs infected during the subclinical phase may remain carriers of this pathogen for months to years (Taylor *et al.*, 2007; Waner, 2008). Splenic sequestration of organisms resulting in subclinical continual infection is frequent. Persistent infection may result in lethal chronic ehrlichiosis and may be related to permanent bone marrow damage (Shaw *et al.*, 2001; Waner, 2008). Several persistently infected patients recover spontaneously where others progress to the chronic phase of this disease. At the chronic phase the diagnosis is crucial and consequently, death may follow due to secondary infection or hemorrhage (Taylor *et al.*, 2007; Waner, 2008).

In experimental as well as naturally occurring cases anti-platelet antibodies were documented (Shaw *et al.*, 2001; Waner, 2008). Autoantibodies reduce the life-span of platelets and affect the platelet membrane glycoproteins (Shaw *et al.*, 2001; Taylor *et al.*, 2007). As a result, this leads to inhibition of aggregation between platelets and platelet membrane glycoproteins (Shaw *et al.*, 2001; Waner, 2008). Thrombocytopenia pathogenesis includes factors like splenic sequestration as well as assembly of a cytokine, platelet migration-inhibitor factor. Central nervous system and ocular irregularities may occur when hyperviscosity due to hyperproteinaemia contributes to platelet dysfunction (Shaw *et al.*, 2001; Taylor *et al.*, 2007; Waner, 2008).

Clinical signs

In young animals and certain dog breeds, such as German shepherds, ehrlichiosis is more serious (Shaw *et al.*, 2001; Taylor *et al.*, 2007). Severity of the disease also depends on strain variation, immune status, and coinfection (Shaw *et al.*, 2001). In the acute phase, there is a variety of clinical signs. These signs may be non-specific or mild to life-threatening and severe. Generally, non-specific signs include weight-loss, depression, tachypnoea, lethargy, pyrexia, and anorexia (Taylor *et al.*, 2007). Specific clinical signs of the acute phase include occasional epistaxis, splenomegaly, lymphadenomegaly, as well as ecchymoses and petechiae of the mucous membranes and skin (Taylor *et al.*, 2007; Waner, 2008). Other but less common symptoms of this phase include dyspnoea, purulent or serious oculonasal discharge, and vomiting (Taylor *et al.*, 2007; Blanton, 2016).

During the chronic phase of the disease, clinical symptoms may be similar but more severe to the acute stage. Peripheral oedema, and emaciation, particularly of the scrotum and hind limbs, as well as paleness of the mucous membranes, may be present. Female dogs may display symptoms of neonatal death, infertility, abortion, and extended bleeding during oestrus. Renal failure and interstitial pneumonia may occur due to secondary protozoal and secondary infections (Taylor *et al.*, 2007; Waner, 2008).

In the acute and chronic phase of this illness, ocular signs are often determined. Ocular signs may exhibit as panuveitis, corneal oedema, conjunctivitis, and ecchymoses and petechiae of the iris and conjunctiva. Blindness because of retinal detachment and haemorrhage may be due to hyperviscosity and monoclonal gammopathy. Neurological signs include cranial nerve dysfunction, ataxia, hyperaesthesia, seizures, and paresis, and may be because of meningoencephalitis or meningitis. Systematic manifestations include shock, haemorrhage, and multi-organ failure (Taylor *et al.*, 2007; Waner, 2008).

Diagnosis

Microscopy may be used where members of Ehrlichiae are observed as obligate intracellular Gram-negative cocci (Perez *et al.*, 1996). These pathogens are observed as elementary bodies (0.2 to 0.4 μm in diameter), initial bodies (0.5 to 4 μm in diameter), or large inclusion bodies (4 to 6 μm in diameter) (Taylor *et al.*, 2007). These organisms stain a dark blue to purple color when using Romanovsky's stains, such as Giemsa and Wright's stain (Parola and Raoult, 2001), light red when using Machiavelli, and brown-black when using silver stain (Taylor *et al.*, 2007). Serological testing, including ELISA tests and indirect fluorescent

antibody tests, are generally used for species cross-reactivity, detection of acute infection, and trouble in distinguishing infection from vaccinal titres or previous exposure (Breitschwerdt *et al.*, 1998; Shaw *et al.*, 2001; Suksawat *et al.*, 2001; Taylor *et al.*, 2007). Molecular assays, including PCR and Western immunoblotting, are used to describe and differentiate between different species (Breitschwerdt *et al.*, 1998; Shaw *et al.*, 2001; Suksawat *et al.*, 2001; Taylor *et al.*, 2007).

Treatment

Treatment differs for the clinical syndromes of the *E. canis* genogroup. For thrombocytopenia producing bleeding diathesis, treatment includes using doxycycline or minocycline. Chronic disease or permanent bone marrow damage treatment includes using oxytetracycline or tetracycline. Treatment for polysystemic immune complex disease includes using chloramphenicol, rifampin or fluoroquinolones (Shaw *et al.*, 2001; Taylor *et al.*, 2007).

1.3.4.3 Babesiosis species

Babesia belongs to the class Aconoidasida and the order Piroplasmida (Baneth, 2018). Morphologically canine babesiosis is divided into small and large forms. Both forms exhibit a global distribution. In the large *Babesia* group *B. canis* and another unspecified *Babesia* sp. detected in the USA, and in the small *Babesia* group, *Babesia annae* and *Babesia gibsoni* are described as disease-causing pathogens of dogs (P. T. Matjila *et al.*, 2008; Schoeman, 2009). *Babesia rossi*, *Babesia canis*, and *Babesia vogeli* are three major species included in the large *Babesia* group infecting dogs. These species are antigenically different and vary in pathogenicity, vector choice, and geographic distribution (Penzhorn *et al.*, 2016). *Babesia* spp. may be transmitted transovarially, transstadially, and by blood transfusion (Schoeman, 2009).

Pathogenesis and aetiology

Babesia causes illness in dogs, especially in puppies. Canine babesiosis caused by *B. canis* has an inoculation phase of ten to twenty-one days. Female ticks attach and take a blood meal for nearly a week and detach before the illness develops (Schoeman, 2009). *Babesia* species, presence of coexisting infections, as well as host immune status and age, are factors contributing to the disease severity (Matjila *et al.*, 2004; Taylor *et al.*, 2007). Primary

pathogenic mechanism includes haemolytic anemia. Additional factors may appear including immune-mediated destruction of erythrocytes (Taylor *et al.*, 2007). In general, the infection is classified as complicated or uncomplicated. Uncomplicated babesiosis is often related to hepato-splenomegaly, anemia that is mild to moderate, weakness, and lethargy. Complicated babesiosis signifies symptoms that are not only due to haemolytic crisis alone and is represented by organic dysfunction and serious anemia (Joseph *et al.*, 2016). Death of patients associated with complicated babesiosis may be more than 80% (Taylor *et al.*, 2007).

Clinical signs

Clinical signs differ because of the range of virulence between subspecies and may be per-acute to chronic or subclinical (Skotarczak, 2008). *B. rossi* is extremely virulent and may produce a per-acute and acute illness (Penzhorn *et al.*, 2016). Fever, pale mucous membranes, splenomegaly, depression, weakness, tachycardia, anorexia, and tachypnoea are the clinical symptoms displayed by the host (Taylor *et al.*, 2007; Skotarczak, 2008; Schoeman, 2009).

Dogs infected with *Babesia* species may have anaemia as the pathogen parasitize the red blood cells (Skotarczak, 2008). Extravascular and intravascular haemolysis occur where other mechanisms including poor bone marrow response may take place as well. Extent of parasitemia is not correlated to the anaemia. After parasitocidal treatment health of patients improve, even though haematocrits commonly decrease before increasing (Schoeman, 2009; Joseph *et al.*, 2016).

Babesia spp. mortality rates vary from approximately 1% for *B. vogeli* to about 12% for *B. rossi* (P. T. Matjila *et al.*, 2004; Schoeman, 2009). A severe form of this illness is distinguished by further immune-mediated red blood cell damage, noticeable haemolytic anaemia, cerebral pathology, acid-base irregularities combined with regular secondary multiple organ failure, acute respiratory stress syndrome, hypoglycaemia, hepatopathy with noticeable icterus, and complications like acute renal failure (ARF) (Skotarczak, 2008; Schoeman, 2009).

There is a small subcategory of dogs demonstrating high haematocrits regardless of strong haemolysis. This may be as fluid moves to the extravascular from the intravascular component. When this happens, patients have a bigger risk of cerebral complications, acquiring ARF, and other organ failures (Joseph *et al.*, 2016). Pancreatitis, with a death frequency of 20%, is often related to other complications. Patients assumed to have pancreatitis often exhibit diarrhea, vomiting, icterus, abdominal pain, and melaena (Schoeman, 2009).

The *B. vogeli* produces a moderate, barely noticeable illness in mature patients. Parasitaemia is low in *B. vogeli* and this may result in the infection to be overlooked during a blood smear examination (P. T. Matjila *et al.*, 2004). Adult patients infected with *B. vogeli* frequently have subclinical infections, whereas puppies represent noticeable anemia (Schoeman, 2009). Patients infected with *B. canis* have pathogenicity intermediate relating to *B. vogeli* and *B. rossi* (Penzhorn *et al.*, 2016).

Diagnosis

Acute cases of *Babesia* infection diagnosis is established on parasites found within the red blood cells by means of using stained blood smears (Skotarczak, 2008; Schoeman, 2009). Blood smears are often stained with Romanowsky stains, including Giemsa (Taylor *et al.*, 2007; Skotarczak, 2008). Generally, the large group of babesias is observed as organisms 2.4 by 5 µm in size clustered in pairs of two (Taylor *et al.*, 2007; Schoeman, 2009; Kostro *et al.*, 2015). Occasionally six to eight pyriform to round organisms may also be present in red blood cells (Schoeman, 2009). Red blood cells often appear pitted and vacuolated in anaemic dogs therefore considerable practice and decent staining techniques are needed for identification of small parasites (Schoeman, 2009; Kostro *et al.*, 2015).

Depending on virulence of the *Babesia* species the extent of parasitemia of the total red blood cells may vary from 0.05 to 10%. Parasitaemia may increase in patients co-infected with *Ehrlichia* spp. (Dantas-torres, 2008; Kostro *et al.*, 2015). Since *B. rossi* are highly virulent the existence of them in red blood cells are adequate to verify diagnosis. Parasitaemia of *B. vogeli* and *B. rossi* tends to be more in capillary than in central blood (P. T. Matjila *et al.*, 2004; Schoeman, 2009).

Diagnosis is often difficult in chronic cases where parasitemia may be lower than the standard microscopic detection limit because species like *B. vogeli* and *B. canis* are less virulent (P. T. Matjila *et al.*, 2004; Schoeman, 2009). Here diagnosis is established by means of PCR or positive indirect fluorescent antibody titers, physical examination findings, and historical findings (Skotarczak, 2008). In cases of low parasitemia non-alcohol fixed thick blood smears may be useful (Schoeman, 2009; Kostro *et al.*, 2015). During the preparation of blood smears parasitized red blood cells tend to marginate, thus observing along the periphery of the blood smear may enhance the probability of discovering parasites (Schoeman, 2009).

Demonstration of rising antibody titers over a period of two to three weeks may verify active or recent *Babesia* spp. infection (Schoeman, 2009; Kostro *et al.*, 2015). ELISA based on serology are capable to differentiate infections between *B. canis* and *B. gibsoni* species (Skotarczak, 2008; Schoeman, 2009; Kostro *et al.*, 2015). Bone marrow aspirate smears or cytology needs to be made from red cells under the buffy coat for several sub-clinical infections to determine parasite presence. Diagnosis based on seropositive patients is inadequate as clinically normal patients may be seropositive from or in endemic areas (Kostro *et al.*, 2015).

Treatment

Main objectives of treatment are to get rid of the pathogens and overturn anaemia (Schoeman, 2009; Joseph *et al.*, 2016). Imidocarb dipropionate, trypan blue, and diminazene aceturate are successful to *B. canis* infections (Taylor *et al.*, 2007; Schoeman, 2009). To avoid the spread of diseases to non-endemic areas it is preferred to make dogs non-infective for ticks by sterilizing the infection. Imidocarb dipropionate given at 7 mg/kg twice daily for fourteen days or 7.5 mg/kg once off is used for sterilization of *Babesia* infections. Use of trypan blue and diminazene in non-endemic areas is not recommended as it is unable to sterilize *Babesia* infections (Schoeman, 2009).

When using conventional therapy these parasites are hard to eliminate and results in patients frequently having recurring episodes of babesiosis or turn out to be chronic carriers. Based on the seriousness of the case-patients that are anaemic or displaying signs of the previously mentioned complications may need supportive treatments (Schoeman, 2009). These

treatments include blood transfusions, crystalloid and colloidal fluid support, and corticosteroid drug administration for damage of immune-mediated red cells (Taylor *et al.*, 2007). Patients with cases of pulmonary oedema because of acute respiratory distress syndrome requires assisted respiration (Schoeman, 2009).

1.3.4.4 Rickettsioses species

Rickettsioses are one of the best-studied arthropod diseases (Azad and Beard, 1998). Obligate intracellular bacteria belonging to the genus *Rickettsia* are the disease-causing agents (Azad and Beard, 1998; Parola and Raoult, 2001). Mediterranean spotted fever or boutonneuse fever was first described in Tunis in 1910 by Conor and Bruch. Part of transmission of this disease by *R. sanguineus* was recognized during the 1930s (Parola and Raoult, 2001). Ixodid ticks are implicated as vectors of this disease (Parola and Raoult, 2001; Alberdi *et al.*, 2012). Rickettsiae in ticks are maintained by means of transovarial and transstadial transmission (Azad and Beard, 1998; Parola and Raoult, 2001).

Pathogenesis and aetiology

Rickettsial species may infect and multiply in nearly every organ of ticks. They are especially present in the salivary glands as it enables the transmission of *Rickettsia* from ticks to vertebrate hosts during the taking of a blood meal (Azad and Beard, 1998; Parola and Raoult, 2001). Humans are not implicated as reservoirs because humans are rickettsemic for brief periods and are rarely parasitized by ticks (Parola and Raoult, 2001).

Clinical signs

Clinical signs may vary depending on the rickettsial species involved. Clinical signs manifest six to ten days after a tick bite and include a headache, fever, rash, muscle pain, one or quite a few inoculation eschars, and local lymphadenopathy. In general hepatic enzyme levels increase and irregular leukocytes and thrombocytopenia counts are frequent (Parola and Raoult, 2001; Blanton, 2016).

Diagnosis

Diagnosis is done by means of using serological tests, cell culture isolation of organisms, molecular detection methods, immunodetection of organisms using tissue and blood samples (Parola and Raoult, 2001; P. T. Matijila *et al.*, 2008; Alberdi *et al.*, 2012). Microscopy is often used where members of the genus *Rickettsia* consist of Gram-negative, intracellular bacteria viewed as short rods that maintain basic fuchsin when using the Gimenez staining method (Azad and Beard, 1998; Parola and Raoult, 2001; Alberdi *et al.*, 2012).

Growth of these bacteria under laboratory conditions require cell cultures (MRC5 cells, L929, Vero, or human embryonic lung) or living host cells (embryonated eggs, or animal models). In addition to the rickettsial antigens rOmpA and rOmpB, which are two high-molecular-mass surface proteins, contain species-specific epitopes. Highly immunogenic antigens, found on the lipopolysaccharide layer, are highly cross-reactive with other bacteria and members of the *Rickettsia* subgroup. Rickettsiae may be identified by comparative microimmunofluorescence where rOmpA and rOmpB proteins are the sources for rickettsial serotyping (Parola and Raoult, 2001). Protein analysis by using species-specific monoclonal antibodies or SDS-PAGE may also be used for species identification (Azad and Beard, 1998). PCR products and sequence analysis from various samples such as ticks, skin biopsies, and blood samples remain a convenient, sensitive, and rapid technique for species identification (Wardrop *et al.*, 2005; Chomel, 2011).

Treatment

Treatment normally commences before laboratory confirmation diagnosis by using 200 mg doxycycline per day for one to seven days, depending on the degree of infection. Other antibiotics may also be used for this disease (Parola and Raoult, 2001).

1.3.4.5 *Coxiella burnetii*

The *C. burnetii* is the causative agent of Q fever (Woldehiwet, 2004; Kazar, 2005; Raoult, Marrie and Mege, 2005; Angelakis and Raoult, 2010). Q fever was first reported amongst slaughterhouse workers by Derrick in 1937. Here it was recognized that *C. burnetii* is harboured by numerous free-living animals and that there was an association between slaughterhouses and this disease (Woldehiwet, 2004; Angelakis and Raoult, 2010).

The *C. burnetii* is described as an obligate intracellular bacterium. This bacterium is non-motile and is located in the phagosomes of infected cells (Raoult, Marrie and Mege, 2005). This organism has an endospore form making the organism resistant to hostile environmental conditions. Even though this bacterium is Gram-negative it does not stain well with a Gram stain (Angelakis and Raoult, 2010). Reason for this is as *C. burnetii* exhibits antigenetic variation that causes variations in the lipopolysaccharides layer (Woldehiwet, 2004).

This bacterium infects a wide range of hosts. These hosts consist of humans, domestic- and free-living mammals, including dogs, cats, mice sheep, cattle, goats, rabbits, pigs, camels, buffaloes, and horses (Woldehiwet, 2004). Birds, including turkeys, chickens, geese, ducks, pigeons, and chickens are also reported hosts. Infections were also reported in several ticks, flies, fleas, mites, and various other arthropods (Angelakis and Raoult, 2010).

Q fever has been reported globally, except New Zealand and Antarctic regions (Raoult, Marrie and Mege, 2005; Angelakis and Raoult, 2010). Infections also seem to be more common in tropical regions (Raoult, Marrie and Mege, 2005).

Pathogenesis and aetiology

There are several ways *C. burnetii* may be acquired by hosts. Several ticks and arthropods may transmit *C. burnetii* when taking a blood meal. Ticks are vital sources for the conservation of *C. burnetii* in nature but are not critical vectors when it comes to human and animal infections (Woldehiwet, 2004; Angelakis and Raoult, 2010). After withering of the primary source, infection of *C. burnetii* also occurs when aerosols produced from contaminated dust,

infected body fluids, or infected placenta are inhaled (Raoult, Marrie and Mege, 2005). Hosts, especially humans, may also be infected during the consumption of contaminated food or affected milk (Van den Brom *et al.*, 2015).

The *C. burnetii* is extremely contagious, this is as little as one organism is required to infect a host. Inside the regional lymph nodes, primary multiplication takes place. Subsequently, bacteraemia lasts for a period of five to seven days. The *C. burnetii* is then confined to the placenta, in the case of pregnant animals, and the mammary glands. Acute infection in humans is usually indicated by hepatitis and atypical pneumonia. Transient bacteraemia may also be present. Infection of the reproductive tract, liver, bone marrow, spleen, and other organs occurs due to haematogenous spread. Subsequently, granulomatous lesions in the bone marrow and the liver is formed. This is followed by endocarditis affecting the mitral and aortic valve (Woldehiwet, 2004; Kazar, 2005).

It is widely recognized that bacterial LPS are vital in pathogenesis of the illness in both animals and humans (Kazar, 2005). Chronic infections of *C. burnetii* may be due to the outcome produced by immunological defects or specific reactions (Angelakis and Raoult, 2010). This gives *C. burnetii* the ability to infect, develop, and multiply inside phagolysosomes to guarantee persistent infection (Woldehiwet, 2004). When *C. burnetii* are released from the phagolysosomes, macrophages are activated by the immune system of the host. Gamma interferon restricts *C. burnetii* in vitro multiplication and increases elimination of the organisms by means of apoptotic mechanisms facilitated by the tumour necrosis factor (Kazar, 2005).

Persistent infections are maintained as antigens of *C. burnetii* by phagolysosomes infested with the acute form of this disease is more expressed than the chronic form of this disease (Woldehiwet, 2004). This makes the chronic form of this disease less immunologically detectable (Angelakis and Raoult, 2010). If *C. burnetii* infection remains after the initial acute phase, in a pregnant animal, the organisms will multiply and are released in great quantities. During the early period and up to 13 weeks of the pregnancy period, *C. burnetii* may be detected in the intestine, liver, lymph nodes, bone marrow, and kidneys (Woldehiwet, 2004). Just before giving birth the amniotic, other fluids, and placenta becomes infected. When giving birth aerosols are released making the surrounding environment contaminated with *C. burnetii* (Van den Brom *et al.*, 2015). It is also during this stage where antibody levels, most likely IgG,

increases (Raoult, Marrie and Mege, 2005). In the pregnancies that follow *C. burnetii* is released in small quantities or not at all. Nevertheless, *C. burnetii* may be present in the milk of the animal for extended time periods. This is especially in the case of cattle (Van den Brom *et al.*, 2015).

When humans are infected with *C. burnetii* there may be various degrees of chronic endocarditis, pneumonia, granulomata of bone marrow, and granulomatous hepatitis (Woldehiwet, 2004).

Clinical signs

Animals, especially domestic animals, infected with *C. burnetii* generally display limited or no clinical signs. Infections of *C. burnetii* in humans display various clinical signs. These signs include non-specific fever to neurological manifestations, atypical pneumonia, hepatitis, and endocarditis. In general, infections occur as an acute feverish condition. Severe headaches are often associated with acute Q fever pneumonia (Woldehiwet, 2004; Raoult, Marrie and Mege, 2005). Major symptoms representing chronic cases of *C. burnetii* infection include endocarditis. Other symptoms of chronic cases include night sweats, fever, fatigue, weight loss, malaise, hepatomegaly, and splenomegaly (Woldehiwet, 2004; Angelakis and Raoult, 2010). Q fever in animals is often represented by abortion and reduced fertility (Woldehiwet, 2004; Raoult, Marrie and Mege, 2005).

Diagnosis

Amniotic fluid and placenta of aborting animals are generally good sources to determine *C. burnetii* infections (Van den Brom *et al.*, 2015). These organisms are often detected by using histological sections or impression smears of pathological material stained with modified Ziehl Neelsen or Machiavello stains. When red-colored large masses of coccobacilli are present it may be a good indicator of *C. burnetii* infection (Woldehiwet, 2004; Angelakis and Raoult, 2010). However, abortions due to *C. burnetii* must be distinguished from abortions caused by *Brucella abortus* or *Chlamydophila abortus* (Woldehiwet, 2004; Van den Brom *et al.*, 2015).

Immunoperoxidase and direct- or indirect immunofluorescence techniques may also be used on histological sections and impression smears to determine the presence of antigens (Woldehiwet, 2004; Angelakis and Raoult, 2010). Molecular techniques, such as PCR, are also used to determine the presence of *C. burnetii* (Samuel, Kiss and Varghees, 2003). Serological tests where enzyme-linked immunosorbent assay (ELISA), complement-fixation test (CFT), immunofluorescence (IF), radioimmunoassay, and microagglutination are generally used (Woldehiwet, 2004; Angelakis and Raoult, 2010).

Treatment

Treatment with fluoroquinolones, doxycycline, erythromycin, chlorotetracycline, rifampin or chloramphenicol is used for acute infections. Sulfamethoxazole and trimethoprim are generally prescribed for children and pregnant women. Continuous treatment with multiple antibiotics is generally used for chronic infections. Individuals at risk may require vaccinations (Woldehiwet, 2004; Angelakis and Raoult, 2010).

1.4 Microbiota of ticks

Assembly of pathogenic, commensal, and symbiotic microorganisms present in different locations of an organism is referred to as the microbiome (Finney *et al.*, 2015). Currently, the emphasis of the microbiome is placed on eubacterial members (Narasimhan and Fikrig, 2015). Microbiome also consists of viruses, archaea, and eukaryotic microorganisms like fungi, protozoa, and nematodes (de la Fuente, Villar, *et al.*, 2016; Esteve-gassent *et al.*, 2016). Interactions of these members inside and throughout kingdoms may cause changes in human and animal health (de la Fuente, Waterhouse, *et al.*, 2016).

Literature contains various examples of associations between arthropods and the associated microbiota. These associations alter vital characteristics of the arthropod life cycle, such as vectorial competence, survival chances, and reproductive fitness (Finney *et al.*, 2015; Narasimhan and Fikrig, 2015; de la Fuente, Waterhouse, *et al.*, 2016).

Developmental stage, taking a blood meal, geographical location and season of sample collection may be factors influencing the microbiome composition (Dantas-torres, 2008). Determination of microbiome composition may be used as biomarkers of the prevalence of infection in ticks existing in endemic areas (Esteve-gassent *et al.*, 2016). Recognising how ticks obtain the microbiota and how the microbiome is altered by genetic factors and environmental conditions is vital to utilize the tick's microbiota to manage tick prevalence and overturn tick-borne pathogen transmission (de la Fuente, Waterhouse, *et al.*, 2016).

Previous metagenomic studies in ticks conducted by Carpi *et al.* (2011), Hawlena *et al.* (2013), Budachetri *et al.* (2014), Narasimhan *et al.* (2014), Qiu *et al.* (2014), Narasimhan and Fikrig (2015), as well as Van Treuren *et al.* (2015) detected and identified a wide diversity of bacterial genera. While these genera may be strongly affected by specific parameters to a study there are some patterns that emerge. Predominating bacterial members are of the phylum Proteobacteria, followed by members of Actinobacteria, Firmicutes, and Bacteroidetes. In several studies members of the phyla Cyanobacteria, Acidobacteria, Fusobacteria, and Saccharibacteria are present. Gram-negative bacteria are often predominant, with aerobic and anaerobic bacteria also present. Some bacterial genera are also present regardless of the geographic locations of the ticks (Narasimhan and Fikrig, 2015) (Figure 1-7).

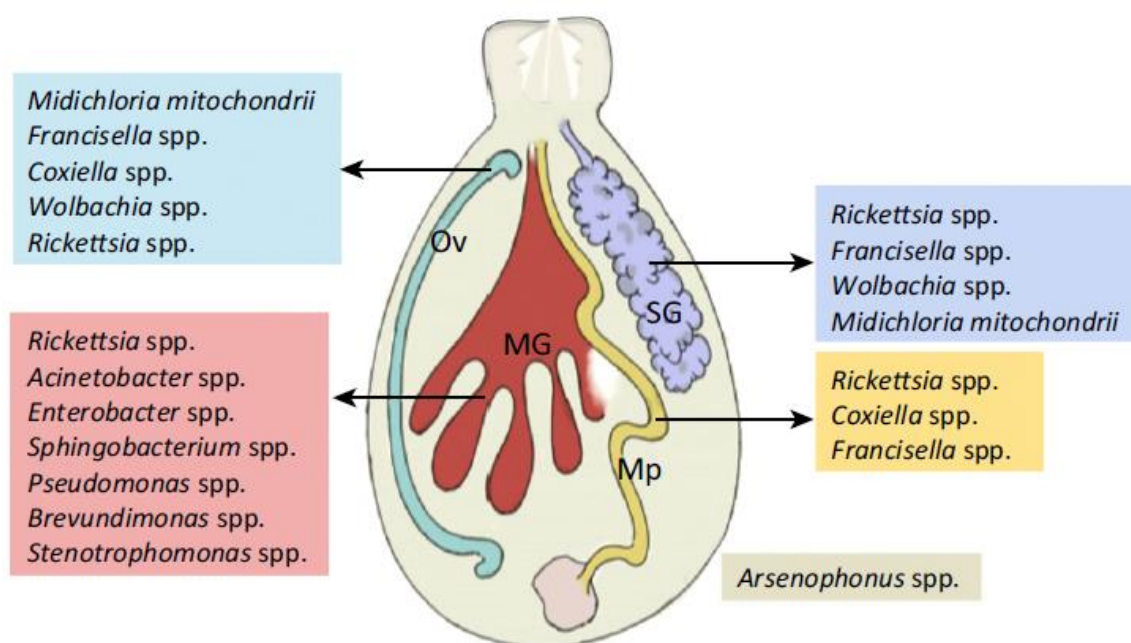


Figure 1-7: Bacterial members frequently present in the Malpighian tubules (Mp), midgut (MG), salivary glands (SG), and ovaries (Ov) of ticks (Narasimhan and Fikrig, 2015)

1.4.1 Symbiotic bacteria of ticks

Various bacterial species have been reported to be associated with arthropods referred to as symbionts, endosymbionts, or microsymbionts (Hunter *et al.*, 2015; Papa *et al.*, 2017). These bacterial species may have a positive, negative, or neutral influence on the arthropod host (Rynkiewicz *et al.*, 2015; de la Fuente, Villar, *et al.*, 2016).

Predicting if tick symbionts may be pathogenic to humans may be impractical. Animal models have been used previously to determine if tick symbionts if found in humans, may be pathogenic, but this technique has been proved to be inaccurate with certain pathogens (Esteve-gassent *et al.*, 2016). An example is bacteria such as *Rickettsia rickettsii* T-type strain which is pathogenic to humans but merely triggers mild disease in guinea pigs. Pathogenicity of tick-borne bacteria in humans may not be determined by inoculation of volunteers as it has ethical implications. Isolating the organisms from humans appearing with signs of disease is the only way to determine pathogenicity (Parola and Raoult, 2001).

1.4.2 Functional role of tick microbiome

Advancing literature suggests that microbial symbionts may have important functions in connection to the host (de la Fuente, Villar, *et al.*, 2016; de la Fuente, Waterhouse, *et al.*, 2016). Some of these functions include the vital role that symbionts have in host resistance against biotic enemies and abiotic conditions (Finney *et al.*, 2015; Esteve-gassent *et al.*, 2016). Impact of the majority microbial connections as well as the functional role in ticks is currently limited (Rynkiewicz *et al.*, 2015).

A microbial community may influence host activity and have a direct impact on disease-causing pathogens. Limited evidence of studies conducted on tick microbiota suggests that the microbial community or specific microorganisms may influence virulence, acquisition, and transmission of disease-causing pathogens. These pathogens may be emerging pathogens or well-studied pathogens (Beati and Keirans, 2001; Clay and Fuqua, 2010; de la Fuente, Villar, *et al.*, 2016; de la Fuente, Waterhouse, *et al.*, 2016; Esteve-gassent *et al.*, 2016).

Transmission and colonization of tick-borne pathogens may be influenced by the illness severity, interactions amid microbes, and co-infections of other pathogens (Finney *et al.*, 2015; de la Fuente, Villar, *et al.*, 2016; Esteve-gassent *et al.*, 2016). Communities of microbes may influence the functions of other microbes or manage activities in hosts by means of cell-cell communication. These mechanisms may be related to interactions taking place between tick-borne pathogens and endosymbionts often having an impact on pathogen transmission and prevalence (Clay and Fuqua, 2010; de la Fuente, Villar, *et al.*, 2016; Esteve-gassent *et al.*, 2016).

1.4.3 Metagenomics

Cultivation of microorganisms from natural environments remains the most commonly used method for microbial identification (Bonnet *et al.*, 2014). This method, however, disregards the discovery of an extensive range of microbes (Khoo *et al.*, 2016). Certain arthropods, including ticks, harbour pathogens or commensals that are nearly impossible to cultivate or are obligate intracellular organisms. Use of traditional histological staining and microscopy may possibly identify microbes. This information is, however, often limited and uncertain (Clay and Fuqua, 2010; de la Fuente, Waterhouse, *et al.*, 2016; Esteve-gassent *et al.*, 2016).

More powerful culture-independent molecular techniques enable the detection and identification of microbes. Small subunit ribosomal RNAs, like the bacterial 16S rRNA, are often the most informative and frequently applied (Bonnet *et al.*, 2014; Budachetri *et al.*, 2017). This is especially useful when investigating arthropod-microbe interactions (de la Fuente, Villar, *et al.*, 2016). Various arthropod-associated microbes present in a variety of systems have been discovered by molecular analysis. Majority of these microorganisms remain uncultured and their detection or identification remains impossible without the use of culture-independent methods. In addition, the identification, comparison, and differentiation of pathogens present in various tick species and pathogen populations gradually depend on molecular detection approaches (Duan and Cheng, 2017).

Innovation in RNA and DNA sequencing platforms and tools of data analysis has proved to be vital to reveal detailed tick microbiome composition (Berggoetz *et al.*, 2014a; de la Fuente,

Waterhouse, *et al.*, 2016). Studies conducted have used various tick developmental stages from different geographical locations targeting several hypervariable regions, including V1 to V3, V4, V5, or V6 regions, of the 16S rDNA (Duan and Cheng, 2017; Sperling *et al.*, 2017). Majority of these studies use the entire tick instead of dissecting specific tissues or organs. A downfall of using whole ticks is that it does not define the tissue-specific microbiome (Berggoetz *et al.*, 2014a; Narasimhan and Fikrig, 2015; Zolnik *et al.*, 2016).

Ticks contain a difficult but fascinating system to study microbiota and microbial interaction (de la Fuente, Waterhouse, *et al.*, 2016). This is as ticks require a blood meal before molting to the next developmental stage, treatment and inoculation of their symbionts are challenging, and the genomes are complex. In addition, the microbiota within ticks is nearly impossible to work with since their detection and identification requires culture independent cultivation techniques (Hunter *et al.*, 2015; Swei and Kwan, 2016; Duan and Cheng, 2017).

1.4.4 Next-generation sequencing technology

Introduction of next-generation sequencing (NGS) technologies, also known as high-throughput sequencing technologies has overcome several limitations of existing molecular approaches for exploration of microorganisms (Greay, 2014; Duan and Cheng, 2017). NGS technologies have the capability to acquire hundreds of thousands of distinctive sequences in one run (Finney *et al.*, 2015). This offers incredible power to investigate a wide range of microbial communities (Greay, 2014; Khoo *et al.*, 2016). When analysing microbial communities, small segments of the 16S rRNA gene are amplified by means of PCR (Bonnet *et al.*, 2014; Finney *et al.*, 2015; Budachetri *et al.*, 2017). This is done with specific primer sets to produce 16S tags. Amplified tags are subsequently subjected to high-throughput sequencing. Specific identifier sequences, also known as bar codes, located on the outside of the region of the primer are included in the initial tag amplification primers that anneal to the target sequence. These barcodes permit correlation of the sequences to be traced back to each specific sample. Phylogenetic information about every microorganism is given when sequence databases are used to analyse each sequence (Finney *et al.*, 2015). Amount of sequences corresponding to a specific taxon are also given and this gives information of the relative abundance of the microorganism present in a specific original sample. Molecular approaches also display the effectiveness of examining tick host-microbiome interactions (Beati and Keirans, 2001).

1.4.5 Illumina MiSeq sequencing platform and microbiome analysis

Generally, the microbiome detection is done by using an Illumina MiSeq platform targeting the hypervariable V3 to V4 regions of the prokaryotic 16S rRNA gene. This process consists of several steps including first stage PCR, first stage PCR clean-up, second stage PCR, second stage PCR clean-up, library quantification, library normalization, library pooling, library denaturation, loading of samples into MiSeq, denaturing and PhiX control, as well as combining the amplicon library and PhiX control. Afterwards two paired-end reads are produced of the raw Illumina sequence for each individual sample (Illumina, 2013).

Due to practical mistakes that may occur during the Illumina MiSeq sequencing process the quality of the reads are checked and bases at the end of reads with low quality bases are removed (Schmieder and Edwards, 2011; Patel and Jain, 2012). The two reads are then combined to produce multiple fasta files of the quality-controlled merged reads for individual samples (Masella *et al.*, 2012; Cole *et al.*, 2014). Several command-line programmes used for metagenomic analysis, such as QIIME, requires a metadata file, also referred to as a mapping file, that is specific to an experiment containing the relevant information of each sample to use for downstream analysis. Information in the mapping file is used to generate a single file containing the sequences of all the individual samples (Kuczunski *et al.*, 2012). The aforementioned file is used to assign operational taxonomic units (OTU's) in the form of bacterial phyla, classes, orders, families, and genera. Operational taxonomic units are normally not assigned on kingdom and species levels. This is as the prokaryotic 16S rRNA gene will only target the bacterial kingdom and as there is too much variation in the V3 and V4 regions to allow OTU picking to species level (Mizrahi-Man *et al.*, 2013; Mysara *et al.*, 2017). Three different strategies are available for picking OTU's including *de novo* OTU picking, closed-reference OTU picking and open-reference OTU picking. *De novo* OTU picking consists of generated reads compared without using a reference database and are assigned into OTU's. Closed-reference OTU picking process consist of the comparison of generated reads to a reference database where matching sequences are clustered in OTU's and mismatching sequences are disregarded from further analysis. Open-reference OTU picking process consists of assigning OTU's where generated reads are compared to a reference database and any mismatching reads are clustered *de novo* (Nguyen *et al.*, 2016). Afterwards an OTU table is generated to use for visualisation of alpha- and beta diversity analysis, such as barcharts, heatmaps and PCoA ordination plots (Sudarikov *et al.*, 2017).

The current study was mainly aimed at identifying ticks infesting dogs in an urban area of Potchefstroom in JB Marks municipality of the North-West Province. Furthermore, this study sought to document pathogens harboured by these dog ticks as well as the gut, salivary glands and whole tick microbiota.

CHAPTER 2

JUSTIFICATION OF THE STUDY, AIM AND OBJECTIVES

2.1 Justification of the study

On a global scale, ticks are of economic, veterinary and medical importance (Rajput *et al.*, 2006). This is because they affect the health of livestock, wild and companion animals, as well as humans (Varela-stokes *et al.*, 2017). Currently, the tick species reported to be infesting dogs in South Africa are *Rhipicephalus sanguineus* and *Haemaphysalis elliptica* (Horak *et al.*, 2018). Some of the most common disease-causing agents infecting dogs in South Africa transmitted by *R. sanguineus* and *H. elliptica* include *Rickettsia rickettsii* (causing Rocky Mountain spotted fever), *Anaplasma phagocytophilum* (causing human granulocytic anaplasmosis), *Ehrlichia canis* (causing canine ehrlichiosis), *Babesia rossi* and *B. vogeli* (causing canine babesiosis) and *Coxiella burnetii* (causing Q fever) (Schorderet-weber *et al.*, 2017). Matjila *et al.* (2004) reported infestation rates ranging from 2 to 29% and 2 to 32% for *Babesia vogeli* and *B. rossi* infecting dogs in various provinces of South Africa, respectively. Kolo *et al.* (2016) reported respective infestation rates of 16 and 70% for *Ehrlichia canis* and *Rickettsia* sp. from blood and ticks collected from dogs in Mpumalanga, South Africa. Mtshali *et al.* (2017) reported an overall infestation rate of 37% for *Rickettsia* sp., 19% for *E. canis*, and 18% for *Anaplasma phagocytophilum* in ticks collected from dogs in four provinces of South Africa.

In the absence of preferred hosts ticks may infest humans and transmit tick-borne pathogens. This is because humans are often in close association with companion animals and ticks are well adapted to survive in urban environments (Vatansever *et al.*, 2008; Varela-stokes *et al.*, 2017). By 2017 it was estimated that there was a population of 87.6 million domestic dogs in South Africa. The dog population will likely continue to increase along with the tick-borne pathogens and associated diseases (Otranto *et al.*, 2017). In addition to the transmission of tick-borne pathogens ticks may also cause host paralysis or allergic reactions due to toxins released when taking a blood meal (Magnarelli, 2009; Karki *et al.*, 2017; Stafford III, Williams and Molaei, 2018).

Ixodid ticks are adapted to survive in warm climates and dry environmental conditions (Magnarelli, 2009; Baneth, 2014). Seasonal variations and yearly average temperature increases resulted in rapid growth of tick populations (de Oliveira, Gazeta and Gurgel-gonçalves, 2017). Ticks are also often introduced to areas due to traveling or migrating hosts. This allows ticks to create new niches and to populate non-endemic areas. As a result, co-infections of tick-borne pathogens in a single host may occur due to ticks present in overlapping geographical distributions (Baneth, 2014; Stafford III, Williams and Molaei, 2018). Clinical signs resulting from co-infections are often difficult to diagnose and require more advanced diagnostic techniques such as molecular diagnostic tools or serological tests (Magnarelli, 2009). Molecular diagnostic tools including PCR, DNA sequencing, and similar methods allow for the detection of tick-borne pathogens and to differentiate between species or strains of organisms (Baneth, 2014).

Tick-borne pathogens are in close association with other non-pathogenic organisms (Baneth, 2014). These organisms may influence the vector competence of recognized tick-borne pathogens (Varela-stokes *et al.*, 2017). Collectively all of these organisms found within ticks are referred to as the microbiome (Sperling *et al.*, 2017). Detection of the majority of these organisms requires cultivation or the use of culture-independent techniques (Davis, 2014). Next-generation sequencing is one of the culture-independent techniques applied for the detection and identification of tick microbiota and determination of tick-host-pathogen interactions (Kazimírová and Štibrániová, 2013).

With lack of information on tick-borne pathogens and microbiota harboured by urban dog ticks in Potchefstroom, this study sought to fill in the information gap by characterising ticks infesting urban dogs admitted at Potchefstroom Animal Welfare Society and detecting tick-borne pathogens of veterinary, medical, and economic importance as well as microbiota they are harbouring.

2.2 Aim

The aim of the study was to identify tick species infesting dogs at the Potchefstroom Animal Welfare Society (PAWS) located in Potchefstroom and to detect the tick-borne pathogens and bacterial communities associated with the dog ticks.

2.3 Objectives

- To characterize ticks morphologically by microscopy and by amplification of the ITS2 and CO1 genes by PCR, sequencing and phylogenetic analysis.
- To detect tick-borne pathogens microscopically by Giemsa-stained blood smears and by DNA based PCR assays.
- To conduct metagenomic diagnosis of microbiota associated with ticks infesting dogs using next-generation sequencing platform.
- To determine an effective sterilization method for the elimination of external bacteria on tick surface.

2.4 Hypothesis

- There are various tick species infesting urban dogs in Potchefstroom.
- Ticks infesting urban dogs in Potchefstroom harbour various pathogenic and non-pathogenic organisms.

2.5 Dissertation outline

Chapter 1: Introduction and literature review

This chapter contains a short introduction relevant to this study. Literature review includes an overview of Ixodid ticks, with the focus on ticks infesting dogs in South Africa. Role of these ticks as vectors and the transmission of tick-borne pathogens is also described, with emphasis on *A. phagocytophilum*, *E. canis*, *B. canis*, *C. burnetii*, and *Rickettsia* spp. Bacteria harboured by ticks, gut microbiota, metagenomics and NGS technologies are also reviewed.

Chapter 2: Justification of the study, aims, and objectives

Study justification explaining the importance of ticks, the associated tick-borne pathogens, bacterial communities harboured by ticks and significance of this study. Aim and objectives of this study are also given in this chapter.

