

Molecular characterization of protozoan parasites and *Ehrlichia* in domestic animals from uMkhanyakude district of KwaZulu-Natal

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ABSTRACT

Protozoan and ehrlichial diseases are a major threat to domestic animals in tropical and sub-tropical regions of Africa. Economically important animal diseases in sub-Saharan Africa include theileriosis, babesiosis, trypanosomosis, hepatozoonosis, toxoplasmosis, besnoitiosis and ehrlichiosis. These diseases have a considerable impact on the country's economic security and impact negatively on poor communities who are depended on livestock production as their source of income and nutritional needs, and as labour for fieldwork and transport. As such, it is documented that the occurrence of protozoan parasites in South African domestic animals hinders the development of livestock industry, which contributes for up to 49% of the agricultural yield. It is important to keep up to date data on occurrence of these diseases using modern molecular diagnostic techniques. Therefore, this study was aimed at improving the current knowledge about the occurrence and genetic diversity of protozoan parasites and *Ehrlichia* in domestic animals from north eastern KwaZulu-Natal (KZN).

A total of 208 blood samples collected from apparently healthy domestic animals (cattle, dogs, goats and sheep) in three different municipalities of uMkhanyakude district, (KZN) were screened using genus and species-specific PCR techniques for the detection of *Besnoitia besnoiti*, *Theileria* spp., *Babesia* spp., *Hepatozoon canis*, *Trypanosoma* spp., *Toxoplasma gondii*, and *Ehrlichia canis* species-specific genes. The PCR amplicons were sequenced for detected species confirmation and phylogenetic analysis. The maximum likelihood trees were constructed to evaluate genetic diversity between protozoan parasites and *Ehrlichia* sequences of randomly selected isolates. Overall infection rates of *T. ovis* in sheep, *B. bigemina*, *B. bovis* in cattle and *Trypanosoma* spp. in cattle, *T. gondii* in cattle and *Ehrlichia canis* in dogs were 3 (30%), 33 (30.3%), 24 (22.2%), 20 (18.35%), 5 (4.58%), 20 (40.8%), respectively. The co-infection of two pathogens were detected in 4 (3.7%) for *B. bovis* and *B. bigemina*. The generated nucleotide sequences were confirmed to correspond with GenBank strains of respective PCR positive species. Analysis of phylograms constructed with RAP-1, B1, 18S and 16S sequences of *B. bovis*, *T. gondii* and *E. canis* indicated a close relationship between isolates detected in this study and GenBank strains. On the other hand, a tree constructed with SpeI-AvaI restriction fragment sequences revealed a high degree of polymorphism among the *B. bigemina* isolates investigated in this study. Taken together, the results of the current

study indicated that protozoan parasites are prevalent in domestic animals from uMkhanyakude district of KZN province. A large scale epidemiological study covering the rest of the district municipalities in KZN province is needed, in order to provide a clearer picture of the prevalence of these protozoan and ehrlichial pathogens in domestic animals. Ultimately, this prevalence data will contribute in formulation of control strategies against diseases caused by these pathogens.

Key Terms: *Ehrlichia canis*, *Toxoplasma gondii*, *Trypanosoma*, *Babesia*, *Theileria*, *Besnoitia*, uMkhanyakude district, Phylogeny, Domestic animals.

RESEARCH OUTPUTS

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TABLE OF CONTENTS

1.1.	Background	1
1.1.1.	Trypanosoma.....	2
1.1.1.1	Classification of trypanosomes	2
1.1.1.2	General life cycle of trypanosomes	2
1.1.1.3	Characterization of trypanosomes	3
1.1.1.4	Epidemiology of African animal trypanosomosis	4
1.1.1.5	Pathogenesis and clinical signs of trypanosomosis	5
1.1.1.6	History of nagana in KwaZulu-Natal province	5
1.1.2.	Apicomplexans	6
1.1.2.1.	Piroplasmids.....	7
1.1.2.2.	Clinical courses and life cycles of piroplasmida	8
1.1.2.2.1.	<i>Theileria</i>	8
1.1.2.2.1.2.	January disease (Zimbabwe theileriosis)	9
1.1.2.2.2.	Life cycle of <i>Theileria</i>	9
1.1.2.2.3.	<i>Babesia</i>	10
1.1.2.2.3.1.	Canine babesiosis	11
1.1.2.2.3.2.	Bovine babesiosis.....	11
1.1.2.2.3.3.	Ovine babesiosis	12
1.1.2.3.	Epidemiology of piroplasmosis	13
1.1.2.4.	Coccidians.....	14
1.1.2.4.3.	<i>Besnotia besnoiti</i>	14

1.1.2.4.3.1.	Transmission and clinical signs of bovine besnoitiosis.....	15
1.1.2.4.3.2.	. Life cycle of <i>Besnoitia</i>	15
1.1.2.4.3.3.	. Epidemiology of besnoitiosis.....	16
1.1.2.4.4.	<i>Toxoplasma gondii</i>	17
1.1.2.4.4.1.	.Transmission and clinical signs of <i>T. gondii</i>	18
1.1.2.4.4.2.	Life cycle of <i>Toxoplasma gondii</i>	18
1.1.2.4.4.3.	. Epidemiology of toxoplasmosis.....	20
1.1.2.4.5.	<i>Hepatozoon</i>	21
1.1.3.	Diagnosis of protozoan parasites and Ehrlichia	25
1.1.3.2.	Polymerase chain reaction (PCR).....	25
1.1.3.3.	Loop mediated isothermal amplification (LAMP)	26
1.1.4.	Genotyping and population dynamics of haemoparasites	27
2.1.	AIM.....	32
2.2.	Objectives	32
2.3.	Hypotheses	32
2.4.	Outline of the dissertation	33
3.1.	Study area	34
3.2.	. Sample collection	35
3.3.	. Experimental procedures.....	36
3.3.2.	DNA Extraction: Salting out method (Nasiri et al. 2005).....	36
3.3.3.	PCR detection of protozoan parasites and Ehrlichia DNA from blood samples	37
3.3.3.1.	Amplification of <i>Besnoitia besnoiti</i> DNA	37

3.3.3.2.	Amplification of <i>Toxoplasma gondii</i> DNA	37
3.3.3.3.	Amplification of <i>Trypanosoma</i> DNA	38
3.3.3.4.	Amplification of Babesia/Hepatozoon/Theileria DNA.....	39
3.3.3.4.1.	Amplification of Theileria DNA	39
3.3.3.4.2.	Amplification of <i>Hepatozoon canis</i> DNA.....	40
3.3.3.4.3.	Amplification of <i>Babesia</i> spp. DNA	40
3.3.4.	PCR product purification	43
3.3.5.	Sequencing of Purified PCR product	43
3.3.6.	Phylogenetic analysis	43
3.4.	Data analysis.....	44
4.1.	Overall infection rates	45
4.2.	Infection rate of piroplasm.....	47
4.2.1.	Infection rate based on host.....	47
4.2.1.1.	Cattle	47
4.2.1.2.	Sheep and Goats.....	48
4.2.1.3.	Dogs.....	49
4.2.2.	Infection rates based on the three municipalities	50
4.2.3.	Mixed infections.....	50
4.2.4.	Comparative analysis	52
4.2.5.	Phylogenetic analysis	57
4.3.	Infection rate of <i>Toxoplasma gondii</i>	73
4.3.1.	Comparative sequence analysis	73
4.3.2.	Phylogenetic analysis	74

4.4.	Infection rate of <i>Trypanosoma</i> spp.	84
4.5.	Infection rate of <i>Ehrlichia canis</i>	84
4.5.1.	Comparative analysis	84
4.5.2.	Phylogenetic analysis	87
5.2.	Bovine piroplasms.....	97
5.3.	Ovine piroplasms	100
5.4.	Canine piroplasms and <i>Hepatozoon canis</i>	101
5.5.	<i>Toxoplasma gondii</i>	102
5.6.	<i>Besnoitia besnoiti</i>	104
5.7.	<i>Trypanosoma</i> spp.....	104
5.8.	<i>Ehrlichia canis</i>	105
5.9.	Conclusion.....	105
5.10.	Recommendation	106

LIST OF TABLES

Table 3.1. Sequences of primers used for bovine <i>Babesia</i> PCR amplification	42
Table 4.1. Overall prevalence of protozoan parasites and <i>Ehrlichia</i> from different municipalities	45
Table 4.2. Overall prevalence of protozoan parasites and <i>Ehrlichia</i> from different hosts	46
Table 4.3. Prevalence of ovine piroplasm in sheep and goats	49
Table 4.4. Name of the municipalities and nested PCR results obtained with species specific primers.....	51
Table 4.5. BLASTn results for <i>RAP-1 Babesia bovis</i> sequences	53
Table 4.6. BLASTn results for Spel-Aval <i>B. bigemina</i> sequences	54
Table 4.7. BLASTn results for 18S rRNA <i>T. ovis</i> sequences	55
Table 4.8. Nucleotide composition of <i>B. bovis</i> RAP-1 gene sequences	60
Table 4.9. Estimates of evolutionary divergence between the <i>RAP-1</i> sequences	61
Table 4.10. Maximum Likelihood Estimate of Substitution Matrix	63
Table 4.11. Test of homogeneity of substitution patterns between <i>B. bovis</i> sequences. P-values are shown below the diagonal and the disparity index per site are shown for each sequence above the diagonal.....	64
Table 4.12. Nucleotide composition from Spel-aval restriction fragment between <i>B. bigemina</i> strains from the study and GenBank	65
Table 4.13. Estimates of evolutionary divergence between the <i>B. bigemina</i> Spel-Aval restriction fragment sequences	66
Table 4.14. Maximum Likelihood Estimate of Substitution matrix	68
Table 4.15. Test of homogeneity of substitution patterns between <i>B. bigemina</i> sequences. P-values are shown below the diagonal and the disparity index per site	

are shown for each sequence above the diagonal. P-values less than 0.05 are highlighted	69
Table 4.16. BLASTn results of <i>T. gondii</i> B1 gene	76
Table 4.17. <i>Nucleotide composition of B1 T. gondii sequences</i>	78
Table 4.18. Estimates of evolutionary divergence between the B1 gene sequences	79
Table 4.19. Maximum Likelihood Estimate of Substitution Matrix	81
Table 4.20. Test of homogeneity of substitution patterns between <i>T. gondii</i> sequences. P-values are shown below the diagonal and the disparity index per site are shown for each sequence above the diagonal. P-values less than 0.05 are highlighted	82
Table 4.21. BLASTn results of 16S rRNA <i>Ehrlichia canis</i> sequences	85
Table 4.22. Nucleotide composition of 16S rRNA <i>E. canis</i> sequences	89
Table 4.23. Estimates of evolutionary divergence between the 16S rRNA gene sequences....	90
Table 4.24. Maximum composite likelihood estimate of the pattern of nucleotide substitution	93
Table 4.25. Test of homogeneity of substitution patterns between <i>E.canis</i> sequences. P-values are shown below the diagonal and the disparity index per site are shown for each sequence above the diagonal. P-values less than 0.05 are highlighted.	94

LIST OF FIGURES

Figure 1.1: General life cycle of salivarian trypanosomes (Hendricks <i>et al.</i> 2000).....	3
Figure 1.2: Life cycle of <i>Theileria parva</i> in cattle and the ixodid tick vector	10
Figure 1.3: Simplified general life cycle of <i>Babesia</i> species (Mehlhorn and Piekarski 2002).	13
Figure 1.4: Life cycle and transmission of <i>Besnoitia besnoiti</i> (Álvarez-Garcia <i>et al.</i> 2013)	16
Figure 1.5: Life cycle of <i>Toxoplasma gondii</i> with three different transmission stages of the parasite (Hunter and Sibley 2012).....	20
Figure 1.6: Life cycle of <i>Hepatozoon</i> (Ewing and Panciera 2003).....	24
Figure 3.1: Map showing the sampled area. A) KwaZulu-Natal Province. (B) Umkhanyakude district with its local municipalities	35
Figure 4.1: PCR amplification of <i>Theileria/Babesia</i> genus DNA from cattle using RLB primers. M is the molecular marker, -ve is for the no template negative control, +ve is <i>B. bigemina</i> and <i>T. parva</i> positive control. Lane 1-8 shows positive piroplasmas samples 430 bp.....	47
Figure 4.2: PCR amplification of <i>B. bovis</i> DNA from cattle using group I primers. M is the molecular marker, -ve is for the no template negative control, +ve is <i>B.</i> <i>bovis</i> positive control. Lane 1,4,5,7 & 8 shows positive samples for <i>B.</i> <i>bovis</i> at 298 bp.....	48
Figure 4.3: PCR amplification of <i>B. bigemina</i> DNA from cattle using group I primers. M is the molecular marker, -ve is for the no template negative control, +ve is <i>B. bigemina</i> positive control. Lane 1, 4,6,7,9 & 10 shows positive samples for <i>B. bigemina</i> at 170 bp.	48
Figure 4.4: PCR amplification of <i>Theileria/ Babesia</i> DNA from sheep using P1 and P2 primers. M is the molecular marker, -ve is for the no template negative control. Lane 1, 3 & 4 shows positive samples for <i>T. ovis</i> at approximately 430 bp.....	49

- Figure 4.5: BLASTn results showing the alignment of *B. bovis* RAP-1 gene sequence from this study which was from a cattle sample from Big 5 hlabisa local municipality. The subject sequence (*B. bovis* isolate CQ (Rap-1a) gene), accession no: KT318580.1 covered 91% of the query sequence (KZN_10MAY-B9 Bovine) and it had 99% identity with one gap. The red star indicates the gap between sequences..... 56
- Figure 4.6: BLASTn results showing the alignment of *B. bigemina* isolate Spel-Aval gene sequence from this study which was from a cattle sample from Big 5 hlabisa local municipality. The subject sequence (*B. bigemina* hypothetical protein partial mRNA), accession no: XM012911573.1 covered 88% of the query sequence (KZN-Hlabisa B9 Bovine) and it had 89% identity with no gaps. The black stars indicate transitions and transversions that occurred between sequences..... 56
- Figure 4.7: BLASTn results showing the alignment of *T. ovis* 18S rRNA sequences from this study which was from a sheep sample from Big 5 False Bay local municipality. The subject sequence (*Theileria ovis* isolate al-lyfs), accession no: JN412663.1 covered 99% of the query sequence (KZN-Big 5-B1 ovine) and it had 99% identity with no gaps. The black stars indicate transitions and transversions that occurred between sequences..... 57
- Figure 4.8: Alignment of *B. bovis* RAP-1 nucleotide sequences (319 bp). The gray shaded area represent conserved regions..... 62
- Figure 4.9: Alignment of *B. bigemina* Spel-Aval nucleotide sequences (98 bp). The gray shaded area represent conserved regions 67
- Figure 4.10: Phylogenetic tree based on *rap-1* gene sequences of *B. bovis* isolates identified in this study (Indicated with bullets) and those of strains whose sequences were retrieved from GenBank. The tree was constructed using maximum likelihood method, with bootstrap values (expressed as percentages of 10000 replications) superimposed at branching points. The horizontal bar represents the number of substitutions per sites. *Babesia orientalis* was used as an outgroup 70
- Figure 4.11: Phylogenetic tree based on *Spel-Aval* gene sequences of *B. bigemina* isolates identified in this study (Indicated with bullets) and those of strains whose sequences were retrieved from GenBank. The tree was constructed using

maximum likelihood method, with bootstrap values (expressed as percentages of 10000 replications) superimposed at branching points. Only values above 50% are shown. The horizontal bar represents the number of mutations per sites. *Babesia microti* was used as an outgroup. 71

Figure 4.12: Phylogenetic tree analysis of *Theileria ovis* based on 18S rRNA gene, the tree was constructed with maximum likelihood method, with bootstrap values (expressed as percentages of 10000 replications) superimposed at branching points. The sequences produced in this study are shown with bullet points. The evolutionary distances were computed using the p-distance method (Kumar et al 2016). *Theileria luwenshuni* was used as an outgroup..... 72

Figure 4.13: Agarose gel showing PCR amplification of *Toxoplasma gondii* DNA from cattle using B1 gene nested primers. M is the molecular marker, -ve is for the no template negative control, +ve is for the positive control. Lane 4, 5 & 6 shows positive samples for *T. gondii* at approximately 73

Figure 4.14: BLASTn results showing the alignment of *T. gondii* isolate with one of the sequences from the study which was from cattle in Hlabisa municipality. The subject sequence (*T. gondii* isolate B1 gene) accession no: KX270388.1 covered 100% of the query cover and it had 98% identity with one gap. Black stars indicate nucleotide polymorphisms that occurred between the sequences. A gap is indicated by a red star. 77

Figure 4.15: Alignment of B1 *T. gondii* nucleotide sequences. The gray shaded area represent conserved regions. 80

Figure 4.16: Maximum likelihood tree created from B1 nucleotide sequences of *T. gondii* determined in this study (with bullets) and those retrieved from Gen Bank with accession numbers. The numbers at the branching points are bootstrap values expressed as percentages of 10, 000 replications. The horizontal scale bar indicates the number of nucleotide substitutions per site. The origin of published sequences is indicated after the isolate name. *Besnoitia besnoiti* was used as an outgroup 83

Figure 4.17: BLAST results showing the alignment of *E. canis* isolate and one of the sequences from this study which was from a dog sample from Big 5

hlabisa local municipality. The subject sequence (*E. canis* clone M), accession no: MH686052.1 covered 93% of the query sequence (KZN-dog-Hlabisa) and it had 89% identity with one gap. The black stars indicates transitions and transversions that occurred between sequences 86

Figure 4.18: Alignment of 16S rRNA *E. canis* nucleotide sequences. The gray shaded area represent conserved regions 92

Figure 4.19: Maximum likelihood tree created from 16S rRNA nucleotide sequences of *E.canis* determined in this study (with bullets) and those retrieved from Gen Bank with accession numbers. The numbers at the branching points are bootstrap values expressed as percentages of 10, 000 replications. The horizontal scale bar indicates the number of nucleotide substitutions per site. The origin of published sequences is indicated after the isolate name. *Babesia rossi* was used as an outgroup. 95

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1. Background

Protozoan and rickettsial parasites are defined as organisms that inhabit the cells and other tissues of their vertebrate host for all or part of their life cycle and metabolic requirements (Perry and Randolph 1999). They are a phylogenetically diverse group of organisms related to a range of vertebrate hosts globally, and are important human and animal pathogens (Concannon et al. 2005). From the late 1800s, a number of protozoan parasites have been identified in domestic as well as in wild animals (Uilenberg 1995; Pfitzer and Kohrs 2005). Some of these parasites developed together with wild ungulates over numerous years resulting in a state of equilibrium because of reciprocal adaptations (Jongejan and Uilenberg 2004). Akande et al (2010) have documented that most of the protozoan parasites have proven to be responsible for destruction of red blood cells, which results in anorexia, infertility, anaemia, and jaundice, (Akande et al. 2010). Most of these parasitic disease agents including *Apicomplexa* and a number of trypanosome species have shown to be transmitted by various insect vectors such as tabanids, tsetse flies and ticks.