Chapter 3: Materials and methods

This chapter gives a description of the study area and sample collection, identification of ticks, detection of tick-borne pathogens and tick microbiota by PCR and metagenomics. Description of the statistical analysis is also given in this chapter.

Chapter 4: Results

This chapter presents the results including the abundance of ticks, morphological and molecular identification, and phylogenetics. Data on tick-borne pathogens and microbiota harboured by ticks is also presented in this chapter.

Chapter 5: Discussion, conclusion and recommendations

This chapter contains the interpretation of the results regarding the abundance of tick species, morphological and molecular identification of the ticks and tick-borne diseases, phylogenetic analysis, and the metagenomic diagnosis. Results obtained from this study is also compared to previous studies. This chapter also contains conclusion and recommendations for future studies.

CHAPTER 3

MATERIALS AND METHODS

3.1 Study area

3.1.1 North-West Province

South Africa consists of nine provinces where the North-West province is the sixth biggest covering an overall area of approximately 104 822 km² or 9.5% of South Africa (Figure 3-1). It is surrounded by the Republic of Botswana on the north, the Free State province on the south-east, the Northern Cape on the west, and Limpopo and Gauteng provinces on the northeast (Bik *et al.*, 2012).

Majority of the North-West Province, which is approximately 71%, forms part of the Savanna Biome. Remaining 29% forms part of the Grassland Biome (North West Department of Rural, Environmental and Agricultural Development, 2014). North-West Province is divided into four main ecological zones. First zone is the Highveld located towards the South-east. Second zone is the Bushveld located towards the north-east. Third zone includes the Middleveld which is a narrow zone located between the Bushveld and the Highveld. Last zone includes the Kalahari Desert located towards the west (Bik *et al.*, 2012).

Rainfall and climate of the North-West Province may fluctuate from the semi-desert plains of the Kalahari located towards the west to the eastern region which is a wetter and more mountainous area. In general, the climate of the province consists of distinct seasons including sunny, but windy, winters and warm summers. Rain season normally starts from October and ends in March (North West Department of Rural, Environmental and Agricultural Development, 2014).

North-West Province consists of a population of about 3.7 million, where 65% resides in rural areas (Davoren, 2017). Ga-Rankuwa, Rustenburg, and Brits, located towards the eastern region of the Province, are categorized as the more populated industrial regions. Mafikeng is the provincial capital. Potchefstroom, Vryburg, Klerksdorp, Ventersdorp, and Lichtenberg are other major towns located in this province (Bik *et al.*, 2012). North-West Province consists of

four district municipalities, as well as eighteen local municipalities (Figure 3-2) (Main, 2017). Dr Kenneth Kaunda District Municipality sits on the border of Gauteng province and is situated 65 km from Johannesburg in a south-west direction (Figure 3-3). This district municipality makes up 14% of the North-West province. Maquassi Hills, JB Marks, and the City of Matlosana are the three local municipalities included in the Dr Kenneth Kaunda District Municipality (Main, 2017).

JB Marks Local Municipality covers an area of approximately 6 398 km² and is the biggest of three local municipalities in the Dr Kenneth Kaunda District Municipality (Figure 3-4). This municipality was established in August 2016 by the merging of Tlokwe local municipality and the Ventersdorp Local Municipality. JB Marks Local Municipality includes the cities of Potchefstroom and Ventersdorp and it has a population of 243 527, and includes 80 572 households (Main, 2017).



Figure 3-1: Map of South Africa showing different provinces (Bik *et al.*, 2012)

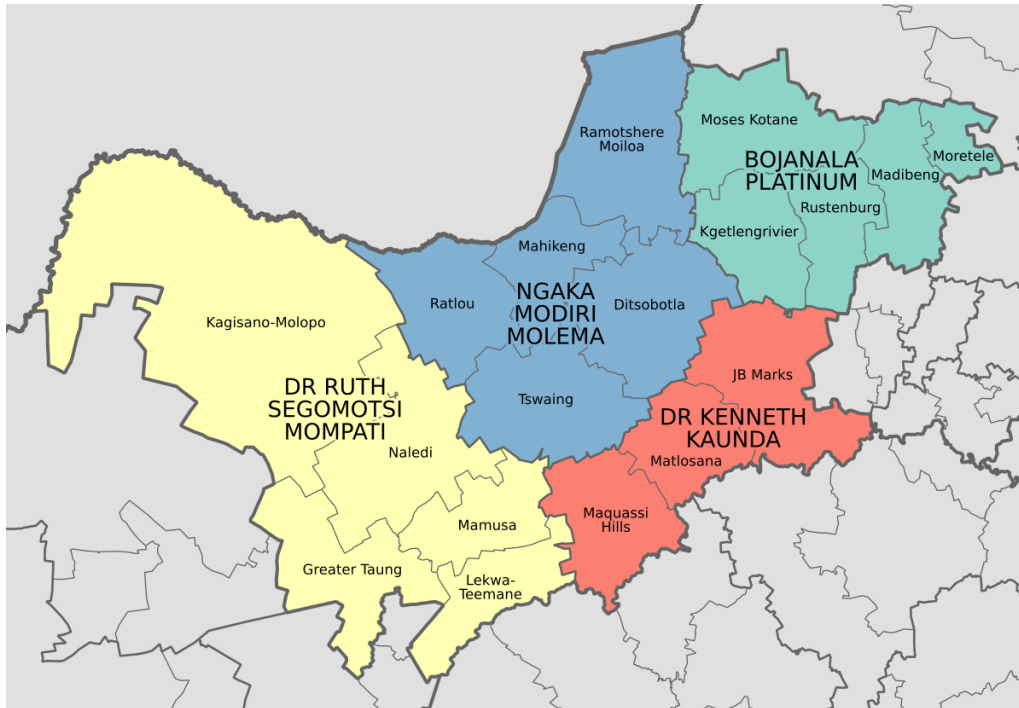


Figure 3-2: Map of North-West Province showing district and local municipalities (Main, 2017)



Figure 3-3: Map of Dr Kaunda District Municipality (Main, 2017)



Figure 3-4: Map of JB Marks Local Municipality (Main, 2017)

3.1.2 Origin of dogs within the study area

All of the tick specimens used in this study were collected by the Potchefstroom Animal Welfare Society (PAWS) from dogs residing in the kennels or from stray dogs rescued from various suburbs located in the JB Marks Local Municipality and incidentally the Merafong City and Emfuleni Local Municipalities. Dogs originated from the Mieder park (26° 45' 11.362" S, 27° 5' 17.6428" E), Die Bult (26° 42' 52.3069" S, 27° 5' 49.371" E), Ikageng (26° 43' 32.3738" S, 27° 2' 59.9492" E), Baillie Park (26° 42' 58.1173" S, 27° 6' 58.2487" E), Kanonnierspark (26° 41' 43.0631" S, 27° 4' 19.3904" E), Boskop (26° 33' 51.9836" S, 27° 7' 44.0036" E), Potchindustrie (26° 43' 7.37" S, 27° 4' 14.0952" E) (Figure 3-5). Samples from one and four dogs were incidentally collected from Fochville (26° 28' 37.4059" S, 27° 29' 27.1457" E) and Boipatong (26° 44' 48.0476" S, 27° 1' 51.492" E), respectively (Figure 3-6).



Figure 3-5: Map of sampling areas in the JB Marks Local Municipality (adapted from SA Venues, 2013). Red stars indicate origin of dogs from whom tick specimens were collected.

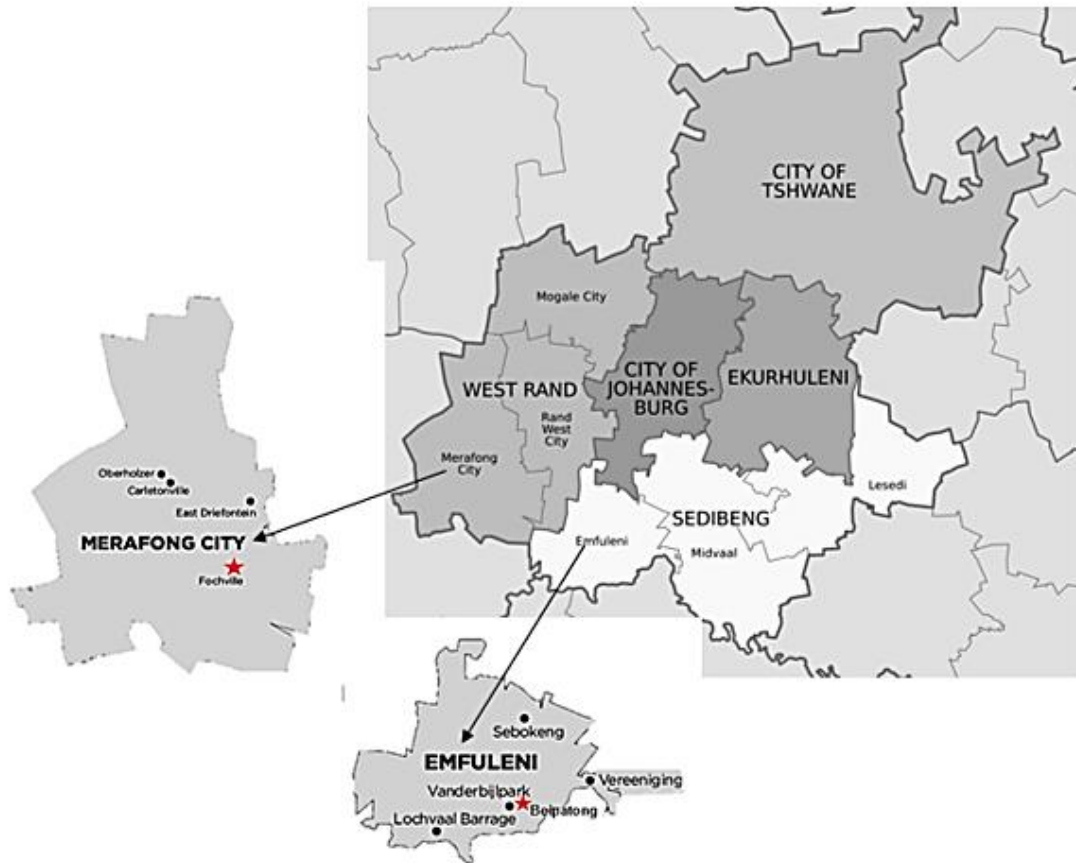


Figure 3-6: Map indicating incidental sampling areas in Merafong City and Emfuleni Local Municipalities (adapted from Main, 2017)

3.2 Sample collection

Tick specimens were collected on a weekly basis from PAWS during 2017 from February to November. Specimens were collected from dogs inside the PAWS kennels as well as from stray dogs rescued and delivered to PAWS. Ticks were also collected from vegetation in surrounding areas of the PAWS offices and kennels by using the flagging and dragging collection method as described by Barker and Walker (2014). After collection, ticks were transferred to collection vials and transported to the North-West University for further processing.

3.3 Morphological identification of ticks

Morphological identification of ticks to species level was done by using the stereo microscope and tick identification keys published by Barker and Walker (2014) and Walker *et al.* (2014). Morphological characteristics used for tick species identification in this study are described in Table 3-1. In addition to tick species the sex, life stage, host, and location of sample collection was also noted. After morphological identification tick samples were stored in 70% ethanol. In cases where ticks laid eggs within collected vials after collection, eggs were collected and stored in 70% ethanol. Tick specimens were also sent to the Gertrud Theiler tick Museum, located at Onderstepoort Veterinary Institute to confirm morphological identification and voucher specimens were issued.

Table 3-1: Morphological characteristics for identification of ticks infesting dogs in South Africa (Barker and Walker, 2014; Walker *et al.*, 2014)

Tick species	Morphological characteristics
<i>Rhipicephalus</i> genus	Short to medium size mouthparts. Commonly basis capituli has a hexagonal shape. Presence of eyes. Presence of festoons. In males, adanal and accessory adanal plates are often present.
<i>R. sanguineus</i>	Size of interstitial punctuation ranges from small to medium. Indistinguishable setiferous punctuations. Eyes are convex. Spiracle plate size is half the size of adjacent festoons and has slender tails. Females: Sharp lateral angles of basis capituli. Wide separation of porose areas. Small palp pedicles. Straight and big shape of cervical fields. Margin of posterior end of the scutum is partly sinuous. Colour of the scutum is often pale. Steep scapular grooves. Texture of the servical fields consists of wrinkled areas. Posterior lips of genital aperture consist of a broad U Shape or broad V shape. Males: Anterior spurs of coxae 1 are dorsally unnoticeable. Indent of cervical fields are ambiguous and texture does not consist of wrinkles. Color of conscutum is often pale. Large accessory adanal plates. Shape of the adanal plates is trapezoid and narrow. Fed males have a broad caudal appendage. Posterior grooves appear to have a deep and wide appearance with wrinkled texture. Lateral grooves are apparent with a smooth texture.
<i>Haemaphysalis</i> genus	Broad and short mouthparts. Basis capituli appear to be rectangular. Inornate scutum. Absence of eyes. Presence of festoons. Males lack adanal plates.
<i>H. elliptica</i>	Large lateral extension of palp articles 2 and appear as a distinctive conical shape. Eleven festoons are present. Dense punctuation distribution. Females: Presence of dorsal spur on palp articles 2 and ventral spur on palp articles 3. Length of spurs of coxae 1 to 3 is medium. Every three festoons are enclosed by a lateral groove. Males: Presence of dorsal and ventral spur on palp articles 2. Length of spurs on coxae 4 is medium. Every two festoons are enclosed by a lateral groove. Long cornua length.

3.4 Molecular analysis of ticks

Molecular identification was done to supplement morphological identification of the collected tick samples.

3.4.1 DNA extraction

Prior to DNA extractions ticks were sterilized with 70% ethanol and double distilled water to eliminate surface contamination. Legs of the ticks were removed and used for molecular identification of the ticks. DNA was extracted by using a modified salting out method as described by Rivero *et al.* (2006). Tick legs were placed in a 1.5 ml Eppendorf tube and crushed by using a sterilized metal pistol. Subsequently, 480 µl of DNA extraction buffer (consisting of 10 mM Tris-HCl [pH 8.0], 10 mM EDTA, and 1% sodium dodecyl sulphate), as well as 10 µl Proteinase K was added to the Eppendorf tube. Contents were mixed for 10 minutes by using the TissueLyser LT (Qiagen, DE) and samples were incubated for 1 hour at 56°C. After incubation another 10 µl Proteinase K was added and the samples were further incubated overnight at 56°C. After overnight incubation, the contents were centrifuged for 5 minutes at 12 000 rpm and the supernatant was transferred to a new 1.5 ml Eppendorf tube. Afterward, 180 µl of 5 M NaCl was added to the supernatant, vortexed for 30 seconds and centrifuged for 5 minutes at 13 500 rpm. Supernatant was then transferred to a new 1.5 ml Eppendorf tube. Subsequently, 420 µl cold isopropanol was added. Reaction mixture was mixed by inverting the Eppendorf tubes fifty times. In order to precipitate the DNA, the reaction mixture was centrifuged for 15 minutes at 13 500 rpm at 4°C. After centrifugation, the supernatant was discarded, and the DNA pellet was washed with 250 µl of 70% ethanol, briefly vortexed and centrifuged for 5 minutes at 4°C. Supernatant was discarded and washed again with 250 µl of 70% ethanol, briefly vortexed and centrifuged for 5 minutes at 13 500 rpm. Supernatant was discarded and samples were left to air dry for 1 hour at room temperature. DNA was dissolved in 50 µl double distilled water. Quantity of DNA was confirmed by using the PCRmax Lambda 3-1 spectrophotometer (Vacutec, ZA). DNA was stored at -35°C until use.

3.4.2 Polymerase chain reaction

Polymerase chain reaction for molecular identification of ticks was performed targeting the internal transcribed spacer 2 (ITS2) and the cytochrome oxidase subunit 1 (CO1) genes by using primers listed in Table 3-2. Each reaction mixture consisted of 12.5 µl AmpliTaq Gold

360 Master Mix (Applied Biosystems, US) 1 µl of 10 µM forward primer, 1 µl of 10 µM of reverse primer and 2 µl template DNA, and 8.5 µl double distilled water to make a total reaction mixture of 25 µl. During PCR 2 µl of the template DNA were added independent of concentration to minimise loss of PCR products during the purification step. Double distilled water was used as the no template control. The *H. longicornis* DNA obtained from the National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Japan was used as positive control. PCR conditions were as follows: Initial denaturation at 95°C for 10 minutes, 35 cycles of denaturation at 95°C for 30 seconds, annealing at 47°C (CO1 gene) and 50°C (ITS2 gene) for 30 seconds, and extension at 72°C for 60 seconds, followed by a final extension at 72°C for 7 minutes and final hold at 4°C ∞, using the ProFlex PCR System (Thermo Fisher Scientific, US). PCR amplicons were visualized using a 1% (w/v) agarose electrophoresis gel stained with ethidium bromide and size fractioned using a 100 bp DNA ladder (New England Biolabs, US). The gel was visualized under UV light using the ENDURO GDS Gel Documentation System (Labnet International, Inc., US).

Table 3-2: Oligonucleotide primers used to amplify ITS2 and CO1 gene for molecular identification of ticks

Target gene	Primer sequence (5'-3')	Fragment size (bp)	Annealing temperature (°C)	Reference
ITS2	Forward 5'- YTG CGA RAC TTG GTG TGA AT -3'	900-1200	50	Muruthi, (2015)
	Reverse 5'- TAT GCT TAA RTT YAG SGG GT -3'			
CO1	LCO1490: 5'- GGT CAA CAA ATC ATA AAG ATA TTG G -3'	710	47	Licari <i>et al.</i> (2017)
	HCO2198: 5'- TAA ACT TCA GGG TGA CCA AAA AAT CA -3'			

3.4.3 Purification and sequencing of amplicons

PCR amplicons were purified using the QIAquick Gel Extraction Kit (Qiagen, DE). Amplified PCR products were cut out of a 1% (w/v) agarose gel stained with electrophoresis by using a sterilized scalpel and transferred to a 1.5 ml Eppendorf tube. Subsequently, 600 µl Buffer QG was added according to the manufacturers instructions. Samples were then incubated at 50°C for 10 minutes. During incubation, Eppendorf tubes were vortexed every 2 minutes to dissolve the gel. After incubation 10 µl of 3 M sodium acetate was added. Afterward, 300 µl isopropanol was added and the mixture was transferred to a QIAquick spin column and 2 ml collection tube and centrifuged for 1 minute at 13 000 rpm. Supernatant gathered in the collection tube was discarded and 500 µl of Buffer QG was added, the mixture was centrifuged for 1 minute at 13 000 rpm and the supernatant was discarded. Subsequently, 750 µl Buffer PE was added, the mixture was centrifuged for 1 minute at 13 000 rpm and the supernatant was discarded. Spin column was centrifuged for an additional 1 minute at 13 000 rpm and the supernatant was discarded. QIAquick column was transferred to a 1.5 ml Eppendorf tube. DNA was eluted by adding 50 µl Buffer EB and centrifuged for 1 minute at 13 000 rpm. After purification purified PCR amplicons were visualized by using a 1% (w/v) agarose electrophoresis gel stained with ethidium bromide to determine the presence of samples.

3.5 Morphological identification of tick-borne pathogens

Babesia canis, *E. canis*, *A. phagocytophilum*, and *Rickettsia* spp. were morphologically detected and identified using Giemsa stained blood smears from engorged ticks.

Thin blood smears were prepared of engorged ticks by using a modified method described by Poostchi *et al.*, (2016). The abdomens of the engorged ticks were punctured by using a sterilized scalpel and a drop of blood was deposited on one side of a microscope slide (specimen slide). A new clean microscope slide (spreader slide) was held towards the drop of blood at a 45° angle on the specimen slide. Blood was spread along the entire width of the spreader slide. Blood smears on the specimen slides were left to air-dry and were fixed with 100% methanol. Thin blood smears were stained with Giemsa's Stain Solution (Merck & Company, Inc., US) by using the rapid (10%) method by using a Pasteur pipette (World Health Organization and Center for Disease Control, 2010). Stain was left for an exposure time of 10 minutes, thereafter the stain was washed with water and the specimen slide was left to air-dry. Giemsa stained blood smears were observed using a light microscope under the oil immersion lens (1000x objective). Detection

and identification were based on the presence of morphological features of tick-borne pathogens as described in Table 3-3.

Table 3-3: Morphological features of pathogens stained with Giemsa stain solution

Tick-borne pathogen	Morphological features	Reference
<i>A. phagocytophilum</i>	Blue cytoplasmic presences within granular leucocytes and monocytes. Usually 1.5 to 5 µm in diameter.	Hartelt <i>et al.</i> , 2004.
<i>E. canis</i>	Dark blue or purple cocci presences within leucocytes. Observed as elementary bodies (0.2 to 0.4 µm in diameter), initial bodies (0.5 to 4 µm in diameter), or large inclusion bodies (4 to 6 µm in diameter).	Perez <i>et al.</i> , 1996; Parola and Raoult, 2001; Taylor <i>et al.</i> , 2007.
<i>B. canis</i>	Organisms 2.4 by 5 µm in size clustered in pairs of two within red blood cells.	Taylor <i>et al.</i> , 2007; Schoeman, 2009; Kostro <i>et al.</i> , 2015.
<i>Rickettsia</i> sp.	Gram-negative, intracellular bacteria observed as short rods.	Azad and Beard, 1998; Parola and Raoult, 2001; Alberdi <i>et al.</i> , 2012.

3.6 Molecular detection of tick-borne pathogens

Molecular identification of tick-borne pathogens from collected ticks and tick eggs was done to supplement morphological identification.

3.6.1 DNA extraction

Ticks samples were pooled according to species, location, and life stage. Egg batches were grouped according to species and location. Prior to DNA extraction ticks were washed with 10% (w/v) Tween 20 for 1 hour, rinsed with 70% ethanol followed by rinsing with double distilled

water to eliminate surface contamination. DNA was extracted by using a modified salting out method as described in section 3.4.1. Quantity of DNA was confirmed by using the PCRmax Lambda 3-1 spectrophotometer (Vacutec, ZA). DNA was stored at -35°C.

3.6.2 Polymerase chain reaction

PCR was performed for screening of tick-borne pathogens using primers shown in Table 3-4. PCR was performed by using *Taq* PCR kit (New England Biolabs, US). Each reaction mixture consisted of 2.5 µl of 10X Standard *Taq* Reaction Buffer, 0.5 µl of 10 mM dNTPs, 0.5 µl of 10 µM forward primer, 0.5 µl of 10 µM reverse primer, 0.125 µl of *Taq* DNA polymerase, 2 µl template DNA, and 18.875 µl double distilled water to make a total reaction mixture of 25 µl. Double distilled water was used as the no template control. *A. phagocytophilum* positive control was obtained PCR positive horse from Northern Cape used in the study by Mlangeni (2016). Positive controls for *E. canis*, *R. Africae* and *C. burnetii* positive controls were acquired from the Centre of Zoonosis Control, Hokkaido University, Japan. Positive control of *B. canis* was obtained from Whitehead Scientific (Pty) Ltd, Cape Town, South Africa. PCR conditions were as follows: Initial denaturation at 95°C for 30 seconds, 30 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C (*B. canis*, *B. rossi*, and *B. Vogeli*), 52°C (*Rickettsia* sp.), 57°C (*C. burnetii*), and 60°C (*A. phagocytophilum* and *E. canis*) for 60 seconds and extension at 68°C for 60 seconds followed by a final extension at 68°C for 5 minutes and final hold at 4°C ∞, using the ProFlex PCR System (Thermo Fisher Scientific, US). PCR amplicons were visualized as described above in section 3.4.2.

Table 3-4: Oligonucleotide primers used for molecular identification of tick-borne pathogens

Tick-borne pathogen	Primer sequence (5'-3')	Fragment size (bp)	Annealing temperature (°C)	Reference
<i>A. phagocytophilum</i>	EHR-521: 5'- TGT AGG CGG TTC GGT AAG TTA AAG -3' EHR-747: 5'- GCA CTC ATC GTT TAC AGG GTG -3'	250	60	Welc-Falęciak <i>et al.</i> (2009)
<i>E. canis</i>	E.c 16S fwd: 5'- TCG CTA TTA GAT GAG CCT ACG T -3' E.c 16S rev: 5'-GAG TCT GGA CCG TAT CTC AGT -3'	154	60	Peleg <i>et al.</i> (2010)
<i>B. canis</i>	BAB1: 5'- GTG AAC CTT ATC ACT TAA AGG -3' BAB3: 5'- CTA CAC AGA GCA CAC AGC C -3'	746	50	Duarte <i>et al.</i> (2008)
<i>B. vogeli</i>	BAB1: 5'- GTG AAC CTT ATC ACT TAA AGG -3' BAB4: 5'- CAA CTC CTC CAC GCA ATC G -3'	590	50	Duarte <i>et al.</i> (2008)
<i>B. rossi</i>	BAB1: 5'- GTG AAC CTT ATC ACT TAA AGG -3' BAB5: 5'- AGG AGT TGC TTA CGC ACT CA -3'	342	50	Duarte <i>et al.</i> (2008)
<i>Rickettsia</i> spp.	Rp877p: 5'- GGG GAC CTG CTC ACG GCG G -3' Rp1258n: 5'- ATT GCA AAA AGT ACA GTG AAC A -3'	381	52	Parola <i>et al.</i> (2003)
<i>C. burnetii</i>	IS1111aF: 5'- CAT CAC ATT GCC GCG TTT AC -3' IS1111aR: 5'- GGT TGG TCC CTC GAC AAC AT -3'	104	57	Roest <i>et al.</i> (2011)

3.7 Metagenomic diagnosis of tick microbiota

Metagenomic diagnosis was performed on adult ticks, as well as on salivary glands, midguts, and eggs of tick specimens.

3.7.1 Dissections of ticks

Ticks were washed with 70% ethanol and rinsed with double distilled water to eliminate surface contamination. Adult ticks were dissected and the salivary glands and midguts were removed following a method described by Patton *et al.*, (2012). Numerous drops containing 25 µl phosphate buffered saline (PBS) were deposited on a sterilized microscope slide. Entire tick was transferred into one of the PBS pools by using sterilized forceps. Tick was viewed under a dissection microscope using the 25X objective. Sterilised fine-tipped forceps were inserted into the posterior part of the tick and the dorsum was severed to reveal the desired organs. Midgut of the tick was removed and transferred to a drop of PBS on the microscope slide. Midgut was then isolated and transferred to a new drop of PBS on the microscope slide. This step was repeated three times to remove unwanted debris. Once midgut was free of unwanted debris it was transferred to a 1.5 ml Eppendorf tube containing 1 ml of PBS. Salivary glands were also removed and washed by following the above-mentioned steps. Midguts and salivary glands were stored at -35°C.

3.7.2 DNA extractions

DNA was extracted from whole tick samples, salivary glands, midguts, and eggs of the different tick species. Midguts and salivary glands were pooled according to the tick species, location of collection, and host from dissected adult ticks as described in section 3.4.1. Each pool consisted of ten midguts or salivary glands. Eggs were pooled in the same manner as the midguts and salivary glands, where each pool consisted of fifty eggs. Each sample representing a whole tick consisted of one adult tick specimen. DNeasy Blood & Tissue kit (Qiagen, DE) was used for DNA extractions. Tick specimens, midguts, salivary glands, and eggs were placed into separate 1.5 ml Eppendorf tubes and crushed using a sterilized metal pistol. Afterward, 180 µl Buffer ATL and 20 µl proteinase K was added to each tube. Tubes were placed into the TissueLyser LT (Qiagen, DE) for 10 minutes. Samples were incubated for 10 minutes at 56°C and were vortexed after every 2 minutes during incubation. After incubation 200 µl of Buffer AL was added to the reaction mixture and vortexed. Subsequently, 200 µl of 99.5% ethanol was added followed by vortexing. Reaction mixture was transferred

into a DNeasy Mini spin column positioned in a 2 ml collection tube, centrifuged for 1 minute at 8 000 rpm, and the supernatant was discarded. Afterward, 500 µl Buffer AW1 was added, reaction mixture was centrifuged for 1 minute at 8 000 rpm and the supernatant was discarded. This was followed by the addition of 500 µl Buffer AW2. Reaction mixture was centrifuged for 3 minutes at 14 000 rpm and the supernatant was discarded. Spin column was transferred to a new 1.5 ml Eppendorf tube. DNA was eluted by adding 50 µl of Buffer AE to the center of the spin column membrane and incubated for 1 minute at room temperature. Tubes were centrifuged for 1 minute at 8 000 rpm. DNA was stored at -35°C.

3.7.3 16S rRNA library preparation

3.7.3.1 Amplicon PCR (First stage PCR)

Amplicon primers consisted of gene-specific sequences with added Illumina adapter overhanging sequences targeting the 16S V3 and V4 region. Primers used during amplicon PCR included the 341F forward primer (5'- TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG -3') and 805R reverse primer (5'- GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C -3') (Klindworth *et al.*, 2013). Forward overhanging (TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG) and Reverse overhanging (GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G) adapters were used (Illumina, 2013). Each PCR reaction mixture consisted of 12.5 µl of 2x KAPA HiFi Hotstart ReadyMix [consisting of KAPA HiFi HotStart DNA Polymerase (1 U/µl), Fidelity Buffer (5X), GC Buffer (5X), and high-quality dNTPs (10 mM each)] (KAPA Biosystems, Massachusetts, USA), 5 µl of 1 µM Amplicon PCR Forward Primer, 5 µl of 1 µM Amplicon PCR Reverse primer, and 2.5 µl microbial DNA. Each PCR reaction mixture had a final volume of 25 µl inside a 96-well 0.2 ml PCR plate (Illumina, 2013). No negative control was included during the processing of the amplicon PCR. PCR conditions were as follows: Initial denaturation at 95°C for 3 minutes, 25 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 30 seconds, followed by a final extension at 72°C for 5 minutes and final hold at 4°C ∞. Size of PCR product was visualized by running 1 µl of PCR product using the Bioanalyzer DNA 1000 chip (Agilent Technologies, DE).

3.7.3.2 First stage PCR clean-up

Amplicon PCR plate was centrifuged for 1 minute at 3 500 rpm at a temperature of 20°C for condensation collection and the seal was removed. AMPure XP beads were left to reach room temperature and vortexed for 30 seconds to guarantee even dispersal of the beads (Illumina, 2013). A multichannel pipette was used to transfer AMPure XP beads to each individual well in the Amplicon PCR plate. A ratio of 0.8 µl AMPure XP beads were added for each µl sample used. After the addition of AMPure XP beads, the mixture was resuspended by pipetting the entire volume upwards and downwards ten times. Samples were then incubated for 5 minutes at room temperature with no shaking. Plate was positioned on a magnetic stand for 2 minutes until the supernatant was clear. Supernatant was then removed and discarded by using a multichannel pipette. Beads were washed twice while the Amplicon PCR plate was positioned on a magnetic stand. During washes 200 µl of newly prepared 80% ethanol was added to each sample well, incubated for 30 seconds, and the supernatant was removed and discarded. Amplicon PCR plate was left to air-dry for 10 minutes and removed from the magnetic stand. A multichannel pipette was used to add 52.5 µl of 10 mM Tris with a pH of 8.5 to each individual amplicon located in the wells of the Amplicon PCR plate. Mixture was gently resuspended and incubated for 2 minutes at room temperature. Again, the plate was positioned on a magnetic stand for 2 minutes, until the supernatant was clear. Afterward, 50 µl supernatant was transferred to a new, clean 96-well PCR plate.

3.7.3.3 Index PCR (Second stage PCR)

From the 50 µl purified PCR amplicons 5 µl was transferred to a new 96-well plate. Remaining 45 µl purified PCR amplicons were stored for further uses. The 96-well plate has horizontally labelled columns from 1 to 12 and vertically labelled rows from A to H. Index 1 and 2 primers were arranged as follows: Index 1 primer tubes, with orange caps and yellow solution, were horizontally aligned with columns 1 to 12, where Index 2 primer tubes, with white caps and clear solution, were vertically aligned with rows A to H in the TruSeq Index Plate Fixture (Illumina, 2013). The 96-well PCR plate containing 5 µl PCR amplicons was positioned in the TruSeq Index Plate Fixture. A PCR reaction mixture with a final volume of 50 µl consisting of 25 µl 2x KAPA HiFi Hotstart ReadyMix [consisting of KAPA HiFi HotStart DNA Polymerase (1 U/µl), Fidelity Buffer (5X), GC Buffer (5X), and high-quality dNTPs (10 mM each)] (KAPA Biosystems, Massachusetts, USA), 10 µl of PCR grade water, 5 µl of Nextera XT Index Primer 1 (N7xx), 5 µl of Nextera XT Index Primer 2 (S5xx), and 5 µl DNA (PCR amplicons from First stage PCR). Reaction was resuspended by pipetting upwards and downwards ten times.

Afterward, the plate was sealed with a Microseal 'A' and centrifuged for 1 minute at 3 500 rpm at 20°C. PCR conditions were as follows: Initial denaturation at 95°C for 3 minutes. Denaturation consisted of 8 cycles at 95°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 30 seconds, followed by a final extension at 72°C for 5 minutes, and a final hold at 4°C ∞.

3.7.3.4 Second stage PCR clean-up

Index PCR plate was centrifuged for 1 minute at 2 500 rpm at a temperature of 20°C for condensate collection. As performed in the First stage PCR cleanup AMPure XP beads were vortexed for 30 seconds to guarantee even dispersal of the beads (Illumina, 2013). A multichannel pipette was used to transfer AMPure XP beads to each individual well in the Index PCR plate. A ratio of 1.15 µl AMPure XP beads were added for each µl sample used. After the addition of AMPure XP beads, the mixture was resuspended by pipetting the entire volume upwards and downwards ten times. Samples were then incubated for 5 minutes at room temperature with no shaking. Plate was positioned on a magnetic stand for 2 minutes until the supernatant was clear. Supernatant was then removed and discarded by using a multichannel pipette. Beads were washed twice while the Index PCR plate was positioned on a magnetic stand. During washes 200 µl of newly prepared 80% ethanol was added to each sample well, incubated for 30 seconds, and the supernatant was removed and discarded. Index PCR plate was left to air-dry for 10 minutes and removed from the magnetic stand. A multichannel pipette was used to add 27.5 µl of 10 mM Tris with a pH of 8.5 to each individual amplicon located in the wells of the Index PCR plate. Mixture was gently resuspended and incubated for 2 minutes at room temperature. Again, the plate was positioned on a magnetic stand for 2 minutes until the supernatant was clear. Afterward, 50 µl supernatant was transferred to a new, clean 96-well PCR plate.

3.7.3.5 Library quantification, normalization, and pooling

By using the Agilent Technologies 2100 Bioanalyzer trace (Agilent Technologies, Waldbronn, Germany) the DNA concentration in nanomolar was determined. Calculation used to determine DNA concentration was as follows:

$$\frac{\text{Concentration in ng/}\mu\text{l}}{660 \text{ g/mol} \times \text{average library size}} \times 10^6 = \text{Concentration in nM}$$

After DNA concentrations were determined DNA was diluted to reach a concentration of 4 nM for preparation of the library (Illumina, 2013). This was done by using 10 mM Tris with pH 8.5. Pooling libraries with unique indices were done by mixing 5 μl DNA that was diluted from each individual library.

3.7.3.6 Library denaturation and loading of samples into MiSeq

Heat block with the ability to contain 1.7 ml microcentrifuge tubes was set to a temperature of 96°C. MiSeq reagent cartridge (Illumina, USA) was exposed to room temperature and ice-water bath containing 1 part water and 3 parts ice was made. Afterward, in a microcentrifuge tube, 5 μl of the 4 nM pooled DNA library and 5 μl newly diluted 0.2 N NaOH was mixed, vortexed, and centrifuged for 1 minute at 2 500 rpm. Samples were then incubated for 5 minutes at room temperature to allow the DNA to denature into single strands (Illumina, 2013). Afterward, a volume of 990 μl of pre-chilled HT1 was mixed with 10 μl of the denatured DNA. Denatured DNA was put on ice to prepare for the final dilution step.

3.7.3.7 Denaturing and dilution of PhiX control

PhiX library was diluted to 4 nM by mixing 3 μl of 10 mM Tris pH 8.5 and 2 μl of 10 nM PhiX library. Afterward, 5 μl of the 4 nM PhiX library was combined with 5 μl of 0.2 N NaOH inside a microcentrifuge tube. Microcentrifuge tube containing the mixture was vortexed and incubated for 5 minutes at room temperature in order for the PhiX library to denature to make single strands (Illumina, 2013). In order to prepare 20 pM PhiX library, 990 μl of pre-chilled HT1 and 10 μl of denatured PhiX library was combined in a microcentrifuge tube, inverted numerous times, pulse centrifuged, and placed on ice.

3.7.3.8 Combination of amplicon library and PhiX control

Two libraries were combined where 570 µl of the denatured and diluted amplicon library was mixed with 30 µl of the denatured and diluted PhiX control. These combined libraries were put on ice before performing the heat denaturation step (Illumina, 2013). Afterward, the heat denaturation step was performed where PhiX control and combined library was incubated at 96°C for 2 minutes. Tubes containing the mixture were inverted twice to ensure mixing and placed on ice for 5 minutes. Samples were placed into the MiSeq flow cell (Illumina, USA) and examined with MiSeq Reporter Software (MRS) by making use of the metagenomic workflow option from the MiSeq system.

3.7.4 Metagenomic data analysis

3.7.4.1 Sequence preparation

Prior to metagenomic data analysis, VirtualBox 5.2.8 and QIIME 1.9.1 64 bit was installed. Additionally, before data analysis, PAired-end Assembler for Illumina sequences (PANDAsseq), SILVA 128 reference database, usearch61, and FastQC version 0.11.7 was installed. Metagenomic sequences from samples were produced from the MiSeq system and retrieved in FASTQ format. FastQC version 0.11.7 (Schmieder and Edwards, 2011; Patel and Jain, 2012) was used to perform quality control of the forward and reverse sequence reads of each sample. Open source bioinformatics pipeline Quantitative Insights Into Microbial Ecology (QIIME) (Caporaso *et al.*, 2010; Nguyen *et al.*, 2016) was used for metagenomic analysis.

3.7.4.2 Merging sequence reads

PANDAsseq was used to merge the forward and reverse reads of each sample (Masella *et al.*, 2012; Cole *et al.*, 2014). Minimum and maximum length of the assembled reads were 250 and 475 bp, respectively. Minimum and maximum overlap between the forward and reverse reads were 75 and 200 bp, respectively. Sequences with a threshold of less than 0.7 were considered as low quality and were removed, to ensure that the final sequences had fewer unmached bases (Masella *et al.*, 2012). All sequences containing uncalled nucleotides from unpaired regions were also removed. A Bayesian classifier was used to ensure all sequences were from prokaryotes.

3.7.4.3 Combining merged sequence labels

A mapping file was created consisting of a spreadsheet where the first four columns consisted of information of the sample name, barcode sequence, primer sequence, and the name of the merged sequences, respectively. Afterward, the mapping file was saved as a CSV tab delimited file. Mapping file was then validated and necessary corrections were made. By using the mapping file all of the individuals merged sequences were combined into a single FNA file used for OTU picking.

3.7.4.4 Operational taxonomic units (OTU) picking

For this part of metagenomic data analysis sequences with 97% or above similarity were clustered together into OTU's. Open-reference OTU picking was done where sequences were clustered using a reference database and non-matching sequences were clustered *de novo* (Bik *et al.*, 2012) Open-reference OTU picking was done by using SILVA 128 reference database (Quast *et al.*, 2013; Yilmaz *et al.*, 2014) and usearch61 (Edgar, 2010; Kopylova *et al.*, 2016).

3.8 Statistical analysis

Confidence interval (CI) of an average of 95% was used to determine tick prevalence. Distribution of ticks was determined on Rstudio by using the Pearson's chi-square test. Sequences obtained from DNA extracted from tick legs to determine tick species from Inaba Biotechnological Industries (Pty) Ltd. were viewed and edited using Molecular Evolutionary Genetics Analysis (MEGA) version 7.0. During editing of sequences degenerate nucleotides (H, D, B, W, S, Y, R, M, and K) were replaced with a suitable nucleotide (A, C, G, and T) determined by color peaks produced by the chromatogram (Ma *et al.*, 2015). Basic Local Alignment Search Tool (BLAST) for nucleotides was used to compare DNA of ticks relevant to the study to other tick sequences in the National Center for Biotechnical Information (NCBI) database to confirm identification. Homologous sequences of other related tick species were obtained from the NCBI database. All of the sequences were added to an Alignment explorer in MEGA 7 where the names of the sequences were edited to represent the tick species, country collected, and accession number in the case of reference sequences. Sequences were aligned by ClustalW. Phylogenetic trees were then constructed by using the maximum likelihood statistical method at 10 000 bootstrap replications. Nucleotide substitution models

were determined by using the lowest Bayesian Information Criterion (BIC) score. Rates among sites were treated as uniform rates and gaps or missing data were deleted during phylogenetic analysis.

Alpha and Beta diversity analysis of metagenomic data were conducted and visualised using QIIME and Rstudio. Alpha- and beta diversity was performed on QIIME simultaneously by performing core diversity analysis. This was done using a sampling depth of 1000 sequences to minimise the loss of identification of bacterial communities in the case of significant loss of sequences during merging and alignment. Alpha diversity was calculated for samples by different tick components (whole ticks, eggs, midguts, and salivary glands) and tick species (*R. sanguineus* and *H. elliptica*) by using detected OTUs. Shannon entropy of counts, Simpson's index, and chao1 richness estimators were used. Beta diversity analysis was performed to determine differentiation between the different tick components.

3.9 Sterilisation methods for the elimination of ticks surface bacteria

Five different washing procedures were tested on *R. sanguineus* and *H. elliptica* ticks to determine which method was more effective for the removal of surface bacteria. For the first washing method ticks were surface sterilized with 70% ethanol and rinsed with double distilled water. Second, third, fourth, and fifth washing method consisted of surface sterilization of the specimens with 10% bleach, 10% Tween 20, 10% Triton X, and PBS, respectively. These sterilization methods were used on tick samples for a period of 1 hour, 2 hours, and 3 hours, respectively. After surface sterilization ticks were surface sterilized again by using 70% ethanol and rinsed with double distilled water.