Ticks are the most important ectoparasites in many African countries and are involved in the transmission of various haemoparasitic infections causing theileriosis, babesiosis and anaplasmosis (Makala et al. 2003). Tsetse flies are the most important vectors which are mainly involved in the transmission of bovine trypanosomiasis (Laohasinnarong et al. 2011). Mechanical transmission of the protozoan parasites, such as *Besnoitia besnoiti* and trypanosome species by tabanids have also been reported in susceptible hosts when interrupted during feeding (Taioe et al. 2017). Ellis et al. (2003) documented that in developing countries, human and animal health have shown to be weakened by these parasitic infections. They have a global distribution, stretching from the polar circle to the equator. This is due to the fact that their vectors; ticks and blood sucking flies are also globally distributed (Okorafor and Nzeako 2014). The most important protozoan parasites and *Ehrlichia* of interest for the current study include species of the genus *Trypanosoma*

and Apicomplexans of genera *Babesia*, *Theileria*, *Hepatozoon*, the species *Toxoplasma gondii*, and *Ehrlichia canis*.

1.1.1. Trypanosoma

1.1.1.1 Classification of trypanosomes

Trypanosomes are members of phylum Sarcomastigophora and genus *Trypanosoma* (Stevens and Brisse 2004). They are a monophyletic group of unicellular parasitic flagellated protozoans that are capable of surviving for a prolonged time in the bloodstream of their vertebrate host (Hamilton et al. 2004). These parasites are usually transmitted by arthropod vectors such as tsetse and tabanid flies (Taioe et al 2017). Important diseases such as Human African Trypanosomiasis (HAT) in humans and Animal African Trypanosomiasis (AAT) in animals are known to be caused by a number of trypanosome species (Hamilton et al. 2004). Because of the parasite transmission mode by the vector arthropods, trypanosomes of pathogenic and economic importance which infects mammals are separated into two clusters: (i) the stercoraria (Subgenera *Schizotrypanum*, *Megatrypanum* and *Herpetosoma*) in which the development in the arthropod vector ends with infective trypanosomes forming in the posterior part of the digestive tract and transmission occurs through the faeces of the insect and (ii) the salivaria (Subgenera *Duttonella*, *Nannomonas*, *Pycnomonas* and *Trypanozoon*), the usual mode of transmission is inoculative, through the biting mouthparts of the vector (Stevens and Brisse 2004). Trypanosomes belonging to subgenus *Herpetosoma* (*T. lewis*, *T. musculi* and *T. microtis*) parasitize rodents with an exception of a few human cases. The subgenus *Pycnomonas* contains only one species, *T. suis*, which has received limited attention because of its little economic importance. In Africa, the salivaria species characteristically possess a variant surface glycoprotein (VSG) gene and are the only trypanosomes to show antigenic variation, and this lead to them being the most prevalent species (Stevens and Brisse 2004).

1.1.1.2 General life cycle of trypanosomes

Two hosts are required for the completion of a typical trypanosome life cycle which is made up of different developmental forms (Figure 1.1). The stumpy bloodstream form of

T. brucei are ingested by tsetse flies when they feed upon the blood of infected host. In the midgut, the bloodstream forms change into procyclic trypanomastigotes that enter the peritrophic membrane and in the long run enter the proventriculus where they change into mesocyclic trypanomastigotes. These relocate to the salivary glands where they transform into epimastigotes. The latter forms then duplicate and differentiate into metacyclic shapes which are prepared to be transmitted by a tsetse fly when it feeds on a mammalian host (Vickerman et al.1988; Hendricks et al. 2000). The developmental stages of *T. congolense* in a tsetse fly are like those of *T. brucei* except that the epimastigotes duplicate and differentiate into metacyclic trypanomastigotes in the proboscis.

Dissimilar to the two specified species, none of the developmental stages of *T. vivax* occur in the midgut, rather, the stumpy bloodstream forms change into procyclic trypanomastigotes, at that point into epimastigotes in the foregut. These then move to the proboscis where they differentiate into metacyclic trypanomastigotes (Vickerman et al.1988). metacyclics which matured in either the proboscis or salivary glands are transferred during a blood meal into the dermis of the skin with the saliva (Hendricks et al. 2000). In the vertebrate host the metacyclics differentiate into different bloodstream forms such as the slender, intermediate and then stumpy forms one and two. At this stage the life cycle of a trypanosome would be complete, and the stumpy forms can be ingested by a tsetse fly and undergo cyclical changes once more (Hendricks et al. 2000).

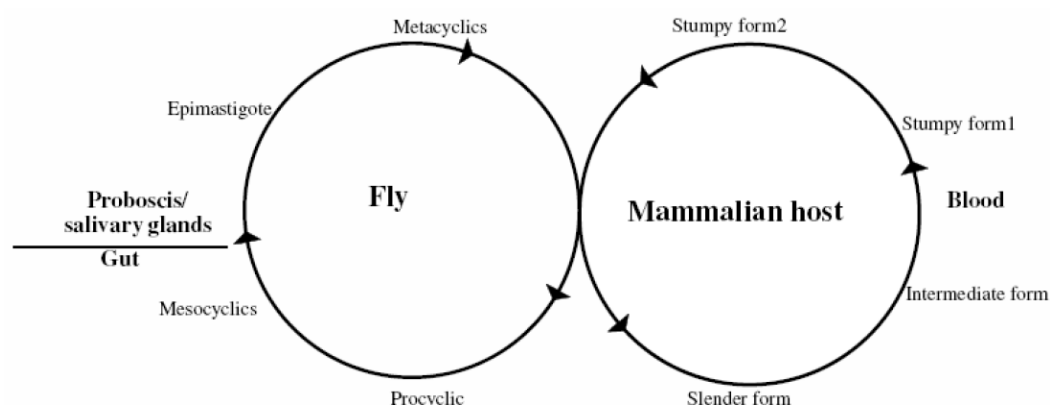


Figure 1.1: General life cycle of salivarian trypanosomes (Hendricks *et al.* 2000)

1.1.1.3 Characterization of trypanosomes

Before the development of genetic methods, trypanosomes were initially characterised morphologically and by host competency (Fisher et al. 2013), and it is challenging to

distinguish parasites merely on their morphological features. For example, it is morphologically difficult to differentiate *T. evansi* from *T. brucei* or *T. congolense* from *T. simiae* as they are very identical. More precise identification approaches for these flagellates are emerging due to the fact that molecular markers are acknowledged and their use cultivated (Auty et al. 2012). The position and determination of parasite motility of trypanosomes in the proboscis, gut or salivary gland of tsetse flies can be sufficient, and to some degree be used for characterization of trypanosomes (Godfrey 1961). Be that as it may, the characterization of trypanosomes in infected tsetse flies by viewing tissue localisations is insufficiently precise to give a reliable diagnosis. This is because mixed infections occur under natural conditions. According to Hamilton et al. (2004), detailed phylogenetic studies of trypanosomes have been possible due to molecular tools, and our understanding of evolutionary and taxonomic relationships have been improved due to the integration of genetic information and morphological characters. This tools include isoenzyme typing, orthogonal field alteration gel electrophoresis (OFAGE), DNA hybridization and PCR (Masiga et al. 1992; Majiwa et al 1994; Desquesnes & Davila 2002).

1.1.1.4 Epidemiology of African animal trypanosomosis

The epidemiology of trypanosomosis is very complex and influenced by three elements such as the distribution of the vectors, the virulence of the parasite and the response of the host (Urquhart et al. 1996). When dealing with tsetse- transmitted trypanosomiasis, much relies upon the distribution and vectorial capacity of the *Glossina* species responsible for transmission. Three types of African animal trypanosomiasis (AAT) exist. These include nagana which is known to affect a number of ruminants including cattle, pigs and horses (Taylor and Authie 2004). The *T. vivax*, *T. simiae*, *T. uniforme*, *T.b. brucei* and *T. congolense* are important causative agents of nagana in Africa, with tsetse flies acting as vectors for the cyclic transmission of the disease in domesticated animals (Steverding 2008). Trypanosomes which are considered not to be pathogenic such as *T. theileri* and *T. ingens* which are commonly found in both domesticated and wild animals can be harboured by African mammals (Biryomumaisho et al. 2013). The *T. evansi* is responsible for the occurrence of the second animal disease known as surra which is known to occur in Asia, South America and Africa. Blood sucking insects from genera *Tabanus* and *Stomoxys* as well as vampire bats such as *Desmodus* and *Rotondus* acts

as vectors of *T. theileri* (Taylor and Authie 2004). The *T. equiperdum* is a causative agent of the third diseases known as dourine. As compared to the other two disease, dourine has a wider geographical distribution, it is sexually transmitted and commonly affects horses (Taylor and Authie 2004).

1.1.1.5 Pathogenesis and clinical signs of trypanosomosis

The primary replication of trypanosomes take place at the site of immunization in the skin causing chancre and swelling. Enlargement of the lymphoid, spleen and plasma cell develop associated with deficiency in B-cells which lead to elevated levels of gamma globulin (Urquhart et al. 1996). Leak (1999) documented that one of the major cardinal effects of infection with pathogenic trypanosomes is anaemia. The pathogenesis of the disease develop in two stages, the chronic and acute stage. The degree of the two stages of the disease is determined by various factors such as the complete tolerance in the case of game animals, virulence of the *Trypanosoma* species and the level of parasitemia (Steverding 2008). The acute stage normally occurs shortly after the infection. This is characterized by high level of parasites in the blood, and a rapid fall of packed cell volume (PCV) because of the destruction of erythrocytes. Erythrophagocytosis is activated which result in fever and within 10 days, death usually occurs in susceptible animals (Connor and Bossche 2004). According to Itty (1996), the chronic stage of the disease can persist for a couple of months and this is typical for indigenous breeds in which infected animals loose condition, becoming increasingly anaemic and drowsy. Due to the fact that concurrent infection with more than one trypanosome species and with other haemoparasites frequently occurs, it is difficult to say which clinical signs are caused by a given parasites (Nyeko et al. 1990). Even though it is difficult to attribute clinical signs to a specific parasite, it was documented by Leak (1999) and OIE (2013) that the most clinical symptoms associated with African animal trypanosomiasis include fever, emaciation, alopecia, lethargy and eventually death.

1.1.1.6 History of nagana in KwaZulu-Natal province

From the past, South Africa occupies an essential position regarding the incidence of African animal trypanosomosis (Nguyen et al 2014). In north eastern KwaZulu- Natal, South Africa, The occurrence of nagana, was recorded for the first time in the 1880's (Steverding 2008; de Beer et al 2016). Bruce (1895) stated that game animals were the

reservoir hosts of the pathogenic trypanosome species, and that tsetse flies were responsible for the transmission of these parasites to their vertebrate host. The most predominant and important vector of AAT in north eastern KZN at that time was *Glossina pallidipes*. On certain occasions, large numbers of trapped flies indicated the abundance of this species (Harris 1932). Between 1942 and 1946, an extreme outbreak of nagana was observed, which was attributed to the existence of these large numbers of *Glossina* spp. (Du Toit 1954).

In the 1950s, the South African government initiated aggressive campaign which involved aerial spraying to control and eliminate the population of the vectors and successfully eradicated *Glossina pallidipes* (Du Toit 1954). Du Toit (1954) also documented that the eradication of *Glossina brevipalpis* from the Hluhluwe-iMfolozi Park was another advantage of this campaign (Du Toit 1954). From 1955, the cases that were reported thereafter were sporadic and they were diagnosed in cattle, horses and dogs (Kappmeier et al. 1998). However, an extreme epidemic of the disease was again reported in the north eastern parts of KZN in 1990. This outbreak showed that AAT had remained a significant debilitating constraint in KZN with co-infection of *T. congolense* and *T. vivax* (Bagnall 1993). Seasonal screening of cattle revealed that the most prevalent species while the least prevalent species was *T. vivax* in areas infested by tsetse flies (Mamabolo et al. 2009; Motloang et al. 2014). In a recent study, Taioe (2014) documented the detection of *T. theileri* and *T. Brucei* by aligning the 18S rRNA gene sequences, and they matched with published sequences of trypanosomes sequences in the National Centre for Biotechnology Information (NCBI). At present; only *G. brevipalps* and *G. austeni* remain restricted to the north eastern Kwa-Zulu Natal (KZN) province (Kappmeier et al. 1998). The incidence of livestock trypanosomiasis (nagana) in KZN has always been related with tsetse flies. After game protection law enforcements that took place in 1879 in Zululand, the number of wildlife increased resulting in livestock trypanosomiasis. The diseases was then effectively managed through treatment with homidium bromide and diminaze aceturate in cattle. This was combined with dipping of cattle in pyrethroid and cyhalothrin twice a month (Kappmeier and Nevill 1999).

1.1.2. Apicomplexans

Apicomplexans are known as a large and diverse group of intracellular protists with a wide geographical. Their characterization depend on the presence of an evolutionary

apical complex, and they are known to consist of motile invasive stages (Moore et al. 2008). Most of the apicomplexans are pathogenic to humans and livestock, even though it is believed that all animals reservoir host to at least one apicomplexan species (Moore et al. 2008). From now on, apicomplexans are acknowledged to be closely associated with the dinoflagellates and ciliates and forms the taxonomic group known as the alveolata (Yoon et al. 2008). It is traditionally considered that the phylum Apicomplexa consists of four clearly distinct groups including the coccidians (*Besnoitia*, *Hepatozoon*, *Toxoplasma*), the gregarines, the haemosporodian and the piroplasmids (*Theileria* and *Babesia*) (Ellis et al. 1998). Phenotypic characteristics such as their associated hosts and vectors, and which particular tissues they live in, are what distinguish these groups. (Perkins et al. 2000). The evolutionary relationship between the apicomplexan groups and how their subsequent taxonomy is arranged remains unclear. The present characterization of the phylum is as a matter of fact a conservative one and does not consider molecular information (Kaya 2001). For the current study, the main focus is on piroplasmids and coccidians.