To determine the effectiveness of the washing methods several samples were prepared, and photos were taken to determine if surface bacteria was removed. For sample preparation samples were transferred to 99.6% ethanol and left overnight. This was done twice to remove excess water. Afterward, samples were placed in holders with perforations in 99.6% ethanol. By using liquid carbon dioxide (CO₂) the samples were critical point dried (Weibel and Ober, 2003). During critical point drying the perforated holders containing the samples were transferred to a critical point dryer. Here, under high pressure, the 99.6% ethanol was substituted with CO₂. Samples were transferred to a stub containing double sided-conductive

tape and the stub was placed inside an IB2 Ion Coater (EIKO Engineering Co. Ltd., Ibaraki, Japan) and coated with a layer of gold.

PCR molecular assays were performed to determine if there was possible damage to samples. DNA was extracted by using a modified salting out method as described in section 3.4.1. PCR was performed targetting the 18S rRNA gene by using the 18S-F (5'-CAT TAA ATC AGT TAT GGT TCC-3') and 18S-R (5'-CGC CGC AAT ACG AAT GC-3') primers (Lv, Wu, Zhang, Zhang, *et al.*, 2014). Each reaction mixture consisted of 12.5 μ l AmpliTaq Gold 360 Master Mix (Applied Biosystems, US), 1 μ l of 10 μ M forward primer, 1 μ l of 10 μ M of reverse primer, 2 μ l template DNA, and 8.5 μ l double distilled water to make a total reaction mixture of 25 μ l. Double distilled water was used as negative control. The *H. longicornis* DNA obtained from the National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Japan was used as positive control. PCR conditions were as follows: Initial denaturation at 95°C for 10 minutes, 35 cycles of denaturation at 95°C for 30 seconds, annealing at 52°C for 30 seconds and extension at 72°C for 60 seconds, followed by a final extension at 72°C for 7 minutes and final hold at 4°C ∞ , using the ProFlex PCR System (Thermo Fisher Scientific, US). PCR amplicons were visualized using a 1% (w/v) agarose electrophoresis gel stained with ethidium bromide and size fractioned using a 100 bp DNA ladder (New England Biolabs, US). The gel was visualized as described under section 3.4.2.

CHAPTER 4

RESULTS

4.1 Tick specimens

A total of 592 ticks were collected from 61 dogs brought to PAWS, in Potchefstroom, JB Marks Local Municipality. Few dogs at PAWS were from the Merafong City and Emfuleni Local Municipalities. From the JB Marks Local Municipality the origin of dogs from which ticks were collected were from Miederpark (38.9%), followed by Ikageng (14.5%), Potchindustrial (5.4%), Boskop (4.6%), Baillie Park (3.7%), Die Bult (3.2%), and Kannonierspark (0.5%) suburbs. Incidental tick samples were from dogs originating from Merafong City Local Municipality, Fochville (1.5%), and Emfuleni Local Municipality, Boipatong (7.3%). Twenty percent of the sampled dogs were admitted at PAWS having been collected in Potchefstroom, but their exact locations of origin was unknown (Table 4-1). There was a significant difference [$p < 2.2e-16$ ($X^2 = 229.6$, $df = 9$)] in distribution of dog ticks collected from various suburbs within and surrounding the JB Marks Local Municipality.

Table 4-1: Tick specimens collected from dogs originating from various locations

Location	Tick species		Total number of ticks per location	Overall occurrence of ticks per location (%)
	<i>Haemaphysalis elliptica</i>	<i>Rhipicephalus sanguineus</i>		
Unknown locations	4	117	121	20.4
Potchindustrie	7	25	32	5.4
Boskop	21	6	27	4.6
Die Bult	11	8	19	3.2
Baillie park	4	18	22	3.7
Miederpark	133	97	230	38.9
Boipatong*	38	5	43	7.3
Ikageng	4	82	86	14.5
Fochville*	9	0	9	1.5
Kannonierspark	0	3	3	0.5
Total number of ticks	231	361	592	

* Not part of J.B. Marks Local Municipality

4.2 Morphological identification of ticks

Two tick species, namely, *Haemaphysalis elliptica* (Plate 4-1) and *Rhipicephalus sanguineus* (Plate 4-2) were identified as species infesting sampled dogs. These tick specimens were submitted to the tick museum at ARC-Onderstepoort Veterinary Research where identification was verified and voucher specimen was issued (Appendix 1). Distinctive morphological features that were used for identification of tick species were based on keys published by Barker and Walker (2014) and Walker *et al.* (2014) (Plate 4-3 and 4-4).

The *H. elliptica* had an overall occurrence of 39% (231/592) and *R. sanguineus* had an overall occurrence of 61% (361/592) (Figure 4-1). The *H. elliptica* was most abundant in Miederpark (22.47%) followed by Boipatong (6.42%), Boskop (3.55%), Die Bult (1.86%), Fochville (1.52%), Potchindustrie (1.18%), Baillie park (0.68%), Ikageng (0.68%), and from unknown locations (0.68%). The *R. sanguineus* was most abundant in unknown locations (19.76%), Miederpark (16.39%), and Ikageng (13.85%). This was followed by Potchindustrie (4.22%),

Baillie Park (3.04%), Die Bult (1.35%), Boskop (1.01%), Boipatong (0.84%), and Kannonierspark (0.51%) (Figure 4-2).



Plate 4-1: *Haemaphysalis elliptica*



Plate 4-2: *Rhipicephalus sanguineus*

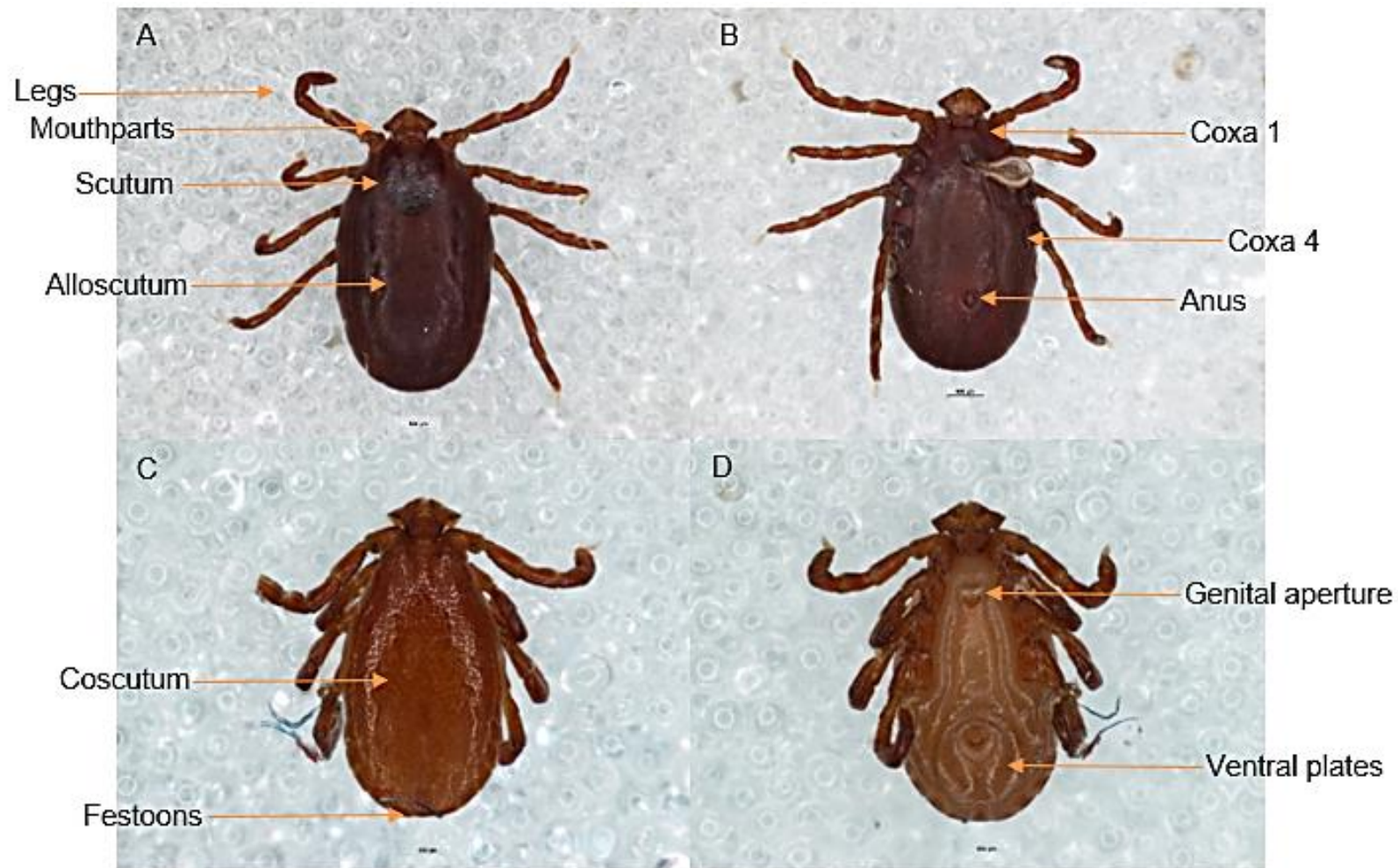


Plate 4-3: *Haemaphysalis elliptica* tick. Female dorsal view (A) and ventral view (B). Male dorsal view (C) and ventral view (D)

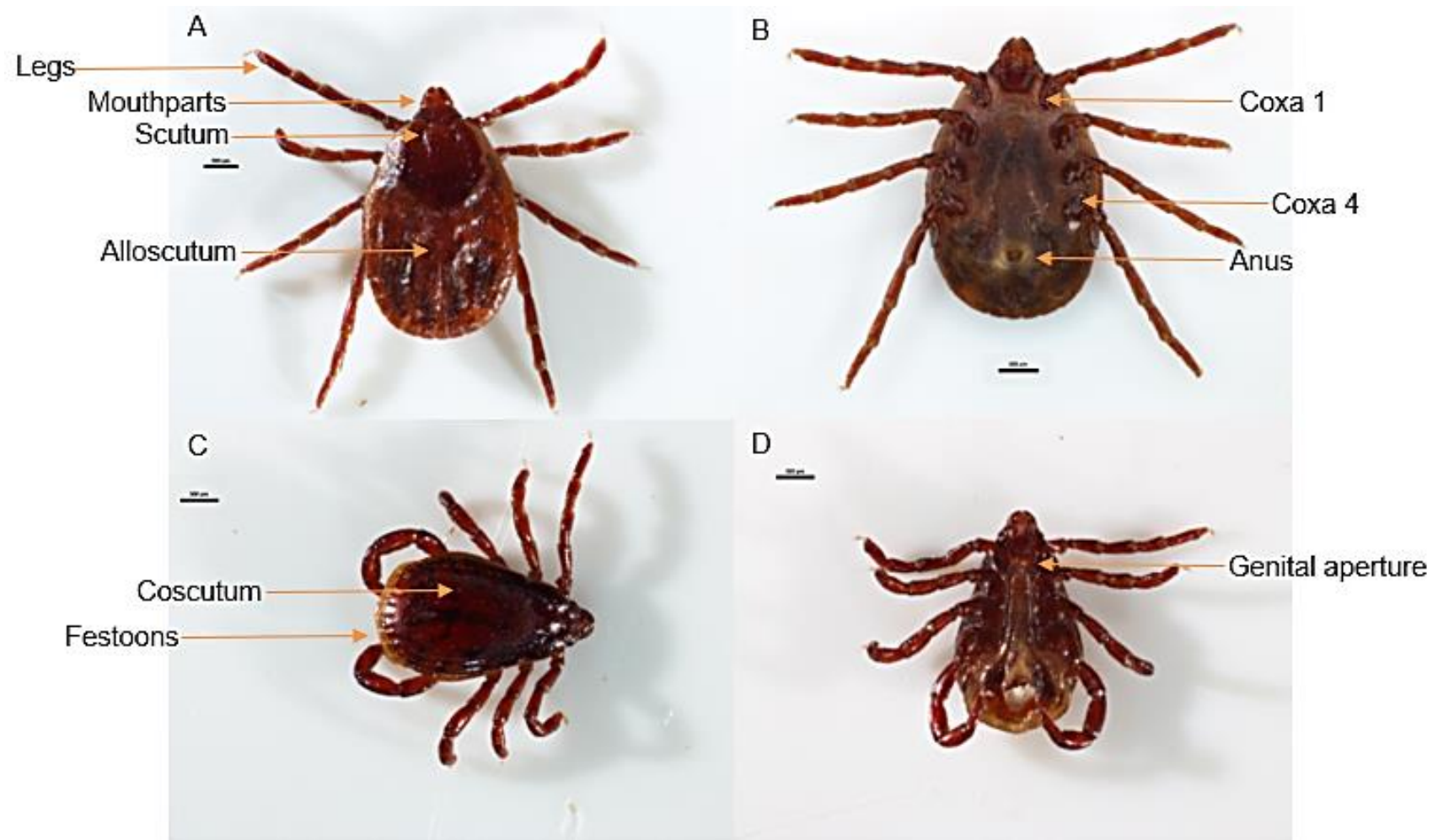


Plate 4-4: *Rhipicephalus sanguineus* tick. Female dorsal view (A) and ventral view (B). Male dorsal view (C) and ventral view (D)

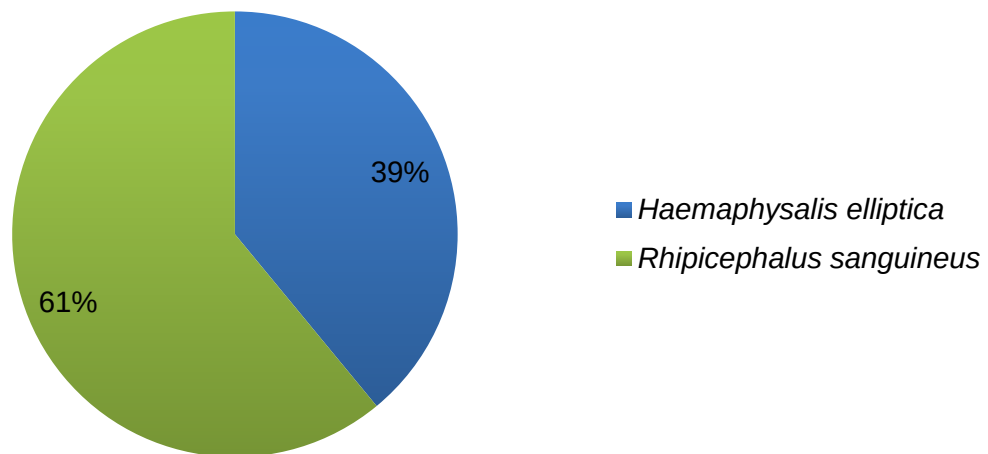


Figure 4-1: Overall occurrence of tick species collected from dogs at PAWS

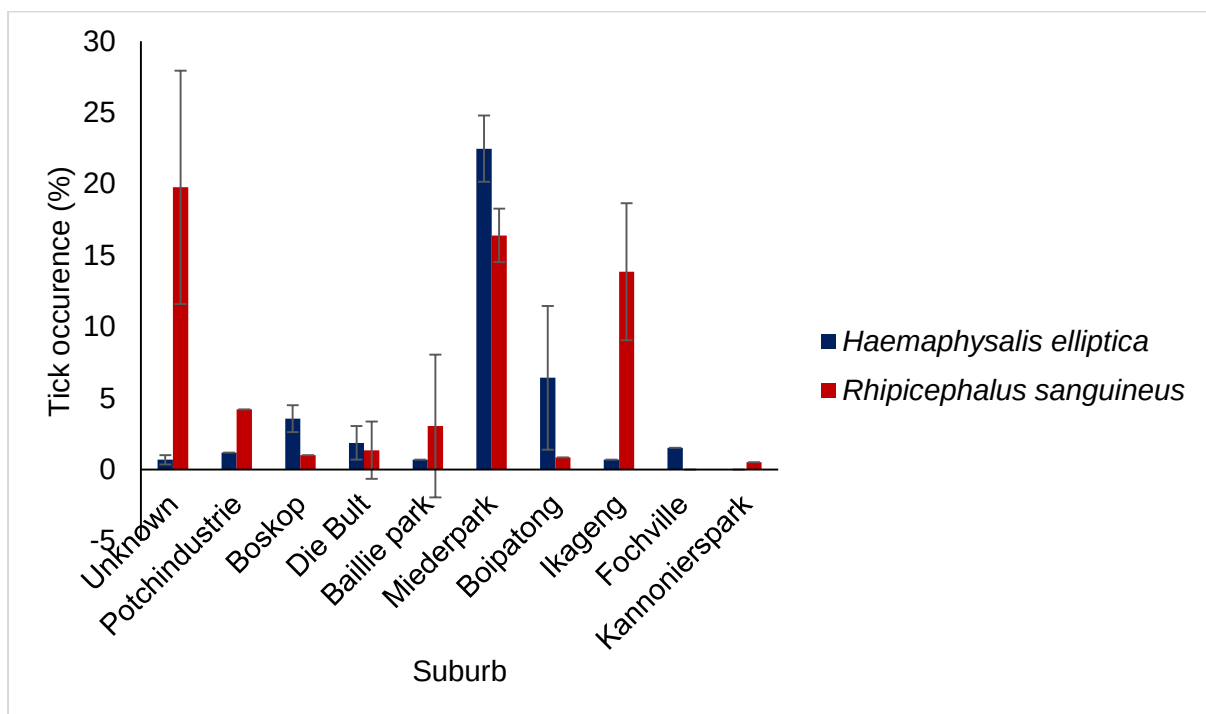


Figure 4-2: Overall occurrence of tick species infesting dogs admitted at PAWS originating from various suburbs

4.3 Molecular identification of ticks

Molecular identification was done to supplement morphological identification of the tick species. This was done by DNA extraction of the tick legs and amplification of the CO1 and ITS2 genes. Extracted DNA concentrations ranged from 0.831 to 4.991 ng/μl.

4.3.1 CO1 gene of dog ticks

Amplification of the ticks DNA targeting the CO1 gene was successful with a PCR product size of 710 bp (Figure 4-3). Average sequenced length ranged between 684 and 712 bp (Table 4-2). BLASTn search was performed where sequences of this study were compared to other sequences on the NCBI database. Similar sequences returned on the NCBI database with the top ranked hits were highly regarded as identification of tick species. BLASTn search results of *R. sanguineus* (Sample ID: R1) of this study were similar to *R. sanguineus* sequences (GenBank accession numbers: JQ737084.1 and MF425993.1) on the NCBI database with query covers of 98%, matching identifications of 99% and E-values of 0. For *H. elliptica* no reference sequences were available on the NCBI database. However, BLASTn results did reveal that *H. elliptica* (Sample ID: H1 and H2) sequences obtained from this study were similar to those of an *Ixodidae* sp. (GenBank accession number: KR262491.1) and other species of the *Haemaphysalis* genus (GenBank accession numbers: KU880573.1 and KY364906.1) on the NCBI database with query covers ranging from 87 to 99%, matching identifications ranging from 87 to 99% and E-values of 0.

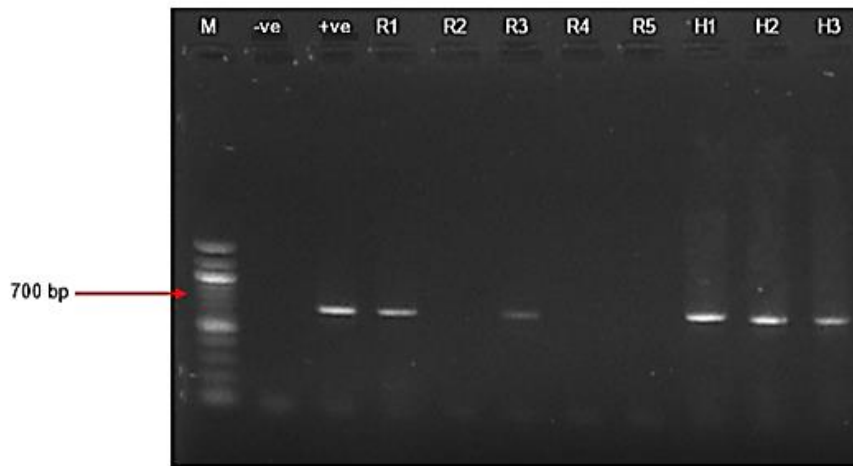


Figure 4-3: The 1% agarose gel electrophoresis of DNA amplicons from ticks targeting the CO1 gene with an expected product size of 710 bp. Lane 1 (M): 100 bp DNA molecular marker. Lane 2 (-ve): No template control. Lane 3 (+ve): Positive control. Lane 4 to 8 (R1 to R5): Amplified DNA from *R. sanguineus*. Lane 9 to 11 (H1 to H3): Amplified DNA from *H. elliptica*

Table 4-2: BLASTn results of the CO1 gene of *H. elliptica* and *R. sanguineus* ticks

Sample ID	Gene size (bp)	Morphological identification	BLAST description	Maximum score	Total score	Query cover	E-value	Identity	Accession number
R1	684	<i>Rhipicephalus sanguineus</i>	<i>Rhipicephalus sanguineus</i>	1192	1192	98%	0.0	99%	JQ737084.1
			<i>Rhipicephalus sanguineus</i>	1190	1190	98%	0.0	99%	MF425993.1
			<i>Rhipicephalus sanguineus</i>	1184	1184	96%	0.0	99%	KT906186.1
			<i>Rhipicephalus sanguineus</i>	1181	1181	98%	0.0	98%	MF425996.1
			<i>Rhipicephalus sanguineus</i>	1181	1181	98%	0.0	98%	MF425994.1
H1	711	<i>Haemaphysalis elliptica</i>	<i>Ixodidae</i> sp.	1134	1134	87%	0.0	99%	KR262491.1
			<i>Haemaphysalis erinacei</i>	791	791	99%	0.0	87%	KU880573.1
			<i>Haemaphysalis concinna</i>	763	763	99%	0.0	86%	KY364906.1
			<i>Rhipicephalus compositus</i>	758	758	99%	0.0	86%	AF132834.1
			<i>Haemaphysalis flava</i>	741	741	99%	0.0	86%	JQ737097.1
H2	712	<i>Haemaphysalis elliptica</i>	<i>Ixodidae</i> sp.	1123	1123	87%	0.0	99%	KR262491.1
			<i>Haemaphysalis erinacei</i>	785	785	97%	0.0	87%	KU880573.1
			<i>Haemaphysalis concinna</i>	754	754	94%	0.0	87%	KR108860.1
			<i>Haemaphysalis qinghaiensis</i>	725	725	99%	0.0	85%	JQ737099.1
			<i>Haemaphysalis flava</i>	730	730	99%	0.0	85%	JQ737097.1

Phylogenetic analysis was done by using the Maximum likelihood (ML) method with sequences of the *Haemaphysalis* and *Rhipicephalus* genera, where *Myialges* sp. was used as an outgroup. Phylogenetic analysis revealed two major clades with bootstrap support values of more than 50%. Clade I consisted of *Rhipicephalus* species and Clade II consisted of *Haemaphysalis* species. *Rhipicephalus sanguineus* sequences of ticks from the JB Marks Local Municipality clustered with other *R. sanguineus* species forming a clade with a bootstrap support value of 100%. *Haemaphysalis elliptica* sequences of ticks from the JB Marks Local Municipality clustered with other Species of the *Haemaphysalis* genus forming a clade with a bootstrap support value of 69% (Figure 4-4).

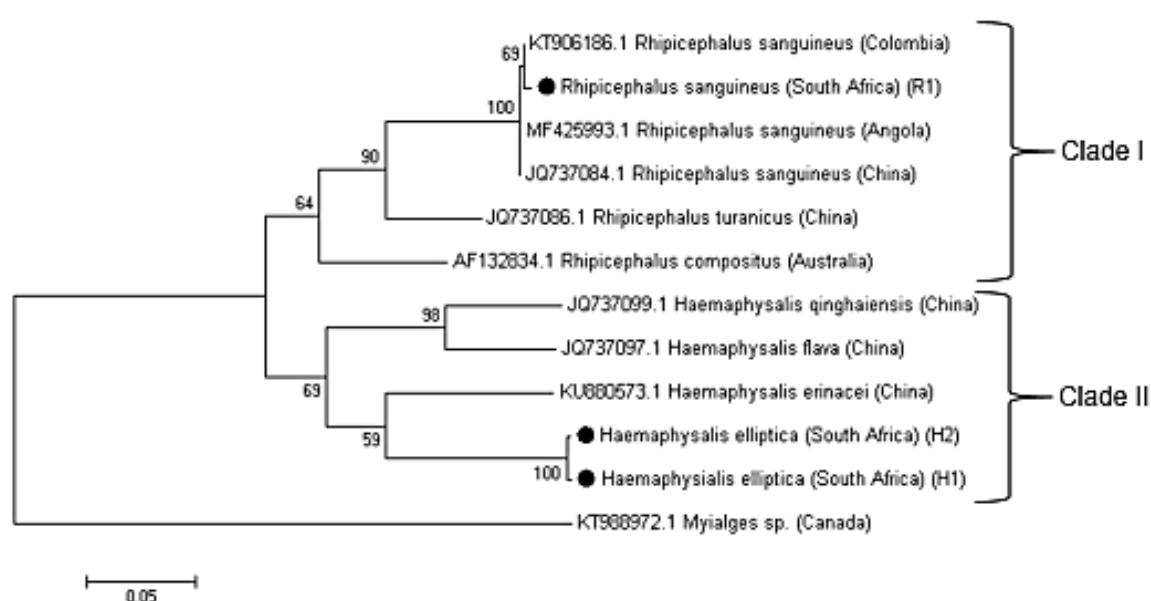


Figure 4-4: Phylogenetic analysis of CO1 gene by using the Maximum Likelihood (ML) method based on the General Time Reversible (NTR) model (Nei and Kumar, 2000). Bootstrap percentage of 10 000 replicates, in which the associated taxa are clustered together is displayed next to the branch nodes. Twelve nucleotide sequences were used for data analysis. Sequences of the phylogenetic tree are displayed as GenBank accession number, species, and country of collection. Sequences of this study are indicated by a black bullet and also contain the sample ID. Phylogenetic analysis was done by using MEGA 7 (Kumar *et al.*, 2016)

For phylogenetic analysis, the final dataset consisted of 534 positions, where missing data and gaps were removed. Table 4-3 shows the base substitutions per site of the sequences used in this study. For *H. elliptica* the sequence divergence among other species of the *Haemaphysalis* genera was between 0.188 and 0.250. For *R. sanguineus* the sequence divergence among other *R. sanguineus* species was 0.004 and 0.006. The p-distance matrix for sequences of the *Haemaphysalis* and *Rhipicephalus* genera indicated strong resolution power ranging between 0 to 0.328% (Table 4-4).

Table 4-3: Number of base substitutions per site of sequences of the CO1 gene

	1	2	3	4	5	6	7	8	9	10	11	12
[1] KT988972.1 <i>Myialges</i> sp. (Canada)		4.000	3.956	3.556	3.721	3.700	3.850	3.850	3.850	3.851	4.078	4.087
[2] JQ737099.1 <i>Haemaphysalis qinghaiensis</i> (China)	0.841		0.578	1.441	1.597	1.711	1.624	1.625	1.625	1.625	1.486	1.480
[3] JQ737097.1 <i>Haemaphysalis flava</i> (China)	0.784	0.106		2.404	1.557	1.430	1.580	1.581	1.581	1.581	1.554	1.548
[4] AF132834.1 <i>Rhipicephalus compositus</i> (Australia)	0.691	0.205	0.208		1.853	1.441	2.419	2.419	2.419	2.993	1.504	1.498
[5] JQ737086.1 <i>Rhipicephalus turanicus</i> (China)	0.721	0.241	0.228	0.149		1.605	2.554	2.302	2.302	2.641	1.907	1.555
[6] KU880573.1 <i>Haemaphysalis erinacei</i> (China)	0.739	0.199	0.218	0.202	0.243		1.675	1.675	1.675	1.676	1.170	2.790
[7] KT906186.1 <i>Rhipicephalus sanguineus</i> (Colombia)	0.776	0.240	0.225	0.179	0.117	0.254		0.002	0.002	0.003	1.648	1.656
[8] MF425993.1 <i>Rhipicephalus sanguineus</i> (Angola)	0.776	0.244	0.228	0.179	0.115	0.251	0.002		0.000	0.004	1.648	1.655
[9] JQ737084.1 <i>Rhipicephalus sanguineus</i> (China)	0.776	0.244	0.228	0.179	0.115	0.251	0.002	0.000		0.004	1.648	1.655
[10] <i>Rhipicephalus sanguineus</i> (South Africa) (R1)	0.798	0.249	0.233	0.186	0.123	0.263	0.004	0.006	0.006		1.649	1.657
[11] <i>Haemaphysalis elliptica</i> (South Africa) (H2)	0.851	0.231	0.250	0.226	0.242	0.188	0.262	0.259	0.259	0.270		0.003
[12] <i>Haemaphysalis elliptica</i> (South Africa) (H1)	0.841	0.230	0.249	0.225	0.241	0.188	0.269	0.266	0.266	0.278	0.004	

*Black numbers indicate number of base substitutions per site and blue numbers indicate standard errors.

Table 4-4: Pairwise (p) distance analysis of sequences of the CO1 gene

	1	2	3	4	5	6	7	8	9	10	11	12
[1] KT988972.1 <i>Myialges</i> sp. (Canada)		0,019	0,019	0,019	0,019	0,019	0,019	0,019	0,019	0,019	0,020	0,020
[2] JQ737099.1 <i>Haemaphysalis qinghaiensis</i> (China)	0,326		0,012	0,015	0,016	0,015	0,016	0,016	0,016	0,016	0,015	0,016
[3] JQ737097.1 <i>Haemaphysalis flava</i> (China)	0,309	0,090		0,015	0,016	0,014	0,015	0,016	0,016	0,016	0,016	0,016
[4] AF132834.1 <i>Rhipicephalus compositus</i> (Australia)	0,301	0,144	0,146		0,013	0,014	0,015	0,015	0,015	0,015	0,015	0,015
[5] JQ737086.1 <i>Rhipicephalus turanicus</i> (China)	0,303	0,165	0,159	0,116		0,016	0,013	0,013	0,013	0,013	0,016	0,016
[6] KU880573.1 <i>Haemaphysalis erinacei</i> (China)	0,309	0,146	0,155	0,144	0,165		0,015	0,015	0,015	0,015	0,014	0,014
[7] KT906186.1 <i>Rhipicephalus sanguineus</i> (Colombia)	0,316	0,165	0,155	0,133	0,097	0,169		0,002	0,002	0,002	0,016	0,016
[8] MF425993.1 <i>Rhipicephalus sanguineus</i> (Angola)	0,316	0,167	0,157	0,133	0,096	0,167	0,002		0,000	0,003	0,016	0,016
[9] JQ737084.1 <i>Rhipicephalus sanguineus</i> (China)	0,316	0,167	0,157	0,133	0,096	0,167	0,002	0,000		0,003	0,016	0,016
[10] <i>Rhipicephalus sanguineus</i> (South Africa) (R1)	0,320	0,169	0,159	0,137	0,101	0,172	0,004	0,006	0,006		0,016	0,016
[11] <i>Haemaphysalis elliptica</i> (South Africa) (H2)	0,328	0,161	0,170	0,157	0,165	0,140	0,176	0,174	0,174	0,180		0,003
[12] <i>Haemaphysalis elliptica</i> (South Africa) (H1)	0,326	0,161	0,170	0,157	0,165	0,140	0,180	0,178	0,178	0,184	0,004	

*Black numbers indicate p-distances and blue numbers indicate standard errors.

	ATT	CGA	ATA	GAA	TTA	GGA	CAA	ACC	GGG	TAC	ATT	AAT	TGG	AAA	TGA	CCA	AAT	TTA	TAA	TGT
#Haemaphysalis_elliptica (South Africa) (H1)	..C	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
#Haemaphysalis_elliptica (South Africa) (H2)	..C	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
#JQ737059.1_Haemaphysalis_qinghaiensis (China)	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
#KU880573.1_Haemaphysalis_erinacei (China)	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
#JQ737057.1_Haemaphysalis_flava (China)	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
#Rhhipicephalus_sanguineus (South Africa) (R1)	..C	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
#AF132834.1_Rhipicephalus_compositus (Australia)	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
#JQ737086.1_Rhipicephalus_turanicus (China)	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
#KT906186.1_Rhipicephalus_sanguineus (Colombia)	..C	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
#MF425993.1_Rhipicephalus_sanguineus (Angola)	..C	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
#JQ737084.1_Rhipicephalus_sanguineus (China)	..C	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
#KT988972.1_Myalges_sp. (Canada)	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
#Haemaphysalis_elliptica (South Africa) (H1)	AAT	TGT	AAC	CGC	TCA	CGC	ATT	CAT	TAT	AAT	TTT	TTT	CAT	AGT	TAT	ACC	TAT	TAT	AAT	TGG
#Haemaphysalis_elliptica (South Africa) (H2)	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
#JQ737059.1_Haemaphysalis_qinghaiensis (China)	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
#KU880573.1_Haemaphysalis_erinacei (China)	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
#JQ737057.1_Haemaphysalis_flava (China)	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
#Rhhipicephalus_sanguineus (South Africa) (R1)	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
#AF132834.1_Rhipicephalus_compositus (Australia)	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
#JQ737086.1_Rhipicephalus_turanicus (China)	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
#KT906186.1_Rhipicephalus_sanguineus (Colombia)	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
#MF425993.1_Rhipicephalus_sanguineus (Angola)	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
#JQ737084.1_Rhipicephalus_sanguineus (China)	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
#KT988972.1_Myalges_sp. (Canada)	TG	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
#Haemaphysalis_elliptica (South Africa) (H1)	GGG	GTT	TGG	AAA	TTG	ACT	AAT	TCC	TTT	AAT	GTT	AGG	AGC	ACC	AGA	TAT	AGC	TTT	TCC	AGC
#Haemaphysalis_elliptica (South Africa) (H2)	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
#JQ737059.1_Haemaphysalis_qinghaiensis (China)																				

Table 4-5 shows the rates of base substitutions for the nucleotide pair. Average nucleotide frequencies, where gaps and missing data were removed, were 29.95% adenine, 39.08% thymine, 17.13% cytosine, and 13.84% guanine.

Table 4-5: CO1 gene rates of base substitutions for nucleotide pairs

From\To	A	T	C	G
A	-	0,088993088	0,017478771	0,042881078
T	0,068201971	-	0,191219745	0
C	0,030548052	0,436078544	-	0,014221815
G	0,092772028	0	0,017604908	-

4.3.2 ITS2 gene of dog ticks

Amplification of the tick DNA targeting the ITS2 gene was successful with a PCR product size ranging from 900 to 1200 bp (Figure 4-6). Average sequenced length ranged between 430 and 1194 bp (Table 4-6). BLASTn search was performed where sequences of this study were compared to other sequences on the NCBI database. Similar sequences returned on the NCBI database with the top ranked hits were highly regarded as identification of tick species. BLASTn search results of *R. sanguineus* (Sample ID: R1, R2, and R3) of this study were similar to *R. sanguineus* sequences (GenBank accession numbers: JQ737127.1 and JQ412127.1) on the NCBI database with query covers of 94 and 96%, matching identifications of 94 and 97%, and E-values of 0. For *H. elliptica* no reference sequences were available on the NCBI database. However, BLASTn results did reveal that *H. elliptica* (Sample ID: H1) sequences obtained from this study were similar to other species of the *Haemaphysalis* genus (GenBank accession numbers: JQ625703.1, KC853414.1, and KU364288.1) on the NCBI database with query covers ranging from 85 to 96%, matching identifications ranging from 79 to 82% and E-values of 3E-113, 9E-101, and 1E-98.

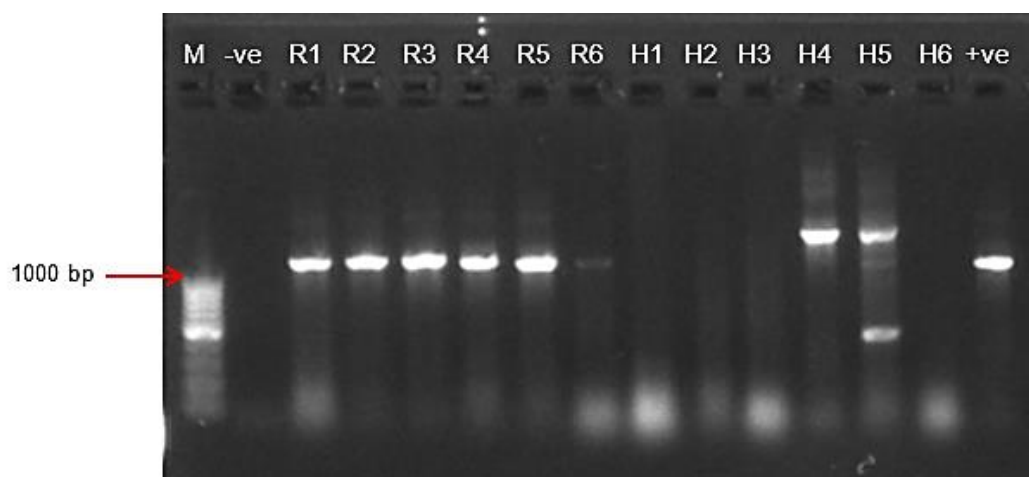


Figure 4-6: The 1% agarose electrophoresis gel of DNA amplicons of ticks targeting the ITS2 gene with an expected product size of 900 to 1200 bp. Lane 1 (M): 100 bp DNA molecular marker. Lane 2 (-ve): No template control. Lane 3 to 8 (R1 to R6): Amplified DNA from *R. sanguineus*. Lane 9 to 14 (H1 to H6): Amplified DNA from *H. elliptica*. Lane 15 (+ve): Positive control

Table 4-6: BLASTn results of ITS2 gene of *H. elliptica* and *R. sanguineus* ticks

Sample ID	Gene size (bp)	Morphological identification	BLAST description	Maximum score	Total score	Query cover	E-value	Identity	Accession number
H1	430	<i>Haemaphysalis elliptica</i>	<i>Haemaphysalis longicornis</i>	419	419	98%	3E-113	81%	JQ625703.1
			<i>Haemaphysalis bispinosa</i>	378	378	94%	9E-101	79%	KC853414.1
			<i>Haemaphysalis erinacei</i>	370	370	85%	1E-98	82%	KU364288.1
			<i>Haemaphysalis japonica</i>	363	363	93%	2E-96	81%	AB861940.1
			<i>Haemaphysalis doentzi</i>	360	360	98%	2E-95	79%	JQ346685.1
R1	1128	<i>Rhipicephalus sanguineus</i>	<i>Rhipicephalus sanguineus</i>	1799	1883	96%	0.0	97%	JQ737127.1
			<i>Rhipicephalus sanguineus</i>	1770	1854	96%	0.0	97%	JQ412127.1
			<i>Rhipicephalus sanguineus</i>	1757	1834	96%	0.0	96%	KF958400.1
			<i>Rhipicephalus sanguineus</i>	1754	1838	96%	0.0	96%	KY945496.1
			<i>Rhipicephalus sanguineus</i>	1754	1831	96%	0.0	96%	KF958408.1
R2	1194	<i>Rhipicephalus sanguineus</i>	<i>Rhipicephalus sanguineus</i>	1633	1633	94%	0.0	94%	JQ412127.1
			<i>Rhipicephalus sanguineus</i>	1627	1627	94%	0.0	94%	JQ737127.1
			<i>Rhipicephalus sanguineus</i>	1624	1624	94%	0.0	93%	KF958400.1
			<i>Rhipicephalus sanguineus</i>	1618	1618	94%	0.0	93%	KY945496.1
			<i>Rhipicephalus sanguineus</i>	1618	1618	94%	0.0	93%	KF958408.1
R3	1130	<i>Rhipicephalus sanguineus</i>	<i>Rhipicephalus sanguineus</i>	1902	2016	99%	0.0	98%	JQ737127.1
			<i>Rhipicephalus sanguineus</i>	1882	1997	99%	0.0	98%	JQ412127.1
			<i>Rhipicephalus sanguineus</i>	1856	1959	98%	0.0	97%	KF958400.1
			<i>Rhipicephalus sanguineus</i>	1855	1970	99%	0.0	97%	KY945496.1
			<i>Rhipicephalus sanguineus</i>	1855	1970	99%	0.0	97%	JF758643.1

Phylogenetic analysis was done by using the Maximum likelihood (ML) method with sequences of both the *Haemaphysalis* and *R. sanguineus* genera, where *Dermanyssus* sp. was used as an outgroup. Phylogenetic analysis revealed two major clades with bootstrap support values of more than 90%. Clade I consisted of *Haemaphysalis* species and Clade II consisted of *R. sanguineus*. *Haemaphysalis elliptica* sequences of ticks from the JB Marks Local Municipality clustered with other species of the *Haemaphysalis* genus forming a clade with a bootstrap support value of 85%. *Rhipicephalus sanguineus* sequences of ticks from the JB Marks Local Municipality clustered with other *R. sanguineus* species forming a clade with a bootstrap support value of 97%. (Figure 4-7).

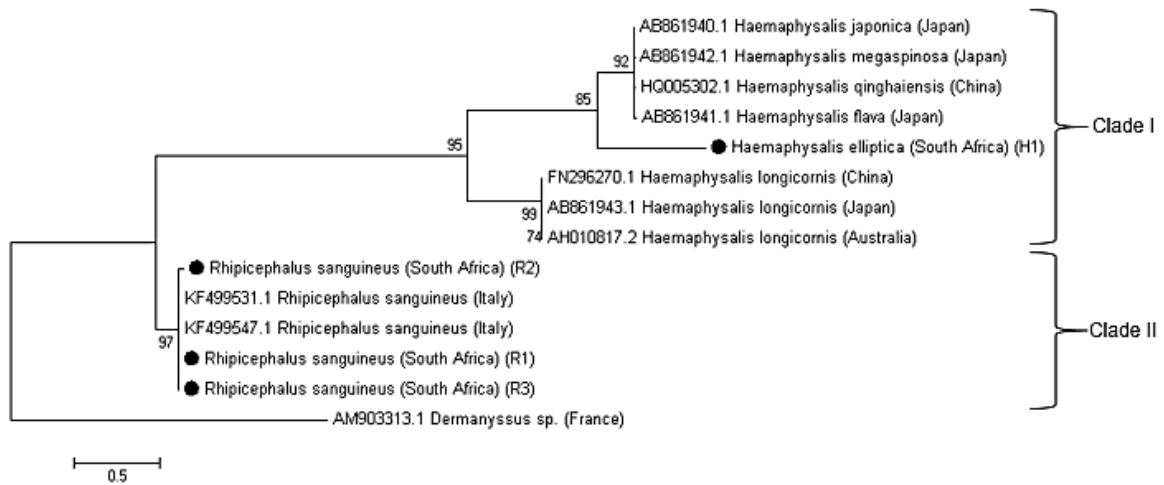


Figure 4-7: Phylogenetic analysis of ITS2 gene using the Maximum Likelihood (ML) method based on the Kimura 2-parameter model (Kimura, 1980). Bootstrap percentage of 10 000 replicates, in which the associated taxa are clustered together is displayed next to the branch nodes. Fourteen nucleotide sequences were used for data analysis. There was a total of 174 positions in the final dataset. Sequences of the phylogenetic tree are displayed as the GenBank accession number, species, and country of collection. Sequences of this study are indicated by a black bullet and also contain the sample ID. Phylogenetic analysis was done by using MEGA 7 (Kumar *et al.*, 2016)

For phylogenetic analysis, the final dataset consisted of 174 positions, where missing data and gaps were removed. Table 4-7 shows the base substitutions per site of the sequences used during this study. For *H. elliptica* the sequence divergence among other species of the *Haemaphysalis* genera was between 1.005 and 2.238. For *R. sanguineus* the sequence divergence among other *R. sanguineus* species was between 0 and 0.03. The p-distance matrix for sequences of the *Haemaphysalis* and *R. sanguineus* genera indicated strong resolution power ranging between 0 to 0.718% (Table 4-8).

Table 4-7: Number of base substitutions per site of sequences of the ITS2 gene

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1) AB861940.1 <i>Haemaphysalis japonica</i> (Japan)		0.010	0.006	38.164	38.164	110.618	38.164	0.009	80.683	80.683	80.683	80.689	80.683	26.480
2) AB861941.1 <i>Haemaphysalis flava</i> (Japan)	0.017		0.012	38.109	38.109	110.249	38.109	0.014	80.756	80.756	80.756	80.758	80.756	26.474
3) AB861942.1 <i>Haemaphysalis megaspinoso</i> (Japan)	0.006	0.023		38.150	38.150	110.578	38.150	0.011	80.655	80.655	80.655	80.662	80.655	26.470
4) AB861943.1 <i>Haemaphysalis longicornis</i> (Japan)	1.601	1.632	1.649		0.000	101.547	0.006	38.226	70.853	70.853	70.853	70.955	70.853	58.802
5) AH010817.2 <i>Haemaphysalis longicornis</i> (Australia)	1.601	1.632	1.649	0.000		101.547	0.006	38.226	70.853	70.853	70.853	70.955	70.853	58.802
6) AM903313.1 <i>Dermanyssus</i> sp. (France)	4.902	5.028	5.029	4.391	4.391		101.547	110.455	83.615	83.615	83.615	83.500	83.615	127.674
7) FN296270.1 <i>Haemaphysalis longicornis</i> (China)	1.601	1.632	1.649	0.006	0.006	4.391		38.226	70.853	70.853	70.853	70.955	70.853	58.802
8) HQ005302.1 <i>Haemaphysalis qinghaiensis</i> (China)	0.012	0.029	0.017	1.587	1.587	4.902	1.587		80.751	80.751	80.751	80.750	80.751	26.450
9) KF499531.1 <i>Rhipicephalus sanguineus</i> (Italy)	3.842	3.992	4.016	3.044	3.044	3.494	3.044	3.842		0.000	0.000	0.014	0.000	76.299
10) KF499547.1 <i>Rhipicephalus sanguineus</i> (Italy)	3.842	3.992	4.016	3.044	3.044	3.494	3.044	3.842	0.000		0.000	0.014	0.000	76.299
11) <i>Rhipicephalus sanguineus</i> (South Africa) (R1)	3.842	3.992	4.016	3.044	3.044	3.494	3.044	3.842	0.000	0.000		0.014	0.000	76.299
12) <i>Rhipicephalus sanguineus</i> (South Africa) (R2)	3.681	3.819	3.843	3.221	3.221	3.620	3.221	3.681	0.030	0.030	0.030		0.014	76.127
13) <i>Rhipicephalus sanguineus</i> (South Africa) (R3)	3.842	3.992	4.016	3.044	3.044	3.494	3.044	3.842	0.000	0.000	0.000	0.030		76.299
14) <i>Haemaphysalis elliptica</i> (South Africa) (H1)	1.005	1.013	1.035	2.238	2.238	5.346	2.238	1.019	3.501	3.501	3.501	3.516	3.501	

*Black numbers indicate number of base substitutions per site and blue numbers indicate standard errors.

Table 4-8: Pairwise (p) distance analysis of sequences of the ITS2 gene

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
[1] AB861940.1 <i>Haemaphysalis japonica</i> (Japan)		0.010	0.006	0.036	0.036	0.031	0.036	0.008	0.031	0.031	0.031	0.032	0.031	0.037
[2] AB861941.1 <i>Haemaphysalis flava</i> (Japan)	0.017		0.011	0.036	0.036	0.031	0.036	0.012	0.032	0.032	0.032	0.032	0.032	0.037
[3] AB861942.1 <i>Haemaphysalis megaspinoso</i> (Japan)	0.006	0.023		0.036	0.036	0.031	0.036	0.009	0.031	0.031	0.031	0.032	0.031	0.037
[4] AB861943.1 <i>Haemaphysalis longicornis</i> (Japan)	0.621	0.626	0.626		0.000	0.032	0.006	0.036	0.034	0.034	0.034	0.034	0.034	0.035
[5] AH010817.2 <i>Haemaphysalis longicornis</i> (Australia)	0.621	0.626	0.626	0.000		0.032	0.006	0.036	0.034	0.034	0.034	0.034	0.034	0.035
[6] AM903313.1 <i>Dermanyssus</i> sp. (France)	0.776	0.787	0.782	0.747	0.747		0.032	0.031	0.034	0.034	0.034	0.034	0.034	0.029
[7] FN296270.1 <i>Haemaphysalis longicornis</i> (China)	0.621	0.626	0.626	0.006	0.006	0.747		0.036	0.034	0.034	0.034	0.034	0.034	0.035
[8] HQ005302.1 <i>Haemaphysalis qinghaiensis</i> (China)	0.011	0.029	0.017	0.615	0.615	0.776	0.615		0.032	0.032	0.032	0.032	0.032	0.037
[9] KF499531.1 <i>Rhipicephalus sanguineus</i> (Italy)	0.759	0.753	0.759	0.701	0.701	0.707	0.701	0.759		0.000	0.000	0.012	0.000	0.033
[10] KF499547.1 <i>Rhipicephalus sanguineus</i> (Italy)	0.759	0.753	0.759	0.701	0.701	0.707	0.701	0.759	0.000		0.000	0.012	0.000	0.033
[11] <i>Rhipicephalus sanguineus</i> (South Africa) (R1)	0.759	0.753	0.759	0.701	0.701	0.707	0.701	0.759	0.000	0.000		0.012	0.000	0.033
[12] <i>Rhipicephalus sanguineus</i> (South Africa) (R2)	0.753	0.747	0.753	0.707	0.707	0.707	0.707	0.753	0.029	0.029	0.029		0.012	0.033
[13] <i>Rhipicephalus sanguineus</i> (South Africa) (R3)	0.759	0.753	0.759	0.701	0.701	0.707	0.701	0.759	0.000	0.000	0.000	0.029		0.033
[14] <i>Haemaphysalis elliptica</i> (South Africa) (H1)	0.489	0.489	0.494	0.655	0.655	0.805	0.655	0.494	0.718	0.718	0.718	0.713	0.718	

*Black numbers indicate p-distances and blue numbers indicate standard errors.

Figure 4-8 shows the nucleotide differences found among the ITS2 gene sequences that were used for phylogenetic analysis.

#Rhhipicephalus_sanguineus_(South_Africa)_(R3)	CAA CCG ACT GCA CCG AGG TGA -TG GCA CCA ACA AAG ACC GCC GCA GGA CTC -GA ATC GGA
#Rhhipicephalus_sanguineus_(South_Africa)_(R2)	...
#Rhhipicephalus_sanguineus_(South_Africa)_(R1)	...
#KF499581.1_Rhhipicephalus_sanguineus_(Italy)	...
#KF499547.1_Rhhipicephalus_sanguineus_(Italy)	...
#Haemaphysalis_elliptica_(South_Africa)_(H1)	G-. TGC GT. C.C .G. CCC .CT CGC A.C GGC GTG TT. GGA AGT CTG TTC GCG C.C GCG AAG
#AB861940.1_Haemaphysalis_japonica_(Japan)	G-. TGC GT. C.C .GA CCC CC- -C .G .TG G.G TT. GGA AGT CTG ATC G.G C.- --- AAG
#AB861941.1_Haemaphysalis_flava_(Japan)	G-. TGC GT. C.C .GA CCC CC- -C .G .TG G.G TT. GGA AGT CTG ATC G.G C.- --- AAG
#AB861942.1_Haemaphysalis_megaspinoso_(Japan)	G-. TGC GT. C.C .GA CCC CC- -C .G .TG G.G TT. GGA AGT CGG ATC G.G C.- --- AAG
#AB861942.1_Haemaphysalis_longicornis_(Japan)	TGG .TT T.C CGC TGA GCC C.- --- .TT GT. .GT GTG .GG CTC .CG G.A CA. --- .AG
#AH010817.2_Haemaphysalis_longicornis_(Australia)	TGG .TT T.C CGC TGA GCC C.- --- .TT GT. .GT GTG .GG CTC .CG G.A CA. --- .AG
#FN296270.1_Haemaphysalis_longicornis_(China)	TGG .TT T.C CGC TGA GCC C.- --- .TT GT. .GT GTG .GG CTC .CG G.A CA. --- .AG
#HQ005302.1_Haemaphysalis_qinghaiensis_(China)	GG. TGC GT. C.C .GA CCC CC- -C .G .TG G.G TT. GGA AGT CTG ATC G.G C.- --- AAG
#AM903212.1_Dermanyssus_sp._(France)	.T. TGC TGC ATT TGT TTC A.T A.A TAT .A. TGT .A. TTT .T. .TT ACT A.T GCT TGA -A.
#Rhhipicephalus_sanguineus_(South_Africa)_(R3)	ATG A-G CCT GCA GGG AAA GCG CGG -CG AGG TAG CGT ACT CCT CTC TGC GAC CGA GGG CGC
#Rhhipicephalus_sanguineus_(South_Africa)_(R2)	...
#Rhhipicephalus_sanguineus_(South_Africa)_(R1)	...
#KF499581.1_Rhhipicephalus_sanguineus_(Italy)	...
#KF499547.1_Rhhipicephalus_sanguineus_(Italy)	...
#Haemaphysalis_elliptica_(South_Africa)_(H1)	CG. AT .GC C.T TC. .TG ATT ... ATA TTC G.A G.. TGC GT- --- GA. CC. GCG .AA GA.
#AB861940.1_Haemaphysalis_japonica_(Japan)	CG. GAT .GC C.T TC. .G CGT .C. GAA TC. GGC G.C G.. GAA TCG GAT TCG AAG .T. .TT
#AB861941.1_Haemaphysalis_flava_(Japan)	CG. GAT .GC C.T TC. .G CGT .C. GAA TC. GGC G.C G.. GAA TCG GAT TCG AAG .T. .TT
#AB861942.1_Haemaphysalis_megaspinoso_(Japan)	CG. GAT .GC C.T TC. .G CGT .C. GAA TC. GGC G.C G.. GAA TCG GAT TCG AAG .T. .TT
#AB861942.1_Haemaphysalis_longicornis_(Japan)	.GT TGT .GC ATC C.. .GG C.. TTT TGC CT. .A GC. T.C G.G .TT GA. CGG GA. CTT TT.
#AH010817.2_Haemaphysalis_longicornis_(Australia)	.GT TGT .GC ATC C.. .GG C.. TTT TGC CT. .A GC. T.C G.G .TT GA. CGG GA. CTT TT.
#FN296270.1_Haemaphysalis_longicornis_(China)	.GT TGT .GC ATC C.. .GG C.. TTT TGC CT. .A GC. T.C G.G .TT GA. CGG GA. CTT TT.
#HQ005302.1_Haemaphysalis_qinghaiensis_(China)	CG. GAT .GC C.T TC. .G CGT .C. GAA TC. GGC G.C G.. GAA TCG GAT TCG AAG .T. .TT
#AM903212.1_Dermanyssus_sp._(France)	GCA .TA GTA TA. ACT GTT CGC GAT CAC .A. .GT GA. .TC GA. .TT GCG TTA TAT .AC GTG
#Rhhipicephalus_sanguineus_(South_Africa)_(R3)	AAA --T CTG CGA GAG ACT CGG ACG CGC AAC TGG AAC GCA CGG TGC ATA CGG GGA GCG AAA
#Rhhipicephalus_sanguineus_(South_Africa)_(R2)	...
#Rhhipicephalus_sanguineus_(South_Africa)_(R1)	...
#KF499581.1_Rhhipicephalus_sanguineus_(Italy)	...
#KF499547.1_Rhhipicephalus_sanguineus_(Italy)	...
#Haemaphysalis_elliptica_(South_Africa)_(H1)	G.. CGC .GA TCG AGT G.. TTC GG. GAA .GA .C TC. C.G GC. CT. G.G .CT CTG A.. .C.
#AB861940.1_Haemaphysalis_japonica_(Japan)	G.G CAC G.. .A. .C GAC GCC GGA T.A GCG C.T GGA A.G G.A GCG GAT GCA TTC C.T GCG
#AB861941.1_Haemaphysalis_flava_(Japan)	G.G CAC G.. .A. .C GAC GCC GGA T.A GCG C.T GGA A.G G.A GCG GAT GCA TTC C.T GCG
#AB861942.1_Haemaphysalis_megaspinoso_(Japan)	G.G CAC G.. .A. .C GAC GCC GGA T.A GCG C.T GGA A.G G.A GCG GAT GCA TTC C.T GCG
#AB861942.1_Haemaphysalis_longicornis_(Japan)	C.G GTC GC. TTG .T GAA GCC .T .TG .C- .TT GG. CAT .C. CCG G.. .TC C.G TTT C.G
#AH010817.2_Haemaphysalis_longicornis_(Australia)	C.G GTC GC. TTG .T GAA GCC .T .TG .C- .TT GG. CAT .C. CCG G.. .TC C.G TTT C.G
#FN296270.1_Haemaphysalis_longicornis_(China)	C.G GTC GC. TTG .T GAA GCC .T .TG .C- .TT GG. CAT .C. CCG G.. .TC C.G TTT C.G
#HQ005302.1_Haemaphysalis_qinghaiensis_(China)	G.G CAC G.. .A. .C GAC GCC GGA .A GCG C.T GGA ATG G.A GCG GAT GCA TTC C.T GCG
#AM903212.1_Dermanyssus_sp._(France)	T.T CTG AAA TC. AGT GTG AC. .C ... TGA ACT T.A ... T-A .CA ... A.C GA ...

Figure 4-8: Nucleotide differences found among the ITS2 gene sequences of Ixodes ticks. Full stops (.) indicates nucleotides similar to the top sequence. Hyphens (-) indicate the presence of an alignment gap

Table 4-9 shows the rates of base substitutions for the nucleotide pair. Average nucleotide frequencies, where gaps and missing data were removed, were 25% for adenine, thymine, cytosine, and guanine, respectively.

Table 4-9: ITS2 gene rates of base substitutions for every nucleotide pairs

From\To	A	T	C	G
A	-	0,050686817	0,050686817	0,148626366
T	0,050686817	-	0,148626366	0,050686817
C	0,050686817	0,148626366	-	0,050686817
G	0,148626366	0,050686817	0,050686817	-

4.4 Morphological detection of tick-borne pathogens infecting sampled ticks

A total of 204 Giemsa-stained blood smears from engorged ticks were examined for the presence of tick-borne pathogens including; *Anaplasma* sp., *Rickettsia* sp., *Babesia* sp., and *Ehrlichia* sp. based on morphological features as described by Perez *et al.* (1996), Azad and Beard (1998), Parola and Raoult (2001), Hartelt *et al.* (2004), Taylor *et al.* (2007), Schoeman (2009), Alberdi *et al.* (2012), and Kostro *et al.* (2015). Ticks of dogs originating from the JB Marks Local Municipality had infestation rates of 0% (0/204) for *Ehrlichia* sp., 0.5% (1/204) for *Babesia* sp., 1% (2/204) for *Anaplasma* sp., and 22% (45/204) for *Rickettsia* sp. (Table 4-10). There was a statistical difference [$p = 0.005935$ ($X^2 = 38$, $df = 19$) and $p < 2.2e-16$ ($X^2 = 261.22$, $df = 19$)] in detection of *Anaplasma* sp. and *Rickettsia* sp. respectively from ticks of dogs originating from various locations in and around the JB Marks Local Municipality. There was no statistical significance for detection of *Babesia* sp. [$p = 0.4568$ ($X^2 = 19$, $df = 19$)]. *Rickettsia* sp. was detected in 29% (32/112) of *H. elliptica* and 14% (13/92) *R. sanguineus* ticks. *Anaplasma* sp. was only detected in 2% (2/112) of *H. elliptica* ticks. *Babesia* sp. was only detected in 1% (1/92) of *R. sanguineus* ticks.

Rickettsia sp. infections were abundant in ticks of dogs originating from Boipatong (52.9%), followed by Miederpark (35%) and unknown locations (29.5%). Whereas ticks positive with *Anaplasma* sp. and *Babesia* sp. were from dogs originating from Boipatong (5.9%) and Die Bult (20%), respectively. Mixed infections of *Anaplasma* and *Rickettsia* sp. were detected from ticks of dogs originating from Boipatong (Table 4-10).

Table 4-10: Overall occurrence of tick-borne pathogens detected in blood smears of engorged adult ticks

Location	Species	<i>Anaplasma</i> sp. (%)*	<i>Rickettsia</i> sp. (%)*	<i>Babesia</i> sp. (%)*	<i>Ehrlichia</i> sp. (%)*	Total blood smears examined
Baillie park	<i>H. elliptica</i>	0 (0)	0 (0)	0 (0)	0 (0)	4
	<i>R. sanguineus</i>	0 (0)	0 (0)	0 (0)	0 (0)	4
Boipatong	<i>H. elliptica</i>	2 (5.9)	18 (52.9)	0 (0)	0 (0)	34
	<i>R. sanguineus</i>	0 (0)	0 (0)	0 (0)	0 (0)	1
Die Bult	<i>H. elliptica</i>	0 (0)	0 (0)	0 (0)	0 (0)	4
	<i>R. sanguineus</i>	0 (0)	0 (0)	1 (20)	0 (0)	5
Fochville	<i>H. elliptica</i>	0 (0)	0 (0)	0 (0)	0 (0)	1
	<i>R. sanguineus</i>	0 (0)	0 (0)	0 (0)	0 (0)	0
Ikageng	<i>H. elliptica</i>	0 (0)	0 (0)	0 (0)	0 (0)	4
	<i>R. sanguineus</i>	0 (0)	0 (0)	0 (0)	0 (0)	14
Kannonierspark	<i>H. elliptica</i>	0 (0)	0 (0)	0 (0)	0 (0)	0
	<i>R. sanguineus</i>	0 (0)	0 (0)	0 (0)	0 (0)	1
Miederpark	<i>H. elliptica</i>	0 (0)	14 (35)	0 (0)	0 (0)	40
	<i>R. sanguineus</i>	0 (0)	0 (0)	0 (0)	0 (0)	21
Boskop	<i>H. elliptica</i>	0 (0)	0 (0)	0 (0)	0 (0)	19
	<i>R. sanguineus</i>	0 (0)	0 (0)	0 (0)	0 (0)	2
Potchindustrial	<i>H. elliptica</i>	0 (0)	0 (0)	0 (0)	0 (0)	3
	<i>R. sanguineus</i>	0 (0)	0 (0)	0 (0)	0 (0)	0
Unknown	<i>H. elliptica</i>	0 (0)	0 (0)	0 (0)	0 (0)	3
	<i>R. sanguineus</i>	0 (0)	13 (29.5)	0 (0)	0 (0)	44
Total		2	45	1	0	204
Overall percentage		1%	22%	0.5%	0%	

*Indicates average percentages of pathogens detected by Giemsa-stained blood smears

4.5 Molecular detection of tick-borne pathogens

Ticks sharing the same host, location of collection, species, and life stage were pooled together for DNA extraction. Tick groups consisting of less than three ticks were split up and treated as individuals for DNA extraction. Tick egg batches were also pooled according to the same host, tick species, and location of collection. In total there was 104 tick pools and 31 egg pools. Tick DNA pools were screened for the presence of transstadially (Table 4-11) transmitted tick-borne pathogens whilst egg DNA pools were screened for transovarially transmitted tick-borne pathogens (Table 4-12). Extracted DNA concentrations ranged from 0.345 to 20.65 ng/μl. Mixed infections of *A. phagocytophilum* and *Rickettsia* sp. were detected from ticks of dogs originating from Boipatong. Mixed infections of *C. burnetii*, *E. canis*, and *Rickettsia* sp. were detected from dogs originating from Miederpark (Table 4-11).

Table 4-11: Overall occurrence of tick-borne pathogens capable of transstadial transmission detected by PCR

Location	Species	Life stage	<i>A. phagocytophilum</i>	<i>Rickettsia</i> sp.	<i>B. canis</i>	<i>B. vogeli</i>	<i>B. rossii</i>	<i>E. canis</i>	<i>C. burnetii</i>	Total pools screened
Baillie park	<i>H. elliptica</i>	Adult	0	0	0	0	0	0	0	1
	<i>R. sanguineus</i>	Nymph	0	2	0	0	0	0	0	2
		Adult	0	0	0	0	0	0	1	2
Boipatong	<i>H. elliptica</i>	Nymph	0	0	0	0	0	0	0	1
		Adult	1	1	0	0	0	0	0	2
	<i>R. sanguineus</i>	Nymph	0	0	0	0	0	0	0	1
		Adult	0	0	0	0	0	0	0	2
Die Bult	<i>H. elliptica</i>	Nymph	0	0	0	0	0	0	0	2
		Adult	0	0	0	0	0	0	0	2
	<i>R. sanguineus</i>	Nymph	0	0	0	0	0	0	0	2
		Adult	0	0	0	0	0	0	0	1
Fochville	<i>H. elliptica</i>	Nymph	0	0	0	0	0	0	0	1
		Adult	0	0	0	0	0	0	1	1
Ikageng	<i>H. elliptica</i>	Adult	0	0	0	0	0	0	0	4
	<i>R. sanguineus</i>	Nymph	0	0	0	0	0	0	0	6
		Adult	0	0	0	0	0	0	0	4
Kannonierspark	<i>R. sanguineus</i>	Nymph	0	0	0	0	0	0	0	1
		Adult	0	0	0	0	0	0	1	1
Miederpark	<i>H. elliptica</i>	Nymph	0	1	0	0	0	2	5	7
		Adult	0	5	0	0	0	2	4	18
	<i>R. sanguineus</i>	Nymph	0	0	0	0	0	1	0	13
		Adult	0	0	0	0	0	0	1	10

Table 4.11 (continue): Overall occurrence of tick-borne pathogens capable of transstadial transmission detected by PCR

Location	Species	Life stage	<i>A. phagocytophilum</i>	<i>Rickettsia</i> sp.	<i>B. canis</i>	<i>B. vogeli</i>	<i>B. rossi</i>	<i>E. canis</i>	<i>C. burnetii</i>	Total pools screened
Boskop	<i>H. elliptica</i>	Nymph	0	0	0	0	0	0	1	1
		Adult	0	0	0	0	0	0	0	4
	<i>R. sanguineus</i>	Nymph	0	0	0	0	0	1	0	1
		Adult	0	0	0	0	0	0	0	1
Potchindustrie	<i>H. elliptica</i>	Adult	0	0	0	0	0	0	0	1
	<i>R. sanguineus</i>	Nymph	0	0	0	0	0	0	0	1
		Adult	0	0	0	0	0	0	0	1
Unknown	<i>H. elliptica</i>	Adults	0	0	0	0	0	0	0	4
	<i>R. sanguineus</i>	Nymph	0	1	0	0	0	0	0	3
		Adult	0	0	0	0	0	0	0	3
Total			1	10	0	0	0	6	14	104
Overall occurrence			1%	9.6%	0%	0%	0%	5.8%	13.5%	

Table 4-12: Overall occurrence of tick-borne pathogens capable of transovarial transmission detected by PCR

Location	Species	<i>Rickettsia sp.</i>	<i>B. canis</i>	<i>B. vogeli</i>	<i>B. rossi</i>	<i>C. burnetii</i>	Total pools screened
Boipatong	<i>H. elliptica</i>	0	0	0	0	1	2
Ikageng	<i>H. elliptica</i>	0	0	0	0	0	1
	<i>R. sanguineus</i>	0	0	0	0	0	4
Kannonierspark	<i>R. sanguineus</i>	0	0	0	0	0	1
Miederpark	<i>H. elliptica</i>	0	0	0	0	1	11
	<i>R. sanguineus</i>	0	0	0	0	0	2
Boskop	<i>H. elliptica</i>	0	0	0	0	0	3
	<i>R. sanguineus</i>	0	0	0	0	0	1
Potchindustrie	<i>R. sanguineus</i>	0	0	0	0	0	1
Unknown	<i>H. elliptica</i>	0	0	0	0	0	2
	<i>R. sanguineus</i>	0	0	0	0	0	3
Total		0	0	0	0	2	31
Overall occurrence		0%	0%	0%	0%	6.5%	

4.5.1 *Anaplasma phagocytophilum*

Detection of *A. phagocytophilum* was conducted by PCR amplifying the 16s *rRNA* gene with an expected product size of 250 bp (Figure 4-9).

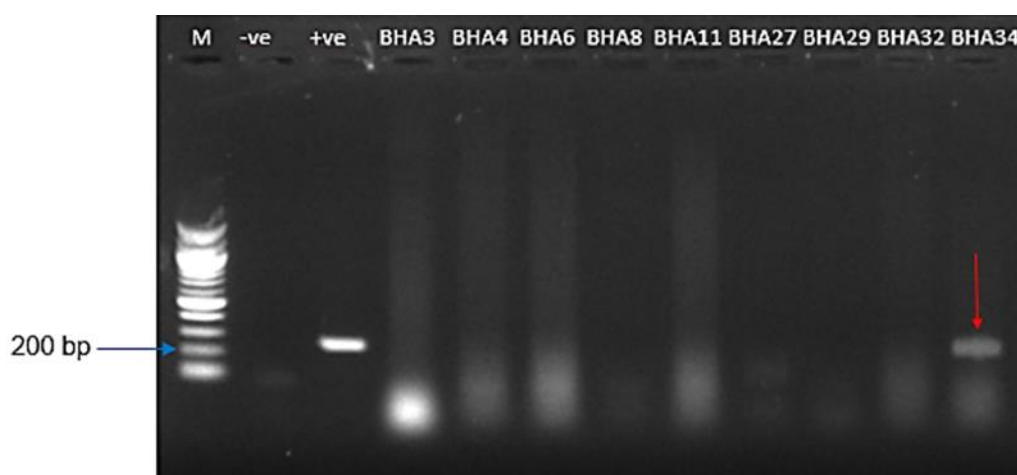


Figure 4-9: The 1% agarose electrophoresis gel of DNA amplicons for the screening of *A. phagocytophilum* with an expected product size of 250 bp. Lane 1 (M): 100 bp DNA molecular marker. Lane 2 (-ve): No template control. Lane 3 (+ve): Positive control. Lane 4 to 12 (BHA3 to BHA 34): Amplified DNA of *A. phagocytophilum* from *H. elliptica* adult ticks. Red arrow indicates the presence of tick borne-pathogens

The average sequenced length of the amplicon was 205 bp (Table 4-13). The BLASTn search results of *A. phagocytophilum* (Sample ID: BHA34) of this study were similar to *A. phagocytophilum* sequences on the NCBI database (GenBank accession numbers: AY623650.1 and MF787270.1) with query covers of 96 and 98%, matching identity of 99%, and E-values of 6.00E-93 and 2.00E-92. Sequence alignment showing matches of the nucleotides between *A. phagocytophilum* from dog ticks collected from PAWS, JB Marks Local Municipality, and from a reference sequence on the NCBI database is shown in Figure 4-10.

The overall occurrence of *A. phagocytophilum* collected from ticks infesting dogs from the JB Marks Local Municipality was 1% (1/104) (Table 4-11). There was no significant statistical difference [$p = 0.4662$ ($X^2 = 33$, $df = 31$)] in infestation rate of *A. phagocytophilum* in and

around various locations of the JB Marks Local Municipality. The *H. elliptica* tick had an infestation rate of 2% (1/49), whereas no *A. phagocytophilum* infections were detected in *R. sanguineus* tick. *Anaplasma phagocytophilum* was only detected from ticks of dogs originating from Boipatong [*H. elliptica* adults (n=1 pool)].

Table 4-13: BLASTn results of *A. phagocytophilum*

Sample ID	Gene size (bp)	BLAST description	Maximum score	Total score	Query cover	E-value	Identity	Accession number
BHA34	205	<i>Anaplasma phagocytophilum</i>	351	351	98%	6,00E-93	99%	AY623650.1
		<i>Anaplasma phagocytophilum</i>	349	349	96%	2,00E-92	99%	MF787270.1
		<i>Anaplasma phagocytophilum</i>	349	349	96%	2,00E-92	99%	MF787269.1
		<i>Anaplasma phagocytophilum</i>	349	349	96%	2,00E-92	99%	KX810088.1
		<i>Anaplasma phagocytophilum</i>	349	349	96%	2,00E-92	99%	KP062963.1

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A. phagocytophilum 3  TGGTAGCTGCTTTTAATACTGCCAGACTCGAGTCCGGAAGAGGATAGCGGAATTCCTAGT 62
AY623650.1 18  TGGTAGCTGCTTTTAATACTGCCAGACTAGAGTCCGGAAGAGGATAGCGGAATTCCTAGT 77
A. phagocytophilum 63  GTAGAGGTGAAATTCGTAGATATTAGGAGGAACACCAGTGGCGAAGGCGGCTATCTGGTC 122
AY623650.1 78  GTAGAGGTGAAATTCGTAGATATTAGGAGGAACACCAGTGGCGAAGGCGGCTATCTGGTC 137
A. phagocytophilum 123 CGGTACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCCTGGTAGT 182
AY623650.1 138 CGGTACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCCTGGTAGT 197
A. phagocytophilum 183 CCACCCCTGTAAACGATGAGTGC 204
AY623650.1 198 CCACGCTGTAAACGATGAGTGC 219

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Figure 4-10: Fragment of the BLASTn alignment between *A. phagocytophilum* of this study and a corresponding sequence. First strand represents *A. phagocytophilum* detected from ticks collected from the JB Marks local municipality. Second strand represents a reference sequence from NCBI. Red arrows indicate where nucleotides mismatch

4.5.2 *Rickettsia* sp.

Detection of *Rickettsia* sp. was conducted by PCR with universal *Rickettsia* primers which were targeting the *gltA* gene with an expected product size of 381 bp (Figure 4-11).

Agarose gel electrophoresis A



Agarose gel electrophoresis B



Figure 4-11: The 1% agarose gel electrophoresis of PCR amplicons for the screening of *Rickettsia* species infections from dog ticks with an expected product size of 381 bp. **Agarose gel electrophoresis A:** Lane 1 (M): 100 bp DNA molecular marker. Lane 2 (-ve): No template control. Lane 3 (+ve): Positive control. Lane 4 to 21 (BHA3 to BHA38): Amplified DNA of *Rickettsia* from *H. elliptica* adult ticks. Lane 22 to 25 (BHN29 to BHN37A): Amplified DNA of *Rickettsia* from *H. elliptica* nymphs. **Agarose gel electrophoresis B:** Lane 1 (M): 100 bp DNA molecular marker. Lane 2 (-ve): No template control. Lane 3 (+ve): Positive control. Lane 4 to 22 (BRN1 to BRN57): Amplified DNA of *Rickettsia* from *R. sanguineus* nymphs. Red arrows indicate the presence of tick borne-pathogens

Average sequenced lengths of the amplicons ranged between 342 and 395 bp (Table 4-14). BLASTn search results of *A. phagocytophilum* (Sample IDs: BHA34, BHA35, BHA36B, BHN36, and BRN45) of this study were similar to *Rickettsia conorii* sequences (GenBank accession numbers: DQ821855.1, MF002509.1, and KY640399.1) on the NCBI database with query covers ranging between 94 and 99%, matching identities ranging between 98 and 100%, and E-values ranging between 2.00E-172 and 8.00E-458.

Overall occurrence of *R. conorii* on ticks infesting dogs originating from the JB Marks Local Municipality was 9.6% (10/104) (Table 4-11). The infestation rates of *R. conorii* on ticks of dogs originating in and around various locations of the JB Marks Local Municipality was statistically significant [$p = 5.081\text{e-}08$ ($X^2 = 92.4$, $df = 31$)]

Heamaphysalis elliptica tick had an infestation rate of 14.3% (7/49) and *R. sanguineus* tick had an infestation rate of 5.5% (3/55). *Rickettsia conorii* infections were most abundant in ticks of dogs originating from Miederpark [*H. elliptica* adults (n=5 pools) and nymphs (n=1 pool)], followed by ticks of dogs originating from Baillie park [*R. sanguineus* nymphs (n=2 pools)], ticks of dogs originating from Boipatong [*H. elliptica* adults (n=1 pool)], and ticks of dogs with unknown location [*R. sanguineus* nymphs (n=1 pool)].

No *Rickettsia* sp. were detected by PCR from the dog tick eggs (Table 4-12). Sequence alignment showing matches of the nucleotides between *R. conorii* detected in this study and a reference sequence from the NCBI database is shown in Figure 4-12.

Table 4-14: BLASTn results of *Rickettsia*

Sample ID	Gene size (bp)	BLAST description	Maximum score	Total score	Query cover	E-value	Identity	Accession number
BHA34	347	<i>Rickettsia conorii</i>	618	618	99%	5,00E-173	99%	DQ821855.1
		<i>Rickettsia conorii</i>	614	614	99%	6,00E-172	99%	MF002509.1
		<i>Rickettsia conorii</i>	614	614	99%	6,00E-172	99%	KY069261.1
		<i>Rickettsia conorii</i>	614	614	99%	6,00E-172	99%	KY640399.1
		<i>Rickettsia conorii</i>	614	614	99%	6,00E-172	99%	AE006914.1
BHA35	342	<i>Rickettsia conorii</i>	616	616	99%	2,00E-172	100%	DQ821855.1
		<i>Rickettsia conorii</i>	610	610	99%	7,00E-171	99%	MF002509.1
		<i>Rickettsia conorii</i>	610	610	99%	7,00E-171	99%	KY069261.1
		<i>Rickettsia conorii</i>	610	610	99%	7,00E-171	99%	KY640399.1
		<i>Rickettsia conorii</i>	610	610	99%	7,00E-171	99%	AE006914.1
BHA36B	348	<i>Rickettsia conorii</i>	612	612	99%	2,00E-171	99%	DQ821855.1
		<i>Rickettsia conorii</i>	607	607	99%	9,00E-170	99%	MF002509.1
		<i>Rickettsia conorii</i>	607	607	99%	9,00E-170	99%	KY069261.1
		<i>Rickettsia conorii</i>	607	607	99%	9,00E-170	99%	KY640399.1
		<i>Rickettsia conorii</i>	607	607	99%	9,00E-170	99%	AE006914.1
BHN36	346	<i>Rickettsia conorii</i>	612	612	99%	2,00E-171	99%	DQ821855.1
		<i>Rickettsia conorii</i>	609	609	99%	3,00E-170	99%	MF002509.1
		<i>Rickettsia conorii</i>	609	609	99%	3,00E-170	99%	KY069261.1
		<i>Rickettsia conorii</i>	609	609	99%	3,00E-170	99%	KY640399.1
		<i>Rickettsia conorii</i>	609	609	99%	3,00E-170	99%	AE006914.1
BRN45	395	<i>Rickettsia conorii</i>	569	569	93%	2,00E-158	98%	DQ821855.1
		<i>Rickettsia conorii</i>	567	567	94%	8,00E-158	98%	KY640399.1
		<i>Rickettsia conorii</i>	567	567	93%	8,00E-158	98%	AB872795.1

```

R. conorii 18 AAAGAATTCGGTAAGTTCTGAGTATATTCCTAAATATATAGCTAAAGCTAACCGATAAAA 77
DQ821855.1 46 AAAGAATTCGGTAAGTTCTGAGTATATTCCTAAATATATAGCTAAAGCTAACCGATAAAA 103
R. conorii 78 ATGATCCATTTAGATTAAATGGGTTTTGGTCATCGTGTGTATAAAACTATGATCCGCGTG 137
DQ821855.1 104 ATGATCCATTTAGATTAAATGGGTTTTGGTCATCGTGTATATAAAACTATGATCCGCGTG 163
R. conorii 138 CCGCAGTACTTAAAGAAACGTGCAAAAGAGTATTAAAGGAACTCGGGCAGCTAGACAACA 197
DQ821855.1 164 CCGCAGTACTTAAAGAAACGTGCAAAAGAGTATTAAAGGAACTCGGGCAGCTAGACAACA 223
R. conorii 198 ATCCGCTCTTACAAATAGCAATAGAACTTGAAGCTATCGCTCTTAAAGATGAATATTTTA 257
DQ821855.1 224 ATCCGCTCTTACAAATAGCAATAGAACTTGAAGCTATCGCTCTTAAAGATGAATATTTTA 283
R. conorii 258 TTGAGAGAAAAATTATATCCAAATGTTGATTTTATTGGGGTATTATCTATAAAGCTATGG 317
DQ821855.1 284 TTGAGAGAAAAATTATATCCAAATGTTGATTTTATTGGGGTATTATCTATAAAGCTATGG 343
R. conorii 318 GTATACCGTCGCAAAATGTTCACTGTACTTTTGCAT 354
DQ821855.1 344 GTATACCGTCGCAAAATGTTCACTGTACTTTTGCAT 380

```

Figure 4-12: Fragment of the BLASTn alignment between *R. conorii* of this study and a corresponding sequence. First strand represents *R. conorii* detected from ticks collected from the JB Marks local municipality. Second strand represents a reference sequence from NCBI. Red arrows indicate where nucleotides mismatch

4.5.3 *Babesia* sp.

PCR for detection of *B. canis*, *B. rossi*, and *B. vogeli* was conducted with primers targeting the *18S rRNA* gene. None of the screened DNA samples were positive for the presence of *B. canis*, *B. rossi*, or *B. vogeli*, furthermore, these protozoan parasites were also not detected by PCR from DNA of tick eggs (Table 4-11 and 4-12).

4.5.4 *Ehrlichia canis*

Detection of *E. canis* was conducted with PCR targeting the *16S rRNA* gene with an expected product size of 154 bp (Figure 4-13).

Agarose gel electrophoresis A



Agarose gel electrophoresis B



Figure 4-13: The 1% agarose gel electrophoresis of PCR amplicons for the screening of *E. canis* with an expected product size of 154 bp. **Agarose gel electrophoresis A:** Lane 1 (M): 100 bp DNA molecular marker. Lane 2 (-ve): No template control. Lane 3 (+ve): Positive control. Lane 4 to 15 (BHA3 to BHA38): Amplified DNA of *E. canis* from *H. elliptica* adult ticks. Lane 16 to 22 (BHN29 to BHN41): Amplified DNA of *E. canis* from *H. elliptica* nymphs. **Agarose gel electrophoresis B:** Lane 1 (M): 100 bp DNA molecular marker. Lane 2 (-ve): No template control. Lane 3 (+ve): Positive control. Lane 4 to 10 (BHN42 to BHN52): Amplified DNA of *E. canis* from *H. elliptica* nymphs. Lane 11 to 20 (BRN10 to BRN47): Amplified DNA of *E. canis* from *R. sanguineus* nymphs. Red arrows indicate the presence of tick borne-pathogens

Average sequenced lengths of the amplicons ranged between 89 and 100 bp (Table 4-15). BLASTn search results of *E. canis* (Sample IDs: BHA24, BHA36B, BRN16, and BRN42) of this study were similar to *E. canis* sequences (GenBank accession numbers: DQ494536.1, JQ976640.1, and MF153965.1) on the NCBI database with query covers ranging between 75 and 96%, matching identifications ranging between 85 and 100%, and E-values ranging between 2.00E-28 and 7.00E-22. Sequence alignment showing matches of the nucleotides between *E. canis* detected in this study and a reference sequence from the NCBI database is shown in Figure 4-14.

The overall occurrence of *E. canis* infecting dog ticks tested in this study was 5.8% (6/104) (Table 4-11). There was significant statistical difference of *E. canis* occurrence in tick collected from dogs originating from various locations [$p = 0.03045$ ($X^2 = 47.333$, $df = 31$)]. *Heamaphysalis elliptica* had an infestation rate of 8.2% (4/49) and *R. sanguineus* had an infestation rate of 3.6% (2/55). The *E. canis* infections were abundant in ticks of dogs originating from Miederpark [*H. elliptica* adults (n=2 pools) and nymphs (n=2 pools), as well as *R. sanguineus* adults (n=1 pool)]. *Ehrlichia canis* was also detected in ticks of dogs originating from Boskop [*R. sanguineus* nymphs (n=1 pool)].