1.1.2.1. Piroplasmids

Piroplasmids are the second most common haemoparasites following the trypanosomes, and they are known to infect a wide range of mammals across the world. (Telford et al. 1993). As of now, two genera of piroplasms have been identified as *Babesia* and *Theileria*. The *Babesia* are characterized as parasites that enter specifically into erythrocytes of the host after injection. In contrast, *Theileria* sporozoites don't at first infect erythrocytes but infiltrate white blood cells or, then again macrophage in which they transform into schizonts (Uilenberg 2006). While some of the piroplasms known in domestic and wild animals seem to be host species specific, it was proven that others are able to cross the host species barrier. These include *Theileria parva*, *Theileria taurotragi* and *Babesia bigemina* as well as *Theileria equi* (De Waal and Van Heerden 1994; Lawrence and Williamson 2005). In domestic animals, *Babesia* and *Theileria* cause some of the most economically and veterinary important diseases such as babesiosis and theileriosis respectively (Burridge 1975; De Vos et al. 2005).

1.1.2.2. Clinical courses and life cycles of piroplasmida

1.1.2.2.1. *Theileria*

Theileriosis caused by *Theileria* species infections is responsible for the high mortality of exotic and crossbred cattle, however, in endemically unstable areas, the indigenous bovines are also infected (Perry and Young 1995). *Theileria parva* is an apicomplexan protozoan parasite known to cause January disease, Corridor disease and East Coast fever (ECF) in bovines (Uilenberg et al. 1982; Perry et al. 1991). It was probably originated as a parasite of African buffalo and became adapted to cattle. In the 19th century, ECF was introduced in southern Africa and eventually got eradicated after the initiation of expensive campaign that involved quarantine of infected farms and slaughtering of infected herds (Anonymous 1981). After ECF got eradicated, corridor disease emerged as the most significant form of theileriosis in South Africa. In areas where common crazing among cattle and infected buffalo occur and where there is an abundance of tick vector species (*Rhipicephalus appendiculatus* and *R. zambeziensis*), the disease still pose a serious threat (Uilenberg 1999).

1.1.2.2.1.1 East coast fever

It is lethal disease of bovines caused by *Theileria parva* with significant economic importance in the development of livestock industry in Africa (Lawrence et al. 1994). Cattle-to-cattle transmission is the major route of transmission of the parasite with high grade cattle being particularly susceptible. Pulmonary oedema is the most common sign of east coast fever (Perry et al. 1991). The disease is also characterized by lymphadenopathy, fever arises and continues throughout the course of infection. There are marked bruises on the skin as a result of tiny haemorrhage on most of mucous membranes of the conjunctiva and the buccal cavity, anorexia develops and it is followed by loss of condition (Lawrence et al. 1994). Additional clinical signs may include secretion of tears, corneal opacity, nasal discharge, shortness of breath and diarrhoea (Lawrence et al. 1994). Recovered adult animals may remain unproductive while the disease result in stunted growth in calves.

Corridor disease is an acute, typically lethal illness of cattle that bear a resemblance to the ECF. The causative agent of the infection is a buffalo-derived *Theileria parva* strains which are transmitted by ticks from African buffaloes (Lawrence et al. 1994). It was first

diagnosed in 1953 in a corridor of land between Hluhluwe and iMfolozi Game Reserves in South Africa, hence the name Corridor disease (Neitz *et al.* 1955). The disease shows similar clinical signs as those of ECF apart from the fact that the course is usually shorter and characterized by low schizont parasitosis and piroplasm parasitaemia (Lawrence *et al.* 1994). Advanced signs of ECF such as emaciation, diarrhoea and regression of lymph nodes are not commonly seen in corridor disease. Severe pulmonary oedema leads to death.

1.1.2.2.1.2. January disease (Zimbabwe theileriosis)

January disease is an acute, often deadly disease caused by the cattle-derived *T. parva* parasite formerly known as *T. parva bovis* (Uilenberg *et al.* 1982). It emerged after the eradication of ECF. The fact that the disease occurs seasonally between December and March contributed to the name January disease (Matson 1967). This seasonal occurrence concurs with the seasonal distribution of the adult vector. The clinical signs, pathogenesis and pathology of the disease are very similar to those of (Lawrence *et al.* 1994). There is no evidence that suggests the occurrence of the disease in South Africa as there are no current clinical signs observed (Lawrence *et al.* 1994).

1.1.2.2.2. Life cycle of *Theileria*

The life cycle *Theileria* is very complex and consists of several developmental stages which are morphologically different in the tick and the vertebrate host cells (Figure 1.2). Shaw (2003) documented that the ability of different invasive stages, the sporozoites and merozoites in the vertebrate host, the zygote and kinete in the tick vector to recognise and invade specific host cells are what drives the transmission and survival of the parasite. In the vertebrate host, the transmission of the parasite is mainly achieved by infected ticks when feeding at the time that the sporozoites mature in its salivary gland (Stagg *et al.* 1981). The matured sporozoites will then invade the white blood cells and transform into schizonts inducing the production of lymphocytes in excessive quantities (Stagg *et al.* 1980). The schizonts will later transform into merozoites which will attack the erythrocytes where they develop into piroplasms, the stage of the parasite infective to vector species. In the red blood cells of the vertebrate host, piroplasms are ingested at the time the tick feed (Melhorn and Schein 1985). Transformation of piroplasms into macro and micro gametes seems to take place in the gut of the vector. After the fusion of gametes, zygote move in to the cells of the tick gut epithelium and mature into motile

kinetes which are released into the hemocel of the tick. Sprogony takes place in the c-cells in type III acini (Faweett et al. 1982).

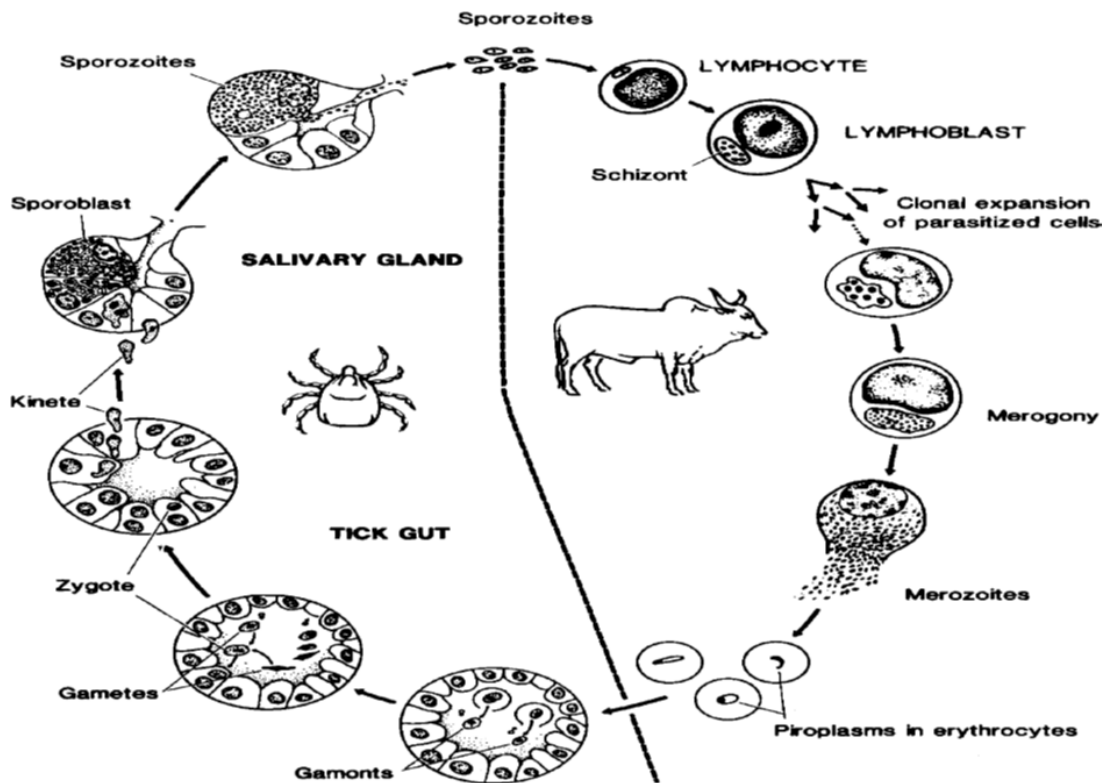


Figure 1.2: Life cycle of *Theileria parva* in cattle and the ixodid tick vector *Rhipicephalus appendiculatus* (Bishop et al. 2004).

1.1.2.2.3. *Babesia*

Babesiosis is caused by various intraerythrocytic protozoa of the genus *Babesia*. The genus belongs to the order Piroplasmida of the phylum Apicomplexa (Homer et al. 2000). Ticks are responsible for the transmission of the *Babesia* parasites infect a variety of animals and humans. Victor Babes discovered microorganisms in the erythrocytes of bovines at the end of the 19th century, and he associated them with red water fever (Babes 1888). Ever since the discovery, the emergence of newly documented babesial pathogens continue to increase globally and their significant impact on the health of livestock and human is continuing (Collett 2000). So far, over 100 species of *Babesia* which infect a range of mammals and some avian species have been identified (Gray and Weiss 2008). Traditionally, the genus was classified according to their morphology, host

specificity, and life cycle (Homer et al. 2000). Practically, they are divided into two groups: Small babesias which include *B. microti*, *Babesia gibsoni* and *B. rodhaini* and large babesias which include and *B. canis*, *B. caballi*, *Babesia bovis* . Of the species affecting domestic animals *B. ovis*, *B. motasi*, *B. canis*, *Babesia bigemina* and *B. bovis* are the most economically important species in most countries across the world (Terkawi et al. 2011).

Canine babesiosis, bovine babesiosis and ovine babesiosis are amongst the most significant diseases caused by *Babesia* infections in dogs, bovines and ovines, respectively. The diseases result from tick transmission of the parasite from infected host to a susceptible host.

1.1.2.2.3.1. Canine babesiosis

It is a veterinary significant disease of canines caused by a group of intraerythrocytic protozoan parasites (Collett 2000). It was detected in canines between 1988 and 1993 at an average of 11.69% annually (Shakespeare 1995). According to Matjila et al. (2004), two species of canines, *B. rossi* and *B. vogelli* are endemic to South Africa. The clinical signs of *B. vogelli* has not yet been estimated and this led to *B. rossi* being considered as the most prevalent species in South Africa as it causes severe, often fatal disease (Jacobson 2006). The *B. rossi* causes the disease that is classified into two categories, complicated and uncomplicated. The disease is said to be uncomplicated if the clinical changes consists of a mild or moderate anaemia with no clinical signs of organ failure (Jacobson and Clark 1994). The disease is complicated when there is evidence of organ failure and where anaemia itself is life threatening. Clinical signs of complicated disease include acute renal failure, coagulopathy, jaundice, pulmonary oedema and pancreatitis (Jacobson and Lobetti 1996). Wright et al. (1988) documented that there have been recognised similarities between *B. rossi* induced canine babesiosis and the pathogenesis of bovine babesiosis.

1.1.2.2.3.2. Bovine babesiosis

Bovine babesiosis is defined as a veterinary and medically important haemoparasitic disease of bovines in tropical and subtropical regions worldwide (McCosker 1981). *Babesia bovis* and *Babesia bigemina* are the two main causative agents of the disease. The clinical signs fluctuate with the species and strain of the parasite and the animal's

age. Normally, inappetence and a high fever are common in bovines infected with *B. bigemina*. Typical symptoms of the disease in subacute cases would include jaundice, diarrhoea, constipation and respiratory distress syndrome with dyspnea in severely affected animals (Bock et al. 2004). Infections associated with *B. bovis* are the same as accompanied by icterus, anaemia and fever in infected bovines but more severe than *B. bigemina*. This is because of its tendency to adhere to vascular epithelia (Ristic 1981). Due to the sequestration of the infected red blood cells in cerebral capillaries which result in low parasitemia in the circulating blood, the disease is associated with nervous symptoms (Ristic 1981; Bock et al. 2004).

1.1.2.2.3.3. Ovine babesiosis

Ovine babesiosis is a tick-borne disease transmitted by hard ticks and predominantly affects ovines across the world. According to Soulsby (1986), the clinical symptoms of the disease include fever, jaundice, anaemia and haemoglobinuria in ovine. Three species infecting of ovines that are known to cause disease include *B. ovis*, *B. motasi* and *B. crassa* (Soulsby 1986; Uilenberg et al.1982). The acute and/or chronic phase of the disease can be attributed to *B. motasi*. In contrast, infection associated with *B. ovis* is commonly not extreme as compared to *B. motasi* whereas *B. crassa* seems to be less or not pathogenic (Soulsby 1986; Morel 1989). The classification of these pathogens is established on the classical methods of morphology, pathogenicity, antigenicity, host specificity, tick vectors, mode of transmission and epidemiological data (Liu et al. 2007).

1.1.2.2.2.4. Life cycle of *Babesia*

The life cycles of the different types of *Babesia* parasites are very similar (Figure 1.3). The bite of infected ixodid ticks is the main cause of natural transmission of the parasite (Hunfeld et al. 2008). The presence of transovarial transmission in some species and the absence in others such as *B. microti* is the key difference between the life cycles (Hunfeld et al. 2008). During the tick feeding, sporozoites are inoculated directly in the infected red blood cells of the host (Uilenberg 2006). According to Uilenberg (2006), this occurrence distinguishes *Babesia spp.* from *Theileria spp.*, where sporozoites do not infect the erythrocytes but primarily penetrate the white blood cells or the macrophages and develop into schizonts. the *Babesia* sporozoites mature into piroplasms inside the

infected red blood cells of the vertebrate host, which results into two or four daughter cells. The daughter cells leave the host cell to infect other red blood cells until the host dies or the immunity of the host clears the parasite (Gray and Weiss 2008).

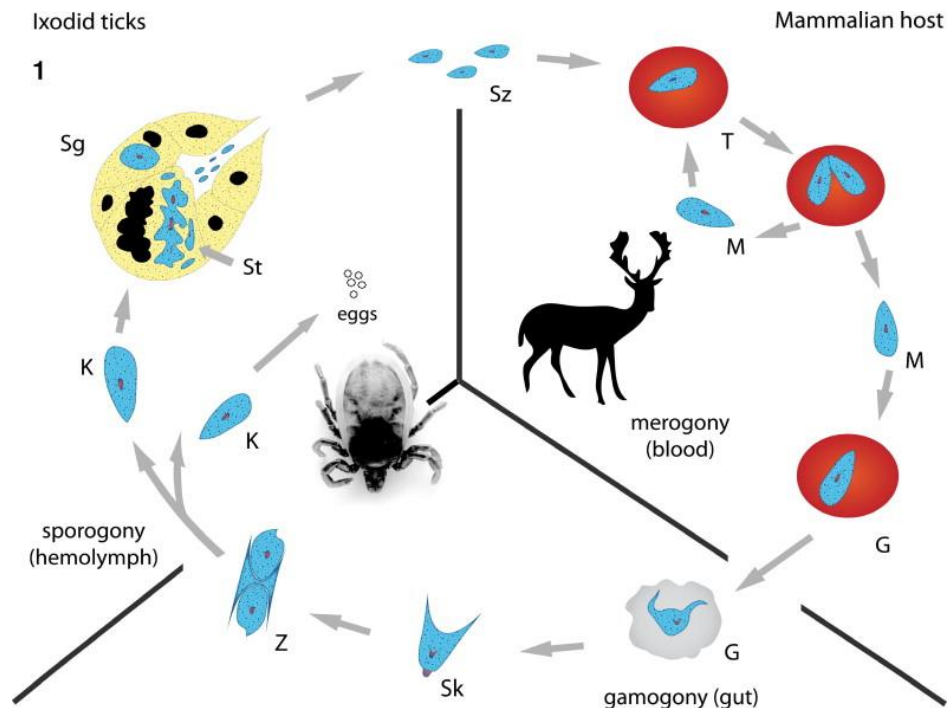


Figure 1.3: Simplified general life cycle of *Babesia* species (Mehlhorn and Piekarski 2002).

1.1.2.3. Epidemiology of piroplasmosis

Information on the epidemiology of piroplasmosis is of great importance as it influences the choice of approaches for control in a production system (Gachohi et al. 2013). The prevalence of infection, occurrence and effect of the disease are determined by the intricate interactions between the environment (e.g. temperature, co-grazing and management practices), host characteristics (e.g. acquired immunity, susceptibility and population dynamics), tick vector (e.g. vector competence, abundance and seasonality) and the pathogen (e.g. virulence, infection rate and antigenic variation) (Norval et al. 1992). According to Kocan et al. (1992), factors such as global change and resistance to chemotherapeutics and acaricides are also significant. Tick population dynamics and the transmission possibilities of both *Babesia* and *Theileria* are influenced by differences in climatic conditions which create a variety of epidemiological situations in various areas (Lessard et al. 1990).