Table 4-15: BLASTn results of *E. canis*

Sample ID	Gene size (bp)	BLAST description	Maximum score	Total score	Query cover	E-value	Identity	Accession number
BHA24	97	<i>Ehrlichia canis</i>	98,7	98,7	75%	7,00E-22	90%	DQ494536.1
		<i>Ehrlichia canis</i>	93,3	93,3	87%	3,00E-20	86%	MF059353.1
		<i>Ehrlichia canis</i>	93,3	93,3	62%	3,00E-20	93%	MF153971.1
		<i>Ehrlichia canis</i>	93,3	93,3	87%	3,00E-20	85%	DQ494537.1
		<i>Ehrlichia canis</i>	91,5	91,5	76%	1,00E-19	88%	JQ976640.1
BHA36B	99	<i>Ehrlichia canis</i>	120	120	86%	2,00E-28	91%	MF153965.1
		<i>Ehrlichia canis</i>	120	120	84%	2,00E-28	92%	JQ976640.1
		<i>Ehrlichia canis</i>	120	120	84%	2,00E-28	92%	JQ976631.1
		<i>Ehrlichia canis</i>	120	120	84%	2,00E-28	92%	DQ494536.1
		<i>Ehrlichia canis</i>	118	118	83%	8,00E-28	92%	KY594915.1
BRN16	89	<i>Ehrlichia canis</i>	156	156	96%	3,00E-39	100%	JQ976640.1
		<i>Ehrlichia canis</i>	156	156	96%	3,00E-39	100%	JQ976631.1
		<i>Ehrlichia canis</i>	154	154	95%	9,00E-39	100%	KY594915.1
		<i>Ehrlichia canis</i>	154	154	95%	9,00E-39	100%	CP025749.1
		<i>Ehrlichia canis</i>	154	154	95%	9,00E-39	100%	KX766395.1
BRN42	100	<i>Ehrlichia canis</i>	147	147	81%	2,00E-36	100%	MF059353.1
		<i>Ehrlichia canis</i>	145	145	80%	6,00E-36	100%	JQ976640.1
		<i>Ehrlichia canis</i>	145	145	80%	6,00E-36	100%	JQ976631.1
		<i>Ehrlichia canis</i>	143	143	79%	2,00E-35	100%	KY594915.1
		<i>Ehrlichia canis</i>	143	143	79%	2,00E-35	100%	CP025749.1

```

E. canis 13 AGGGGGAATGGCCTACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGACGATCAGCCACA 72
MF153985.1 147 AGAGGTAATGGCTTACCAAGGCTATGATCTATAGCTGGTCTGAGAGGACGATCAGCCACA 206
E. canis 73 CTGGGACTGAGATACGGTCCAGACTC 98
MF153985.1 207 CTGGAACTGAGATACGGTCCAGACTC 232

```

Figure 4-14: Fragment of the BLASTn alignment between *E. canis* of this study and a corresponding sequence. First strand represents *E. canis* detected from ticks collected from the JB Marks local municipality. Second strand represents a reference sequence from NCBI. Red arrows indicate where nucleotides mismatch

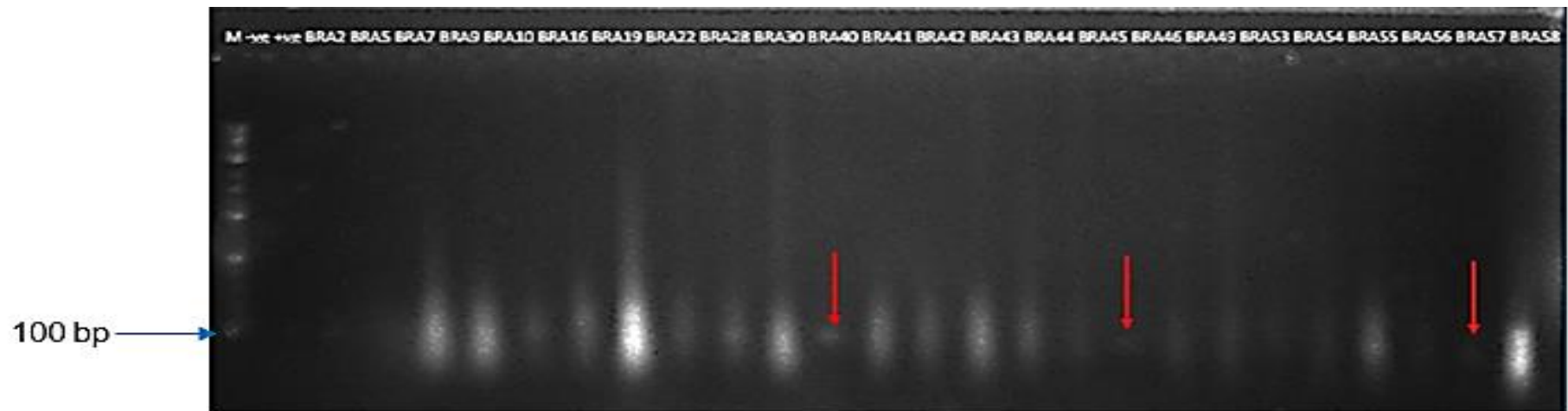
4.5.5 *Coxiella burnetii*

Detection of *C. burnetii* was conducted by PCR targeting the *IS1111* transposase gene with an expected product size of 104 bp (Figure 4-15).

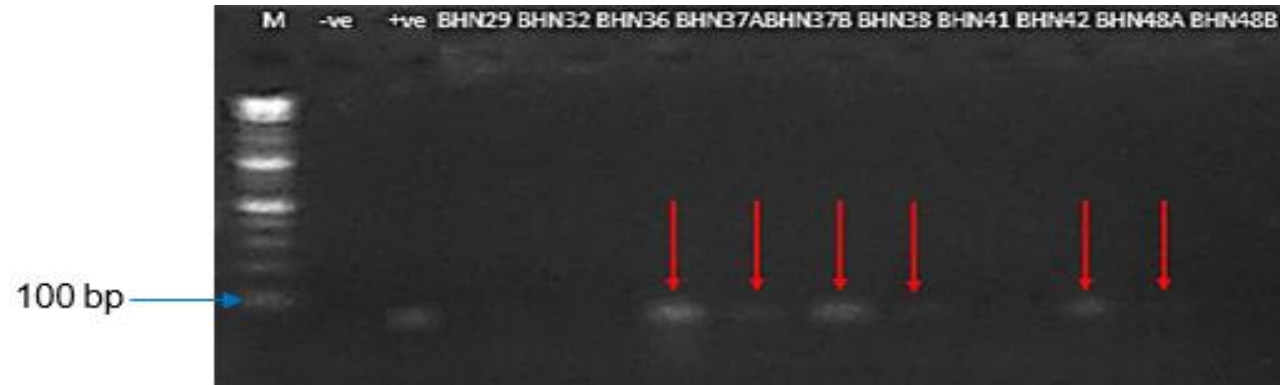
Agarose gel electrophoresis A



Agarose gel electrophoresis B



Agarose gel electrophoresis C



Agarose gel electrophoresis D



Figure 4-15: The 1% agarose electrophoresis gel of DNA amplicons for the screening of *C. burnetii* with an expected product size of 104 bp. **Agarose gel electrophoresis gel A:** Lane 1 (M): 100 bp DNA molecular marker. Lane 2 (-ve): No template control. Lane 3 (+ve): Positive control. Lane 4 to 28 (BHA3 to BHA48): Amplified DNA of *C. burnetii* from *H. elliptica* adult ticks. **Agarose gel electrophoresis gel B:** Lane 1 (M): 100 bp DNA molecular marker. Lane 2 (-ve): No template control. Lane 3 (+ve): Positive control. Lane 4 to 27 (BRA2 to BRA58): Amplified DNA of *C. burnetii* from *R. sanguineus* adult ticks. **Agarose gel electrophoresis gel C:** Lane 1 (M): 100 bp DNA molecular marker. Lane 2 (-ve): No template control. Lane 3 (+ve): Positive control. Lane 4 to 13 (BHN29 to BHN48B): Amplified DNA of *C. burnetii* from *H. elliptica* nymph ticks. **Agarose gel electrophoresis gel B:** Lane 1 (M): 100 bp DNA molecular marker. Lane 2 (-ve): No template control. Lane 3 (+ve): Positive control. Lane 4 to 13 (EH24 to EH47): Amplified DNA of *C. burnetii* from *H. elliptica* eggs. Red arrows indicate the presence of tick borne-pathogens

Average sequenced lengths of the amplicons ranged between 37 and 41 bp (Table 4-16). BLASTn search results of *C. burnetii* (Sample IDs: BHN36, BHN37B, BHN42, and EH38) of this study were similar to *C. burnetii* sequences (GenBank accession numbers: JF970261.1, MH394636.1, and CP014563.1) on the NCBI database with query covers ranging between 82 and 100%, matching identifications ranging between 95 and 100%, and E-values ranging between 2.00E-05 and 7.00E-06. Sequence alignment showing matches of the nucleotides between *C. burnetii* detected from this study and a reference sequence from the NCBI database are shown in Figure 4-16.

The overall occurrence of *C. burnetii* infecting dog ticks was 13.5% (14/104) (Table 4-11). The infestation rate of *C. burnetii* in dog ticks was statistically significant [$p = 7.855\text{e-}08$ ($X^2 = 91.143$ and $df = 31$)] in and around various locations of the JB Marks Local Municipality. *Heamaphysalis elliptica* had an infestation rate of 22.4% (11/49) and *R. sanguineus* had an infestation rate of 5.5% (3/55). *Coxiella burnetii* was abundant in ticks of dogs originating in Miederpark [*H. elliptica* adults (n=4 pools) and nymphs (n=5 pools), as well as *R. sanguineus* adults (n=1 pool)]. *Coxiella burnetii* was also detected in ticks of dogs originating from Baillie park [*R. sanguineus* adults (n=1 pool)], Fochville [*H. elliptica* adults (n=1 pool)], Kannonierspark [*R. sanguineus* adults (n=1 pool)], and Boskop [*H. elliptica* nymphs (n=1 pool)].

The overall occurrence of *C. burnetii* from dog tick eggs was 6.5% (2/31) (Table 4-12). Transovarial transmission of *C. burnetii* in and around various locations of dog origin was not statistically significant [$p = 0.5321$ ($X^2 = 9$, $df = 10$)]. *Heamaphysalis elliptica* had an infestation rate of 10.5% (2/19), whereas *C. burnetii* was not detected in *R. sanguineus* eggs. *Coxiella burnetii* was detected in tick eggs of dogs originating from Miederpark [*H. elliptica* (n=1)] and Boipatong [*H. elliptica* (n=1)].

Table 4-16: BLASTn results of *C. burnetii*

Sample ID	Gene size (bp)	BLAST description	Maximum score	Total score	Query cover	E-value	Identity	Accession number
BHN36	41	<i>Coxiella burnetii</i>	66,2	66,2	87%	2,00E-08	100%	JF970261.1
		<i>Coxiella burnetii</i>	62,6	62,6	82%	2,00E-07	100%	MH394636.1
		<i>Coxiella burnetii</i>	62,6	62,6	82%	2,00E-07	100%	CP014563.1
		<i>Coxiella burnetii</i>	62,6	62,6	82%	2,00E-07	100%	CP014561.1
		<i>Coxiella burnetii</i>	62,6	62,6	82%	2,00E-07	100%	CP014559.1
BHN37B	39	<i>Coxiella burnetii</i>	68	68	94%	4,00E-09	100%	MH394636.1
		<i>Coxiella burnetii</i>	68	68	94%	4,00E-09	100%	CP014563.1
		<i>Coxiella burnetii</i>	68	68	94%	4,00E-09	100%	CP014561.1
		<i>Coxiella burnetii</i>	68	68	94%	4,00E-09	100%	CP014559.1
		<i>Coxiella burnetii</i>	68	204	94%	4,00E-09	100%	CP014557.1
BHN42	38	<i>Coxiella burnetii</i>	57,2	57,2	92%	7,00E-06	97%	JF970261.1
		<i>Coxiella burnetii</i>	55,4	55,4	100%	2,00E-05	95%	MH394636.1
		<i>Coxiella burnetii</i>	55,4	55,4	100%	2,00E-05	95%	CP014563.1
		<i>Coxiella burnetii</i>	55,4	55,4	100%	2,00E-05	95%	CP014561.1
EH38	37	<i>Coxiella burnetii</i>	57,2	57,2	94%	6,00E-06	97%	MH394636.1
		<i>Coxiella burnetii</i>	57,2	57,2	94%	6,00E-06	97%	CP014563.1
		<i>Coxiella burnetii</i>	57,2	57,2	94%	6,00E-06	97%	CP014561.1
		<i>Coxiella burnetii</i>	57,2	57,2	94%	6,00E-06	97%	CP014559.1
		<i>Coxiella burnetii</i>	57,2	171	94%	6,00E-06	97%	CP014557.1

C. burnetii 5 TTATTCCCACTCGAATGTTGTCGAGGGACCAACC 38
 MH394636.1 649 TTATTCCCACTCGAATGTTGTCGAGGGACCAACC 616

Figure 4-16: Fragment of the BLASTn alignment between *C. burnetii* of this study and a corresponding sequence. First strand represents *C. burnetii* detected from ticks collected from the JB Marks local municipality. Second strand represents a reference sequence from NCBI

4.6 Metagenomic diagnosis of tick microbiota

Metagenomic analysis of *H. elliptica* and *R. sanguineus* ticks (n=6), salivary glands (n=6), midguts (n=6), and eggs (n=6), with extracted DNA concentrations ranging from 2.412 to 10.94 ng/μl, produced a total of 1111515 assembled sequences. In total 785080 sequences, with 97 to 100% similarity, were aligned to a reference database and *de novo* (Table 4-17). Final analysis revealed 404 OTUs produced up to genus level (Appendix 2).

Table 4-17: Sequences from tick samples used to generate OTUs

Sample code	Sample information	Sequences assembled	Sequences aligned	Total OTUs produced
RS.SG.1	<i>R. sanguineus</i> salivary gland	92772	79399	404
RS.SG.2	<i>R. sanguineus</i> salivary gland	26662	21943	404
RS.SG.3	<i>R. sanguineus</i> salivary gland	11226	3182	404
HE.SG.1	<i>H. elliptica</i> salivary gland	37207	10396	404
HE.SG.2	<i>H. elliptica</i> salivary gland	16184	4655	404
HE.SG.3	<i>H. elliptica</i> salivary gland	18442	1556	404
RS.MG.1	<i>R. sanguineus</i> midgut	33333	21772	404
RS.MG.2	<i>R. sanguineus</i> midgut	77798	70149	404
RS.MG.3	<i>R. sanguineus</i> midgut	95498	73037	404
HE.MG.1	<i>H. elliptica</i> midgut	50355	50020	404
HE.MG.2	<i>H. elliptica</i> midgut	64406	60728	404
HE.MG.3	<i>H. elliptica</i> midgut	38637	38142	404
RS.EGG.1	<i>R. sanguineus</i> eggs	27990	19918	404
RS.EGG.2	<i>R. sanguineus</i> eggs	69262	59651	404
RS.EGG.3	<i>R. sanguineus</i> eggs	44084	27532	404
HE.EGG.1	<i>H. elliptica</i> eggs	53055	50020	404
HE.EGG.2	<i>H. elliptica</i> eggs	64406	60728	404
HE.EGG.3	<i>H. elliptica</i> eggs	38637	38142	404
RS.WT.1	<i>R. sanguineus</i> whole tick	36001	20492	404
RS.WT.2	<i>R. sanguineus</i> whole tick	31638	12627	404
RS.WT.3	<i>R. sanguineus</i> whole tick	50264	40171	404
HE.WT.1	<i>H. elliptica</i> whole tick	44336	5614	404
HE.WT.2	<i>H. elliptica</i> whole tick	56863	5205	404
HE.WT.3	<i>H. elliptica</i> whole tick	32459	10001	404
Total		1111515	785080	

4.6.1 Sequence alignments, OTUs produced and bacterial classification

There were 25 phyla detected during metagenomic analysis (Figure 4-17). Bacterial phyla that were unassigned or had relative abundances in all of the sample types with less than 1% are labeled as other. These phyla had a total relative abundance of 14.2%. Dominating bacterial phyla detected were Proteobacteria (57.2%), Actinobacteria (17.4%), and Bacteroidetes (12%). This was followed by Firmicutes (7.8%) and Cyanobacteria (2.7%).

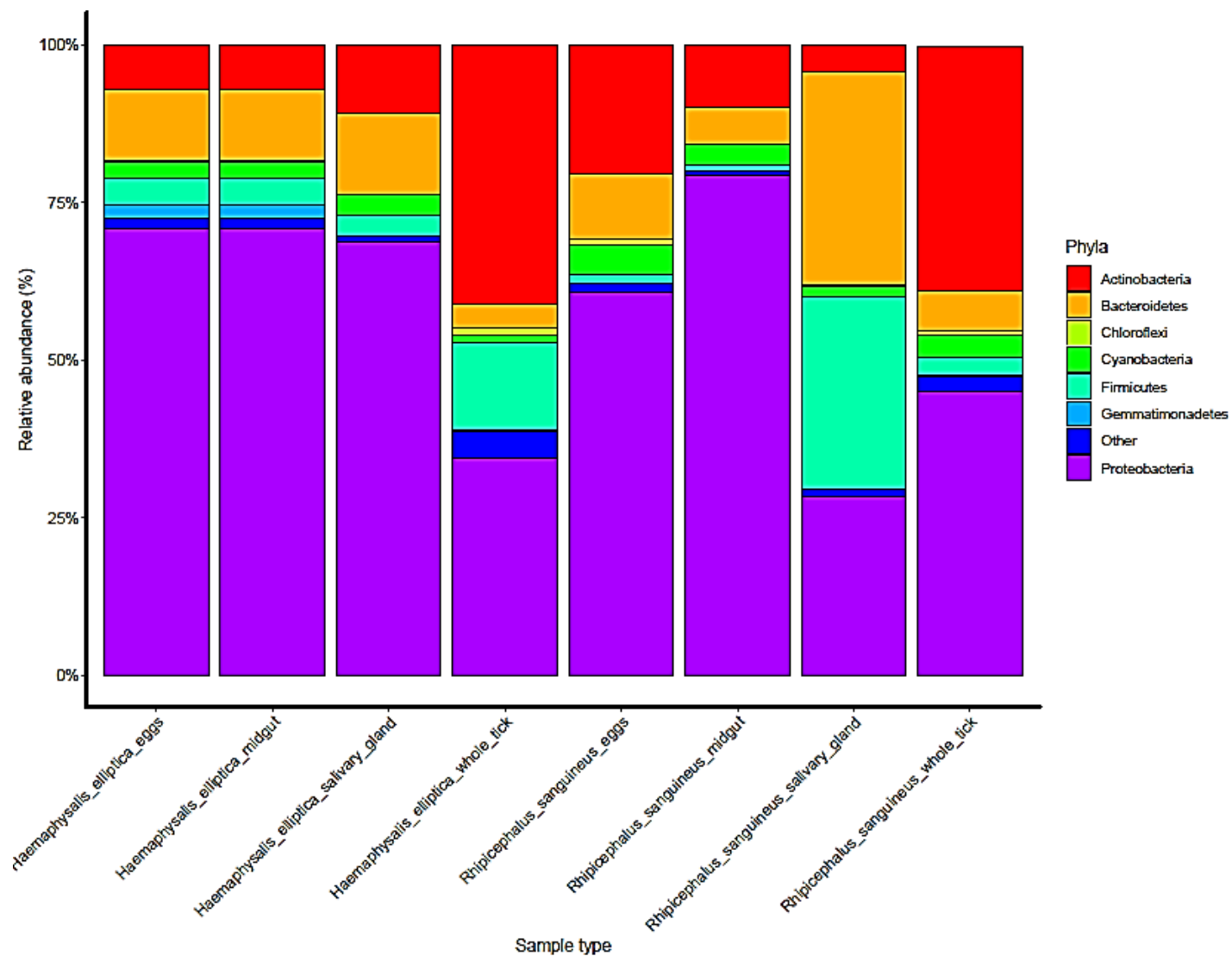


Figure 4-17: Bacterial phyla detected from different tick samples

There were 63 classes detected during metagenomic analysis (Figure 4-18). Bacterial classes that were unassigned or had relative abundances in all of the sample types with less than 1% are labeled as other. These classes had a total relative abundance of 23.9%. Dominating bacterial classes detected were Alphaproteobacteria (56.5%), Clostridia (7.6%), and Bacteroidia (5%). This was followed by Saprospirae (3.6%) and Flavobacteriia (2.4%). Other bacterial classes detected included Cytophagia (0.8%) and Coriobacteriia (0.2%).

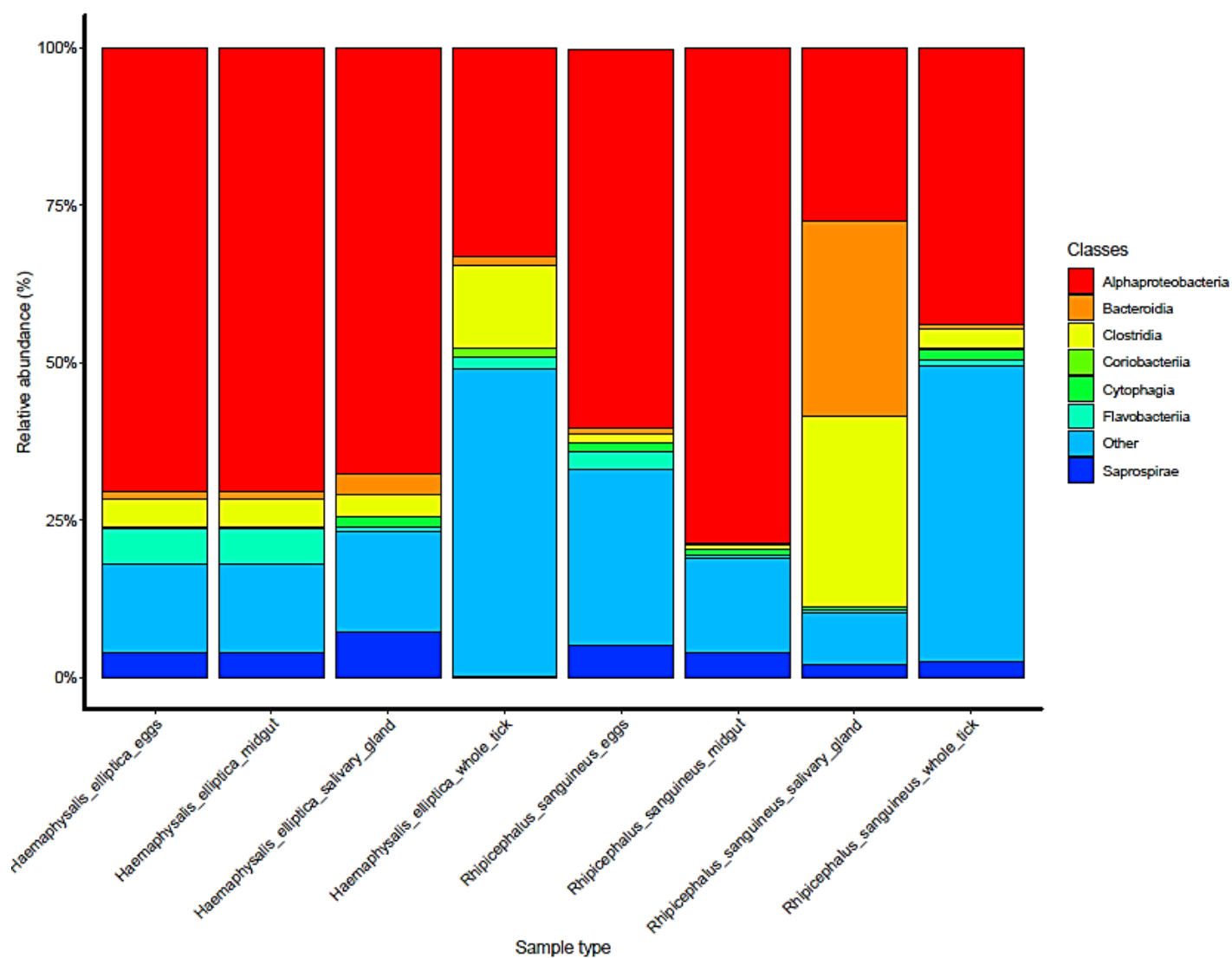


Figure 4-18: Bacterial classes detected from different tick samples

There were 102 orders detected during metagenomic analysis (Figure 4-19). Bacterial orders that were unassigned or had relative abundances in all of the sample types with less than 1% are labeled as other. These orders had a total relative abundance of 8.3%. Dominating bacterial orders detected were Sphingomonadales (23.4%), Rhizobiales (22.5%), and Actinomycetales (16.9%). This was followed by Clostridiales (7.6%), Bacteroidales (5%), Rhodospirillales (3.7%), Saprospirales (3.6%), Caulobacteriales (3.5%), and Flavobacteriales (2.4%). Other bacterial orders detected included Cytophagales (0.8%), Rhodobacterales (0.8%), Rickettsiales (0.7%), Gemmatimonadales (0.5%), and Coriobacteriales (0.2%)

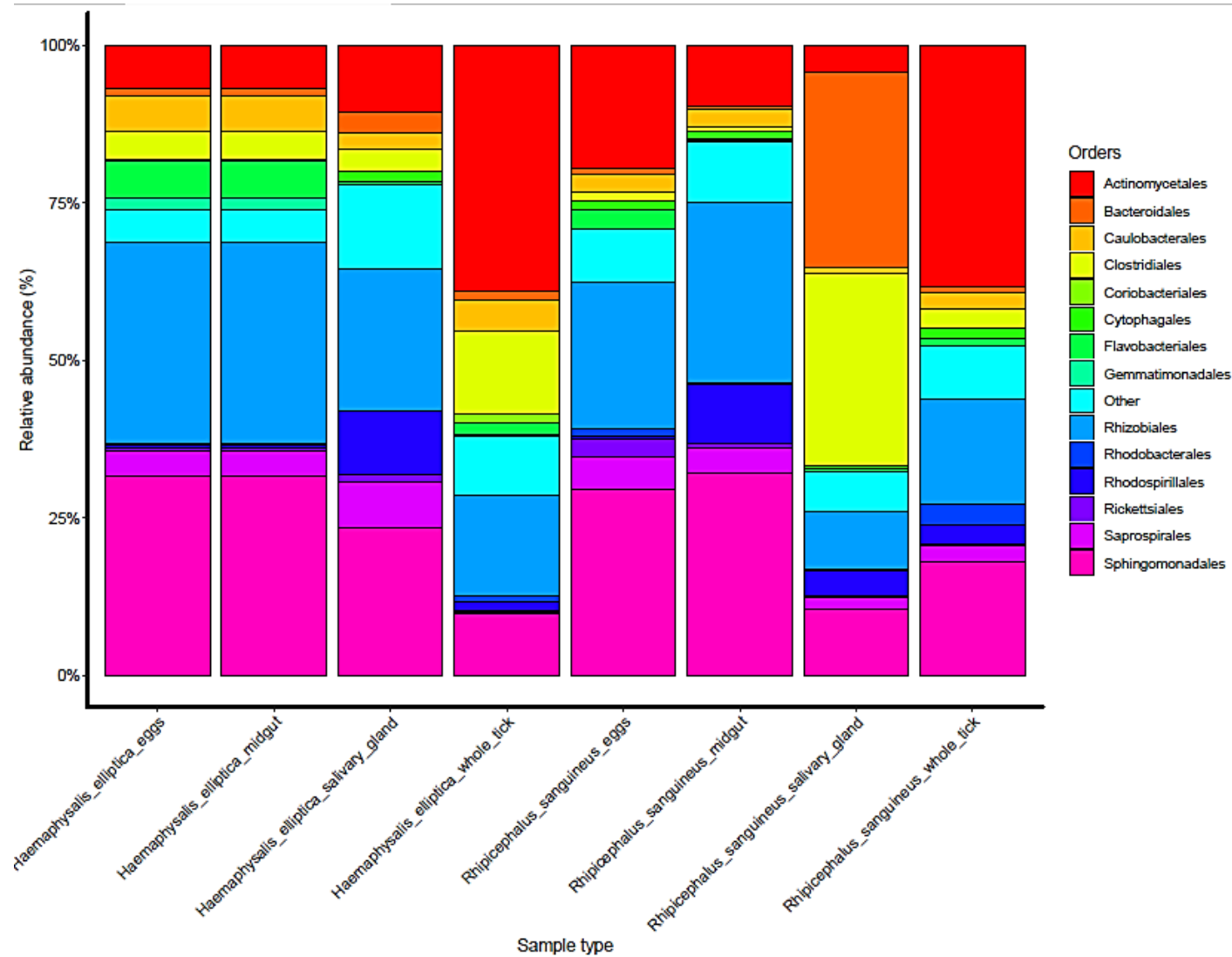


Figure 4-19: Bacterial orders detected from different tick samples

There were 205 families detected during metagenomic analysis (Figure 4-20). Bacterial families that were unassigned or had relative abundances in all of the sample types with less than 1% are labeled as other. Dominating bacterial families detected were Sphingomonadaceae (22.8%) and Bradyrhizobiaceae (12.8%). This was followed by Corynebacteriaceae (5.7%), Propionibacteriaceae (5.0%), Chitinophagaceae (3.6%), Caulobacteraceae (3.5%), Rhodospirillaceae (3.1%), Brucellaceae (2.6%), Rhizobiaceae (2.6%), Ruminococcaceae (2.5%), Hyphomicrobiaceae (2.5%), Rikenellaceae (2.1%), Bacteroidaceae (1.8%), Weeksellaceae (1.8%), Clostridiaceae (1.4%), Micrococcaceae (1.2%), Lachnospiraceae (1%), and Tissierellaceae (1%). Other bacterial families detected included Cytophagaceae (0.8%), Rhodobacteraceae (0.8%), Nocardiopteraceae (0.8%), Microbacteriaceae (0.7%), Methylobacteriaceae (0.7%), Acetobacteraceae (0.6%), Erythrobacteraceae (0.6%), Flavobacteriaceae (0.5%), Mycobacteriaceae (0.3%), Phyllobacteriaceae (0.3%), Dietziaceae (0.2%), Coriobacteriaceae (0.2%), and Anaplasmataceae (0.2%).

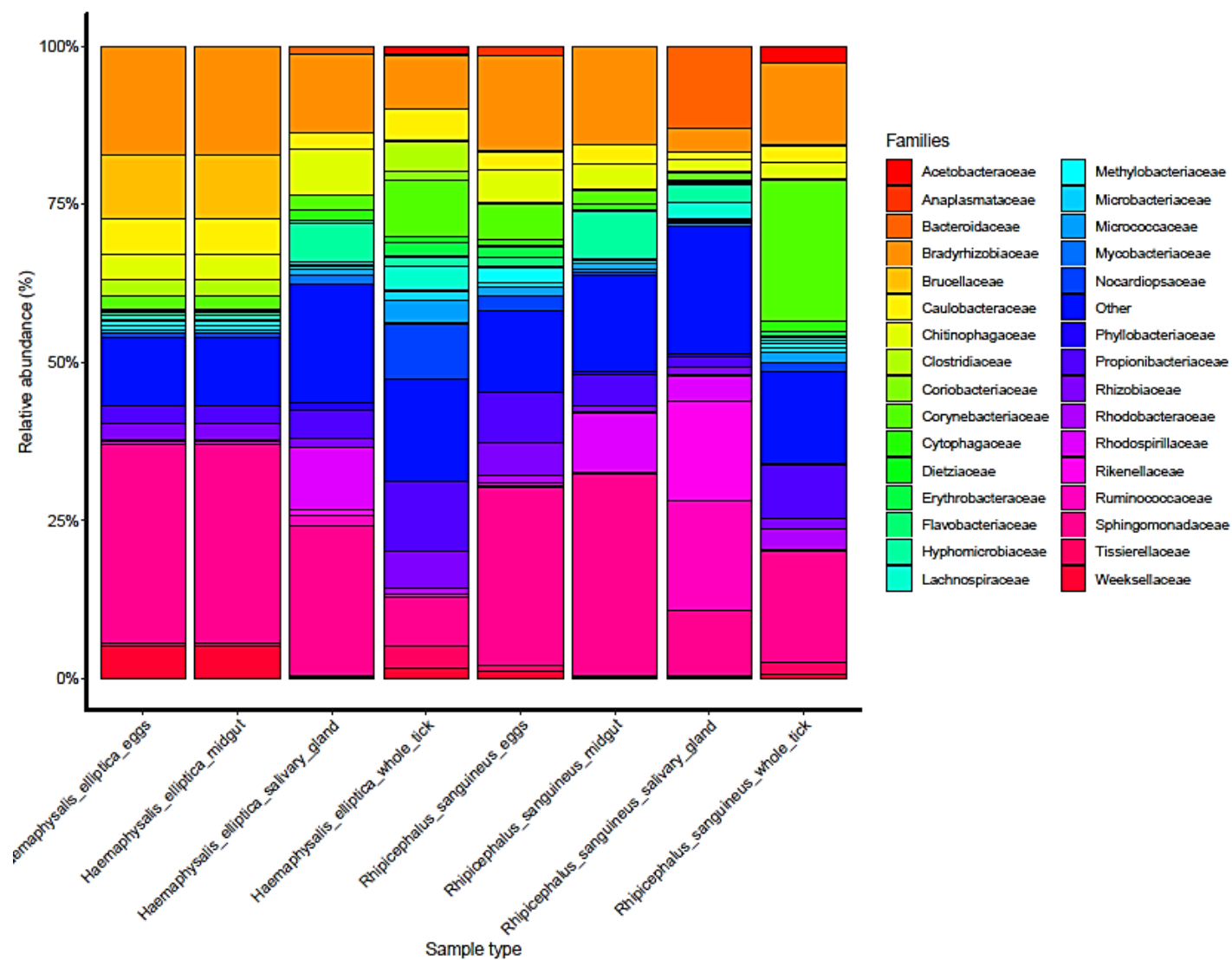


Figure 4-20: Bacterial families detected from different tick samples

There were 404 genera detected during metagenomic analysis (Figure 4-21). Bacterial genera that were unassigned or had relative abundances in all of the sample types with less than 1% are labeled as other. These genera had a total relative abundance of 48.5%. Dominating bacterial genera detected were *Sphingomonas* (19%), *Corynebacterium* (5.7%), and *Propionibacterium* (5.5%). This was followed by *Agrobacterium* (2.2%), *Sphingopyxis* (2.2%), *Bacteroides* (1.7%), *Sediminibacterium* (1.7%), *Chryseobacterium* (1.5%), *Hyphomicrobium* (1.4%), *Clostridium* (1.2%), and *Brevundimonas* (1.1%). Other bacterial genera detected included *Oscillospira* (0.8%), *Micrococcus* (0.7%), *Rhodoplanes* (0.7%), *Paracoccus* (0.7%), *Novosphingobium* (0.6%), *Flavobacterium* (0.5%), *Anaerococcus* (0.5%), *Phenylobacterium* (0.5%), *Rhizobium* (0.4%), *Kaistobacter* (0.4%), *Nocardiopsis* (0.3%), *Blautia* (0.3%), *Ruminococcus* (0.3%), *Finnegoldia* (0.3%), *Dietzia* (0.2%), *Mycobacterium* (0.2%), *Collinsella* (0.2%), *Rikenella* (0.2%), *Faecalibacterium* (0.2%), and *Ehrlichia* (0.2%).

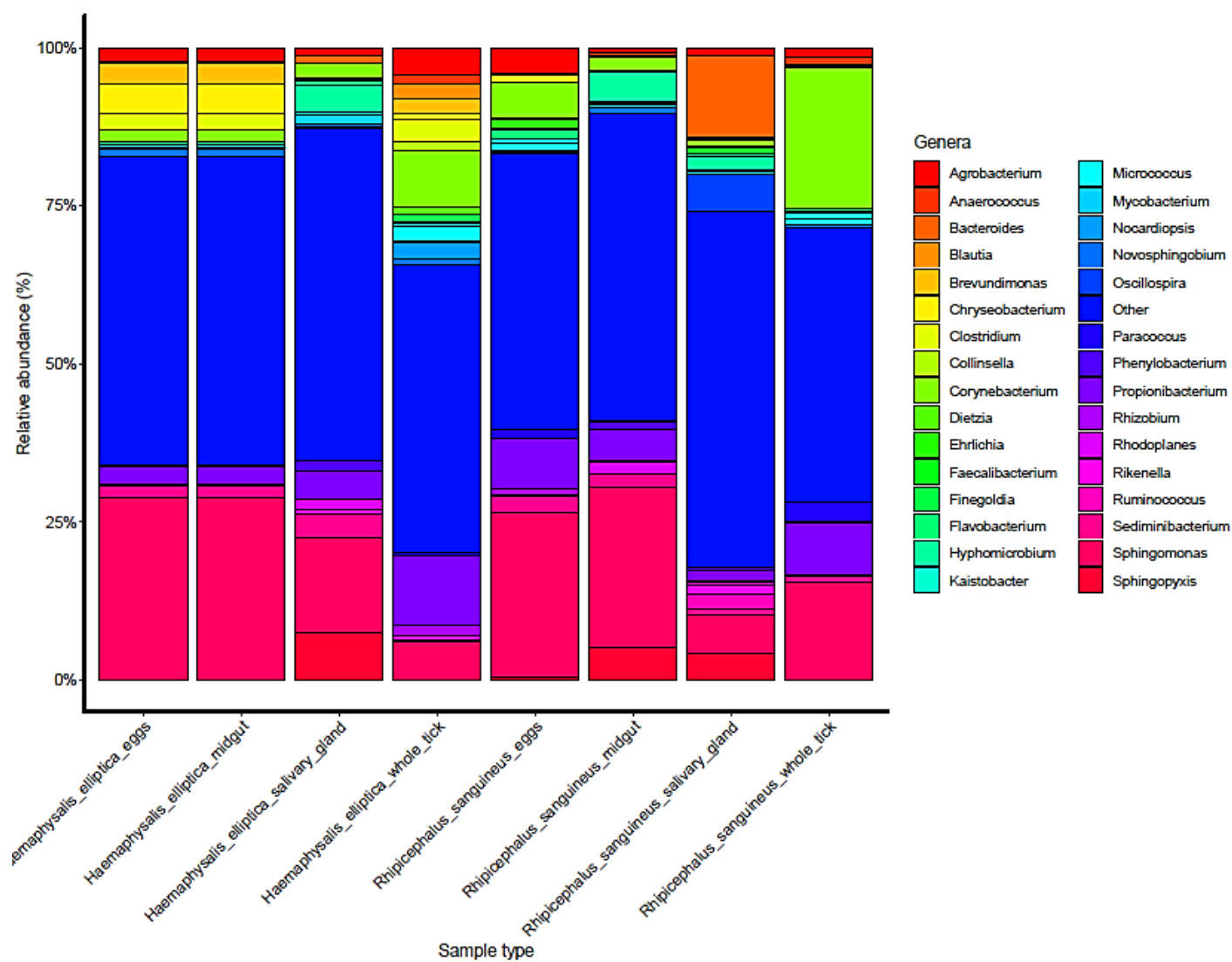


Figure 4-21: Bacterial genera detected from different tick samples

4.6.2 Alpha and beta diversity analysis

Alpha diversity analysis was done to determine the species richness and evenness within each sample type (Table 4-18). After OTU picking the observed OTUs were almost the same as the expected OTUs (as determined by using the chao1 estimator) for all sample types except for the midguts and eggs of *H. elliptica*. For these samples, the expected OTUs ranged between 33682 to 76598, respectively, whereas the observed OTUs ranged between 4122 and 5100, respectively. All of the sample types had good coverage with goods coverage score ranging from 90.6 to 99.8%, respectively. Shannon diversity index values ranged from 6.54 to 7.44, respectively. For all of the sample types, the Simpson diversity index values were less than 1 with respective values ranging between 0.918 and 0.981 indicating no diversity between the bacterial communities of the ticks.

Table 4-18: Alpha diversity indices of tick samples based on data obtained from Illumina Miseq

Sample code	Sample information	Estimated number of OTUs (chao1)	Number of observed OTUs	Shannon diversity	Simpson diversity	Good's coverage (coverage values)
RS.SG.1	<i>R. sanguineus</i> salivary gland	1590	1545	7,44	0,977	99,8%
RS.SG.2	<i>R. sanguineus</i> salivary gland	963	739	6,46	0,976	98,8%
RS.SG.3	<i>R. sanguineus</i> salivary gland	366	259	5,84	0,954	97,1%
HE.SG.1	<i>H. elliptica</i> salivary gland	766	490	6,01	0,968	98,0%
HE.SG.2	<i>H. elliptica</i> salivary gland	530	418	6,55	0,966	97,1%
HE.SG.3	<i>H. elliptica</i> salivary gland	245	189	5,73	0,951	96,0%
RS.MG.1	<i>R. sanguineus</i> midgut	1077	837	6,07	0,964	98,6%
RS.MG.2	<i>R. sanguineus</i> midgut	1306	1168	5,98	0,963	99,6%
RS.MG.3	<i>R. sanguineus</i> midgut	1614	1447	5,65	0,930	99,5%
HE.MG.1	<i>H. elliptica</i> midgut	56645	4204	6,04	0,928	92,6%
HE.MG.2	<i>H. elliptica</i> midgut	76598	5100	5,91	0,918	92,5%
HE.MG.3	<i>H. elliptica</i> midgut	33682	4122	6,03	0,941	90,6%
RS.EGG.1	<i>R. sanguineus</i> eggs	855	632	5,54	0,935	98,9%
RS.EGG.2	<i>R. sanguineus</i> eggs	1075	912	5,77	0,946	99,6%
RS.EGG.3	<i>R. sanguineus</i> eggs	592	501	5,55	0,962	99,4%
HE.EGG.1	<i>H. elliptica</i> eggs	56645	4204	6,04	0,928	92,6%
HE.EGG.2	<i>H. elliptica</i> eggs	76598	5100	5,91	0,918	92,5%
HE.EGG.3	<i>H. elliptica</i> eggs	33682	4122	6,03	0,941	90,6%
RS.WT.1	<i>R. sanguineus</i> whole tick	967	817	6,20	0,957	98,9%
RS.WT.2	<i>R. sanguineus</i> whole tick	882	767	6,89	0,968	98,5%
RS.WT.3	<i>R. sanguineus</i> whole tick	1261	1160	6,72	0,969	99,4%
HE.WT.1	<i>H. elliptica</i> whole tick	410	361	6,05	0,958	98,5%
HE.WT.2	<i>H. elliptica</i> whole tick	525	439	7,06	0,981	97,6%
HE.WT.3	<i>H. elliptica</i> whole tick	573	462	6,61	0,979	98,5%

Beta diversity analysis was done to determine differentiation among the sample types. To determine the relative abundances of the OTUs indicating species richness heatmaps was constructed at class and genus level. Figure 4-22 illustrates that relative abundances at class level between the *H. elliptica* eggs and midguts were very similar. Relative abundances at class level between *H. elliptica* and *R. sanguineus* eggs, midguts, and ticks were also similar. These similarities observed of relative abundances indicate similarities among species richness. Relative abundances at class level between *H. elliptica* and *R. sanguineus* midguts were not similar, indicating differentiation among species richness.

Figure 4-23 illustrates that relative abundances at genus level between the eggs and midguts of both *H. elliptica* and *R. sanguineus* were very similar especially for the *Sphingomonas* genus. Relative abundances between the salivary glands of *H. elliptica* and *R. sanguineus* were somewhat similar. These similarities observed of relative abundances indicate similarities among species richness. Different relative abundances between the bacterial genera were observed between the entire tick samples of *H. elliptica* and *R. sanguineus*. These differences indicate differentiation among species richness. This may be due to bacterial genera present on the external surfaces of the tick

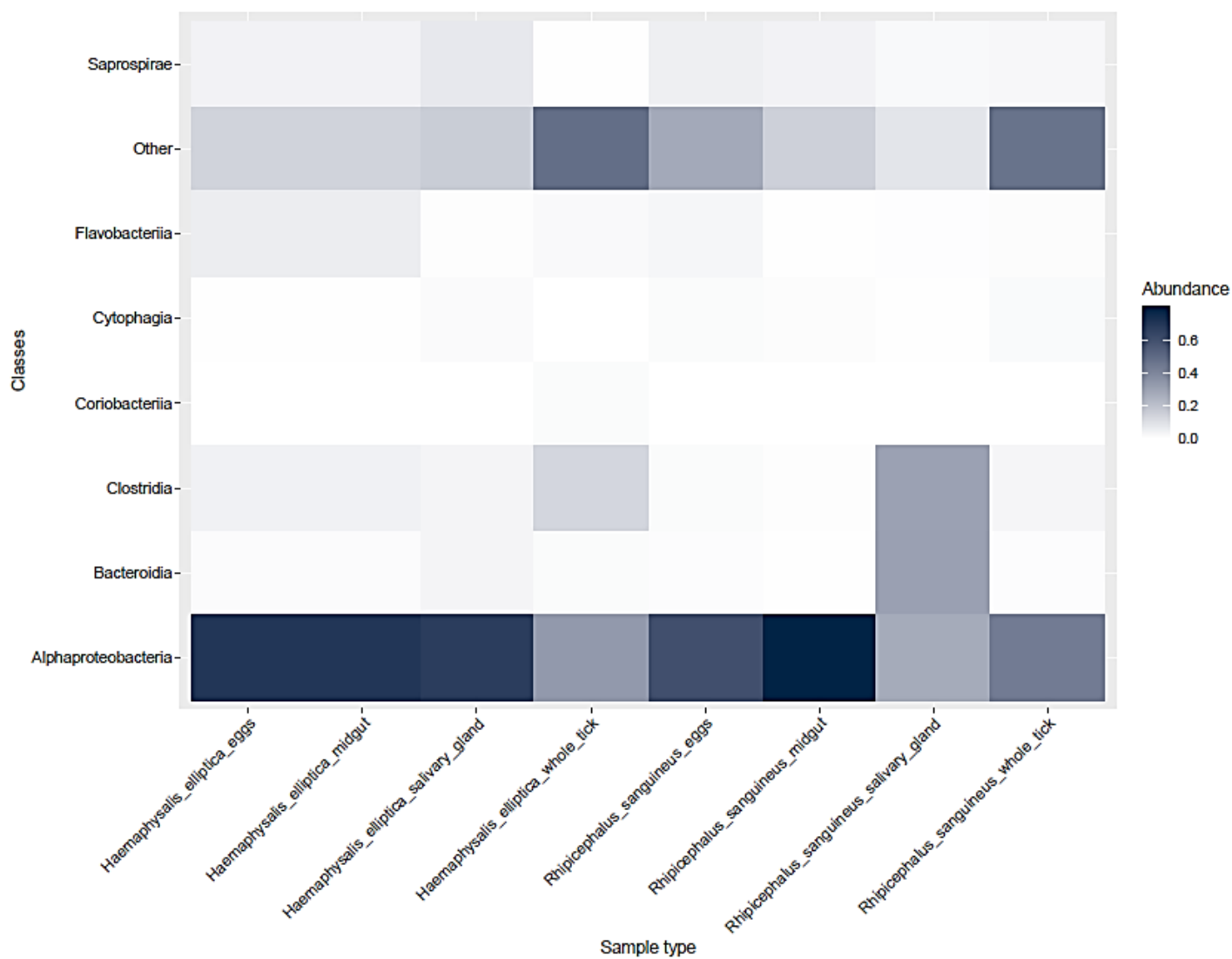


Figure 4-22: Heatmap to indicate bacterial abundance at class level

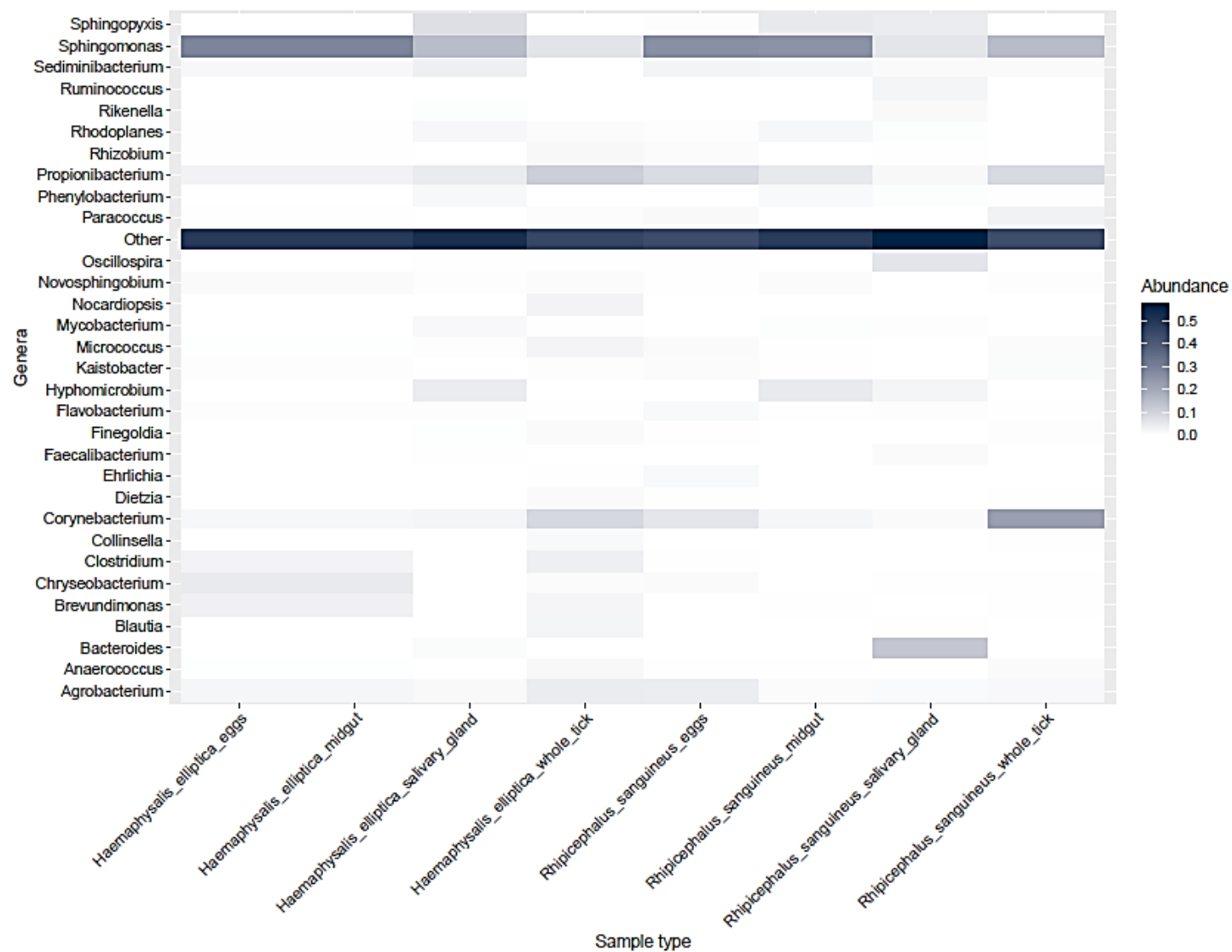


Figure 4-23: Heatmap to indicate bacterial abundance at genus level

Venn diagrams were constructed of the bacterial genera to determine the shared species richness between different sample types. Between *H. elliptica* and *R. sanguineus* ticks, there were 110 shared bacterial genera (Figure 4-24). Between *H. elliptica* samples 63 bacterial genera were shared between the entire tick, eggs, midguts, and salivary glands. Whereas 34 and 26 bacterial genera were shared between the entire tick, midguts, eggs, as well as, the eggs, midguts, and salivary glands, respectively. There was also 96 and 13 bacterial genera shared between the eggs and midguts, as well as the entire tick and the salivary glands, respectively (Figure 4-25). Between *R. sanguineus* samples there were 62 shared bacterial genera between the entire tick, the eggs and, salivary glands. There were also 44 and 11 shared bacterial genera between the entire tick and the eggs, as well as the eggs and salivary glands, respectively (Figure 4-26).

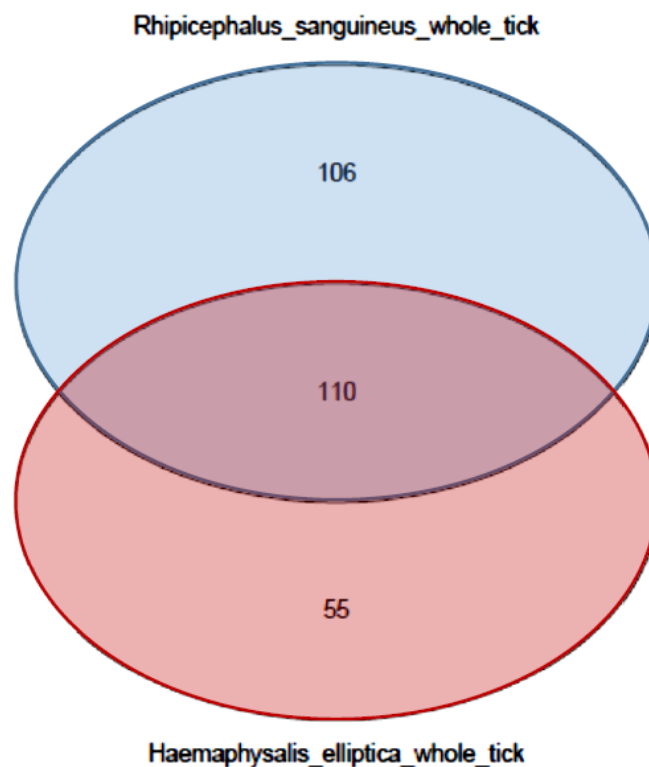


Figure 4-24: Venn diagram indicating the shared number of bacterial genera between *H. elliptica* and *R. sanguineus* ticks

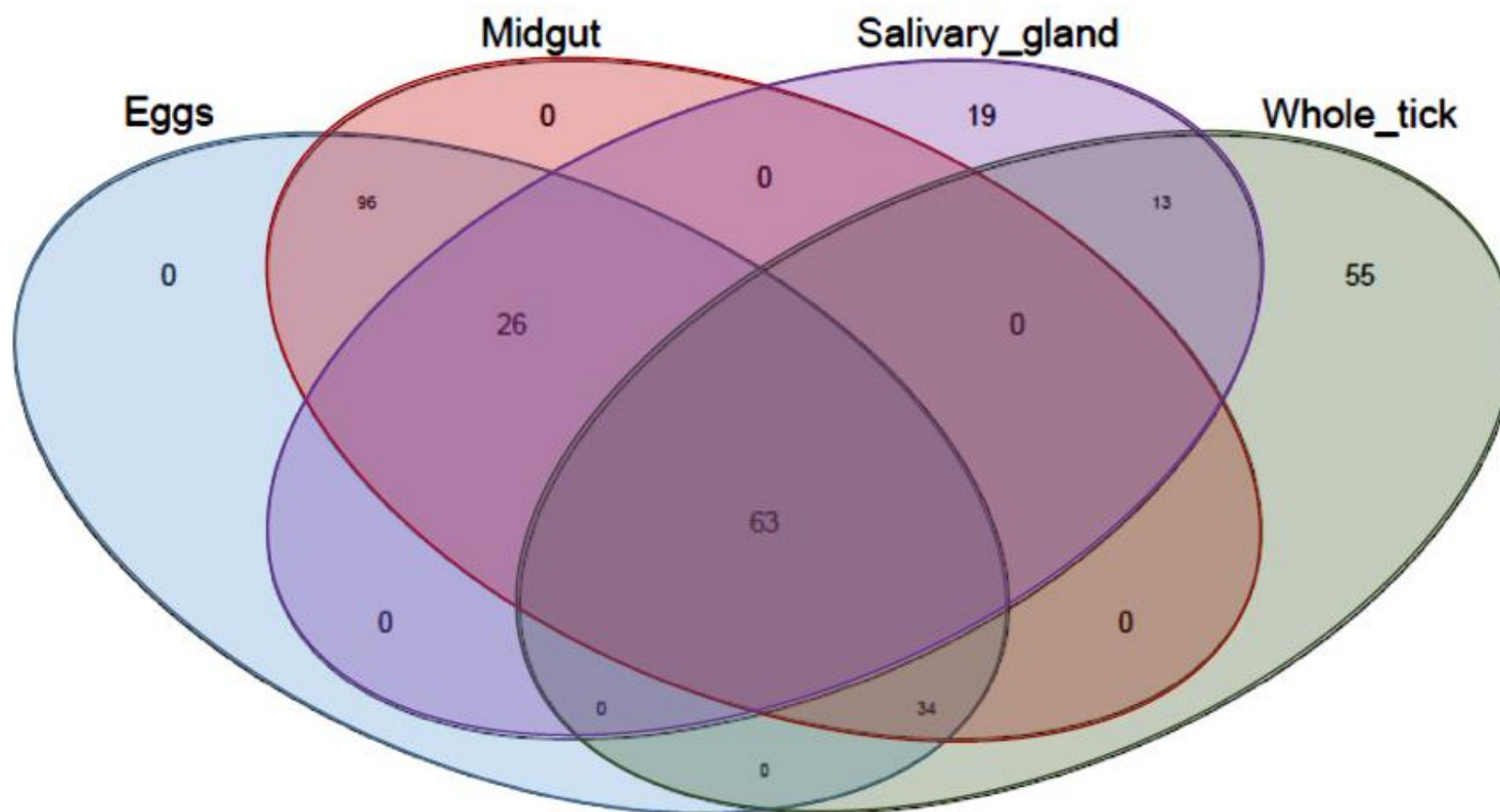


Figure 4-25: Venn diagram indicating the shared number of bacterial genera between *H. elliptica* samples

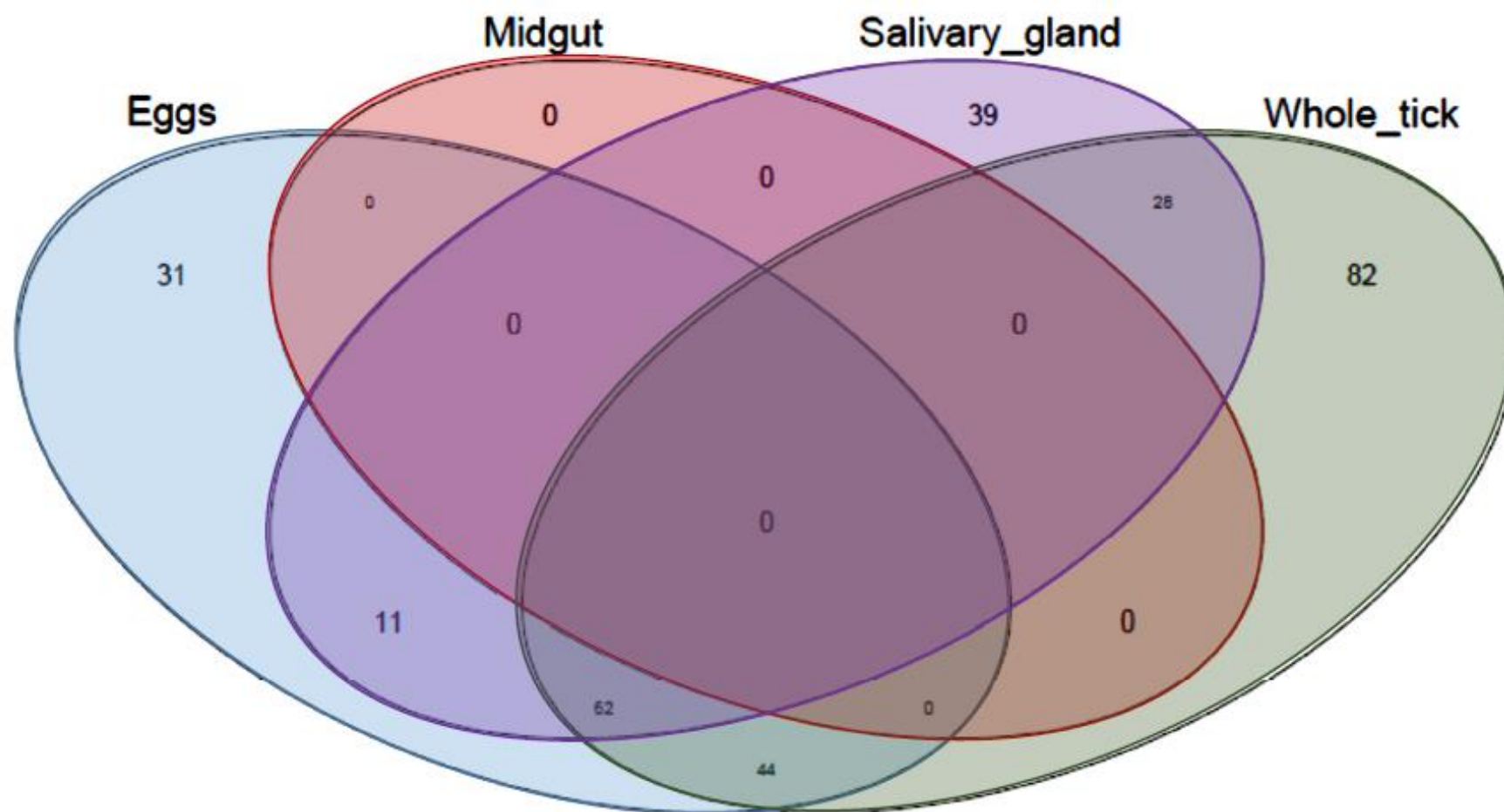


Figure 4-26: Venn diagram indicating the shared number of bacterial genera between *R. sanguineus* samples

Bacterial genera of veterinary, ecological, and medical importance were also detected in lower relative abundances including *Bacillus*, *Staphylococcus*, *Streptococcus*, *Clostridium*, *Ehrlichia*, *Rickettsia*, *Wolbachia*, *Neisseria*, *Campylobacter*, *Proteus*, *Coxiella*, *Borrelia*, and *Pseudomonas*, to mention but a few (Table 4-19).

Table 4-19: Veterinary, environmental and, medical important bacterial genera of *R. sanguineus* and *H. elliptica*

Genus	Significance	Reference
<i>Corynebacterium</i>	Certain species are animal and plant pathogens.	Hernández-jarguín <i>et al.</i> (2018)
<i>Micrococcus</i>	Certain species causes infections in humans.	Yap and Mermel (2003)
<i>Mycobacterium</i>	Causes infections in humans and mammals, such as leprosy and tuberculosis.	Cole <i>et al.</i> (2001); Gomez and McKinney (2004)
<i>Nocardia</i>	Causes infections in humans, associated with Nocardiosis.	Wilson (2012)
<i>Streptomyces</i>	Some species cause infection in humans.	Kapadia, Rolston, and Han (2007)
<i>Bifidobacterium</i>	Symbiotic and commensal bacteria of humans.	Hernández-jarguín <i>et al.</i> (2018)
<i>Bacillus</i>	Associated with various diseases, including anthrax.	Kandi (2016)
<i>Staphylococcus</i>	Opportunistic pathogens associated with septicemia and food poisoning.	Hernández-jarguín <i>et al.</i> (2018)
<i>Lactobacillus</i>	Certain species are identified as potential emerging pathogens of humans.	Cannon <i>et al.</i> (2005)
<i>Streptococcus</i>	Opportunistic pathogens associated with meningitis, septicemia, and pneumonia.	Hernández-jarguín <i>et al.</i> (2018)
<i>Clostridium</i>	Causes a variety of diseases in animals and humans.	Uzal <i>et al.</i> (2018)
<i>Fusobacterium</i>	Associated with several diseases of humans.	Jayasimhan <i>et al.</i> (2017)
<i>Ehrlichia</i>	Tick-borne pathogen responsible for animal and human diseases.	Hernández-jarguín <i>et al.</i> (2018)
<i>Rickettsia</i>	Vector-borne intracellular bacteria, causes typhus and spotted fever in humans.	Hernández-jarguín <i>et al.</i> (2018)
<i>Wolbachia</i>	Mutualistic bacteria found in nematodes and insects.	Hernández-jarguín <i>et al.</i> (2018)
<i>Burkholderia</i>	Causes diseases in humans and animals.	Coenye and Vandamme (2003)
<i>Neisseria</i>	Two species are human pathogens.	Warinner <i>et al.</i> (2014)
<i>Campylobacter</i>	Various species are pathogenic to humans and animals.	Kaakoush <i>et al.</i> (2015)

Table 4.19 (continue): Veterinary, environmental and, medical important bacterial genera of *R. sanguineus* and *H. elliptica*

Genus	Significance	Reference
<i>Proteus</i>	Causes infections in humans.	Ebringer and Rashid (2013)
<i>Coxiella</i>	Associated with Q fever in humans and animals.	Eldin <i>et al.</i> (2017)
<i>Borrelia</i>	Vector-borne pathogen causing relapsing and Lyme disease in mammals.	Hernández-jarguín <i>et al.</i> (2018)
<i>Pseudomonas</i>	Opportunistic pathogens infecting plants and humans. Some species are used to promote plant growth.	Hernández-jarguín <i>et al.</i> (2018)
<i>Actinomyces</i>	Forms abscesses in the gastrointestinal tract, mouth, and lungs in humans.	Hernández-jarguín <i>et al.</i> (2018)
<i>Acinetobacter</i>	Certain species are opportunistic pathogens responsible for human infections.	Hernández-jarguín <i>et al.</i> (2018)
<i>Leptotrichia</i>	Forms part of the human microflora, certain species are responsible for opportunistic infections.	Hernández-jarguín <i>et al.</i> (2018)
<i>Rhodococcus</i>	One species infects animals and causes pneumonia.	Hernández-jarguín <i>et al.</i> (2018)
<i>Sphingobium</i>	Causes infections in humans.	Ryan and Adley (2010)
<i>Helicobacter</i>	Some species of this genus are human pathogens.	van Der Mee-marquet <i>et al.</i> (2017)

4.7 Surface sterilization methods

For the elimination of surface bacteria on the ticks, different washing procedures at different exposure times were tested. PCR was also done with these ticks to determine any damage to the genetic material. Due to the general use of 70% ethanol, this was used as a washing method on its own, followed by rinsing thrice with double distilled water. Surface sterilization was also done with 10% Bleach, 10% Tween 20, Phosphate buffered saline (PBS), and 10% Triton X-100 for 1, 2 and 3 hours, respectively. After the use of these washing procedures, the ticks were rinsed twice with 70% ethanol and thrice with double distilled water.

4.7.1 Visual inspection of surface sterilization methods

During the collection of samples, unwanted bacteria was present on the surface of the ticks (Plate 4-5). Use of 70% ethanol as a washing method on its own did not eliminate all the surface bacteria, as there were microorganisms still visible on the mouthparts, alloscutum, and the ticks leg (Plate 4-6). Respective use of 10% Bleach (Plate 4-7) and 10% Tween 20 (Plate 4-8) was effective washing methods as no surface bacteria was observed. These methods, however, did not completely remove all the particles, as there were still particles visible on the alloscutum and genetic material, due to shredding of the mouthparts, most likely from physical removal of the ticks from the host. In addition, no difference was observed of these washing methods at 1, 2, and 3 hours, respectively. Ticks surface sterilized with PBS for 1, 2, and 3 hours respectively did not eliminate the surface bacteria (Plate 4-9). Ticks surface sterilized with 10% Triton X-100 did eliminate the surface bacteria (Plate 4-10). However, material from the mouthparts, likely due to physical damage, from removal of the tick from the host, was not eliminated. At 3 hours ticks also appeared to have damage on the exterior surface.

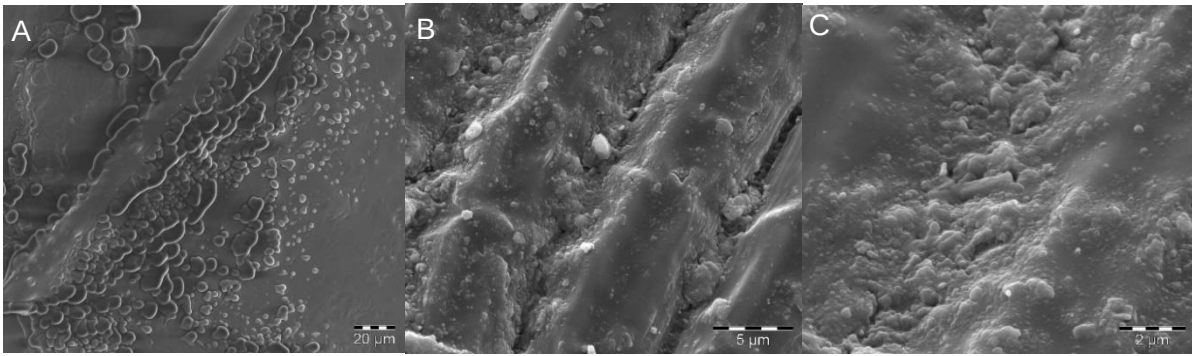


Plate 4-5: TEM image of unwashed tick surface. (A) Alloscutum and (B and C) scutum

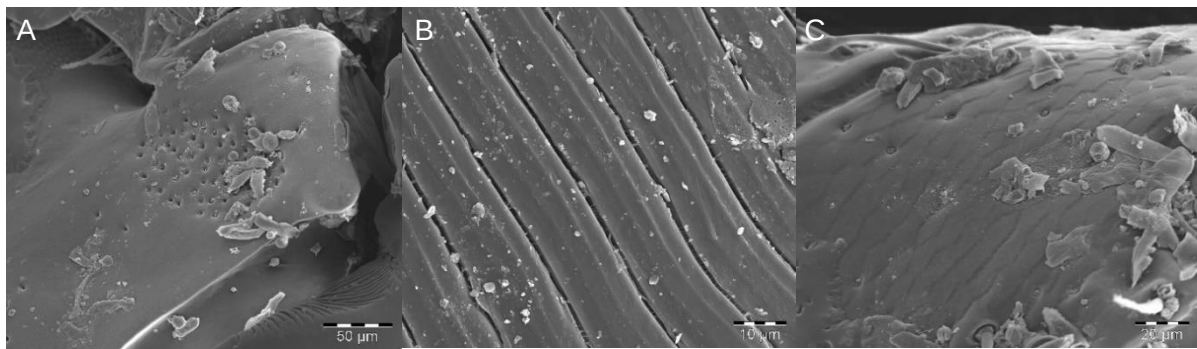


Plate 4-6: TEM image of tick surface sterilized with 70% ethanol. (A) Mouthparts, (B) alloscutum and (C) leg

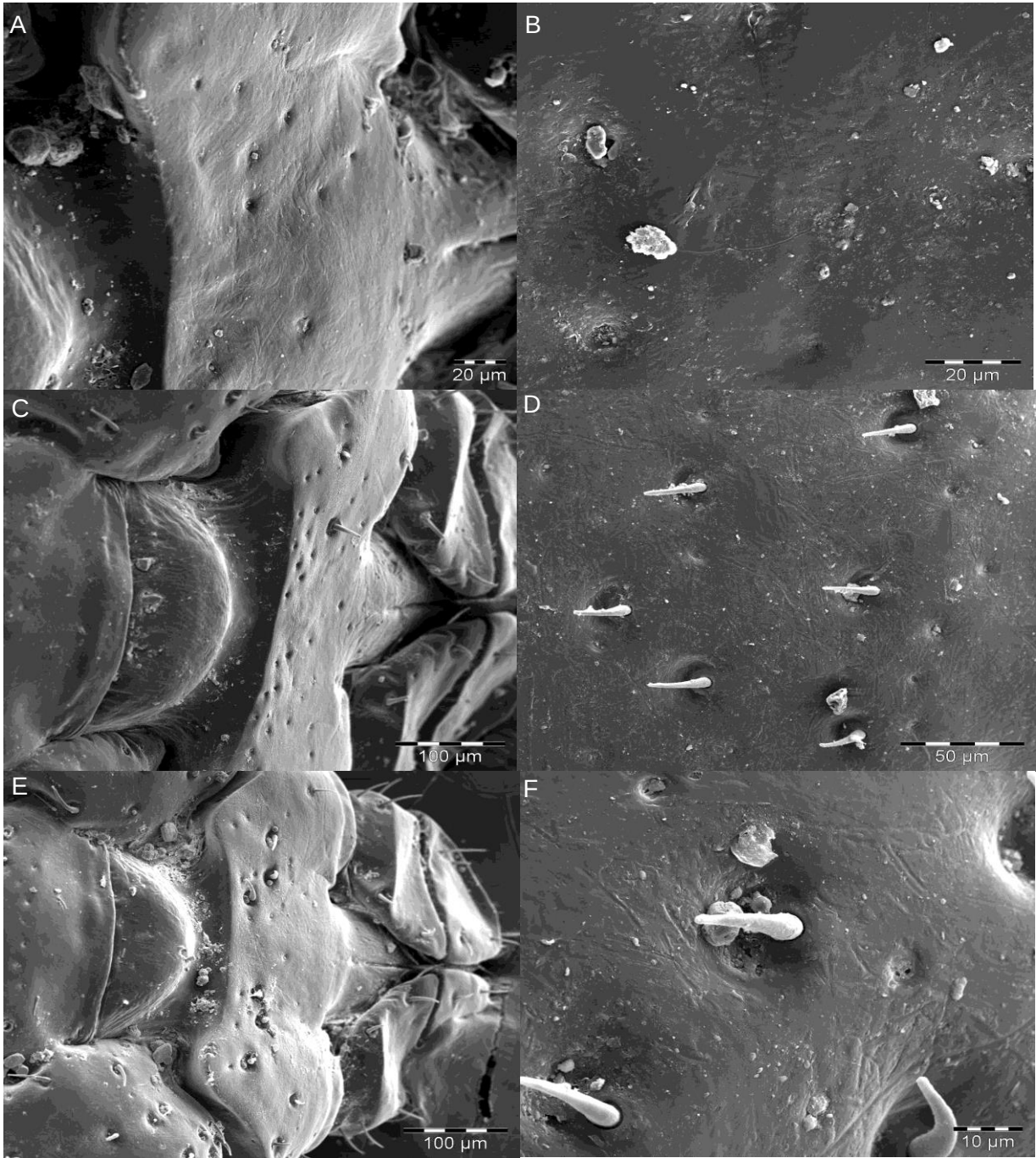


Plate 4-7: TEM image of tick mouthparts (A, C, and F) and alloscutum (B, D, and E) sterilized with 10% bleach for 1, 2 and 3 hours, respectively

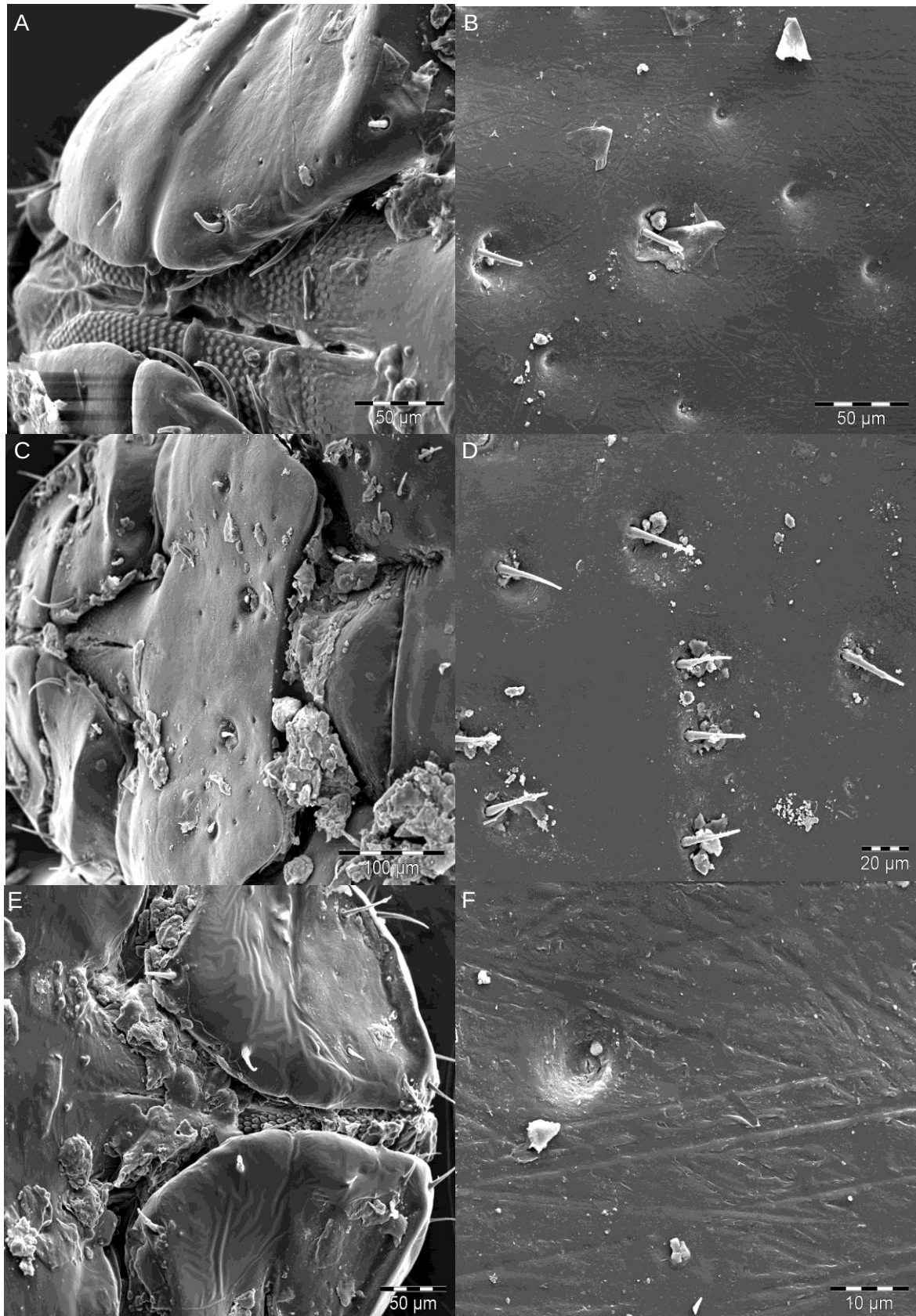


Plate 4-8: TEM image of tick mouthparts (A, C, and F) and alloscutum (B, D, and E) sterilized with 10% Tween 20 for 1, 2 and 3 hours, respectively

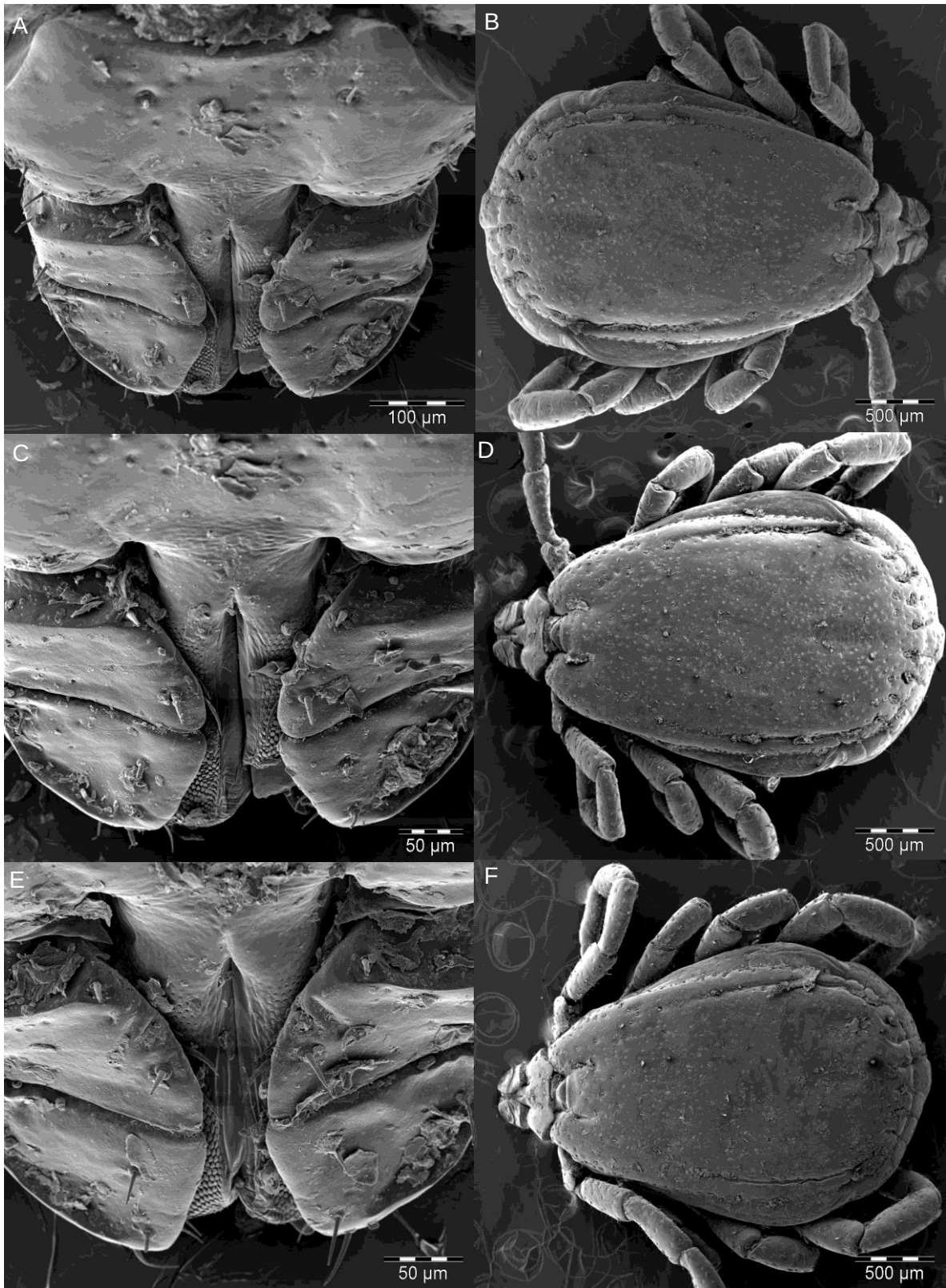


Plate 4-9: TEM image of mouthparts (A, C, and F) and dorsal view (B, D, and E) of tick sterilized with PBS for 1, 2 and 3 hours, respectively

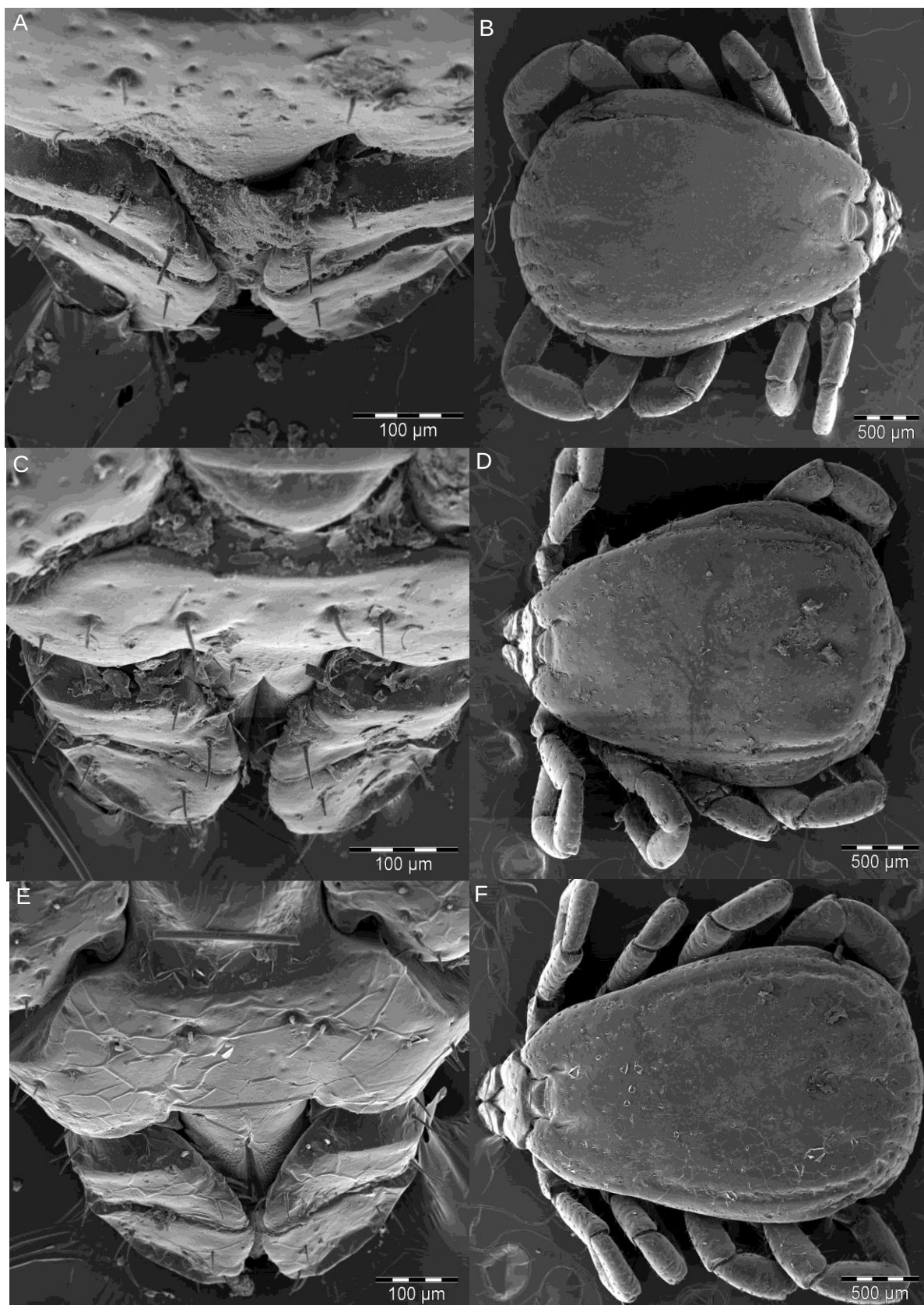


Plate 4-10: TEM image of mouthparts (A, C, and F) and dorsal view (B, D, and E) of tick surface sterilized with 10% Triton X for 1, 2 and 3 hours, respectively

4.7.2 Determination of washing methods effect on tick DNA

To assess the effects of 10% Bleach and 10% Tween 20 on tick DNA, PCR was conducted targeting the *18S rRNA* gene with an expected product size of 780 bp (Figure 4-27). All of the DNA amplified of ticks washed with 10% Tween 20 at the expected product size, whereas 3 ticks washed with 10% Bleach for 1, 2 and 3 hours, respectively, did not amplify.