The different breeds of african cattle population exhibit fluctuating level of resistance and parasite susceptibility (Norval et al. 1992). Bock et al. (2004) documented that the most susceptible breeds of cattle to tick infestation and infections associated with both *Babesia* and *Theileria* species are the *Bos taurus* breeds, whereas indigenous breeds such as *Bos indicus*, Sanga and Zebu types of cattle in endemic area are resistant to the parasites. Additionally, in areas where there is an abundance of tick population, natural exposure normally arise at an early age when these animals are protected and this allows them to acquire immunity and become immune to next challenges as adults (Bock et al. 2004). Animals that recover either naturally or following treatment from the disease remains carriers and acts as reservoirs for the parasite infection during tick feeding (Kariuki et al. 1995).

1.1.2.4. Coccidians

1.1.2.4.3. Besnoitia besnoiti

Besnoitia Besnoiti is a cyst forming coccidian parasite and a member of family *Sarcocystidae* and sub-family *Toxoplasmatinae* which is closely related to *Toxoplasma gondii*, *Neospora caninum* and *Hammondia hammondi* (Basso et al. 2011). So far, up to ten species have been acknowledged in the genus *Besnoitia* (*B. besnoiti*, *B. bennetti*, *B. jellisoni*, *B. wallacei*, *B. tarandi*, *B. darling*, *B. caprae*, *B. akadoni*, *B. neotomofelis* and *B. oryctofelis*) (Dubey and Lindsay 2003). Between these ten species, *B. besnoiti* is known to be the causative agent of bovine besnoitiosis, previously known as globidiosis which is aa extremebut often not lethal disease with an important veterinary impact in some African countries and Asia. The disease started receiving attention at the end of 20th century when its distribution and prevalence increased. Alzieu et al. (2007) documented that the disease was previously encountered in the south west of France and the clinical cases have recently been regularly reported in the French Alps, the Massif Central and occasionally in the Loire region of France. In addition, it was documented by Mehlhorn et al. (2009) that the epidemic of besnoitiosis in bovines was recently reported for the first time in Germany (Mehlhorn et al. 2009). There is little information on how the disease is transmitted and on which meaurs to take in order to prevent and control the spread of besnoitiosis.

1.1.2.4.3.1. Transmission and clinical signs of bovine besnoitiosis

The transmission of the disease can be either through direct and indirect horizontal transmission via both hematophagous and non-biting insect vectors or by close contact (mating or physical among animals with wounds or lacerations) and through the use of one syringe during herd health procedures (Bigalke 1968; Pols 1960; Bigalke and Prozesky 1994). Papadopoulos et al. (2014) indicated that the most significant and currently known mode of disease transmission is mechanical transmission via blood-sucking insects. This is assumed to take place when a fly feeding on an infected host is interrupted, meaning it has to take an additional feed on susceptible host, thus transmitting the parasite via its contaminated mouthparts and through regurgitation (Kasigaz 1994). According to Peteshev et al. (1974), ingestion of mature isosporan-type oocysts shed in faeces of definitive host is an additional route of infection.

Bovine besnoitiosis may cause poor body condition in bovines and also lead to temporary or permanent sterility in male bovines (Basso et al. 2011). The acute stage of the disease is characterized by fever, nose and eye discharge, subcutaneous oedema and inflammation of the testicles whereas the scleroderma stage is mainly characterized by scleroderma, loss of hair and loss of function of the testes (Basso et al. 2011). These and other unrestricted clinical signs may possibly be confused with other infectious diseases such as blue tongue or invasive catarrhal fever (Alzieu et al. 2007). In either acute or scleroderma stage, death may occur. On the other hand, most cases of the disease are subclinical and go undetected (Bigalke 1981).

1.1.2.4.3.2. Life cycle of *Besnoitia*

It is suspected that *Besnoitia besnoiti* has a heterogenous life cycle with a predator as a definitive host and bovine as the intermediate host. In any case, the thorough life cycle of *B. besnoiti* is still obscure and a definitive host, which sheds oocysts after ingestion of infected tissues, has not yet been recognized (Diesing et al. 1988; Rommel 1975). In the intermediate host, The parasite experiences two infective asexual phases of development where they are found in cysts inside subcutaneous connective tissue (Alvarez-Garcia et al. 2013). Previous experimental studies recommended domestic felines or dogs as the final host(s) for *B. besnoiti* (Peteshev et al. 1974; Rommel 1978). Peteshev et al. (1974)

documented that, after ingesting cyst containing tissue the domestic and wild felines shed oocysts and this also happens for other *Besnoitia* species (figure1.4).

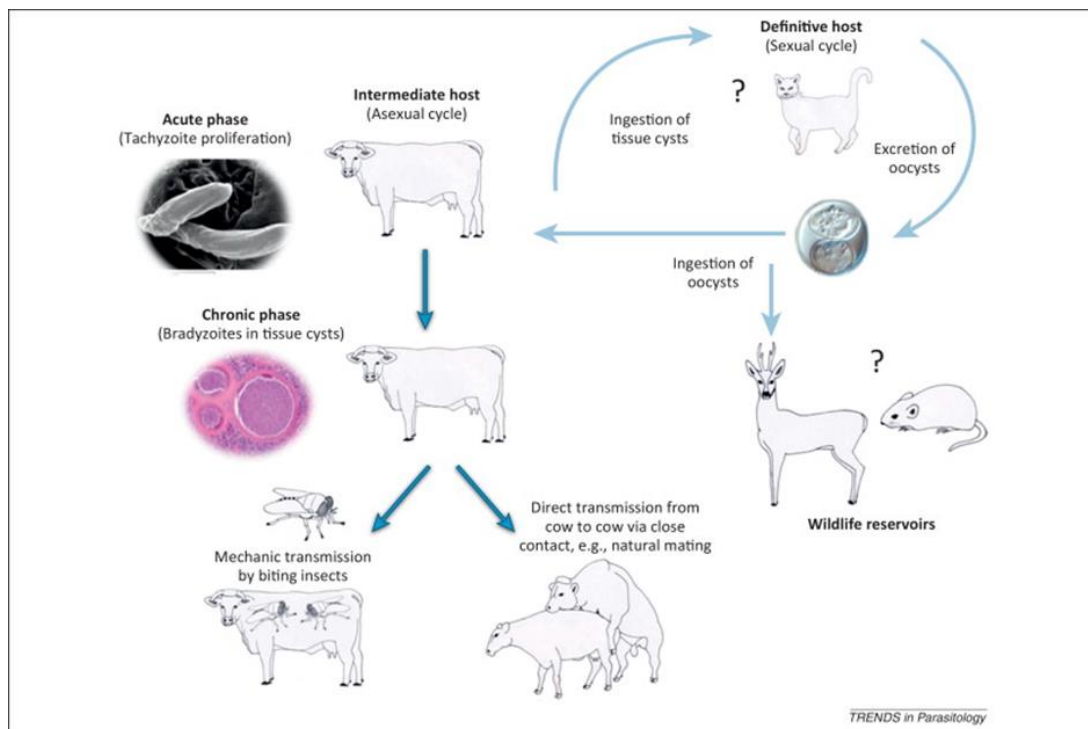


Figure 1.4: Life cycle and transmission of *Besnoitia besnoiti* (Álvarez-Garcia et al. 2013)

1.1.2.4.3.3. . Epidemiology of besnoitiosis

Due to the fact that the spread and geographic expansion of bovine besnoitiosis have been described in Europe, significant effort has been made in understanding the epidemiological aspects of *Besnoitia besnoiti* and to deliver a more circumstantial awareness of population dynamics (Alzieu 2007; Jacquet et al. 2010; Liénard et al. 2011; Basso et al. 2011). According to Pols (1960) and Bigalke (1968), Infection in enzootic and epizootic areas is reported across the world with only a small proportion of infected animals showing clinical symptoms. Usually in enzootic areas, extensiveness of the clinical signs varies from 1 – 10% per year, with incidences fluctuating from 2 – 5% (Legrand 2003). More acute symptoms with a high mortality rate are observed in male than females. In Africa, the disease was documented in quite a lot of countries including South Africa and Zimbabwe (Bigalke 1981; Chatikobo et al. 2013). In South Africa, the

disease is known to be of economic importance in many provinces including KwaZulu-Natal, with few incidences reported in Free State and Northern Cape Province (Bigalke and Prozesky 1994). Insufficient information about the life cycle of *B. besnoiti* makes it complicated to distinctly define the risk factors associated with *B. besnoiti* infection. According to European Food Safety Authority (2010), important factors like seasonality, age, breed, gender, route of infection and sub-clinical carriers play part in the transmission and occurrence of the infection. There is also an insufficient information on how *Besnotia* relates to other cyst forming coccidian such as *Toxoplasma gondii*, but their grouping into the subfamily *Toxoplasmatinae* is frequently well accepted (Frenkel 1977; Tenter and Johnson 1997).

1.1.2.4.4. *Toxoplasma gondii*

Toxoplasma is a coccidian and an obligate intracellular parasite that infect a wide range of warm blooded animals (Howe and Sibley 1995). Its ability to infect all warm blooded animals including humans makes it one of the most successful parasites across the world. Nicolle and Manceaux discovered *T. gondii* in 1908 at the Pasteur Institute in Tunisia while doing research on *Leishmania*. The parasite was discovered from an African, hamster-like rodent named the gundi (*Ctenodactylus gundi*). The first case of human congenital toxoplasmosis was first discovered in 1939 from a newborn baby who suffered from seizures (Wolf et al. 1939). Another case of toxoplasmosis was discovered in the 1950's in the enucleated eyes and was presumed to be a result of congenital transmission of the parasite (Wilder 1952). Be that as it may, current studies have described a great number of ocular toxoplasmosis cases than expected due to postnatally acquired infection (Burnett et al. 1998; Gilbert et al. 1999; Montoya and Remington 1996). Cats were identified as the definitive hosts for the parasite in 1970 when the first description of the sexual development of *T. gondii* in the small intestine of cats was published (Frenkel 1970; Hutchison et al. 1970). This discovery was significant, as forty years on, felids are still acknowledged to be the only definitive host. According to Luft and Remington (1988), another significant part in the history of *Toxoplasma* took place in the 1980's when AIDS patients were found to be susceptible to the parasite. The *T. gondii* was identified as a major opportunistic infection for these immunocompromised patients, where either newly acquired infection or recrudescence of latent infection would frequently cause toxoplasmic encephalitis (Luft & Remington 1992). Furthermore, the emergence of

genetically different strains (atypical strains) of the parasite have been linked to several fatal cases of acquired infection in immuno-competent individuals, further highlighting the potential public health risk of the parasite (Carme et al. 2002; Carme et al. 2009).

1.1.2.4.4.1. .Transmission and clinical signs of *T. gondii*

Toxoplasmosis is worldwide zoonotic infection distributed between warm blooded animals with a high prevalence in humans, particularly children and immune-compromised patients (Dubey 1995). Postnatal infection in humans and animals occurs basically by ingesting raw and undercooked infected meat which contains viable *Toxoplasma* tissue cysts or food or drink contaminated with *Toxoplasma* oocysts excreted from the faeces of infected felids (Dubey 1995). According to Bowie et al. (1997) and Torgerson et al. (2015), this makes toxoplasmosis a most important foodborne and waterborne parasitic disease. Additionally, humans can acquire the infection through blood transfusion or on the other hand by organ transplantation (Singh and Sehgal 2010). Pregnant females with active infection can transplacentally transfer the infection to the developing fetuses (Montoya and Liesenfeld 2004).

The clinical signs of the disease include enlargement of the lymph nodes associated with fever, fatigue, muscle pain, sore throat and headache (Hill and Dubey 2002). The headache may cause a condition which is considered to show signs such as confusion, lack of muscle control and coma. Neuropsychiatric symptoms such as depression and schizophrenia are also seen (Faustina et al. 2017). Most animals infected with toxoplasmosis show no clinical manifestations of the disease. The most commonly indicated sign in adult animals, especially sheep is abortion. Felids which are considered to be the definitive hosts of the infection may show signs of pneumonia or damage to the nervous system or eyes. Affected canines may show signs of encephalitis such as seizures, head tilt, tremors or paralysis. Encephalitis is considered to be the most significant manifestation of the diseases in animals and immuno-compromised patients as it causes severe damage to the patient (Dubey and Beattie 1988).

1.1.2.4.4.2. Life cycle of *Toxoplasma gondii*

The life cycle of *T. gondii* has two different stages; the sexual stage which takes place in the definitive host (felid), and an asexual stage which occurs within a number of warm blooded animals (intermediate hosts) (Tenter et al. 2000). After ingestion of tissue cysts

by the definitive host, proteolytic enzymes in the gut and small intestine break down the cyst wall, releasing bradyzoites which then begin asexual replication (merogony) within the epithelial cells of the small intestine (Frenkel and Dubey 1972). After successful replication, there are five different asexual phases that occur in enterocytes before the formation of gametes take place. Macrogametes fertilize the microgametes in the enterocyte forming zygotes. Around the fertilized zygote, there is a thin wall that releases unsporulated oocysts into the intestinal lumen which are then released to the environment with faeces when the enterocytes rupture (Peterson and Dubey 2001).

Once the oocysts/cysts are released to the environment, they are then ingested by the intermediate host (Bhopale 2003). This will be followed by the degradation of the enzymes, disrupting the walls of the oocysts/ cysts which will release either the sporozoites or bradyzoites into the intestinal lumen. The released bradyzoites or sporozoites invade the surrounding cells and develop into tachyzoites. According to Bhopale (2003), tachyzoites rapidly divide within the host cell, leading to its rupturing which release the parasite into the blood and lymphatic system where they are carried to other cells to invade and begin the process. Figure 1.5 shows diagrammatic representation of the *T. gondii* life cycle.

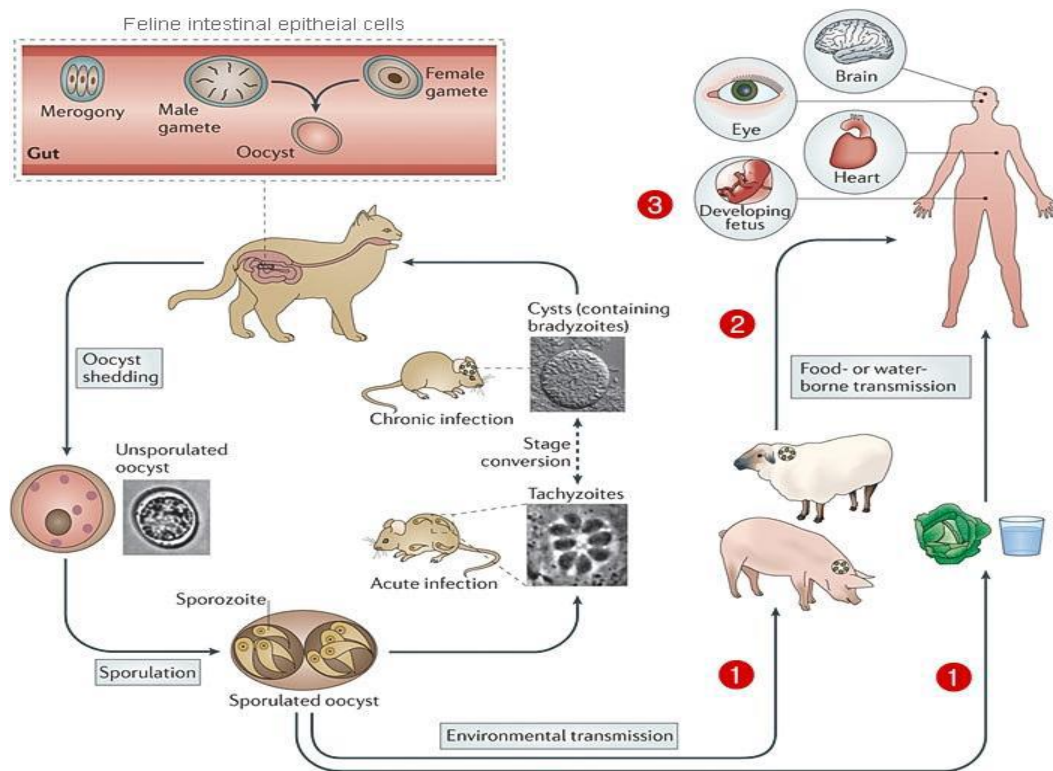


Figure 1.5: Life cycle of *Toxoplasma gondii* with three different transmission stages of the parasite (Hunter and Sibley 2012).