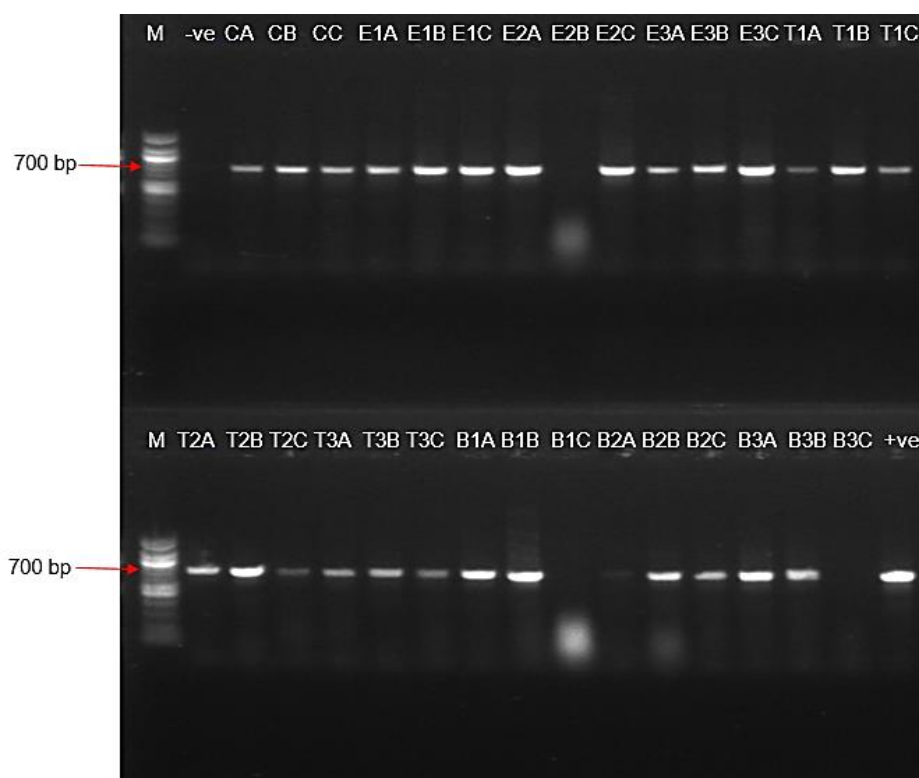


Figure 4-27: The 1% agarose gel electrophoresis of DNA amplified from *R. sanguineus* ticks targeting the *18S rRNA* gene with an expected product size of 780 bp. Lane 1 and 18 (M): 100 bp DNA molecular marker. Lane 2 (-ve): No template control. Lane 3 to 5 (CA to CC): unwashed ticks. Lane 6 to 8 (E1A to E1C): ticks surface sterilized with 70% ethanol for 1 hour. Lane 9 to 11 (E2A to E2C): ticks surface sterilized with 70% ethanol for 2 hours. Lane 12 to 14 (E3A to E3C): ticks surface sterilized with 70% ethanol for 3 hours. Lane 15 to 17 (T1A to T1C): ticks surface sterilized with 10% tween 20 for 1 hour. Lane 19 to 21 (T2A to T2C): ticks washed with 10% tween 20 for 2 hours. Lane 22 to 24 (T3A to T3C): ticks washed with 10% tween 20 for 3 hours. Lane 25 to 27 (B1A to B1C): ticks washed with 10% bleach for 1 hour. Lane 28 to 30 (B2A to B2C): ticks washed with 10% bleach for 2 hours. Lane 31 to 33 (B3A to B3C): ticks washed with 10% bleach for 1 hour. Lane 34 (+ve): positive control

CHAPTER 5

DISCUSSION, CONCLUSION, AND RECOMMENDATIONS

5.1 Discussion

Ticks are well-known ectoparasites with the ability to transmit various pathogenic and non-pathogenic microorganisms to their domestic animal hosts when taking a blood meal. Due to the close association between domestic animals and humans, it is vital to identify these ticks, their associated haemoparasites, and bacterial communities to determine their impact. This study has sought to identify ticks infesting dogs housed at Potchefstroom Animal Welfare Society (PAWS) and to detect pathogens and bacterial communities they are harbouring. In addition, various tick sterilization methods were tested to determine their effectiveness on removing microorganisms on the tick surface.

5.1.1 Morphological identification of ticks

During the current study, tick species collected from dogs housed at PAWS which mainly originated around the JB Marks, Merafong City and Emfuleni Local Municipalities, were identified as *Rhipicephalus sanguineus* and *Haemaphysalis elliptica*. The *R. sanguineus* were the most dominating tick species with an overall occurrence of 61%, whereas *H. elliptica* had an overall occurrence of 39%. Similar results were obtained by a study conducted by Mtshali (2012) where only *R. sanguineus* and *H. elliptica* ticks, with occurrences of 94.9 and 5.0% respectively were collected from dogs originating from the North-West Province. In another study in the North-West Province conducted by Bryson *et al.* (2000) respective occurrences of 96.62% and 2.85% for *R. sanguineus* and *H. elliptica* were also reported. Matthee *et al.* (2010) respectively reported a total of 433 and 3 *R. sanguineus* and *H. elliptica* ticks collected from domestic dogs in the Northern Cape Province, South Africa. In contrast, other studies have reported higher occurrences of *H. elliptica* ticks in other areas of South Africa, including an overall occurrence of 71.37% in the Cape Province and Transvaal (Horak *et al.* 1987). Similarly, Kolo *et al.* (2016) reported 30 of 103 *H. elliptica* and 27 of 103 *R. sanguineus* ticks collected from dogs in the Province of Mpumalanga, South Africa. In another study conducted by Horak (1995) 164 of 395 pure infestations and 140 of 395 mixed infestations of *H. elliptica* and 59 of 395 pure infestations and 87 mixed infestations of *R. sanguineus* ticks infesting dogs were reported in Pretoria, South Africa. Findings by Horak *et al.* (1987), Rautenbach *et al.* (1991), Horak (1995), and Bryson, Horak *et al.* (2000) indicated that the most dominating tick species infesting companion animals originating from South Africa are *R. sanguineus*, *H. elliptica*, and *R. simus*. Outside South Africa

R. sanguineus infestations on domestic animals were reported in Morocco (68%) by Pandey *et al* (1987), from Arkansas and Oklahoma, USA (62%) by Koch (1982) and from Porto Alegre City, Brazil (93.2%) by Ribeiro *et al.* (1997) to mention a few. The *H. elliptica* occurrence has also been recorded outside South Africa, including Zimbabwe, Kenya, Tanzania, Zambia, Democratic Republic of Congo, Mozambique, and Uganda (Apanaskevich *et al*, 2007).

Results obtained from the current study indicated that ticks were the most abundant in Miederpark (38.9%) and Ikageng (14.5%) suburbs. In the Miederpark suburb, where PAWS is located, numerous stray dogs reside in the kennels. PAWS is situated in an isolated area surrounded by dense vegetation which creates the ideal environment for exophilic ticks that spend much of their life cycle in open environments such as vegetation and meadows. In contrast, Ikageng is a resource-poor community where many stray dogs often occur and there is often lack of tick control measures to prevent tick infestations (Bechara *et al.*, 1994; Little *et al*, 2007). The combination of favourable environmental conditions, availability of hosts, and often a lack of tick control measures may be possible explanations for the high occurrences of ticks (Bechara *et al.*, 1994; Horak, 1995; Bryson *et al*, 2000; Little *et al*, 2007).

In the current study and various other studies conducted in South Africa, there seems to be a considerable difference between the occurrences of *R. sanguineus* and *H. elliptica* ticks. Differences in these occurrences may be attributed to different host seeking strategies, differences in preferred hosts when taking a blood meal, and how well the tick species are adapted to the environment (Bechara *et al.*, 1994; Little *et al*, 2007). Horak (1995), as well as Bryson *et al* (2000), reported that occurrences of *H. elliptica* ticks are higher in wealthy communities with access to modern veterinary services. In addition, *R. sanguineus* ticks are also often reported, with high occurrences, in the presence of domestic dogs (Bechara *et al.*, 1994; Little *et al*, 2007). Rautenbach *et al* (1991) suggested that *R. sanguineus* ticks are more likely to be the dominating tick species in resource-poor communities, in the presence of stray dogs where grooming is uncommon. This tick thrives in open environments and is well adjusted to survive in human homes and dog kennels for extended time periods (Bryson *et al.*, 2000; Little *et al.*, 2007). It is also documented that *R. sanguineus* infest other domestic animals, livestock, as well as humans (Bryson *et al.*, 2000; Little *et al.*, 2007; Dantas-torres and Otranto, 2015).

A study conducted by Little *et al.* (2007) demonstrated that *R. sanguineus* has metastriate male adult ticks which are capable of migrating between dogs in close association. This means these

ticks can repeatedly infect a host, seek a female tick, and mate without molting to the next developmental stage. Aforementioned study also revealed that metastriate adult male ticks often changed either the attachment site on one host or to completely move to a new host to seek female tick to mate. This may explain the high occurrences of *R. sanguineus* ticks in the Miederpark and Ikageng suburbs in the current study. In addition results of the study conducted by Little *et al.* (2007) suggests that intrastadial transmitted tick-borne pathogens may be acquired by metastriate ticks from an infected host and may be transmitted to uninfected hosts. This raises concern as metastriate ticks, such as *R. sanguineus*, may feed on companion animals, mate, seek new hosts (including livestock and humans), in effect transmitting intrastadial tick-borne pathogens (Little *et al.*, 2007).

Haemaphysalis elliptica are also frequently reported infesting domestic dogs, but in lower abundances when compared to *R. sanguineus*. A possible reason may be that *H. elliptica* adults are reported to prefer other hosts, such members of the family Canidae, whereas the earlier developmental stages, including larvae and nymphs, prefer murid rodents (Horak *et al.*, 1987; Bryson *et al.*, 2000; Apanaskevich *et al.*, 2007). Horak *et al.* (1987) reported that *H. elliptica* infestations of companion animals, originating from South Africa, tend to follow a general annual climatic pattern, with high occurrences from May to February. In addition, Bryson *et al.* (2000) reported that the occurrence of *H. elliptica* relies greatly on host distribution, especially during the early developmental stages infesting murid rodents. This implies that companion animals in close association with murid rodents may have a higher occurrence of *H. elliptica*. This raises concern as these ticks may infest wild animals during the early developmental stages and infest companion animals, livestock, and possibly humans during the later developmental stages (Bechara *et al.*, 1994; Bryson *et al.*, 2000; Apanaskevich *et al.*, 2007).

In addition to the transmission of tick-borne pathogens, additional effects of tick infestations are commonly reported, such as tick paralysis in companion animals and hide damage on livestock (Bechara *et al.*, 1994). Little *et al.*, (2007) reported that the combination of using acaricidal and repellent products, reducing tick habitats in kennels and urban environments, and avoiding natural areas infested with ticks may reduce tick infestations of companion animals, livestock, and humans.

5.1.2 Molecular identification of ticks

In the current study, molecular identification of tick species was done to supplement morphological identification by using conventional PCR and sequencing. Phylogenetic trees were constructed with the ITS2 and CO1 genes using maximum likelihood (ML) method. On each phylogenetic tree, the presence of two clades was formed supporting the respective monophyly of the ticks from the *Rhipicephalus* and *Haemaphysalis* genera. The p-distance matrix for sequences of these genera indicated strong resolution power. In addition, there were reliable differences that were observed between the numbers of base substitutions per site for the sequences used revealing low levels of genetic differences. Findings of the current study indicate that the CO1 and ITS2 genes are able to determine evolutionary relationships between the *Rhipicephalus* and *Haemaphysalis* genera.

The internal transcribed spacer (ITS) region, forming part of the nuclear gene cluster, are often used for the identification of ticks and mites (Bechara *et al.*, 1994; Zahler *et al.*, 1997; Murrell *et al.*, 2001; Lv, Wu, Zhang, Chen, *et al.*, 2014). This is a cluster made up of three genes, namely the 28S, 5.8S, and 18S *rDNA*, which are transcribed into RNA but are not translated into a protein. Yao *et al.* (2010) suggested that the ITS2 region, located in the nuclear DNA, has enough variation within the primary sequences as well as the secondary structures making it an effective gene for molecular identification of animals. Fukunaga *et al.* (2000) suggested that using the ITS2 sequences is useful to distinguish between ticks that share similar morphological features and synonymized tick species.

CO1 gene forming part of the mitochondria consisting of a sequence of approximately 600 bp long is currently acknowledged as a standard barcode for the identification of animals up to species level (Erpenbeck *et al.*, 2006; Kress and Erickson, 2008; Stoeckle and Hebert, 2008; Lv, Wu, Zhang, Chen, *et al.*, 2014). The CO1 gene and ITS2 gene has similar range of uses but Cruickshank (2002) reported that the CO1 gene evolves at a faster rate than the ITS2 gene. Stoeckle and Hebert (2008) reported that differences in the sequence of mitochondrial DNA are more frequent when compared to cell nucleus DNA. This means the use of segments of mitochondrial DNA will better distinguish between animal species.

Lv, Wu, Zhang, Chen, *et al.* (2014) conducted a study comparing the CO1, ITS2, 12S *rDNA*, and 16S *rDNA* gene sequences for the identification of *Ixodes* and *Rhipicephalus* tick species. Results

of the aforementioned study indicated that the ITS2 gene was preferred for identification of tick species. This was attributed to the fact that ITS2 gene is having the least intra-specific variation and the highest inter-specific divergence among the other compared DNA fragments. The CO1 gene is also suggested for molecular identification for tick species because it is recognized as the standard barcode for identification of animals. These genes should, however, be used in combination with other genes, such the *12S rDNA*, *16S rDNA*, and *18S rDNA*, to give the most reliable results (Lv, Wu, Zhang, Chen, *et al.*, 2014).

5.1.3 Morphological and molecular detection of tick-borne pathogens

In this study, tick pools and egg batches were respectively screened for the detection of transstadially and transovarially transmitted tick-borne pathogens, including *Anaplasma phagocytophilum*, *Rickettsia* sp., *Babesia* sp., *Ehrlichia canis*, and *Coxiella burnetii*. This was performed by using a combination of morphological and molecular approaches.

5.1.3.1 *Anaplasma phagocytophilum*

In the current study, *Anaplasma* sp. was detected in Giemsa stained blood smears of the engorged ticks (1%) as well as by PCR of tick pools (1%). The use of species-specific primers and BLASTn search on the NCBI database identified these species as *A. phagocytophilum*. Mtshali *et al.* (2017) detected the presence of *Anaplasma*-like organisms with an overall occurrence of 18% from ticks infesting companion animals originating from several South African provinces using conventional PCR. In the aforementioned study, it was reported that both *R. sanguineus* and *H. elliptica* ticks were infested with occurrences of 50 and 15% respectively. Specifically, in the North-West Province, an overall occurrence of 63% was reported. In a similar study conducted by Inokuma *et al.* (2005) the presence of *Anaplasma* sp. infesting dogs originating from South Africa was detected and described to be closely related to *A. phagocytophilum* and *A. platys*. In other countries of Africa, Sanogo *et al.* (2003) reported the presence of *A. platys* infesting *R. sanguineus* ticks as well as one dog originating from the Republic of the Congo by PCR. *Anaplasma phagocytophilum* infections in dogs have also been reported outside of Africa, including serological diagnosis reported in Germany (43.2%) by Jensen *et al.* (2007), Italy (38%) by Pennisi *et al.* (2012), and the United States (5.5 to 11.6%) by Bowman *et al.* (2009). Lester *et al.* (2005) also reported an infection in Vancouver Island by using a combination of serology and PCR.

Telfer *et al.* (2010) reported that hosts infected with one parasitic microorganism may influence the infection ability of other parasites. Another reason for the low occurrence of *A. phagocytophilum* in the current study may be due to competitiveness created by other haemoparasites. This is because *A. phagocytophilum* infections activate cytopenias, in turn reducing the amount of white and red blood cells, therefore influencing the infection and survival of other haemoparasites. This was proved by Telfer *et al.* (2010) as hosts infected with *A. phagocytophilum* were reported to have improved susceptibility to cowpox, but reduced susceptibility to members of the *Bartonella* genus.

5.1.3.2 *Rickettsia* sp.

Rickettsia species were detected in Giemsa-stained blood smears of the engorged ticks (22%), as well as by PCR using general *Rickettsia* primers of tick pools (9.6%) in this study. The PCR amplicons were sequenced and subjected to BLASTn search on the NCBI database which identified these species as *Rickettsia conorii*. Additionally, members of the *Rickettsia* genus were detected in the current study during metagenomic diagnosis of the tick microbiota. In South Africa *Rickettsia* infections are commonly reported, as members of the *Rickettsia* genus are described as obligately intracellular parasites or mutualists of arthropods (Ogata *et al.*, 2001). Mtshali *et al.* (2017) reported an overall occurrence of 37% for *R. conorii* and *R. africae* using PCR. In the aforementioned study, *Rickettsia* sp. occurred in 35% of *R. sanguineus* ticks, and specifically the North-West Province had an occurrence of 38%. In a similar study Kolo *et al.* (2016) reported infestation rates of 70% for *Rickettsia* sp. from blood and ticks collected from dogs in Mpumalanga, South Africa. Nicholson *et al.* (2010), as well as Dantas-torres and Otranto (2015), reported that *R. conorii* has great zoonotic potential. The *R. conorii* and *R. africae* infections in humans that reside in or travelled to South African countries were reported by Dupont *et al.* (1995), Fournier *et al.* (1998), Raoult *et al.* (2001), Caruso *et al.* (2002), and Jensenius *et al.* (2003) by using serological methods. *Rickettsia mongolotimonae* infection was also reported by Pretorius and Birtles (2004) from a human originating from South Africa using a combination of Giemsa-stained blood smears and serology.

Outside of South Africa *R. conorii* infections were also reported in *R. sanguineus* ticks collected from dogs originating from Switzerland (40%) by Péter *et al.* (1984), from North America (17 to 93%) by Levin *et al.* (2011), and from the Greek island of Cephalonia by Psaroulaki *et al.* (2006) using a variety of detection methods, including Giemsa-stained blood smears, PCR, and serology. *Rickettsia conorii* infections in dog sera were reported in Italy (72%) by Pennisi *et al.* (2012), and

in Portugal (38.5%) by Alexandre *et al.* (2011) using serology. Levin *et al.* (2011) suggested that the different genetic background of dogs seem to contribute to the susceptibility to infections caused by *R. conorii*, possibly explaining the different infestation rates.

5.1.3.3 *Babesia* sp.

During the current study *Babesia* sp. was detected in Giemsa-stained blood smears of the engorged ticks (0.5%) but was not detected when conventional PCR was used. *Babesia* sp. infections in dogs originating from South Africa are commonly reported. Allan (2016) reported the presence of *B. rossi* and *B. vogeli* with respective infestation rates of 12.7% and 3.2% in dogs originating from Cape Town using a combination of PCR and reverse line blot hybridization assays. Matjila *et al.* (2004 and 2008) also reported the presence of *B. rossi* with respective infestation rates of 75 and 32.1% and *B. vogeli* with respective infestation rates of 3 and 1.8% in dogs originating from Pretoria using a combination of PCR and reverse line blot hybridization assays. In other studies in Pretoria Bryson *et al.* (2000) reported the presence of *Babesia* sp. with an occurrence of 77% from *H. elliptica*, *R. sanguineus*, and *R. simus* ticks, whereas, Horak (1995) reported that the majority of 395 dogs diagnosed with canine babesiosis were previously infested with *H. elliptica* ticks. Rautenbach *et al.* (1991) reported 6 cases of canine babesiosis in dogs originating from Maboloka using blood smears.

Outside South Africa the presence of *B. vogeli* were also reported in Columbia (51.6%) by Vargas-Hernández *et al.* (2012), in Brazil (4.1%) by Annoscia *et al.* (2017), in Thailand by Solano-gallego and Baneth (2011), in Brazil by Malheiros *et al.* (2016), as well as Passos *et al.* (2005), in Eastern Sudan (2 of 78 dogs) by Oyamada *et al.* (2005), in Nigeria (0.6%) by Kamani *et al.* (2013), and in Turkey (2 of 3 dogs) by Gülanber *et al.* (2006) to name a few. Even though literature reports that *B. rossi* infections are limited to South Africa infections were previously reported in Eastern Sudan (5 of 78 dogs) by Oyamada *et al.* (2005) and in Nigeria (6.6%) by Kamani *et al.* (2013).

Although *Babesia* sp. seems to be highly infectious in domestic dogs originating from South Africa the infestation rates between previous studies differ significantly. Schetters *et al.*, (1997), Bryson *et al.*, (2000), Matjila *et al.* (2004), Jacobson (2006), and Yabsley *et al.* (2008) indicated alterations in the strains reported in different countries, including South Africa, Europe, and North America, thus indicating different transmission profiles of *Babesia* species. Reyers *et al.* (1998) and Collett (2000) reported that almost 12% of dogs presented at the Onderstepoort Veterinary Academic

Hospital, Pretoria, on an annual basis were diagnosed with babesiosis and that the inflammatory responses differ. Rautenbach *et al.* (1991), Schetters *et al.* (1997), and Jacobson (2006) also suggested that the detection of acute diseases, including babesiosis, are often difficult due to different virulence of the *Babesia* species. In addition, Collett (2000) suggested that blood smears are often used by veterinary institutions to confirm the presence of *Babesia* sp. but with complicated cases, the majority of these institutions hardly come across these infections. With regard to tick transmission of *Babesia* sp. Horak (1995) and Bryson *et al.* (2000) reported that although ticks, especially *R. sanguineus* and *H. elliptica*, are commonly collected from dogs, originating from South Africa, diagnosed with canine babesiosis, they are not essential vectors of this pathogen.

5.1.3.4 *Ehrlichia canis*

During the current study, the presence of *Ehrlichia* sp. was detected only by PCR on tick pools (5.8%) using species-specific primers as well as by 16S metagenomic diagnosis using NGS. The PCR amplicons were sequenced and subjected to BLASTn search on the NCBI database which confirmed that these species are *E. canis*. Additionally, members of the *Ehrlichia* genus were detected in the current study during metagenomic diagnosis of the tick microbiota. Bryson *et al.* (2000); and Inokuma *et al.* (2005) stated that *E. canis* infections in South Africa are commonly reported, especially in veterinary institutions and resource-poor communities. Mtshali *et al.* (2017) reported *E. canis* with an overall infestation rate of 19% from ticks infesting companion animals originating from South Africa. In the aforementioned study, the presence of *E. canis* was detected in 40% *R. sanguineus* ticks. Van Heerden (1982) reported 120 cases of *E. canis* infections in dogs originating from Pretoria using morphological detection methods. Allan (2016) reported an *E. canis* infestation rate of 12.7% on dogs originating from Cape Town using a combination of PCR and reverse line blot hybridization assay. Pretorius and Kelly (1998) reported *E. canis* with an infestation rate of 42% in dogs originating from Bloemfontein using serology. Rautenbach *et al.* (1991) and Matjila *et al.* (2008) reported an *E. canis* infestation rate of 17.2% and 3% in dogs originating from Maboloka using blood smears and Pretoria using a combination of PCR and reverse line blot hybridization assay, respectively. In Mpumalangs Kolo *et al.* (2016) reported infestation rates of 16% for *E. canis* from canine blood and ticks. Outside of South Africa *E. canis* infections were also reported by Loftis *et al.* (2006) in Egypt (33%), Zimbabwe (33-68%), Senegal (13-78%), and North-Africa (47%). Infections were also reported, respectively, in the United States (1.3 and 24.7%) by Yabsley *et al.* (2008) and Bowman *et al.* (2009), in Italy (46%) by Pennisi *et al.* (2012), and in Oklahoma (3.1%) by Murphy *et al.* (1998). In addition, Dantas-torres and Otranto (2015) reported that *E. canis* has zoonotic potential. An *E. canis* infection was also previously reported in a human in Venezuela (Inokuma *et al.*, 2005).

Rautenbach *et al.* (1991), Pretorius and Kelly (1998), Allsopp and Allsopp (2001), as well as Harrus and Waner (2011) stated that *E. canis* infections are difficult to detect when using morphological detection methods, including blood smears, due to low parasitaemia. This explains why this pathogen was detected when PCR and NGS were used but were absent in Giemsa-stained blood smears in this study.

5.1.3.5 *Coxiella burnetii*

Coxiella burnetii was detected in the current study by PCR on tick pools (13.5%) as well as on egg batches (6.5%). Thus, demonstrating transovarial and transstadial transmission of *C. burnetii*. Additionally, members of the *Coxiella* genus were detected in the current study during metagenomic diagnosis of the tick microbiota. Mtshali *et al.* (2017) reported an overall infestation rate of 41% for *C. burnetii* infecting ticks collected from companion animals originating from South Africa by PCR. In the aforementioned study specifically, the North-West province had an infestation rate of 31%. Of these ticks, *R. sanguineus* had an infestation rate of 44% and *H. elliptica* had an infestation rate of 4%. Duron *et al.* (2015) also reported infections in several tick species investing wildlife originating from South Africa. These infections include but are not limited to *R. decoloratus* ticks collected from Sandvelt, Queenstown, Vaalwater and Lephalale, as well as *R. microplus* ticks collected from Eglinton and Welverdiemda. The *C. burnetii* infections are also frequently reported outside of South Africa where Duron *et al.* (2015) reported *C. burnetii* infections from *Hyalomma impeltatum*, *R. evertsi* and *R. decoloratus* ticks infesting wildlife originating from Zimbabwe. Knobel *et al.* (2013) reported *C. burnetii* infestation rates of 30.9% in humans, 28.3% in cattle, 32% in goats, and 18.2% in sheep originating from Western Kenya using serology. Dantas-torres and Otranto (2015) reported that *C. burnetii* has great zoonotic potential. Baca and Paretsky (1983), Dupont *et al.* (1995), Buhariwalla *et al.* (1996), Zhang *et al.* (1998), Loftis *et al.* (2006) and Duron *et al.* (2015) reported that *C. burnetii* infections in humans are quite common and globally reported, where 4 000 infections were reported in 2007 to 2010 in the Netherlands alone. Heinzen *et al.* (1996), Mediannikov *et al.* (2010), and Duron *et al.* (2015) stated that the majority of *C. burnetii* infections to humans are associated to contact between humans and infected livestock. This suggests that even though *C. burnetii* infections to humans from tick bites are frequently reported, arthropods are not vital in maintaining transmission of *C. burnetii* to humans or other animals.

5.1.3.6 Mixed infections

The current study has detected mixed infections of several tick-borne pathogens. These include mixed infections between *A. phagocytophilum* and *R. conorii*, as well as between *R. conorii*, *E. canis*, and *C. burnetii* on ticks collected from dogs originating from Boipatong and Miederpark, respectively. Matjila *et al.* (2008) reported mixed infections of *E. canis* and *Babesia* sp. on dogs originating from Pretoria, South Africa. Van Heerden (1982) and Pennisi *et al.* (2012) suggested that mixed infections between ticks are common, especially in areas where domestic animals are co-housed. Matjila *et al.* (2004 and 2008) suggested that mixed infections of tick-borne pathogens may be attributed to *R. sanguineus* and *H. elliptica* ticks feeding on the same infected hosts in overlapping regions. Aforementioned is in accordance with results of the current study as the Boipatong suburb is described as a resource-poor community where stray dogs often occur, and the Miederpark suburb is where PAWS is located where dogs are co-housed in kennels.

Špitalská and Kocianová (2003), as well as Dantas-torres and Otranto (2015), reported that the occurrences of tick-borne pathogens differ due to different detection methods applied, the developmental stage of ticks, if ticks are already fed or not, host infection status, sample size, and seasonality of sample collection. Aforementioned may be used to explain differences in occurrences of tick-borne pathogens in the current study using Giemsa-stained blood smears, PCR, and next generation sequencing techniques.

5.1.4 Tick surface sterilization methods

Choosing the correct washing method to eliminate surface bacteria without damaging the host tissue is a challenging task as different methods may inhibit the growth of surface bacteria or eliminate it completely. The destruction of microorganisms, viable spores, and living cells in a given environment is defined as sterilization. On the other hand, the removal of pathogenic microorganisms is defined as disinfection. Budachetri *et al.* (2014) reported that bacterial communities that commonly inhabit soil environments may come into contact on the surface of ticks due to ticks spending long periods in the environments searching for hosts. As a result, these might result in contamination when tick microbiota is examined from tick internal organs. During the current study different surface sterilization methods were tested to determine their effectiveness to remove surface bacteria on the ticks. Afterward, conventional PCR was conducted to determine the effect of the washing procedures on the host tissue.

During the current study the use of 70% ethanol alone did not remove all of the tick surface microorganisms. The 70% ethanol is commonly used to disinfect microorganisms from arthropod surfaces. However, it is well documented that the use of 70% ethanol alone on surfaces only reduces and does not completely remove external microbial populations (Graziano, 2013). Boyce (2018) suggests that 70% ethanol is classified as a low-level disinfectant and needs to be combined with other disinfectants to ensure complete removal of microbial communities from surfaces. In this study the combined use of 10% bleach and 70% ethanol successfully removed the tick surface microorganisms. In addition, PCR of the majority of tick samples were successfully conducted suggesting that there is no damage to DNA. Bleach is classified as a useful and effective disinfectant. Sodium hypochlorite is the active ingredient in bleach and is especially successful for the removal of viruses, fungi, and bacteria. However, the World Health Organization (2014) reported that bleach reacts with other chemicals, it decays under light and heat and is irritant to the skin, mucous membranes, as well as airways. This potentially makes it unsafe and unpractical to use on biological material. During the current study the combined use of 10% tween 20 and 70% ethanol successfully removed the tick surface microorganisms and PCR amplified all tick DNA washed with these reagents. This proves that this method is effective to sterilize the tick surface without causing damage to the host DNA. Johnson (2013) reported that tween 20 is commonly used for disinfecting surfaces. It is a polysorbate surfactant consisting of a relatively long polyoxymethylene chain with a fatty acid ester moiety. It is reported to have no effect on protein activity, suggesting that it causes minimal damage to host tissue. Use of 10% Triton X-100 and 70% ethanol successfully removed the tick surface microorganisms but during visual inspection of ticks surface sterilized for 3 hours tissue damage was observed. Johnson (2013) also that Triton X-100 is also generally used to disinfect surfaces. It does, however, cause damage to host tissue as it is often used to lyse cell and tissue samples for various molecular techniques, such as western blotting and immunocytochemistry. During the current study the use of 70% ethanol and PBS did not remove the tick surface microorganisms. Martin *et al.* (2006) described PBS as a buffered solution consisting of sodium phosphate, sodium chloride, and potassium phosphate. Mainly it prevents osmosis by stabilizing the salt concentration around cells. It is commonly used for washing cells when using cellular techniques such as immunohistochemistry. This suggests that it does not cause damage to host tissue but is not effective in eliminating surface microorganisms.

5.1.5 Metagenomic diagnosis of tick microbiota

Ticks harbour various pathogenic and non-pathogenic microorganisms. These include organisms such as nematodes, protozoa, and bacteria (Dantas-torres and Otranto, 2015). A lot of these microorganisms go undetected and unidentified due to the difficulty to culture them or due to the

use of techniques, like PCR, that is designed for the detection of specific organisms (Klindworth *et al.*, 2013; Zhang *et al.*, 2014; Van Treuren *et al.*, 2015). To overcome this burden metagenomic diagnosis was performed on ticks using Next-generation sequencing technology to detect and identify bacterial communities.

In this study, dominant bacterial phyla detected include Proteobacteria, followed by Actinobacteria, Firmicutes, and Bacteroidetes. Cyanobacteria, Acidobacteria, Fusobacteria, Chloroflexi, Spirochates, Deinococcus-Thermus, Tenericutes, and Planctomycetes were also reported in lower abundances. This is similar to previous studies which detected tick microbiota from other countries (Carpi *et al.* 2011; Hawlena *et al.* 2013; Budachetri *et al.* 2014; Narasimhan *et al.* 2014; Qiu *et al.* 2014; Narasimhan and Fikrig, 2015; Van Treuren *et al.* 2015).

5.1.5.1 Phylum Proteobacteria

The phylum Proteobacteria is the most diverse and largest group of the bacterial kingdom, consisting of more than 450 genera. Members of this phylum are widespread and adapted to various ecological niches. Because of this, various interactions between members of the Proteobacteria phylum, other bacterial communities and hosts are often reported. Ecological, veterinary, medically, and economic importance of members of this phylum are also well documented (Prescott *et al.*, 2005). In the current study genera *Rickettsia*, *Coxiella*, *Hyphomicrobium*, *Caulobacter*, *Rhizobium*, *Agrobacterium*, *Nitrobacter*, *Neisseria*, *Burkholderia*, *Pseudomonas*, *Proteus*, *Bdellovibrio*, *Campylobacter*, *Helicobacter*, *Ehrlichia*, and *Wolbachia* were detected.

Members of the genus *Rickettsia* and *Coxiella* are well-known to such an extent that they are described as obligately intracellular parasites or mutualists. This is because the membrane of members of these genera consists of an adenylate exchange carrier that substitutes ADP for ATP from the host. In addition to ticks, they are also reported in other arthropod blood-sucking vectors, including lice, mites, and fleas. Several members of these genera are pathogenic to domestic animals and humans, including *C. burnetii* (associated with Q fever), *R. rickettsii* (associated with Rocky Mountain Spotted Fever), *R. conorii* (associated with Mediterranean spotted fever), *R. prowazekii* and *R. typhi* (both associated with typhus fever) (Garrity *et al.*, 2005; Prescott *et al.*, 2005; Eldin *et al.*, 2017; Hernández-jarguín *et al.*, 2018). Previous studies of ticks from other countries detected members of *Rickettsia* and *Coxiella* genera from eggs and various tick species

(Yuan 2010; Andreotti *et al.* 2011; Carpi *et al.*, 2011; Vayssier-taussat *et al.*, 2013; Budachetri *et al.*, 2014; Ponnusamy *et al.*, 2014; Qiu *et al.*, 2014; Rynkiewicz *et al.*, 2015; Van Treuren *et al.*, 2015; Duron *et al.*, 2017; and Hernández-jarguín *et al.*, 2018).

Several members of this phylum have the ability to survive and flourish in nutrient-poor environments. An example of this is *Hyphomicrobium* that flourishes in the presence of compounds with one carbon and may grow on ethanol and acetate (Garrity *et al.*, 2005; Prescott *et al.*, 2005). Members of the *Caulobacter* genus are also able to survive in nutrient-poor environments. They are reported to stick to algae, bacteria, as well as various other microorganisms where they take up nutrients from their hosts (Garrity *et al.*, 2005; Prescott *et al.*, 2005).

Members of the genus *Rhizobium* form symbiotic relationships with legumes as nitrogen-fixing bacteria, where they develop in the inside of the cells of root nodules (Prescott *et al.*, 2005). Members of the genus *Agrobacterium* are parasitic to plants as they invade stems, crown, and roots where they convert plant cells into tumor cells (Garrity *et al.*, 2005; Prescott *et al.*, 2005). In general members of the genera, *Nitrobacter* is of ecological importance. If members of this genus develop in the same niche as other nitrifying genera, such as *Nitrosomonas*, a process known as nitrification takes place, where ammonia is transformed to a nitrate (Garrity *et al.*, 2005; Prescott *et al.*, 2005).

Members of the genus *Neisseria* are generally isolated from animal mucus membranes. Some species of this genus are human pathogens associated with gonorrhea and bacterial meningitis (Garrity *et al.*, 2005; Prescott *et al.*, 2005; Warinner *et al.*, 2014). Species of the genus *Burkholderia* have an ecological importance as they have the ability to degrade various organic molecules and play an active role in recovering organic materials in the environment. Certain species of this genus are pathogenic in plants and were previously reported to cause disease in hospitalized patients because of contaminated medication and equipment (Coenye and Vandamme, 2003; Garrity *et al.*, 2005; Prescott *et al.*, 2005). Carpi *et al.* (2011) and Qiu *et al.* (2014) reported the presence of members of the *Burkholderia* genus in several tick species.

Species of the genus *Pseudomonas* have several important impacts. Members of this genus have the ability to degrade several organic molecules making them important key players in the mineralization process in sewage treatment plants and the environment. Some of these members are important in science experiments because of their biochemistry and microbial physiology. Several species are plant and animal pathogens associated with plant wilts and urinary tract infections. Due to their ability to degrade proteins and lipids and develop at 4°C, some species are included in the spoilage of refrigerated seafood, milk, eggs, and meat (Imhoff *et al.*, 2005; Prescott *et al.*, 2005; Hernández-jarguín *et al.*, 2018). Yuan (2010), Andreotti *et al.* (2011), Carpi *et al.* (2011), Qiu *et al.* (2014), Van Treuren *et al.* (2015), and Hernández-jarguín *et al.* (2018) reported species of *Pseudomonas* that were detected in different tick species.

Members of the genus *Proteus* have practical uses as it is generally used to determine contamination of water sources and faecal matter. Certain strains are also documented as human pathogens and are associated with urinary tract infection or gastroenteritis (Imhoff *et al.*, 2005; Prescott *et al.*, 2005; Ebringer and Rashid, 2013). Species of the genus *Bdellovibrio* are documented to have an unusual association with other microorganisms due to their unusual life cycle where it requires other Gram-negative bacteria as prey. During its life cycle, these species fluctuates between a non-developmental predatory phase and intracellular reproductive phase (Garrity *et al.*, 2005; Prescott *et al.*, 2005).

Genus *Campylobacter* consists of both pathogenic and non-pathogenic members of animals and humans. Two well documented pathogenic species of this genus include *C. fetus* and *C. jejuni*. *Campylobacter fetus* causes abortions in livestock, such as cattle and sheep. In humans, *C. fetus* is associated with several diseases varying from enteritis to septicemia. *Campylobacter jejuni* is documented to cause diarrhea in humans and abortions in sheep (Garrity *et al.*, 2005; Prescott *et al.*, 2005; Kaakoush *et al.*, 2015). Members of the genus *Helicobacter* commonly infest the upper intestines and stomachs of domestic animals and humans. Infections are so common it is reckoned that in developing and developed countries 70 to 90% and 25 to 50% populations, respectively, harbour members of this genus. *Helicobacter pylori* is pathogenic to humans and is associated with peptic disease and gastritis (Garrity *et al.*, 2005; Prescott *et al.*, 2005; van Der Mee-marquet *et al.*, 2017).

Members of the *Ehrlichia* genus were also detected by metagenomics diagnosis. It is most likely possible that members of *E. canis* were detected as ticks of this study were collected from dogs

and since conventional PCR, using *E. canis* specific primers, detected the presence of this species. *E. canis* is also of veterinary and medical importance as it is associated with canine monocytic ehrlichiosis (Hernández-jarguín *et al.*, 2018). Hernández-jarguín *et al.* (2018) reported the presence of members of the *Ehrlichia* genus in *Ixodes ricinus* ticks. Members of the *Wolbachia* genus are well-documented symbiotic bacteria of arthropod vectors (Garrity *et al.*, 2005; Hernández-jarguín *et al.*, 2018). Andreotti *et al.* (2011), Duron *et al.* (2017), and Hernández-jarguín *et al.* (2018) reported the detection of members the *Wolbachia* genus from eggs and ticks in several tick species.

5.1.5.2 Phylum Actinobacteria

In the current study genera *Actinomyces*, *Micrococcus*, *Arthrobacter*, *Corynebacterium*, *Mycobacterium*, *Nocardia*, *Rhodococcus*, *Propionibacterium*, *Streptomyces*, *Actinomadura*, *Geodermatophilus*, and *Bifidobacterium* were detected.

Members of the *Actinomyces* genus are reported to inhabit the mucosal surfaces of warm-blooded animals and humans. Several species are documented as human pathogens associated with ocular infections, actinomycoses, and periodontal disease. *Actinomyces bovis* are associated with cattle lumpy jaw (Micheal *et al.*, 2005; Prescott *et al.*, 2005; Hernández-jarguín *et al.*, 2018). Hernández-jarguín *et al.* (2018) reported the presence of members of the *Actinomyces* genus in *I. ricinus* ticks. Members of the *Micrococcus* genus were reported to be frequently found in water, soil, and on the skin of mammals. Due to their wide range of habitat, especially on mammalian skin, they are in symbiotic associations with other microorganisms (Micheal *et al.*, 2005; Prescott *et al.*, 2005). Several species in the *Micrococcus* genus are pathogenic to humans (Yap and Mermel, 2003).

Members of the *Arthrobacter* genus are reported to inhabit a wide range of habitats, including plant surfaces, sewage, fish, and soil, due to their ability to survive nutrient deprivation and desiccation. In soil habitats, they have a significant role in the microbial flora. Members of the *Arthrobacter* genus are nutritionally flexible and are important in the mineralization process of organic molecules, including the degradation of several pesticides and herbicides (Micheal *et al.*, 2005; Prescott *et al.*, 2005). Hernández-jarguín *et al.* (2018) reported the presence of members of the *Arthrobacter* genus in *I. ricinus* ticks. Several members of the *Corynebacterium* genus are reported to be pathogenic to humans and plants (Hernández-jarguín *et al.*, 2018). An example

includes *C. diphtheriae* associated with diphtheria of humans (Micheal *et al.*, 2005; Prescott *et al.*, 2005). Andreotti *et al.* (2011), Budachetri *et al.* (2014), and Qiu *et al.* (2014) reported the detection of *Corynebacterium* in several tick species.

Several members of the *Mycobacterium* genus are well-documented pathogens of animals and humans (Cole *et al.*, 2001; Gomez and McKinney, 2004). *Mycobacterium bovis* is associated with tuberculosis in primates, cattle, other ruminants, and potentially humans. *Mycobacterium leprae* is another pathogenic species of this genus associated with human leprosy (Micheal *et al.*, 2005; Prescott *et al.*, 2005). Carpi *et al.* (2011) reported the presence of members of the *Mycobacterium* genus in *I. ricinus* ticks. Members of the *Nocardia* genus are reported to have a global distribution in soil and aquatic environments. Several *Nocardia* species are significant in the biodeterioration process of rubber joints in sewage pipes and water environments, due to their ability to degrade waxes and hydrocarbons. Several species are pathogenic to humans and animals and are associated with nocardiosis (Micheal *et al.*, 2005; Prescott *et al.*, 2005; Wilson, 2012).