1.1.2.4.4.3. . Epidemiology of toxoplasmosis

Toxoplasma gondii has been detected in many counties across the world in various species of mammals as well as in avian (De Sousa 2009). Up to so far, it is estimated that about 33% of the world human population is chronically infected with only a small percentage of exposed immune-compromised individuals showing clinical signs during acute or chronic phase of the disease (Montoya and Liesenfeld 2004). The prevalence of the parasite is highly unpredictable between different geographic regions and this could be due to the number of risk factors such as: preparation of food, diet, hygiene, environmental conditions, definitive host population and different laboratory techniques used for diagnosis (De Sousa 2009). Risk factors can also be associated with host factors such as age and gender, and the management of the farm (Pinheiro et al. 2009).

Prevalence of *T. gondii* in small ruminants is normally high because of continuous contamination of pastures by the parasite's oocysts (Cenci-Goga et al. 2013).

Seropositivity is particularly lower and more unpredictable in horses, rabbits and poultry. This may reflect epidemiological factors such as different types of confinement, hygiene of stables and different types of feed (Tenter et al. 2000). In contrast, seropositivity is usually high in dogs, indicating their continuous exposure to a natural environment and the cumulative effect of age. According to Tenter et al. (2000), all of these animals may harbour a huge amount of tissue cysts in their organs, including skeletal muscles, and thus have importance in food-borne transmission to humans who consume their meat. Only insufficient epidemiological information is available on the prevalence of *T. gondii* in domestic livestock in some African countries including South Africa. The limited studies carried out to investigate the greatness of *T. gondii* infection in animals in Ethiopia demonstrated high prevalence extending from 22.9%-56% in ovine and 11.6%-82% in goats (Bekele and Kasali 1989; Decononck et al. 1996). Avezza et al. (1993) and El Ridi et al. (1990) documented that tissue cysts are hardly found in beef or buffalo meat, even though antibodies have been indicated up to 92% of cattle and up to 20% of buffaloes as indication of past exposure to the parasite.

1.1.2.4.5. *Hepatozoon*

Hepatozoon canis and *H. americanum* are the two canine pathogens which are hepatozoid apicomplexan protozoan parasites of white blood cells and parenchymal tissues, and they have been reported in many countries across the world (Craig 1990; Baneth 2001). They belong to a diverse group of parasites that consist of more than 300 *Hepatozoon* spp., of which 46 have been described in mammals (Smith 1996). The genus *Hepatozoon* was previously categorized as a member of family Haemogregarinidae, but it now belongs to the family Hepatozoidae of the suborder Adeleorina. This change of families was based on a number of considerations such as host specificity, morphometric and morphological facts, and most recently upon molecular characterization (Barta 1989; Smith 1996; Matthew et al. 2000). According to Barta (1989) and Matthew et al. (2000), a large portion of the data used to reconstruct the evolutionary history of *Hepatozoon* species proposes that they are representative of a paraphyletic genus when associated to other protozoan species. The *H. canis* originated in India as a causative agent of canine hepatozoonosis (James 1905). The host range of vertebrates and invertebrates, and the life cycles in definitive hosts have been studied over the years (Wenyon 1926; Barta 1989). Out of 46 species associated with hepatozoonosis in vertebrates, 2 species are

known to cause disease in dogs. *Hepatozoon .canis* is the most commonly found associated with hepatozoonosis in dogs and carnivores from Europe, Asia and Africa (Barta 1989).

1.1.2.4.3.1. Transmission and clinical signs of *Hepatozoon* infected host

Canine hepatozoonosis is a tick-transmitted disease caused by *H. canis* and *H. americanum* (Baneth et al 2003). The parasite is transmitted by different species of ticks depending on their geographical distribution. In Africa the parasite is known to be transmitted by the brown dog tick (*Rhipicephalus sanguineus*), although other species may also transmit the parasite, and transmitted by *Amblyomma maculatum* in the Southern USA. (Vicent-Johnso et al. 1997; Baneth et al. 2003). Unlike other tick transmitted diseases, ingestion of infected tick by dogs is the main route of transmission rather than the tick biting, however alternative routes have been reported (Ewing and Panciera 2003). Like other coccidia, *Toxoplasma gondii* and *Neospora caninum*, a vertical transmission from the mother to the offspring was observed in *Hepatozoon canis* (Murata et al. 1993). Direct transmission from infected rodents to dogs is at present being investigated. Experimental infections were not effective with parenteral inoculation of tissues or blood from infected canines, however could be accomplished with inoculation of tick tissue emulsion. Conceicao-Silva et al. (1988) documented that there are no feeding studies assessing the infectivity of *H. canis* cysts. Additional ingestion of sporocyst-containing oocysts inside ticks and cystozoites in muscle tissue of rats, transplacental transfer of *H. canis* has been accounted for in Japanese dogs. McCully et al. (1975) considered *H. canis* as a parasite that does not cause any damage to its host. The gametocytes seen were believed to be related to presentation of clinical signs in dogs attributed to concurrent with other pathogens such as *Babesia* and *Ehrlichia* (Ogunkoya et al. 1981; Harmelin et al. 1992). On the other hand, a number of dogs exhibited high parasitemia and severe illness associated with fever, anorexia, weight loss, anaemia, ocular discharge, weakness of the hind limbs and signs of chronic debilitating disease (Mundim et al. 1994; Gondim et al. 1998). Dissimilarly, *H. americanum* infection is thought to be a fatal disease associated with generalised pain, muscle atrophy, weakness and osteopoliferation lessions (Baneth et al. 2003).

1.1.2.4.3.2. Life cycle of *Hepatozoon* species

All species of *Hepatozoon* share a basic life cycle that incorporates sexual development and sporogony in a blood sucking invertebrate definitive host, and merogony accompanied by gamontogony in a vertebrate intermediate host (Figure 1.6) (Ewing and Panciera 2003). Haematophagous insects such as lice, leeches and reduviid bugs are considered to be the definitive hosts of *Hepatozoon* species. The gamont stage is found in the white blood cells of the vertebrate host. following the ingestion of oocysts by the vertebrate host, sporocysts are freed, releasing sporozoites. It is assumed that the sporozoites cross the gut wall and are carried through the lymphatic system or the circulation system to tissues all through the body (Ewing and Panciera 2003). Parasitized host cells have been shown lodged between myofibres in a variety of skeletal muscles not long after trial exposure to infective oocysts (Cummings 2001). The trophozoite found in macrophage-like cells (predominantly in striated muscle) clearly changes the host cell into a mucopolysaccharide-creating substance, bringing about the alleged "onion skin cysts". This appears to shield the parasite from the canine's immune system until the point that merogony is completed and the cystic structure is ruptured (Ewing and Panciera 2003). Mature meronts release merozoites, causing local irritation with a related systemic response and overt illness. Profoundly vascular granulomas develop with parasites in macrophages, where apparently gamogony commences (Panciera et al. 1998). Parasites enter leukocytes, which in this manner circle in the bloodstream as gamonts and might be consumed by haematophagous ixodid ticks (Panciera et al. 1998).

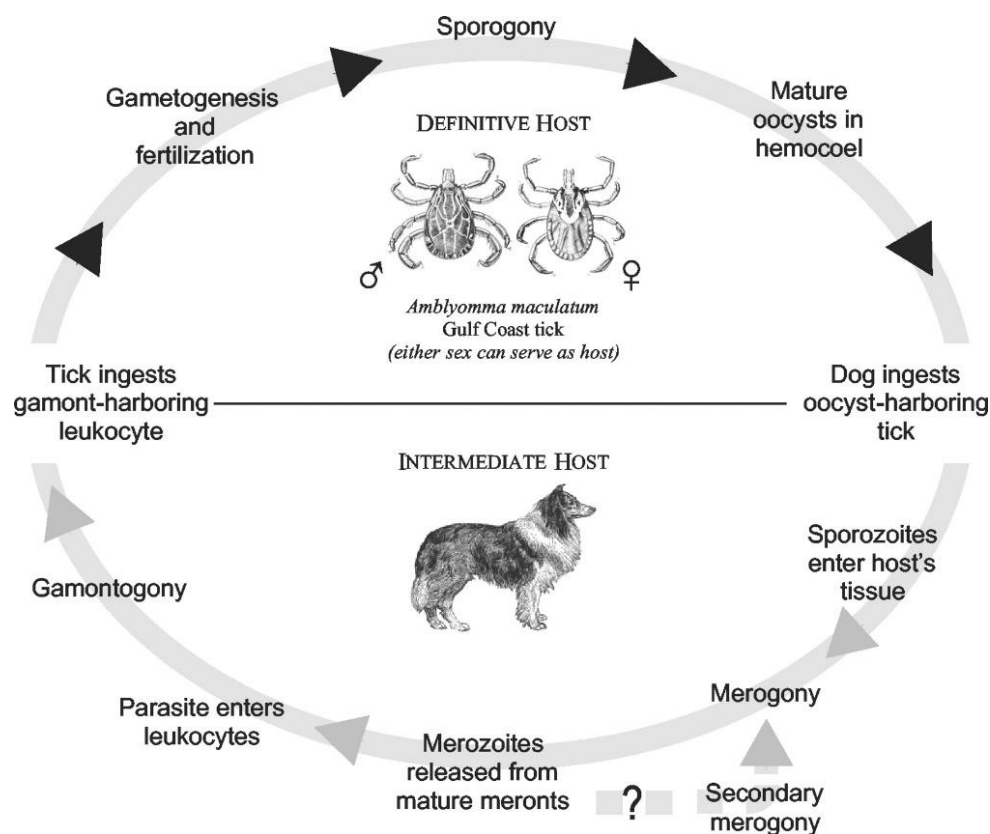


Figure 1.6: Life cycle of *Hepatozoon* (Ewing and Panciera 2003).

1.1.2.4.3.3. Epidemiology of hepatozoonosis

Infection associated with *Hepatozoon canis* between dogs is widespread. Its distribution is synchronized with that of its vector tick *Rhipicephalus sanguineus* in many continents including Africa and South America (Craig 1990; Gevrey 1993; Baneth 2006). This tick vector is known to be adaptable to different environmental conditions and found in warm and temperate regions. Hepatozoonosis is demonstrated as an enzootic infection with variable prevalence. The preliminary investigations on the seroprevalence of *H. canis* in canines demonstrated a variety of percentages in different countries, with 36% in Portugal, 17.6% in Nigeria and 2.3% in Israel (Gevrey 1993). On account of its much of the time asymptomatic course, the assurance of the extensity and intensity of *H. canis* infection require precise, immediate and indirect investigations of this parasite in canines. The occurrence of *H. canis* infection in the rural areas is also extensively varying. Circulating *H. canis* gamonts in blood have been identified in 39% of puppies in the rural areas of Rio de Janeiro state in Brazil, in 22% of puppies in Zaria, Nigeria and in 1.2% of

puppies in Malaysia (Ezekolli et al. 1983; O'Dwyer et al. 2001). Most cases of *H. canis* infections are detected in hot seasons when the movement of the vector is higher. Be that as it may, illnesses are seen during the winter months as well, most likely because of persisting infections (Baneth and Weigler 1997). The capability of *H. canis* to infect likewise other species is extensive and these are potentially species genetically close to domestic dogs. This could be confirmed by experimental transmissions or, then again genetic typization and comparison of acquired isolates (Ezekolli et al. 1983). Current assumptions on the epidemiology of *H. americanum* suggests that Coyotes (*Canis latrans*) have been accounted for to be naturally infected with *H. americanum*, and they might be significant components of the emerging problem in domestic dogs (Davis et al. 1978, Marcer et al. 1988; Kocan et al. 1999). It stays to be decided if both the coyotes and domestic dogs are just being inserted into an already existing enzootic cycle involving Gulf Coast ticks and a vertebrate host such rodents (Kocan et al.1999).

1.1.3. Diagnosis of protozoan parasites and Ehrlichia

The diagnosis of protozoan parasites and *Ehrlichia* developed from the use of light microscopy and the indirect immuno-fluorescent antibody tests (IFATs) to different kinds of molecular based tests which involves the amplification of genes using set of primers and a slot blotting hybridization technique to real time (qPCR) (Collins et al. 2002). IFATs have been widely used in mass screening of animals for the diagnosis of haemoparasites, but a number of constraints about its sensitivity and specificity have limited their everyday use (Terkawi et al. 2011). According to Böse et al. 1990, enzyme-linked immunosorbent assay (ELISA) has been used as an alternative test which proved to offer greater sensitivity and capacity in testing more sera than any other current serological test. Even though light microscopy was widely used for epidemiological surveys of blood parasites, its limitation was that it could not detect carrier animals upon smear examination and could also not differentiate between blood parasites that are morphologically similar (Lawrence 1979). This was therefore improved by the use of PCR detection assays and other molecular techniques that targeted different molecular markers (Bishop et al. 1992).

1.1.3.2. Polymerase chain reaction (PCR)

PCR methods of generates millions of copies of DNA from a small source material that could be either old or of poor quality very quick and fast, and was first developed by Kary

B. Mullis in the 1980s (Kaltenboeck & Wang 2005). The sensitivity and specificity of the technique makes it easy for scientists to detect micro-organisms that are difficult to culture or to confirm their presence by analysis of their products (Kaltenboeck & Wang 2005). When coming to detect agents of infection, convectional PCR has become the most popular choice as a diagnostic tool. Conventional PCR techniques basically specify the presence or absence of parasite DNA when compared to the quantitative PCR methods which are able to give an indication of the level of parasite load (Desquesnes and Dávila 2002; Eisler et al. 2004). Even though this has been the preferred method of diagnosis so far, drawbacks such as the risk of post-amplification contamination are highly possible. However, PCR is highly recommended in various research centres for diagnosis of infectious diseases (Harmon et al. 2007).

The use of quantitative PCR techniques has exhibited to be of potential value for a number of parasitic infections in domesticated animals. Fluorogenic probes that release fluorescent signals during amplification are used when analysing genomes with this technique (Sharma et al. 2012). Due to the fact that there is no need for gel electrophoresis to visualize the PCR products, this makes qPCR convenient, more rapid and advantageous than convectional PCR (Salih et al. 2014). This technique is simple, fast and automatized amplification system responsible for reducing the risk of cross contamination; it has been used to detect various parasites including trypanosomes in many countries across the world (Gasser 2006; Sharma et al. 2012).

Other approaches to investigate the molecular variation between various haemoparasites include techniques such as; restriction enzyme fragment length polymorphism (RFLPs), randomly amplified polymorphic DNAs (RAPD), and loop mediated isothermal amplification (LAMP) (Eisler et al. 2004; Adams et al. 2010). Eisler et al. (2004) documented that these techniques may be effective for characterization of haemoparasites even though they have not been applied largely in the detection of parasites because they usually require large quantities of purified parasite DNA.

1.1.3.3. Loop mediated isothermal amplification (LAMP)

Lamp is an effective strategy for gene amplification which relies on the auto-cycling strand displacement synthesis of target DNA by *Bst* DNA polymerase under isothermal conditions (Notomi et al. 2000). The use of additional primers has helped in improving

the technique as it increased its efficiency and rapidity (Nagamine et al. 2002). Through the addition of fluorescent dyes such as SYBR Green, this technique allows visual detection of amplified products and measurement of turbidity (Mori et al. 2001; Poon et al. 2006). In contrast to PCR, LAMP is usually performed at a temperature range of 60 to 65°C which eliminates the need of a thermal cycler. In addition, the reaction can be carried out without the need of DNA extraction. The method has been successfully developed for detecting quite a lot of haemoparasites (Salih et al. 2008; Liu et al. 2008; Muller et al. 2010; Thekisoe et al. 2010).

1.1.4. Genotyping and population dynamics of haemoparasites

Protozoan parasites are thought to have developed genetic variation to survive the immunologically unfavourable conditions of their hosts (Sivakumar et al. 2012). According to Deitsch et al. (2009), genetic variation frequently result in antigenic variation in parasites, in this manner enabling them to get away from the immune response of their hosts. The advanced mechanisms of genetic and epigenetic variation, like the cases of VESA1 and variable surface glycoprotein (VSG) gene families of *B. bovis* and *Trypanosoma brucei*, respectively, are not frequent in *Theileria* species (Hoeijmakers et al. 1980). Be that as it may, significant genetic variation is detected among parasite field isolates, and these differences are considered to result predominantly from recombination in the tick vectors. *Theileria* parasites use a number of mechanisms to create genetically diverse populations, and recombination can be considered a primary underlying mechanism. According to Callow (1967) and Curnow (1973), *Babesia bigemina* is a heterogeneous parasite and challenges with its isolates shown immunological difference between them, and antigenic differences between the isolates were established by testing homologous antisera with a slide agglutination test. Genetic polymorphism between *B. bigemina* was revealed by the use of various PCR techniques when comparing five geographically distinct isolates from Brazil (Madruga et al. 2002). Fisher et al. (2001) and Suarez et al. (2003) analysed various genetic loci of *B. bigemina* such as the rhoptry associated protein 1-c gene and the merozoite surface antigen gp45 gene. Amplification and sequencing the amplicons of these surface proteins could discriminate different isolates. Vogl (2004) studied the highly polymorphic internal transcribed spacers (ITS) of the rRNA genes, and a dendrogram based on these sequences has shown that *B. bigemina* isolates form clusters which correlates with their geographical origin.

Molecular analysis based on the ITS1, large and small subunit of the nuclear ribosomal DNA have indicated that *Besnoitia* spp are monophyletic and forms a sister group with closely related clade such as genera *Neospora*, *Hammondia* and *Toxoplasma* (Dubey et al. 2004, 2005). Dubey and Lindsay (2003) did not find any significant difference when comparing the morphological description of *Besnoitia* bradyzoites between *B. besnoiti* and *B. caprae*. According to Ellis et al. (2000), the complete identity of ITS-1 supports the fact that *B. besnoiti* and *B. caprae* are a single species. Be that as it may, Njenga et al. (1993) pointed out that, biological properties of *B. caprae* are not similar to those of *B. besnoiti*. No infection occurs in rodents and cattle with *B. caprae* contrary to *B. besnoiti*. For this reason, further studies should be conducted in order to conclude whether these are two different species or two separate populations (Ellis et al. 2000). Two isolates of *B. besnoiti* from cyst from a bull (*Bos taurus*) in Israel and from a blue wildebeest in South Africa were used by Blancq et al. (1986) and they observed distinct strains of eight enzymes by electrophoresis. Bigalke et al. (1967) suggested that this differentiation in strains could be because of host species and geographical variation. It was documented by Sibley et al. (2009) that recombinant and clonal isolates of *T. gondii* exist due to its sexual and asexual replication. New studies from remote geographical region has shown that there is a great genetic variability than previous reports of *T. gondii* isolates obtained from animals and humans (Ajzenberg et al. 2004; Lehmann et al. 2004). Generally it was believed that *T. gondii* had a clonal population structure with three predominant lineages such as type I, II, III which were originated from a range of human and animal sources in Europe and North America (Dubey et al. 2011). Differences within the type of strains are very scarce, which suggest that these three lineage types have undergone clonal expansion (Su et al. 2003). In Africa, Mercier et al. (2010) described a clonal population structure which consists of additional common clonal lineages known as the Africa 1-3 haplogroups, these lineages are thought to coexist with type II and III. In Ethiopia, four ToxoDB (#1, #2, #3 and #20) genotypes of *T. gondii* were identified from feral cats.

Melville et al. (2004) documented that African trypanosomes consist of two genomes with the first one being situated within the nucleus while the other one is enclosed within the kinetoplast. Nuclear DNA bears genes coding for ribosomal RNA and ribosomal DNA citron genes which occur in multiple copies in cycle arrays (Desquesnes and Davila 2002). It was specified by Desquesnes and Davila (2002) that these genes are made up of transcriptional units (TU) and separated by non-transcribed spacers (NTS). The TU is

made up of 18S ribosomal subunit, internal transcribed spacer 1 (ITS-1), 5.8S ribosomal subunit and ITS-2, 28S ribosomal subunit. Successful detection and identification of trypanosomes have been observed with molecular evaluation of the genomic or mitochondrial DNA by the use of RFLP and PCR-RAPD or the utilization of microsatellite and mini-satellite DNA probes (Agbo et al. 2001).

This study sought to detect various protozoan and ehrlichial infections from domestic animals in KwaZulu-Natal province. Furthermore, genetic diversity of these haemoparasites was resolved by phylogenetic analysis.

CHAPTER 2

JUSTIFICATION OF THE STUDY

It is believed that, in South Africa, around 18% of livestock mortalities are attributed to protozoan diseases (Mtshali and Mtshali 2013). These disease have major impact on the economy and also have negative impact on the communities that depends on domestic animals for income, fod and transport. As such it is reported that the occurrence of protozoan parasites in South African domestic animals hinders the development of livestock sector, which contributes about 49% of agricultural yield (Terkawi et al. 2011). Furthermore, the expansion of the human population and growing interest in animal products has pointed out the need to enhance the protective status of domesticated animals. KwaZulu-Natal is of the primary regions farming domesticated animals in South Africa with extensive herds of beef and dairy cattle, and mutton sheep. Goats are generally restricted to communal farming regions and do not form a major part of formal commercial livestock production in the region, despite the fact that their numbers are increasing. Domestic dogs are used in farming as herdsman assistants, as guides for the blind, by law enforcement agencies, hunting and personal security (Abdel-Rhman et al. 2015). Their population as family pets in developing countries is rapidly growing. Most of these countries are located near the equator where the climatic conditions are suitable for the spread of ectoparasites responsible for the transmission of protozoan parasites and *Ehrlichia* (Irwin 2014).

Protozoan infections causes medically and economically important diseases which are constraints to livestock production in developing countries including South Africa (Uilenberg 1995). They are responsible for the high morbidity and mortality in decreased production of meat, milk and other livestock by-products. These parasites are also important constraints on the genetic improvement of indigenous breeds of domestic animals since they prevent the introduction of more productive exotic breeds (Simuunza et al. 2011). The most important vector-borne diseases in sub-Saharan Africa include theileriosis, babesiosis, trypanosomiasis, hepatozoonosis, toxoplasmosis and besnoitiosis. However, despite the considerable economic loss associated with these

haemoparasitic diseases, information on their epidemiology in South Africa is inadequate. It is therefore important for conservation purposes to know the abundance, epidemiology and pathogenicity of parasites infecting domestic animals. Because of the fact that many protozoan parasites, especially piroplasms were isolated from healthy animals, it is presently believed that wild animals - similar to cattle - live in epidemic stability with piroplasms (De Vos *et al.* 2005). It is not clear whether protozoan parasites detected in wildlife are species specific or whether just a few species of parasites can infect a variety of game species. Kabuusu *et al.* (2013) documented that wild ungulates such as the Cape buffalo may act as reservoir host for some parasites. This implies that a “wildlife-livestock interface” may alter the epidemiology of parasite transmission. It is unfortunate that limited access to veterinary diagnostics and molecular information hinders the ability to assess the impact of sylvatic infections on domesticated animals.

A little while back, molecular techniques such as PCR and sequence analysis of four DNA nucleotides (adenine, thiamine, guanine and cytosine) have been used for epidemiological studies and diagnosing haemoparasite infections (Baneth *et al.* 2000). This was because of the fact that they have high sensitivities and specificities for detecting the target pathogens in peripheral blood. The use of these molecular methods allows the detection of haemoparasites to be easily performed at the subspecies level. These methods evolved from DNA probing, combination of DNA probing and PCR, and currently to PCR alone. Accurate detection of haemoparasites in the host blood relies heavily on techniques such as PCR which showed to be effective in characterizing haemoparasites, typing new haemoparasites, in collecting epidemiological data and in the treatment of the animals (Masiga *et al.* 1992).

Therefore, the current study is aimed at improving the current knowledge on the epidemiological status of haemoparasites in the north eastern KwaZulu-Natal province of South Africa. Furthermore, this study is aimed at assessing the genetic diversity and phylogenetic position of South African protozoan parasites and *Ehrlichia* infecting domesticated animals in the study area. This will further assist in understanding the phylogeny of the parasites found in domestic animals in KwaZulu-Natal Province. As a result, this will increase the information on the relatedness of the protozoan parasites found in the study areas as well as other affected countries in Africa and across the world.

2.1. AIM

- To conduct molecular characterization of protozoan parasites and *Ehrlichia* infecting domestic animals (Cattle, sheep, goats and dogs) in north-eastern KwaZulu-Natal.

2.2. Objectives

- To determine the occurrence and genetic diversity of bovine babesiosis, theileriosis and besnoitiosis caused *Babesia bovis*, *Babesia bigemina*, *Theileria parva* and *Besnoitia besnoiti* respectively from cattle in uMkhanyakude district of KwaZulu-Natal Province, South Africa by the use of PCR techniques.
- To determine the occurrence and genetic diversity of bovine trypanosomiasis caused by *Trypanosoma vivax*, *T. congolense* and *T. brucei* from cattle in uMkhanyakude district using PCR techniques.
- To determine the occurrence and genetic diversity of canine babesiosis, ehrlichiosis and hepatozoonosis caused by *Babesia rossi*, *B. vogelli*, *E. canis* and *Hepatozoon canis* respectively in dogs from uMkhanyakude district using PCR techniques.
- To determine the occurrence of ovine babesiosis and theileriosis caused by *Babesia ovis*, *Babesia motasi* and *Theileria ovis* respectively from sheep and goats in uMkhanyakude district by the use of PCR techniques.
- To determine occurrence and genetic diversity of toxoplasmosis caused by *Toxoplasma gondii* from domestic animals (Cattle, Sheep, Goats and Dogs) in uMkhanyakude district of KwaZulu-Natal Province, South Africa using semi-nested PCR.

2.3. Hypotheses

- There are various protozoan parasites infecting domestic animals in uMkhanyakude district.
- There is a high genetic diversity among protozoan parasites and *Ehrlichia* infecting domestic animals in uMkhanyakude district.

2.4. Outline of the dissertation

Chapter 1- Introduction and literature review:

The background of protozoan parasites and *Ehrlichia*, their classifications, life cycles, transmission and clinical signs, and various diagnostic techniques are well described in this chapter.

Chapter 2- Statement of the problem:

This chapter consists of the justification of the study, aim, objectives and the hypothesis.

Chapter 3- Materials and methods:

This chapter entails detailed information of the study area, methodology and how data will be analysed.

Chapter 4- Results:

This chapter includes a full presentation of the data obtained in the study.

Chapter 5- Discussion, conclusion and recommendations:

The obtained data is interpreted with conclusions indicating whether the aim and objectives of the study are achieved or not. Recommendations include suggestions and studies that need to be conducted with reference to the data obtained.

CHAPTER 3

MATERIALS AND METHODS

3.1. Study area

The present study was carried out in three municipalities, namely, Mtubatuba, Big 5 hlabisa, and Umhlabuyalingana of the uMkhanyakude district (28°01'25"89 S, 32°17'30"30 E), KwaZulu-Natal province, South Africa (Figure 3.1). The district is situated in the North Eastern part of KwaZulu-Natal Province with altitude ranging between 450 - 900 m, sharing a boundary with Mozambique in the north, Swaziland in the north-west and the Indian Ocean in the east. The district covers the total land area of approximately 13,859 km² and it was named after the uMkhanyakude Tree (*Acacia xanthophyllous*, Fever tree, which translated to English means "the light in the distance"). Dominating structural vegetation types include dense bushveld thickets and the dominant tree genera are *Afzelia*, *Balanites*, *Combretum*, *Ficus* and *Pseudobersama*, which form canopies that ranges between 5 to 20 meters giving heavy shade, and there is little undergrowth (Pooley 1993; Mucina and Rutherford 2006). Sour grassland is the second dominating vegetation type with *Heteropogon*, *Perotis*, *Setaria* and *Tragus* as dominant grass genera (Esterhuizen et al. 2005; Mucina and Rutherford 2006). According to Esterhuizen (2005), wooded grassland is the third vegetation type and consists of plantations of exotic *Eucalyptus* and *Pinus* trees that are mainly used for wood and paper. The district generally has frequent rains in the summer than in winter with mean annual precipitation ranging between 600 - 1 050 mm (Mucina and Rutherford 2006). Its weather conditions are characterized by hot summers and some frost during the winter months, and lastly it has well-drained as well as shallow soil types (Pooley 1993). However, due to drainage of wetlands for housing and agriculture, competition from invasive alien plants, deforestation as well as commercial farming, conservation in the uMkhanyakude district is crucial (Pooley 1993). According to Statistics South Africa Census (2001), the population of the district consist of 45.2% males and 54.8% females. The major ethnic group in the district is the Zulu people. Most of the area is rural, with the majority of people depending on subsistence agriculture and animal husbandry as sources of income.

protozoan infections such as African animal trypanosomiasis and apicomplexans are one of the leading causes of animal morbidity and mortality in the area. The majority of the people in the area are too poor to afford commercial insect repellents and they use plant materials as repellents against vector bites.

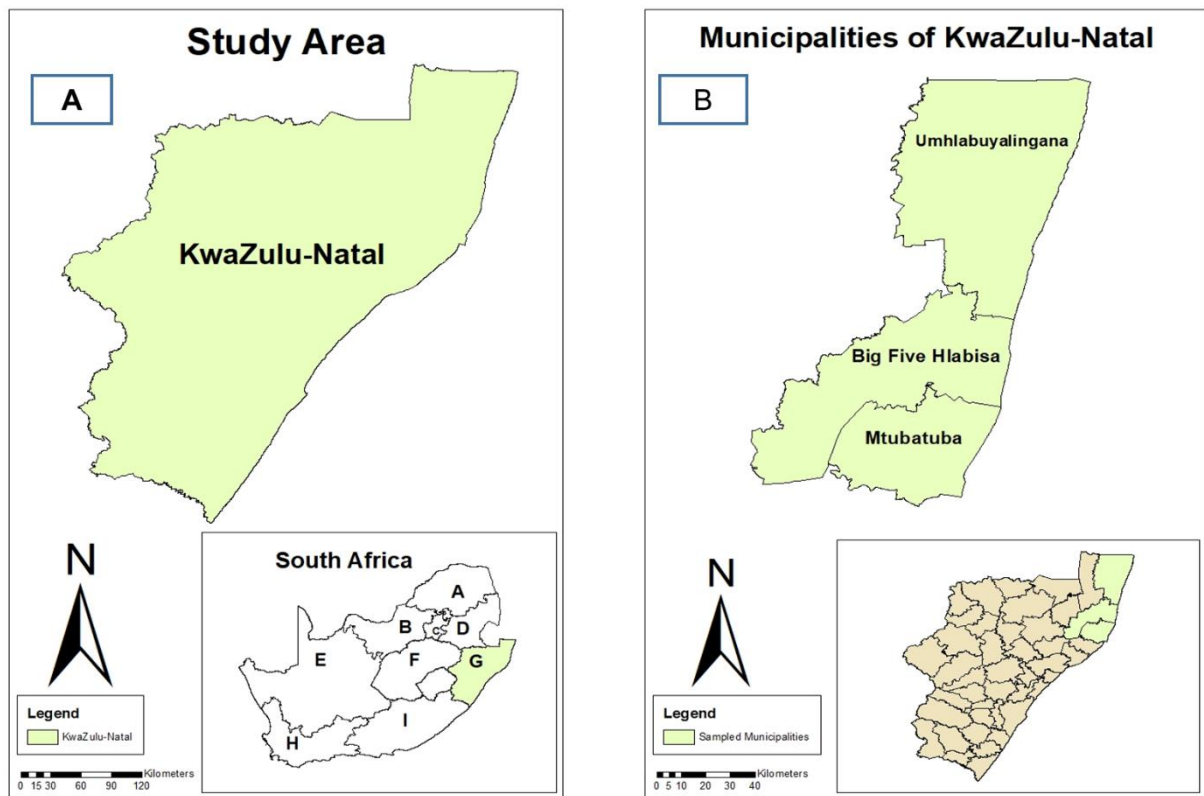


Figure 3.1: Map showing the sampled area. A) KwaZulu-Natal Province. (B) Umkhanyakude district with its local municipalities

3.2. . Sample collection

In the sampling areas, rural communal farming is predominately practised. This is not for commercial purpose, but for family utilization and indication of wealth. The owners of the sample animals did not have any information about the age of the animals, and the breed of goats and sheep. The cattle breed is Nguni. Blood was collected from the jugular or coccygeal vein of live animals into EDTA vacutainer tubes (BD vacutainer system, Oxford, U.K) with 18 gauge needles. The blood samples were then packed in a cooler bag with ice packs at the field before they were sent to the laboratory. A total of 208 blood samples were obtained from cattle (n=109), sheep (n=10), goats (n=40) and dogs (49)

respectively. Sheep are not desired as domestic animals in the province, thus, only few were available during the sampling period.

3.3. . Experimental procedures

3.3.2. DNA Extraction: Salting out method (Nasiri et al. 2005)

A volume of 50 µl of blood was transferred into 1.5 ml Eppendorf tubes. Thereafter 410 µl extraction buffer that contains 10 mM Tris-HCl [pH 8.0], 10 mM EDTA and 1% sodium dodecyl sulphate (SDS). Then 80 µl of 10% SDS was added, followed by 10 µl of proteinase K (Pro-K) and the contents were mixed by finger vortex and then incubated at 50°C - 60°C for an hour for DNA lysis and digestion. After one hour, additional 10 µl of proteinase K was added and the samples were incubated again at 55°C and left overnight for completion of DNA digestion.