Members of the *Rhodococcus* genus are reported to inhabit aquatic and soil environments. Several members of this genus are significant in the biodegradation process, with the ability to degrade several molecules, including detergents, pesticides, and petroleum hydrocarbons (Micheal *et al.*, 2005; Prescott *et al.*, 2005; Hernández-jarguín *et al.*, 2018). Carpi *et al.* (2011) reported the presence of members of the *Rhodococcus* genus in *I. Ricinus* ticks. Members of the *Propionibacterium* genus are reported to grow in the digestive tract and on the skin of animals. Due to this habitat, they are reported to be in symbiotic associations with other organisms. Members of this genus are also reported to inhibit several dairy products and are involved in the process during the production of Swiss cheese (Micheal *et al.*, 2005; Prescott *et al.*, 2005). Carpi *et al.* (2011), Budachetri *et al.* (2014), and Qiu *et al.* (2014) reported members of the *Propionibacterium* genus were detected in various tick species.

Members of the *Streptomyces* genus are reported to inhabit soil environments and are of great medical and environmental significance. Members of this genus are nutritionally flexible and have a vital role in the mineralization process degrading resistant substances, including several antibiotics, latex, keratin, lignin, pectin, chitin, and aromatic compounds. Several species of this genus are pathogenic to animals and plants, including *S. scabies* (associated with scab disease of beets and potatoes) and *S. somaliensis* (associated with actinomycetoma in humans) (Micheal *et al.*, 2005; Prescott *et al.*, 2005; Kapadia *et al.*, 2007). Carpi *et al.* (2011) reported the presence

of members of the *Streptomyces* genus in *I. Ricinus* ticks. Members of the *Actinomadura* genus are reported to be pathogenic and are associated with actinomycetoma (Micheal *et al.*, 2005; Prescott *et al.*, 2005).

Members of the *Geodermatophilus* genus are reported to inhabit soil environments. In these environments, they fix nitrogen from the atmosphere and forms symbiotic relations with several nonleguminous plants (Micheal *et al.*, 2005; Prescott *et al.*, 2005). Members of the *Bifidobacterium* genus are reported to inhabit the intestinal tract and oral cavity of warm-blooded mammals, as well as in insects and sewage. Due to their habitat range, it is in symbiotic associations with other organisms. Although members of this genus are not major pathogens several infections were reported in humans (Micheal *et al.*, 2005; Prescott *et al.*, 2005; Hernández-jarguín *et al.*, 2018).

5.1.5.3 Phylum Firmicutes

During the current study members of the *Clostridium*, *Veillonella*, *Bacillus*, *Staphylococcus*, *Lactobacillus*, and *Streptococcus* genera were detected.

Genus *Clostridium* is of economic and veterinary importance. Members of this genus are implicated with food spoilage, including canned foods, due to their ability to produce heat-resistant endospores and vegetative cells to survive in anaerobic environments. Several species of *Clostridium* are also reported pathogens, including *C. botulinum* (associated with botulism), *C. tetani* (associated with tetanus), and *C. perfringens* (associated with food poisoning and gas gangrene). Certain members of this genus are also used in the industrial industry, such as *C. acetobutylicum* used to produce butanol (Prescott *et al.*, 2005; Vos *et al.*, 2011; Uzal *et al.*, 2018). Carpi *et al.* (2011) reported the presence of members of the *Clostridium* genus in *I. Ricinus* ticks. Members of the genus *Veillonella* was previously reported to develop in the dental plaque and tongue surface of humans since they utilize the lactic acid produced by streptococci and several bacteria in the oral cavity. Several species of the *Veillonella* genus are documented to be parasitic in warm-blooded animals (Prescott *et al.*, 2005; Vos *et al.*, 2011).

Members of the genus *Bacillus* have numerous impacts. Certain species of this genus are used in the production of antibiotics, such as gramicidin, bacitracin, and polymyxin. Some species of

this genus have a parasporal body that is used in the production of insecticides, including *B. thuringiensis* (used to kill moths) and *B. sphaericus* (used to kill mosquitoes, especially those infected with *Plasmodium*). Several species of the *Bacillus* genus are of veterinary and medical importance, including *B. cereus* (associated with food poisoning) and *B. anthracis* (associated with anthrax) (Prescott *et al.*, 2005; Vos *et al.*, 2011; Kandi, 2016). Carpi *et al.* (2011) reported the presence of members of the *Bacillus* genus in *I. ricinus* ticks. Members of the genus *Staphylococcus* generally inhabit the mucous membranes, skin, and skin glands of warm-blooded animals. Several species are reported to be pathogenic to humans. *Staphylococcus epidermidis* is associated with endocarditis and other various infections. *Staphylococcus aureus* is associated with multiple diseases, including food poisoning, abscesses, pneumonia, wound infections, and toxic shock syndrome (Prescott *et al.*, 2005; Vos *et al.*, 2011; Hernández-jarguín *et al.*, 2018). Andreotti *et al.* (2011) reported the detection *S. aureus* and other *Staphylococcus* species from the adults, midguts, and eggs of ticks infesting cattle. Qiu *et al.* (2014) and Hernández-jarguín *et al.* (2018) reported the presence of members of the *Staphylococcus* species from several tick species.

Members of the genus *Lactobacillus* are reported to be common bacteria of the human gut microbiome and are generally found inside the intestinal tract and mouth. They are also reported to be present on the surfaces of plants, sewage, water, meat, dairy products, fruits, and beer. This genus is of great practical importance as they are used in the production process of beverages, dairy products, and food (Prescott *et al.*, 2005; Vos *et al.*, 2011). Several species of the *Lactobacillus* genus are identified as potential emerging pathogens of humans (Cannon *et al.*, 2005). Rynkiewicz *et al.* (2015) reported the presence of members of the genus *Lactobacillus* in *Dermacentor variabilis* and *Ixodes scapularis* ticks. Members of the genus *Streptococcus* are reported to inhabit the upper respiratory tract and oral cavity of several animals and humans. Several species of this genus are pathogenic to humans, including *S. pyogenes* (associated with rheumatic fever and acute glomerulonephritis), *S. pneumoniae* (associated with otitis media and lobar pneumonia), and *S. mutans* (associated with dental caries formation) (Prescott *et al.*, 2005; Vos *et al.*, 2011; Hernández-jarguín *et al.*, 2018). Hernández-jarguín *et al.* (2018) reported the presence of members of the *Streptococcus* genus in *I. ricinus* ticks.

5.1.5.4 Phylum Bacteroidetes

Bacteroidetes are very distinct phylum and was reported to be very closely related to the phylum Chlorobi. Several members of this phylum are reported to inhabit the intestinal tract and oral cavity of animals and humans, as well as the rumen of ruminants. This suggests that they are in a symbiotic relationship with their host. In this study, members of the *Bacteroides* genus were detected. This genus has a major significance as it forms a key component of the hosts' rumen flora, because of its ability to ferment pectin, starch, and several carbohydrates to supply the host with extra nutrition. In contrast, several members of this genus are documented as human pathogens, associated with various diseases affecting organ systems, like the skeletal system and the central nervous system (Prescott *et al.*, 2005; Krieg *et al.*, 2010).

5.1.5.5 Other significant phyla detected

During this study, members of the Cyanobacteria phylum were detected, including members of the *Phormidium* and *Synechococcus* genera. Cyanobacteria are the largest and most distinct phylum of photosynthetic bacteria. Cyanobacteria are metabolically flexible. Even though the majority members of this phylum are obligate photolithoautotrophs several of these members have the ability to develop in the dark by functioning as chemoheteroautotrophs through oxidizing glucose and other sugars. It was previously reported that several strains of the *Synechococcus* genera in marine habitats are capable of movement of about 25 µm per second. Members of the Cyanobacteria phylum has a diverse ecological niche and are tolerant of extreme environmental conditions. It was previously reported that members of this genus are present in various water and soil habitats. Thermophilic species of this phylum have the ability to grow neutral to alkaline hot springs up to 75°C. Several photoautotrophs were reported to develop primary on surfaces or in soil habitats lacking plant growth. Several unicellular forms were also reported to develop in the cracks of desert rocks (Prescott *et al.*, 2005; Garrity, 2012).

Members of the Cyanobacterial phylum is of ecological and veterinary importance as several genera reproduce at a rapid pace produce blooms on the surface of ponds or lakes rich in nutrients. When these blooms decompose large amounts of organic matter are released. Subsequently, this organic matter stimulating the growth chemoheterotrophic bacteria in effect diminishing the available oxygen, leading to the death of higher organisms, such as fish, in the water habitat. In addition, several genera secrete toxins in water habitats leading to the death of livestock and other animals that consume the contaminated water (Prescott *et al.*, 2005; Garrity, 2012). Cyanobacteria are key players in forming symbiotic associations with several organisms.

Associations between Cyanobacteria with fungi, as well as protozoa are well documented. Nitrogen-fixing species of Cyanobacteria are also in symbiotic relationships with several plant species (Prescott *et al.*, 2005; Garrity, 2012).

In this study, the Classes of the Acidobacteria phylum were identified as Acidobacteriia, Solibacteres, and Chloracidobacteria. Members of the Acidobacteria phylum were previously reported to inhabit a variety of habitats, including faeces, soils, marine environments, hot springs, sediments, snow, soil contaminated with metal, and cave environments. Use of culture-independent culturing methods indicates substantial diversity within this phylum, describing several functional roles of members within this phylum (Krieg *et al.*, 2010).

In the current study, members of the Fusobacteria phylum, including members of the *Fusobacterium* and *Leptotrichia* genera, were detected. Members of the *Fusobacterium* genus are reported to inhabit the mucous membranes of animals and humans (Krieg *et al.*, 2010). Several species of this genus are reported to be pathogenic to humans (Jayasimhan *et al.*, 2017). Members of the *Leptotrichia* genus are reported to inhabit the oral cavity of humans and were previously reported from blood from immunocompromised patients diagnosed with endocarditis and neutropenia. Aforementioned indicate that members of the *Leptotrichia* genus may be pathogenic to humans with comprised immune systems. *Leptotrichia buccalis* are associated with periodontal disease and tooth decay (Krieg *et al.*, 2010; Hernández-jarguín *et al.*, 2018).

In this study as well as previous metagenomic detection of bacterial communities studies of ticks, bacterial members of the phylum Chloroflexi were also reported in lower abundances. This phylum consists of non-photosynthetic as well as photosynthetic members. Members of this phylum were previously isolated from soil and freshwater habitats and are reported to be in close association to cyanobacteria (Prescott *et al.*, 2005; Garrity, 2012).

Members of the Spirochaetes phylum has a diverse ecological niche and have reportedly been isolated from various habitats ranging from environmental habitats, such as water and soil, to different tissue of organisms, like the human mouth and digestive tract of several arthropods. Several members of this genus are well-documented to develop symbiotic relationships with other organisms. Several species are located in freshwater sediments and in the hindgut of termites

and where they fix nitrogen. In effect, they are important sources that contribute to the nitrogen nutrition to the given environment (Prescott *et al.*, 2005; Krieg *et al.*, 2010). Only members of the genus *Borrelia* were found in ticks of this study. *Borrelia* is documented as an important pathogen, associated with Lyme disease, previously isolated from mammals and arthropods. In addition, previous whole-genome sequencing studies conducted on *B. burgdorferi* suggested that several plasmid proteins are implicated in bacterial virulence (Prescott *et al.*, 2005; Krieg *et al.*, 2010; Hernández-jarguín *et al.*, 2018). An effect on the bacterial virulence was also suggested by Narasimhan *et al.* (2014), indicating that bacterial communities in the midgut of *I. scapularis* ticks were influenced in such a manner to create a favourable environment for members of the *Borrelia* genus. Andreotti *et al.* (2011), Carpi *et al.* (2011), Ponnusamy *et al.* (2014), Van Treuren *et al.* (2015), and Hernández-jarguín *et al.* (2018) reported the detection of members of the *Borrelia* genus from various tick species.

The Deinococcus-thermus phylum consists of only three genera, namely *Deinococcus*, *Thermus*, and *Meiothermus*, of which all these genera were detected during this study. Of these three genera, *Deinococcus* is the best documented. Although the natural habitat of the *Deinococcus* genus is still to be determined members of this genus were previously isolated from various sources including faeces, meat, freshwater, and air. It is reported that most of these strains have a high tolerance to radiation and desiccation because they repair damaged chromosomes. It is suggested that members of the *Deinococcus* genus may survive radiation of up to 3 to 5 million rad, whereas 100 rad is fatal to humans (Prescott *et al.*, 2005; Garrity, 2012).

During this study, members of the Tenericutes and Planctomycetes phyla were also detected. Members of the genus *Anaeroplasma*, from the Tenericutes phylum, was previously isolated from plants, animals, soil, and compost piles. It is believed that arthropods may be vectors of members of this genus (Prescott *et al.*, 2005; Vos *et al.*, 2011). The phylum Planctomycetes consist of one class, one order, and four genera, where the *Gemmata* and *Pirellula* genera were detected in ticks of this study. The genus *Pirellula* and species *Gemmata obscuriglobus* differ from other bacterial species as they contain a membrane-bounded nuclear body (Prescott *et al.*, 2005; Krieg *et al.*, 2010).

In general, this study detected similar bacterial communities across the different components of the tick species, including the eggs, salivary glands, midguts, and entire tick specimens. However these bacterial communities were present in different relative abundances, indicating different

ecological niches or influencing factors. Andreotti *et al.* (2011), as well as Narasimhan and Fikrig (2015), indicated that several factors may influence the bacterial composition of the tick microbiome. Andreotti *et al.* (2011), Hawlena *et al.* (2013), Ponnusamy *et al.* (2014), and Van Treuren *et al.* (2015) reported that the ecological environment of ticks and egg deposition may influence the composition of the bacterial community. Narasimhan *et al.* (2014) suggested that the midgut of ticks is a vital organ that determines the infection success of pathogenic microorganisms. Hawlena *et al.* (2013) and Zhang *et al.* (2014) determined that the microbiome composition of the midgut of several tick species were modified after a blood meal was taken. Here it was suggested that although the bacterial composition changed the results of the Shannon-diversity index were similar between unfed and fed ticks, suggesting that there was no change in the bacterial diversity. In addition, it was reported by Zhang *et al.* (2014) that *B. burgdorferi* and *A. phagocytophilum* was detected in *I. persulcatus* ticks after a blood meal was taken suggesting that these tick-borne pathogens were transstadially transmitted from rat hosts. Furquim *et al.* (2008) reported that there are various changes that occur in characteristics of tick salivary glands, specifically in female *R. sanguineus* ticks, after the completion of taking a blood meal and starting reproduction. Bacterial communities are bound to change during and after these complex changes. Budachetri *et al.* (2014) and Zhang *et al.* (2014) reported that different tick tissues have distinctive bacterial communities due to different functions of those tissues. In addition, the use of whole individual ticks may increase the detection of rare bacterial communities. Often the sampling depth, also referred to as the refraction depth, selected during alpha- and beta diversity NGS data analysis greatly impact the detection of bacterial communities as different sampling depths influence applied diversity metrics. High sampling depth enable more diversity to be detected in samples where a low sampling depth minimize the loss of samples with a low amount of aligned sequences (Zaheer *et al.*, 2018).

5.2 Conclusions

The aims of this study were to identify ticks infesting dogs and their associated tick-borne pathogens from dogs housed at PAWS but mainly originating from the JB Marks Local Municipality by using a combination of morphological and molecular methods. Bacterial communities that the ticks harbour were also detected by using a next-generation sequencing platform. Different washing procedures were also tested to determine their effectiveness to remove surface bacteria on the ticks.

Two tick species collected from dogs in this study were identified as *R. sanguineus* and *H. elliptica* which is in accordance with literature and other studies where they have been documented as dog ticks. Many of these ticks were collected from dogs originating from the PAWS institution and resource-poor communities. This suggests that ticks are adapted and flourish in environments where dogs are co-housed in kennels or where stray dogs often occur, where grooming of these dogs are rare and there is a lack of tick control measures.

These ticks are notorious for their ability to transmit tick-borne pathogens while taking a blood meal to develop to the next developmental stage. Hard ticks require a minimum of 3 different hosts to complete their life cycle and are able to transmit these pathogens to domestic animals, livestock, and humans. In this study *A. phagocytophylum*, *R. conorii*, *E. canis*, and *C. burnetii*, were detected, by using a combination of morphological and molecular detection methods, indicating that there is a cause of concern for the health of companion animals and humans as most of the above-mentioned species cause zoonotic diseases.

During the detection of tick-borne pathogens from tick specimens collected in this study, it was demonstrated that there are limitations when using only morphological or molecular methods. Using a combination of these methods is suggested to ensure reliable results. In addition, most molecular detection methods are designed only for the identification of a specific genus or species, thus making the determination of an entire bacterial kingdom impossible. To bridge this gap metagenomic diagnosis using a next-generation sequencing platform was used.

Metagenomic diagnosis supported the notion that ticks also harbour various bacterial communities, known as the microbiota. The composition of the microbiota may be influenced by several environmental factors and in turn influences interactions between ticks, hosts, as well as tick-borne pathogens. Several of the bacterial communities detected in this study were reported to have an environmental, veterinary, or medical significance.

Due to ticks spending the majority of their life cycle of their hosts and in the environment, they often come into contact with different microorganisms thus influencing the bacterial composition of the tick surface. Picking a washing method to eliminate these surface microorganisms, for laboratory experiments, without damaging the host tissue is a daunting task. Data obtained in this

study indicate that the combined use of 10% tween 20 and 70% ethanol are effective to eliminate surface microorganisms and cause minimal damage to host tissue.

The hypothesis of the current study was accepted as it was demonstrated that ticks infest urban dogs and that these ticks harbour various pathogenic and non-pathogenic microorganisms. Data generated in the current study adds knowledge of ticks infesting stray and kennel dogs, their associated tick-borne pathogens, and the bacterial communities that they are harbouring, particularly at the JB Marks Local Municipality.

5.3 Limitations and recommendations

- Sample collection should be performed for a longer period to get data of seasonal abundance of urban dog ticks and tick-borne pathogens.
- Further detection of tick-borne pathogens must be conducted from blood collected from these stray and kennel dogs in order to determine their relationship to the ticks.
- Supplementary or alternative screening methods for the detection of tick-borne pathogens should be used, such as Multiplex PCR/Reverse Line Blot Hybridization assay.
- A collaborative project of veterinary and medical sector is necessary in order to determine the prevalence of zoonotic pathogens in humans in relation to tick vectors and dog hosts.
- For metagenomic diagnosis more tick host tissue samples should be considered such as the ovaries, malphigian tubes as well as substances such as tick saliva, faeces and hemolymph in order to elucidate tissue preference of internal tick microbiota.
- In addition, more tick life stages such as the larval and nymphal stages should be subjected to metagenomic diagnosis of their microbiota in order to document whether they share similar bacterial communities with egg and adult stages.

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ANNEXURES

Appendix 1: Identification of ticks submitted as voucher specimens at the ARC-Onderstepoort Veterinary Research.

Tube number	Host	Tick identification	Gender	Life stage	Count	Co-ordinates	OP accession number
7.1	Dog	<i>R. sanguineus</i>	Female	Adult	1	26°42'08.97"S 27°06'41.76"E	OP5078
7.2	Dog	<i>R. sanguineus</i>	Male	Adult	1	26°42'08.97"S 27°06'41.76"E	OP5079
7.3	Dog	<i>R. sanguineus</i>	Female	Engorged Adult	1	26°42'08.97"S 27°06'41.76"E	OP5080
7.9	Dog	<i>R. sanguineus</i>	Male	Adult	1	26°42'08.97"S 27°06'41.76"E	OP5081
7.12	Dog	<i>R. sanguineus</i>	Female	Adult	1	26°42'08.97"S 27°06'41.76"E	OP5082
8.1	Dog	<i>R. sanguineus</i>	Female	Engorged Adult	1	26°44'53.15"S 27°05'31.87"E	OP5083
16.19	Dog	<i>R. sanguineus</i>	Male	Adult	1	26°44'53.17"S 27°05'31.81"E	OP5111
16.20	Dog	<i>R. sanguineus</i>	Male	Adult	1	26°44'53.17"S 27°05'31.81"E	OP5112
17.1	Dog	<i>H. elliptica</i>	Female	Engorged adult	1	26°44'53.17"S 27°05'31.81"E	OP5113
17.2	Dog	<i>R. sanguineus</i>	N/A	Engorged Nymph	1	26°44'53.17"S 27°05'31.81"E	OP5114
20.1	Dog	<i>H. elliptica</i>	Male	Adult	1	26°42'53.19"S 27°05'24.72"E	OP5115
20.2	Dog	<i>H. elliptica</i>	Male	Adult	1	26°42'53.19"S 27°05'24.72"E	OP5116

Appendix 2: OTU picking results.

Kingdom	Phylum	Class	Order	Family	Genus
Unassigned	Other	Other	Other	Other	Other
Archaea	Crenarchaeota	Thaumarchaeota	Nitrososphaerales	Nitrososphaeraceae	<i>Candidatus Nitrososphaera</i>
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	<i>Methanobrevibacter</i>
Archaea	Euryarchaeota	Methanomicrobia	Methanomicrobiales	Methanocorpusculaceae	<i>Methanocorpusculum</i>
Bacteria	Other	Other	Other	Other	Other
Bacteria	Acidobacteria	Acidobacteria-6	iii1-15		
Bacteria	Acidobacteria	Acidobacteriia	Acidobacteriales	Koribacteraceae	
Bacteria	Acidobacteria	Solibacteres	Solibacterales	Other	Other
Bacteria	Acidobacteria	Solibacteres	Solibacterales		
Bacteria	Acidobacteria	Solibacteres	Solibacterales	Solibacteraceae	
Bacteria	Acidobacteria	Solibacteres	Solibacterales	[Bryobacteraceae]	
Bacteria	Acidobacteria	[Chloracidobacteria]	PK29		
Bacteria	Acidobacteria	[Chloracidobacteria]	RB41	Ellin6075	
Bacteria	Actinobacteria	Acidimicrobiia	Acidimicrobiales		
Bacteria	Actinobacteria	Acidimicrobiia	Acidimicrobiales	C111	
Bacteria	Actinobacteria	Acidimicrobiia	Acidimicrobiales	EB1017	
Bacteria	Actinobacteria	Acidimicrobiia	Acidimicrobiales	Iamiaceae	<i>Iamia</i>
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Other	Other
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales		
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Actinomycetaceae	<i>Actinomyces</i>
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Actinomycetaceae	<i>Trueperella</i>

Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Actinosynnemataceae	Other
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Actinosynnemataceae	
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Brevibacteriaceae	<i>Brevibacterium</i>
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Cellulomonadaceae	Other
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Cellulomonadaceae	
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Cellulomonadaceae	<i>Cellulomonas</i>
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Corynebacteriaceae	<i>Corynebacterium</i>
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Dermabacteraceae	Other
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Dermabacteraceae	<i>Brachybacterium</i>
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Dermabacteraceae	<i>Dermabacter</i>
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Dietziaceae	Other
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Dietziaceae	<i>Dietzia</i>
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Geodermatophilaceae	Other
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Geodermatophilaceae	
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Geodermatophilaceae	<i>Geodermatophilus</i>
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Geodermatophilaceae	<i>Modestobacter</i>
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Gordoniaceae	<i>Gordonia</i>
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Intrasporangiaceae	Other
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Intrasporangiaceae	
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Intrasporangiaceae	<i>Janibacter</i>
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Intrasporangiaceae	<i>Knoellia</i>
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Intrasporangiaceae	<i>Phycococcus</i>
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Kineosporiaceae	Other

Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Kineosporiaceae	
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Other
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	<i>Cryocola</i>
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	<i>Curtobacterium</i>
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	<i>Leucobacter</i>
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	<i>Microbacterium</i>
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	<i>Pseudoclavibacter</i>
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	<i>Salinibacterium</i>
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	Other
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	<i>Arthrobacter</i>
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	<i>Kocuria</i>
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	<i>Microbispora</i>
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	<i>Micrococcus</i>
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	<i>Nesterenkonia</i>
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	<i>Rothia</i>
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Micromonosporaceae	
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Micromonosporaceae	<i>Virgisporangium</i>
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Mycobacteriaceae	<i>Mycobacterium</i>
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Nocardiaceae	<i>Nocardia</i>
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Nocardiaceae	<i>Rhodococcus</i>
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Nocardioidaceae	

Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Nocardiodaceae	<i>Aeromicrobium</i>
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Nocardiodaceae	<i>Friedmanniella</i>
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Nocardiodaceae	<i>Kribbella</i>
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Nocardiodaceae	<i>Nocardiodides</i>
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Nocardiodaceae	<i>Pimelobacter</i>
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Nocardiodaceae	<i>Propionicimonas</i>
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Nocardiopsaceae	Other
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Nocardiopsaceae	
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Nocardiopsaceae	<i>Nocardiopsis</i>
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Nocardiopsaceae	<i>Prauseria</i>
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Promicromonosporaceae	Other
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Promicromonosporaceae	<i>Cellulosimicrobium</i>
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Promicromonosporaceae	<i>Xylanimicrobium</i>
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	<i>Microlunatus</i>
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	<i>Propionibacterium</i>
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Pseudonocardiaceae	
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Pseudonocardiaceae	<i>Actinomycetospora</i>
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Pseudonocardiaceae	<i>Pseudonocardia</i>
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Pseudonocardiaceae	<i>Saccharopolyspora</i>
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Sanguibacteraceae	<i>Sanguibacter</i>
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Sporichthyaceae	
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Streptomycetaceae	Other
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Streptomycetaceae	

Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Streptomycetaceae	<i>Streptomyces</i>
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Streptosporangiaceae	<i>Nonomuraea</i>
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Thermomonosporaceae	<i>Actinomadura</i>
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Williamsiaceae	<i>Williamsia</i>
Bacteria	Actinobacteria	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	<i>Bifidobacterium</i>
Bacteria	Actinobacteria	Actinobacteria	Micrococcales		
Bacteria	Actinobacteria	Coriobacteriia	Coriobacteriales	Coriobacteriaceae	<i>Collinsella</i>
Bacteria	Actinobacteria	Coriobacteriia	Coriobacteriales	Coriobacteriaceae	<i>Eggerthella</i>
Bacteria	Actinobacteria	MB-A2-108	0319-7L14		
Bacteria	Actinobacteria	Thermoleophilia	Gaiellales		
Bacteria	Armatimonadetes	0319-6E2			
Bacteria	Armatimonadetes	Armatimonadia	Armatimonadales	Armatimonadaceae	
Bacteria	Armatimonadetes	Armatimonadia	FW68		
Bacteria	Armatimonadetes	SJA-176	RB046		
Bacteria	Armatimonadetes	[Fimbriimonadia]	[Fimbriimonadales]	[Fimbriimonadaceae]	<i>Fimbriimonas</i>
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales		
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	BS11	
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	5-7N15
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	<i>Bacteroides</i>
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	<i>Porphyromonas</i>
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	<i>Prevotella</i>

Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	Other
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	<i>Alistipes</i>
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	<i>Rikenella</i>
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	S24-7	
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	[Barnesiellaceae]	
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	[Odoribacteraceae]	<i>Odoribacter</i>
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	[Paraprevotellaceae]	<i>Paraprevotella</i>
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	[Paraprevotellaceae]	YRC22
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	[Paraprevotellaceae]	<i>[Prevotella]</i>
Bacteria	Bacteroidetes	Cytophagia	Cytophagales		
Bacteria	Bacteroidetes	Cytophagia	Cytophagales	Cyclobacteriaceae	
Bacteria	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	
Bacteria	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	<i>Adhaeribacter</i>
Bacteria	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	<i>Dyadobacter</i>
Bacteria	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	<i>Flectobacillus</i>
Bacteria	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	<i>Hymenobacter</i>
Bacteria	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	<i>Pontibacter</i>
Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Cryomorphaceae	<i>Fluviicola</i>
Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	
Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	<i>Flavobacterium</i>
Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	<i>Tenacibaculum</i>
Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	[Weeksellaceae]	Other

Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	[Weeksellaceae]	
Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	[Weeksellaceae]	<i>Chryseobacterium</i>
Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	[Weeksellaceae]	<i>Cloacibacterium</i>
Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	[Weeksellaceae]	<i>Wautersiella</i>
Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales		
Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	
Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	<i>Pedobacter</i>
Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	<i>Sphingobacterium</i>
Bacteria	Bacteroidetes	[Rhodothermi]	[Rhodothermales]	Rhodothermaceae	
Bacteria	Bacteroidetes	[Saprospirae]	[Saprospirales]	Chitinophagaceae	
Bacteria	Bacteroidetes	[Saprospirae]	[Saprospirales]	Chitinophagaceae	<i>Flavisolibacter</i>
Bacteria	Bacteroidetes	[Saprospirae]	[Saprospirales]	Chitinophagaceae	<i>Niabella</i>
Bacteria	Bacteroidetes	[Saprospirae]	[Saprospirales]	Chitinophagaceae	<i>Sediminibacterium</i>
Bacteria	Bacteroidetes	[Saprospirae]	[Saprospirales]	Chitinophagaceae	<i>Segetibacter</i>
Bacteria	Chloroflexi	C0119			
Bacteria	Chloroflexi	Chloroflexi	AKIW781		
Bacteria	Chloroflexi	Chloroflexi	[Roseiflexales]	[Kouleothrixaceae]	
Bacteria	Chloroflexi	Chloroflexi	[Roseiflexales]	[Kouleothrixaceae]	<i>Kouleothrix</i>
Bacteria	Chloroflexi	Ellin6529			
Bacteria	Chloroflexi	Gitt-GS-136			
Bacteria	Chloroflexi	Ktedonobacteria	B12-WMSP1		
Bacteria	Chloroflexi	Ktedonobacteria	Ktedonobacteriales	Ktedonobacteraceae	
Bacteria	Chloroflexi	SHA-26			

Bacteria	Chloroflexi	TK17			
Bacteria	Chloroflexi	Thermomicrobia	JG30-KF-CM45		
Bacteria	Cyanobacteria	4C0d-2	MLE1-12		
Bacteria	Cyanobacteria	4C0d-2	YS2		
Bacteria	Cyanobacteria	Chloroplast	Chlorophyta		
Bacteria	Cyanobacteria	Chloroplast	Cryptophyta		
Bacteria	Cyanobacteria	Chloroplast	Stramenopiles		
Bacteria	Cyanobacteria	Chloroplast	Streptophyta		
Bacteria	Cyanobacteria	Oscillatoriothycideae	Oscillatoriales	Phormidiaceae	<i>Phormidium</i>
Bacteria	Cyanobacteria	Synechococcophycideae	Synechococcales	Synechococcaceae	<i>Synechococcus</i>
Bacteria	Firmicutes	Bacilli	Bacillales	Other	Other
Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	<i>Bacillus</i>
Bacteria	Firmicutes	Bacilli	Bacillales	Planococcaceae	Other
Bacteria	Firmicutes	Bacilli	Bacillales	Planococcaceae	<i>Lysinibacillus</i>
Bacteria	Firmicutes	Bacilli	Bacillales	Planococcaceae	<i>Solibacillus</i>
Bacteria	Firmicutes	Bacilli	Bacillales	Planococcaceae	<i>Sporosarcina</i>
Bacteria	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	<i>Staphylococcus</i>
Bacteria	Firmicutes	Bacilli	Bacillales	[Exiguobacteraceae]	
Bacteria	Firmicutes	Bacilli	Bacillales	[Exiguobacteraceae]	<i>Exiguobacterium</i>
Bacteria	Firmicutes	Bacilli	Lactobacillales	Other	Other
Bacteria	Firmicutes	Bacilli	Lactobacillales		
Bacteria	Firmicutes	Bacilli	Lactobacillales	Carnobacteriaceae	<i>Trichococcus</i>
Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	<i>Lactobacillus</i>

Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	<i>Streptococcus</i>
Bacteria	Firmicutes	Clostridia	Clostridiales	Other	Other
Bacteria	Firmicutes	Clostridia	Clostridiales		
Bacteria	Firmicutes	Clostridia	Clostridiales	Christensenellaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Other
Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	<i>Caloramator</i>
Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	<i>Clostridium</i>
Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	<i>Oxobacter</i>
Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	<i>SMB53</i>
Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	<i>Thermoanaerobacterium</i>
Bacteria	Firmicutes	Clostridia	Clostridiales	Eubacteriaceae	<i>Pseudoramibacter_Eubacterium</i>
Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Other
Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	<i>Blautia</i>
Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	<i>Butyrivibrio</i>
Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	<i>Coprococcus</i>
Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	<i>Dorea</i>
Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	<i>[Ruminococcus]</i>
Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Other
Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	<i>Clostridium</i>
Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	<i>Peptostreptococcus</i>

Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	<i>Faecalibacterium</i>
Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	<i>Oscillospira</i>
Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	<i>Ruminococcus</i>
Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	<i>Dialister</i>
Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	<i>Veillonella</i>
Bacteria	Firmicutes	Clostridia	Clostridiales	[Mogibacteriaceae]	
Bacteria	Firmicutes	Clostridia	Clostridiales	[Mogibacteriaceae]	<i>Anaerovorax</i>
Bacteria	Firmicutes	Clostridia	Clostridiales	[Mogibacteriaceae]	<i>Mogibacterium</i>
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	1-68
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	<i>Anaerococcus</i>
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	<i>Finegoldia</i>
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	GW-34
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	<i>Gallicola</i>
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	<i>Parvimonas</i>
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	<i>Peptoniphilus</i>
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	<i>Sedimentibacter</i>
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	WAL_1855
Bacteria	Firmicutes	Clostridia	SHA-98		
Bacteria	Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	
Bacteria	Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	<i>Bulleidia</i>
Bacteria	Fusobacteria	Fusobacteriia	Fusobacteriales		

Bacteria	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	<i>Fusobacterium</i>
Bacteria	Fusobacteria	Fusobacteriia	Fusobacteriales	Leptotrichiaceae	<i>Leptotrichia</i>
Bacteria	Fusobacteria	Fusobacteriia	Fusobacteriales	Leptotrichiaceae	<i>Sneathia</i>
Bacteria	Gemmatimonadetes	Gemm-1			
Bacteria	Gemmatimonadetes	Gemmatimonadetes	Other	Other	Other
Bacteria	Gemmatimonadetes	Gemmatimonadetes			
Bacteria	Gemmatimonadetes	Gemmatimonadetes	Gemmatimonadales	Other	Other
Bacteria	Gemmatimonadetes	Gemmatimonadetes	Gemmatimonadales		
Bacteria	Gemmatimonadetes	Gemmatimonadetes	Gemmatimonadales	Ellin5301	
Bacteria	Gemmatimonadetes	Gemmatimonadetes	Gemmatimonadales	Gemmatimonadaceae	<i>Gemmatimonas</i>
Bacteria	Nitrospirae	Nitrospira	Nitrospirales	Nitrospiraceae	<i>Nitrospira</i>
Bacteria	OD1	ZB2			
Bacteria	Planctomycetes	Phycisphaerae	Phycisphaerales		
Bacteria	Planctomycetes	Phycisphaerae	WD2101		
Bacteria	Planctomycetes	Planctomycetia	Gemmatales	Gemmataceae	<i>Gemmata</i>
Bacteria	Planctomycetes	Planctomycetia	Gemmatales	Isosphaeraceae	
Bacteria	Planctomycetes	Planctomycetia	Pirellulales	Pirellulaceae	
Bacteria	Planctomycetes	Planctomycetia	Pirellulales	Pirellulaceae	<i>Pirellula</i>
Bacteria	Proteobacteria	Other	Other	Other	Other
Bacteria	Proteobacteria	Alphaproteobacteria	Other	Other	Other
Bacteria	Proteobacteria	Alphaproteobacteria			
Bacteria	Proteobacteria	Alphaproteobacteria	BD7-3		
Bacteria	Proteobacteria	Alphaproteobacteria	Caulobacterales	Other	Other

Bacteria	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Other
Bacteria	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	
Bacteria	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	<i>Asticcacaulis</i>
Bacteria	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	<i>Brevundimonas</i>
Bacteria	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	<i>Caulobacter</i>
Bacteria	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	<i>Mycoplana</i>
Bacteria	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	<i>Phenylobacterium</i>
Bacteria	Proteobacteria	Alphaproteobacteria	RF32		
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Other	Other
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales		
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Aurantimonadaceae	Other
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Aurantimonadaceae	
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Aurantimonadaceae	<i>Aurantimonas</i>
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bartonellaceae	Other
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bartonellaceae	
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae	Other
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae	
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	Other
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	<i>Balneimonas</i>
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	<i>Bradyrhizobium</i>
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	<i>Nitrobacter</i>
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Brucellaceae	Other

Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Brucellaceae	
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Brucellaceae	<i>Ochrobactrum</i>
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Brucellaceae	<i>Paenochrobactrum</i>
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Brucellaceae	<i>Pseudochrobactrum</i>
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Other
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	<i>Devosia</i>
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	<i>Hyphomicrobium</i>
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	<i>Pedomicrobium</i>
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	<i>Rhodoplanes</i>
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylobacteriaceae	Other
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylobacteriaceae	
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylobacteriaceae	<i>Methylobacterium</i>
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylocystaceae	
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylocystaceae	<i>Methylosinus</i>
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylocystaceae	<i>Pleomorphomonas</i>
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	Other
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	<i>Mesorhizobium</i>
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	<i>Phyllobacterium</i>
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Other
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Agrobacterium</i>

Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Rhizobium</i>
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Shinella</i>
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Other
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	<i>Amaricoccus</i>
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	<i>Paracoccus</i>
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	<i>Rhodobacter</i>
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	<i>Rubellimicrobium</i>
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales		
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	Other
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	<i>Acetobacter</i>
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	<i>Gluconobacter</i>
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	<i>Roseococcus</i>
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	<i>Roseomonas</i>
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	<i>Azospirillum</i>
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	<i>Skermanella</i>
Bacteria	Proteobacteria	Alphaproteobacteria	Rickettsiales		
Bacteria	Proteobacteria	Alphaproteobacteria	Rickettsiales	Anaplasmataceae	<i>Ehrlichia</i>
Bacteria	Proteobacteria	Alphaproteobacteria	Rickettsiales	Rickettsiaceae	
Bacteria	Proteobacteria	Alphaproteobacteria	Rickettsiales	Rickettsiaceae	<i>Rickettsia</i>
Bacteria	Proteobacteria	Alphaproteobacteria	Rickettsiales	Rickettsiaceae	<i>Wolbachia</i>

Bacteria	Proteobacteria	Alphaproteobacteria	Rickettsiales	mitochondria	Other
Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Other	Other
Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales		
Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Erythrobacteraceae	Other
Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Erythrobacteraceae	
Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Other
Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	
Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	<i>Blastomonas</i>
Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	<i>Kaistobacter</i>
Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	<i>Novosphingobium</i>
Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	<i>Sphingobium</i>
Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	<i>Sphingomonas</i>
Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	<i>Sphingopyxis</i>
Bacteria	Proteobacteria	Betaproteobacteria	Other	Other	Other
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Other	Other
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Other
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	<i>Oligella</i>
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	Other
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	<i>Burkholderia</i>
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Other
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	

Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	<i>Comamonas</i>
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	<i>Curvibacter</i>
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	<i>Delftia</i>
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	<i>Methylibium</i>
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Other
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	<i>Janthinobacterium</i>
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	<i>Polynucleobacter</i>
Bacteria	Proteobacteria	Betaproteobacteria	Neisseriales	Neisseriaceae	<i>Neisseria</i>
Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Other
Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	
Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Dok59
Bacteria	Proteobacteria	Deltaproteobacteria	Bdellovibrionales	Bdellovibrionaceae	<i>Bdellovibrio</i>
Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	<i>Bilophila</i>
Bacteria	Proteobacteria	Deltaproteobacteria	Myxococcales	0319-6G20	
Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	<i>Arcobacter</i>
Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	<i>Campylobacter</i>
Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Helicobacteraceae	
Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Helicobacteraceae	<i>Flexispira</i>
Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Helicobacteraceae	<i>Helicobacter</i>
Bacteria	Proteobacteria	Gammaproteobacteria	Other	Other	Other
Bacteria	Proteobacteria	Gammaproteobacteria	Aeromonadales	Succinivibrionaceae	<i>Succinivibrio</i>
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Other

Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	<i>Proteus</i>
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	<i>Serratia</i>
Bacteria	Proteobacteria	Gammaproteobacteria	Legionellales		
Bacteria	Proteobacteria	Gammaproteobacteria	Legionellales	Coxiellaceae	Other
Bacteria	Proteobacteria	Gammaproteobacteria	Legionellales	Coxiellaceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Legionellales	Coxiellaceae	<i>Coxiella</i>
Bacteria	Proteobacteria	Gammaproteobacteria	Legionellales	Legionellaceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Legionellales	Legionellaceae	<i>Legionella</i>
Bacteria	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Alcanivoracaceae	<i>Alcanivorax</i>
Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Other	Other
Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	<i>Acinetobacter</i>
Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	<i>Enhydrobacter</i>
Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	<i>Psychrobacter</i>
Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Other
Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	<i>Pseudomonas</i>
Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Sinobacteraceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	<i>Lysobacter</i>
Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	<i>Stenotrophomonas</i>
Bacteria	Proteobacteria	TA18	PHOS-HD29		

Bacteria	SR1				
Bacteria	Spirochaetes	Spirochaetes	[Borreliales]	[Borreliaceae]	<i>Borrelia</i>
Bacteria	Synergistetes	Synergistia	Synergistales	Synergistaceae	
Bacteria	TM7				
Bacteria	TM7	TM7-1			
Bacteria	TM7	TM7-3			
Bacteria	TM7	TM7-3	CW040		
Bacteria	TM7	TM7-3	CW040	F16	
Bacteria	TM7	TM7-3	EW055		
Bacteria	Tenericutes	Mollicutes	Anaeroplasmatales	Anaeroplasmataceae	<i>Anaeroplasma</i>
Bacteria	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	<i>Akkermansia</i>
Bacteria	WPS-2				
Bacteria	[Thermi]	Deinococci	Deinococcales	Deinococcaceae	<i>Deinococcus</i>
Bacteria	[Thermi]	Deinococci	Thermales	Thermaceae	<i>Meiothermus</i>
Bacteria	[Thermi]	Deinococci	Thermales	Thermaceae	<i>Thermus</i>