On the second day, DNA was extracted as follows: The samples were centrifuged for 5 minutes at 12 000 rpm. By the use of micropipette, 600 µl of the supernatant was transferred to the second set of 1.5 ml Eppendorf reaction tubes and 180 µl of 5 M NaCl was added to the supernatant. The tubes were vortexed for 30 seconds and centrifuged at 13 500 rpm for 5 minutes. The supernatant was transferred to the third 1.5 ml Eppendorf tube where 420 µl of ice-cold isopropanol (Propan-2-ol) was added to the supernatant. The mixture in the tube was mixed by inverting the tube 50 times, followed by centrifugation at full speed (14 000 rpm) for 5 minutes at 4°C for the precipitation of the DNA. Subsequent to centrifugation, the supernatant was discarded and the pellet was washed twice by addition of 250 µl of 75% ethanol. The tubes were vortexed for 30 seconds, centrifuged at full speed for 5 minutes and the supernatant was discarded. The samples were left open for an hour to air dry at a room temperature to evaporate the 75% ethanol. Finally, the DNA was dissolved in 200 µl of double distilled water and incubated at 37°C for 30 minutes. The presence of DNA was confirmed by gel electrophoresis using 1% agarose gel stained with 1 µl ethidium bromide and was visualized under UV light before storage at -20°C for later use.

3.3.3. PCR detection of protozoan parasites and *Ehrlichia* DNA from blood samples

For the detection of haemoparasites in the blood of domestic animals and the determination of their phylogenetic position in comparison with other related taxa, conventional PCR and nested PCR assays were conducted using both genus and species specific primers.

3.3.3.1. Amplification of *Besnoitia besnoiti* DNA

Species-specific Primers Bes-F (5'- ATT CGG ACC GTT TTG TGG-3') and Bes-R (5'- CCT CTC GAG GCT ACA AGT CG-3') that amplifies a 1065 bp of the ITS 1 gene fragment were used as described by Namazi et al. (2010). The genomic DNA of *Besnoitia besnoiti* obtained from PCR positive DNA of tabanid fly by Taioe (2017) was used as positive control, and double distilled water (ddH₂O) as negative control. For PCR assay, 2 µl of the extracted genomic DNA was added into a 25 µl reaction mixture containing 2.5 µl of 10X standard *Taq* Reaction Buffer, 0.5 µl of forward and reverse 10 µM primer, 0.5 µl of 10 mM dNTPs, 0.125 µl of *Taq* DNA polymerase and double distilled water to adjust the final volume to 25 µl. The primary reactions were run in proFlex thermocycler (Applied Biosystems, USA) employing the following thermocycling conditions: initial denaturation at 95°C for 30 seconds, followed by 35 cycles of denaturation at 95°C for 30 seconds. This was followed by annealing at 60°C for 1 minute, extension at 68°C for 1 minute and final extension at 68°C for 5 minutes (Namazi et al. 2010). Following the amplification, 5 µl amplicon was resolved by electrophoresis using 1% agarose gel stained with 10 µl ethidium bromide and visualized under UV light.

3.3.3.2. Amplification of *Toxoplasma gondii* DNA

A nested PCR targeting the *T. gondii* B1 gene was performed to detect possible infection with *T. gondii* (Burg et al. 1989). Two sets of primers that amplified a 194 bp fragment were used for screening of *T. gondii*. The primer set F1 (5'-TCT TTA AAG CGT TCG TGG TC-3') and R1 (5'- GGA ACT GCA TCC GTT CAT GAG-3') was used to amplify the first fragment. The second fragment was amplified with the F2 (5'- GGC GAC CAA TCT GCG AAT ACA CC-3') and R2 (5'- TGC ATA GGT TGC AGT CAC TG-3') primer set. The

synthesized genomic DNA of *Toxoplasma gondii* referred to as g-block (White Sci - IDT, Johannesburg) was used as positive control and double distilled water (ddH₂O) as negative control. The PCRs were conducted in 25 µl volumes containing 2.5 µl of 10X standard *Taq* Reaction Buffer, 0.5µl of forward and reverse (10 µM each), 0.5 µl of 10 mM dNTPs, 2 µl of each individual DNA sample, 0.125 µl of *Taq* DNA polymerase and double distilled water to adjust the final volume to 25 µl. The primary reactions were run in proFlex thermocycler (Applied Biosystems, USA) employing the following thermocycling conditions: initial denaturation at 95°C for 30 seconds, followed by 35 cycles of denaturation at 95°C for 30 seconds. Annealing at 63°C and 57°C for 1 minute respectively, extension at 68°C for 1 minute and final extension at 68°C for 5 minutes (Wang et al. 2017). Following the amplification, 5 µl amplicon was resolved by gel electrophoresis using 1% agarose gel stained with 10 µl ethidium bromide and visualized under UV (ultra violet) light.

3.3.3.3. Amplification of *Trypanosoma* DNA

Genus-specific primers KIN 1 (GCG TTC AAA GAT TGG GCA AT) and KIN 2 (CGC CCG AAA GTT CAC C) that are known to anneal to the conserved region of the 18S and ITS1-5.8S rRNA gene regions were used to detect the presence of trypanosome DNA from the blood samples as described by Desquesness et al. (2002). The genomic DNA of *T. congolense* IL3000, *T. b. brucei* GuTat1.3 and *T. theileri* Japan Isolate were used as positive control, while double distilled water (ddH₂O) was used as negative control. PCR was conducted in 25 µl volume containing 2.5 µl of 10X standard *Taq* Reaction Buffer, 0.5 µl of forward and reverse 10 µM primer, 0.5 µl of 10 mM dNTPs, 2 µl of each individual DNA sample, 0.125 µl of *Taq* DNA polymerase and double distilled water to adjust the final volume to 25 µl. The primary reactions were run in proFlex thermocycler (Applied biosystems, USA) according to the following thermocycling conditions: initial denaturation at 95°C for 30 seconds, followed by 35 cycles of denaturation at 95°C for 30 seconds. This was followed by annealing at 58°C for 1 minute, extension at 68°C for 1 minute and final extension at 68°C for 5 minutes (Desquesness et al. 2002). Following the amplification, 5 µl amplicon was resolved by gel electrophoresis using 1% agarose gel stained with 10 µl ethidium bromide and visualized under ultra violet (UV) light.

3.3.3.4. Amplification of Babesia/Hepatozoon/Theileria DNA

The PCR assay for the amplification of *Babesia*, *Hepatozoon* and *Theileria* parasites as described by Ica et al. (2007), was conducted with RLBF (5'- GAC ACA GGG AGG TAG TGA CAA G-3') and RLBR (CTA AGA ATT TCA CCT CTG ACA GT-3' primers targeting 390-430 bp fragmentation of the hyper variable region V4 of the 18S rRNA. The genomic DNA of *Babesia bigemina* SA strain, *B. bovis* SA strain and *Theileria parva* Muguga strain were used as positive control, while double distilled (ddH₂O) water was used as negative control. The PCR was conducted in 25 µl volume containing 2.5 µl of 10X standard *Taq* Reaction Buffer, 0.5 µl of forward and reverse 10 µM primer, 0.5 µl of 10 mM dNTPs, 2 µl of each individual DNA sample, 0.125 µl of *Taq* DNA polymerase and double distilled water to adjust the final volume to 25 µl. The primary reactions were run in proFlex thermocycler (Applied biosystems, USA) according to the following thermocycling conditions: initial denaturation at 95°C for 30 seconds, followed by 35 cycles of denaturation at 95°C for 30 seconds. This was followed by annealing at 58°C for 30 seconds, extension at 68°C for 1 minute and final extension at 68°C for 5 minutes (Ica et al. 2007).

Additional species-specific PCR and nested PCR were performed for *Theileria*, *Hepatozoon* and *Babesia* species.

3.3.3.4.1. Amplification of Theileria DNA

For the amplification of 1101 bp 18S SSU rRNA gene of *T. parva*, PCR was performed using S5F (5'-ATG ACA AAC ACA GAA GTC GCC CT-3') and S5R (5'-ATT TCA TCC TTC TTC TTG ATT GCG T-3') primers which are known to anneal to the conserved regions between *T. annulata* and *T. parva* S5 ribosomal genes (Mans et al. 2011). The genomic DNA of *T. parva* provided by North West University was used as positive control and double distilled water as negative control. Blind PCR was performed with a pair of primer TSsr170F (5'-TCGAGACCTTCGGGT-3') and TSsr 670R (5'-TCCGGACATTGTAAAACAAA-3') that amplified 520 bp fragment of the small subunit ribosomal RNA (ssu rRNA) gene of *T. ovis*. PCR reaction mixtures were run as described above, annealing at 53°C and 60°C for 30 seconds, respectively.

3.3.3.4.2. Amplification of *Hepatozoon canis* DNA

Species-specific primers Hep-F (5'-ATA CAT GAG CAA AAT CTC AAC-3') and Hep-R (5'-CTT ATT CCA TGC TGC AG-3') that are known to amplify an approximately 625 bp partial sequence of the 18S rRNA gene of *Hepatozoon* spp. were used for the detection of *Hepatozoon canis* (Rubuni et al. 2005). Blind PCR reaction mixtures were run as described in 3.3.2.4, annealing at 57°C for 30 seconds.

3.3.3.4.3. Amplification of *Babesia* spp. DNA

PCR and nested PCR assays with group I primers were conducted for the amplification of 298 bp *RAP-1* genes for *B. bovis* and 170bp *Spei-AvaI* for *B. bigemina* restriction fragments. In order to study the phylogenetic relationship between the isolates of *B. bovis* and *B. bigemina*, PCR amplicons were subjected to nested PCR with group II primers targeting a 1009 bp *RAP-1* (*B. bovis*) and 853 bp *gp45* (*B. bigemina*) fragments. The genomic DNA of *B. bovis* SA strain and *B. bigemina* SA strain obtained from North West University was used as positive control, and double distilled water (ddH₂O) as negative control. The PCRs were conducted in 25 µl volumes containing 2.5 µl of 10X standard *Taq* reaction buffer, 0.5µl of forward and reverse 10 µM primer, 0.5 µl of 10 mM dNTPs, 2 µl of each individual DNA sample, 0.125 µl of *Taq* DNA polymerase and double distilled water to adjust the final volume to 25 µl. The primary reactions were cycled in proFlex thermocycler (Applied biosystems, USA) according to the following thermocycling conditions: initial denaturation at 95°C for 30 seconds, followed by 35 cycles of denaturation at 95°C for 30 seconds. This was followed by annealing temperatures (Table 3.1), extension at 68°C for 1 minute and final extension at 68°C for 5 minutes (Mtshali et al. 2013).

For nested PCR, 1 µl of the primary PCR products was added into a second PCR mixture containing the same reagent composition as described above, except that the nested PCR primers were used instead of the external primers. Reaction mixtures were run as described above. Following the amplification, 5 µl amplicon was resolved by gel

electrophoresis using 1% agarose gel stained with 10 µl ethidium bromide and visualized under UV light.

For the detection of *B. ovis* and *B. motasi*, blind PCR was conducted using P1 (5'-CAC AGG GAG GTA GTG ACA AG-3') and P2 (5'-AAG AAT TTC ACC TAT GAC AG-3') primers that amplified 430 bp of the hypervariable region of 18S rRNA gene of piroplasms (Schnittger et al. 2004). To control the specificity of the PCR products from the 18S rRNA, additional species-specific Semi nested PCR was conducted using P3 (5'-GTC TGC GCG CGG CCT TTG CG-3') and P4 (5'-CGC GAT TCC GTT ATT GGA G-3') primers were used. These primers amplified 186 and 205 bp within the hypervariable region of the V4 of 18S rRNA gene of *B. ovis* and *B. motasi* respectively, and they were paired with P2 primer in the secondary reaction. PCR reaction mixtures were thermally cycled as described above, annealing at 58°C for 1 minute (Shayan et al. 2008).

For canine *Babesia*, semi-nested PCR was conducted using an outer primer pair 455-479F (5'-GTCTTGTAATTGGAATGATGGTGAC-3') and 793-772R (5'-ATGCCCCCAACCGTTCCTATTA-3') that is known to amplify an approximately ~ 340bp fragment of hypervariable region of the 18S rRNA gene for *B. canis vogelli*, *B. canis canis* and *B. canis rossi* (Abdel-Rhman et al. 2015). Then, additional species-specific internal primers BCVF (5'-GTTCGAGTTTGCCATTCGTT-3') and BCCF (5'-TGCGTTGACGGTTTGACC-3') were used for *B. canis vogelli* and *B. canis canis* respectively, and they were paired with the outer reverse primer in the semi-nested secondary reaction to amplify 192 and 198 bp amplicons respectively. The PCR reactions were conducted in 25 µl volumes containing 2.5 µl of 10X standard *Taq* reaction buffer, 0.5µl of forward and reverse 10 µM primer, 0.5 µl of 10 mM dNTPs, 2 µl of each individual DNA sample, 0.125 µl of *Taq* DNA polymerase and double distilled water to adjust the final volume to 25 µl. The primary reactions were cycled in proFlex thermocycler (Applied biosystems, USA) according to the following thermocycling conditions: initial denaturation at 95°C for 30 seconds, followed by 35 cycles of denaturation at 95°C for 30 seconds. This was followed by annealing at 58°C for 30 seconds, extension at 68°C for 1 minute and final extension at 68°C for 5 minutes (Abdel-Rhman et al. 2015).

Table 3.1. Sequences of primers used for bovine *Babesia* PCR amplification

Species	Assay	Primer sequence (5'-3')	Annealing (°C)	Product size	Reference
<i>B. bigemina</i>	PCR	CATCTAATTTCTCTCCATACCCCTCC CCTCGGCTTCAACTCTGATGCCAAAG	55	278 bp	Figueroa et al. 1993.
<i>B. bigemina</i>	nPCR	CGCAAGCCCAGCACGCCCCGGTGC CCGACCTGGATAGGCTGTGTGATG	55	170 bp	Figueroa et al. 1993.
<i>B. bovis</i>	PCR	CACGAGGAAGGAACTACCGATGTTGA CCAAGGAGCTTCAACGTACGAGGTCA	55	360 bp	Figueroa et al. 1993.
<i>B. bovis</i>	nPCR	TCAACAAGGTACTCTATATGGCTACC CTACCGAGCAGAACCTTCTTCACCA	55	298 bp	Figueroa et al. 1993.

3.3.4. PCR product purification

Positive PCR product was purified from the agarose gel according to the manufacturer's PCR purification kit (QIAquick Gel Extraction Kit). Briefly, the DNA fragment from the agarose gel was excised with a clean, sharp scalpel removing extra agarose. Three hundred microliter Buffer QG was added to each gel and incubated at 50°C for 10 minutes. During incubation, the tubes were vortexed after every 2 - 3 minutes to help dissolve the gel. After the gel was dissolved completely, 300 µl of isopropanol was added. A QIAquick spin column was placed in a 2 ml collection tube and In order to bind DNA, 800µl of the mixture was transferred to a QIAquick spin column reservoir and centrifuged for 1 minute. The flow-through was discarded and the QIAquick column was placed back in the same collection tube. To remove all traces of agarose, 500 µl of buffer QG was added to QIAquick column and centrifuged for 1 minute. To wash, 750 µl of buffer PE was added to QIAquick column and let stand for 2 - 5 minutes before centrifuging for 1 minute. The flow-through was discarded and the QIAquick column was centrifuged for an additional 1 minute at 13000 rpm. The QIAquick column was placed into a clean 1.5 ml micro centrifuge tube. Finally, 50 µl of buffer EB was added to the center of the QIAquick membrane and centrifuged for 1 minute in order to elute the DNA.

3.3.5. Sequencing of Purified PCR product

Twenty microlitres of all purified haemoparasite positive PCR products were sent for sequencing at Inqaba Biotechnical Industries (Pty) Ltd in Pretoria, South Africa.

3.3.6. Phylogenetic analysis

Generated sequences of PCR products in this study were analysed using BLASTn to confirm their identities. They were aligned with equivalent sequences of protozoan parasites (*Besnoitia* spp., *Babesia* spp., *Hepatozoon* spp., *Theileria* spp., *Toxoplasma gondii* and *Trypanosoma* spp.) and *Ehrlichia canis* using Clustal W algorithm. Sequences that belonged to a specific species were identified based on being the first and closest match in GenBank, with the identity of >80 up to 100% coverage. Phylogenetic trees were

constructed using the Neighbour-joining analysis, by means of MEGA 7 software with a bootstrap of 10000 replications.

3.4. Data analysis

Data was recorded in an Excel sheet to produce descriptive statistics that was employed in the analysis of the data. The infection prevalence rates among protozoan parasites and their localities were expressed as percentages of total number of animals sampled. This was compared using Prism 7 software. The proportions for 95% confidence intervals (95% CI) were computed as CIs for proportions with binomial data employing no continuity correction. The Chi-square (χ^2) test was used to calculate the significant difference of parasitic infections between localities of their occurrence and their hosts. A *P-value* <0.05 was considered statistically significant.

CHAPTER 4

RESULTS

4.1. Overall infection rates

In the present study, the prevalence of different protozoan parasites and *Ehrlichia canis* in four municipalities of KwaZulu-Natal, South Africa is reported. This was analysed by PCR techniques to detect specific infections. Out of 208 blood samples examined, the total of 103 samples were positive for different protozoan parasites. Five genera of parasites were identified in this study-*Theileria*, *Babesia*, *Trypanosoma*, *Toxoplasma gondii* and *Ehrlichia*. *Ehrlichia* had the highest prevalence of 40.8%, *Babesia* (22.39%), *Trypanosoma* (9.6%), *Toxoplasma gondii* (2.4%), and *Theileria* (1.89%). *Ehrlichia* showed a prevalence of (55.6%), (36.3%) and (30%) at uMhlabauyalingana, Mtubatuba, and Big 5 Hlabisa, respectively (Table 4.1). The overall infection rate of protozoan and ehrlichial parasites by PCR in the tested domestic animals was (18.81%), (7.5%), (0.0%) and (13.61%) in cattle, sheep goats and canines, respectively (Table 4.2)

Table 4.1. Overall prevalence of protozoan parasites and *Ehrlichia* from different municipalities

Municipalities	Total samples	<i>Trypanosoma</i>	<i>Babesia</i>	<i>Theileria</i>	<i>Toxoplasma gondii</i>	<i>Ehrlichia</i>
Big 5 Hlabisa	129	12.64%	6.19%	3.23%	5.38%	30%
uMhlabauyalingana	48	12.5%	3.75%	0.0%	0.0%	55.6%
Mtubatuba	31	0.0%	6.25%	0.0%	0.0%	36.3%

Table 4.2. Overall prevalence of protozoan parasites and *Ehrlichia* from different hosts

Host	Total samples	<i>Trypanosoma</i> spp.	<i>B. bovis</i>	<i>B. bigemina</i>	<i>B. ovis</i>	<i>B. motasi</i>	<i>T. ovis</i>	<i>T. gondii</i>	<i>E. canis</i>
Cattle	109	18.35% (±7.45) ^{a)}	30.3% (±8.60)	22.02% (±7.46)	NS	NS	NS	4.58% (±3.92)	NS
Sheep	10	0.0% (±0.0)	NS	NS	0.0% (±0.0)	0.0% (±0.0)	30% (±28.42)	0.0% (±0.0)	NS
Goats	40	0.0% (±0.0)	NS	NS	0.0% (±0.0)	0.0% (±0.0)	0.0% (±0.0)	0.0% (±0.0)	NS
Dogs	49	0.0% (±0.0)	NS	NS	NS	NS	NS	0.0% (±0.0)	40.8% (±13.72)

a) 95% confidence intervals. NS) not screened

4.2. Infection rate of piroplasm

4.2.1. Infection rate based on host

4.2.1.1. Cattle

A total of 109 cattle samples were screened for the presence of *Babesia bovis*, *Babesia bigemina* and *Theileria parva* DNAs. The PCR amplicons that were positive with RLB primers produced single DNA bands of approximately 430 bp for piroplasms 18S rRNA gene (Figure 4.1). The PCR amplicons that were positive with nested PCR primers (Group I) produced single DNA bands of approximately 298 bp and 170 bp for *B. bovis* RAP-1 genes and *B. bigemina* *SpeI-AvaI* restriction fragments, respectively (Figure 4.2 and 4.3). From the PCR detection it was noted that *B. bigemina* outnumbered *B. bovis* by the percentage of infection. The overall infection rate of the three piroplasm parasites in the blood samples tested was 30.30% (95% CI=±8.60), 22.20% (95% CI=±7.45) and 0.0% for *B. bigemina*, *B. bovis* and *T. parva*, respectively.

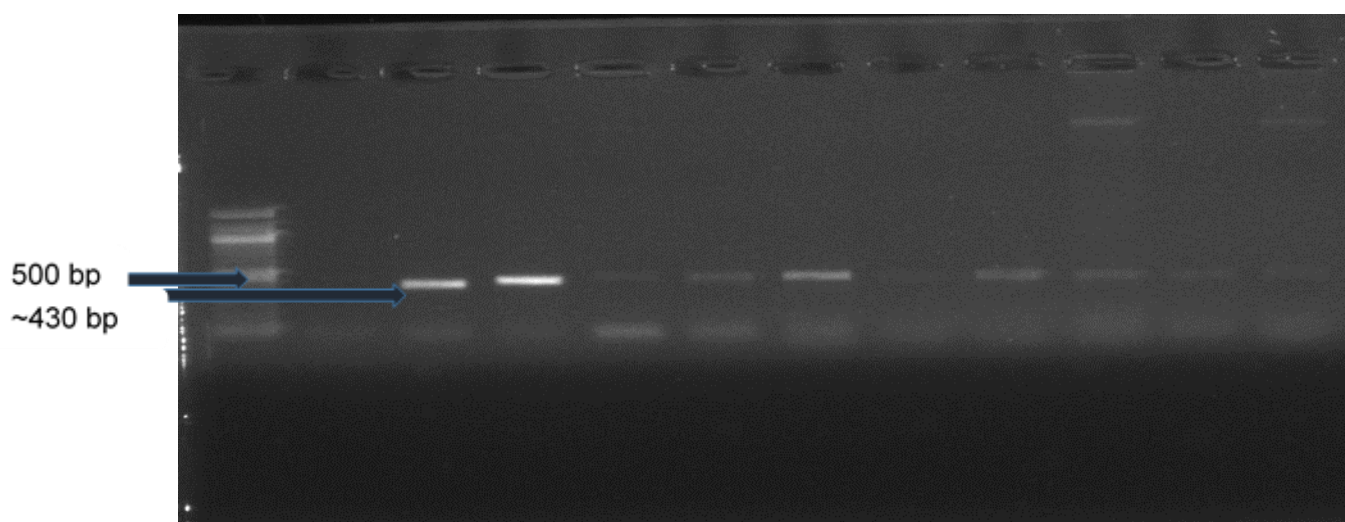


Figure 4.1: PCR amplification of *Theileria/Babesia* genus DNA from cattle using RLB primers. M is the molecular marker, -ve is for the no template negative control, +ve is *B. bigemina* and *T. parva* positive control. Lane 1-8 shows positive piroplasmas samples 430 bp.

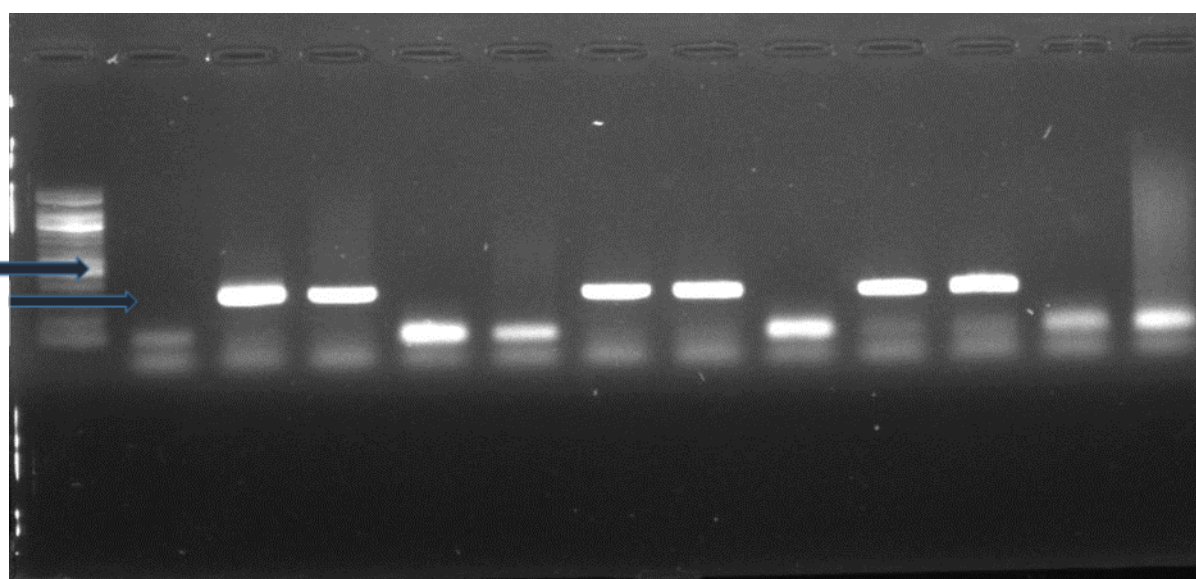


Figure 4.2: PCR amplification of *B. bovis* DNA from cattle using group I primers. M is the molecular marker, -ve is for the no template negative control, +ve is *B. bovis* positive control. Lane 1,4,5,7 & 8 shows positive samples for *B. bovis* at 298 bp.

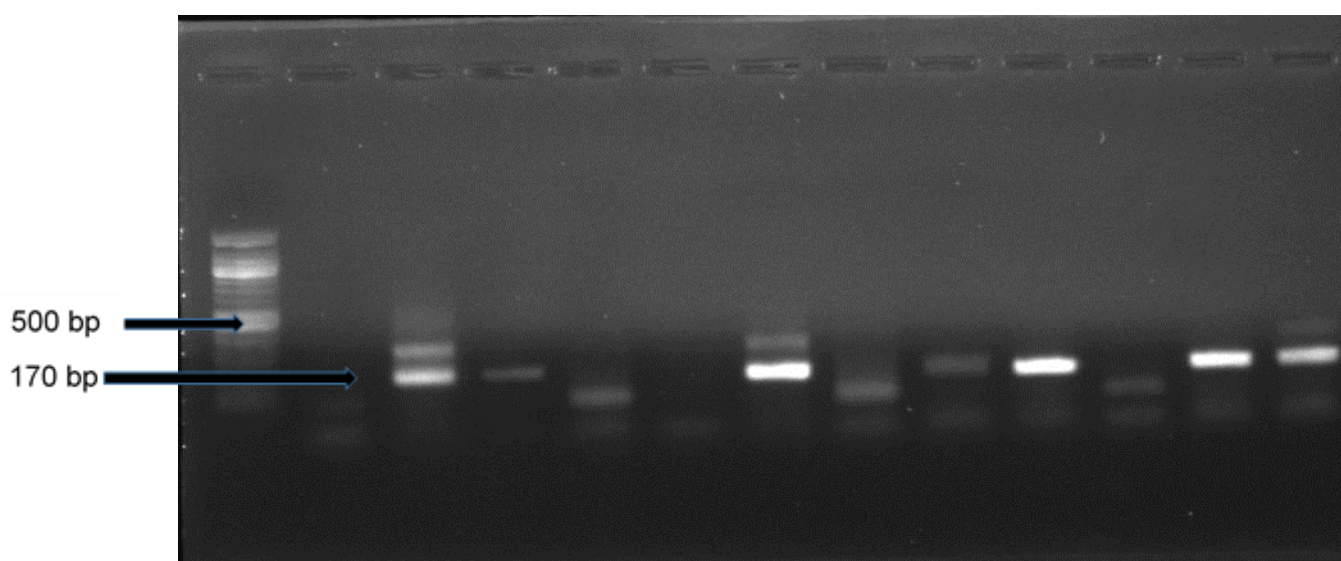


Figure 4.3: PCR amplification of *B. bigemina* DNA from cattle using group I primers. M is the molecular marker, -ve is for the no template negative control, +ve is *B. bigemina* positive control. Lane 1, 4,6,7,9 & 10 shows positive samples for *B. bigemina* at 170 bp.

4.2.1.2. Sheep and Goats

A total of 50 small ruminants (10 sheep and 40 goats) were sampled in this study and all were screened for *Theileria ovis*, *Babesia ovis*, and *Babesia motasi*. The PCR amplicons that were positive with semi nested PCR primers produced single DNA bands of approximately 430 bp for *Theileria/Babesia* 18S rRNA gene (Figure 4.4). PCR detected

T. ovis from DNA of sheep blood only. The infection rate of *T. ovis* was (3/10) 30% from sheep and (0/40) 0% from goats (Table 4.3). No ovine *Babesia* spp. was recorded in the present study.

Table 4.3. Prevalence of ovine piroplasm in sheep and goats

Host	Total number of samples	<i>Theileria ovis</i>		<i>Babesia ovis</i>		<i>Babesia motasi</i>	
		<i>n</i> ^{a)}	Percentage	<i>n</i>	Percentage	<i>n</i>	Percentage
Sheep	10	3	30% (± 28.42) ^{c)}	0	0.0% (± 0.0)	0	0.0% (± 0.0)
Goats	40	0	0.0% (± 0.0)	0	0.0% (± 0.0)	0	0% (± 0.0)

a) *n*=total number of samples positive for *T. ovis*, *B. ovis* and *B. motasi* c) 95% confidence intervals.

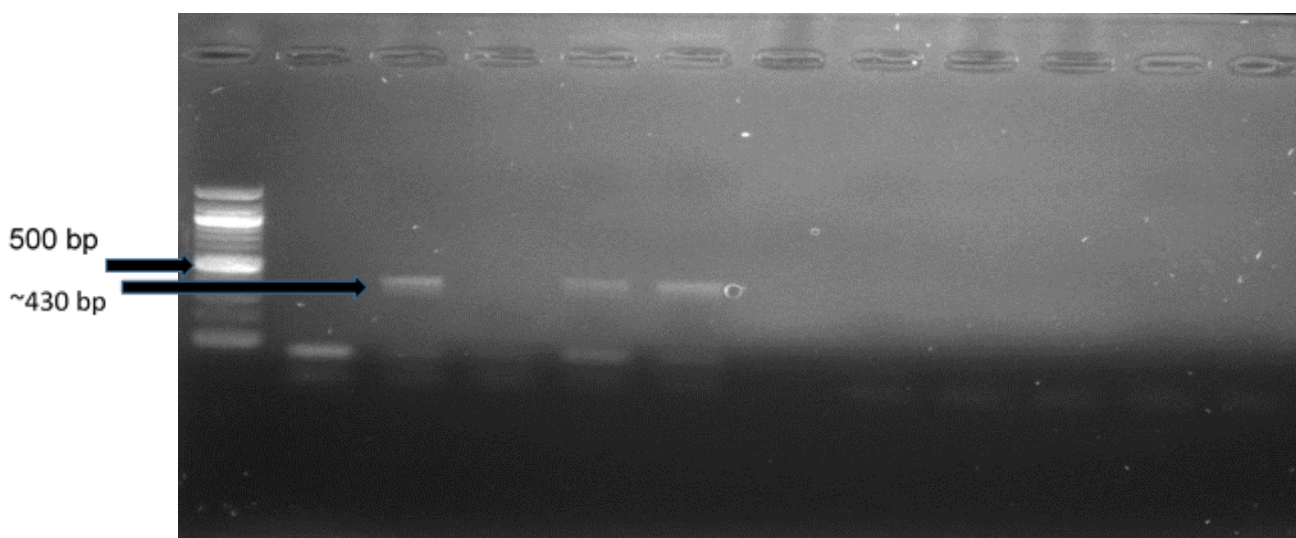


Figure 4.4: PCR amplification of *Theileria/ Babesia* DNA from sheep using P1 and P2 primers. M is the molecular marker, -ve is for the no template negative control. Lane 1, 3 & 4 shows positive samples for *T. ovis* at approximately 430 bp.

4.2.1.3. Dogs

A total of 49 dogs were sampled in the present study and all were screened for *B. vogeli* and *B. rossi*. From the PCR detection results, it was observed that none of the dog

samples was positive for any canine *Babesia*. *Hepatozoon canis* infection was also not detected in domestic dogs.

4.2.2. Infection rates based on the three municipalities

From the PCR detection results, it was observed that cattle infected with piroplasms were distributed across all the three municipalities sampled. The highest occurrence of *B. bigemina* and *B. bovis* was recorded in Big 5 hlabisa municipality in which 34.8% (95% CI=±11.37) and 24.6% (95% CI=±10.19) of animals were infected, respectively. uMhlabaulyalingana municipality had the lowest number (3 out of 20) of cattle infected with both *B. bigemina* and *B. bovis*. The results of PCR amplification are shown in table 4.4. It was also observed that sheep infected with *T. ovis* were from Big 5 hlabisa with the highest occurrence in which 30% (95% CI=±28.42) of animals were infected.

4.2.3. Mixed infections

The mixed infection rate of both *B. bovis* and *B. bigemina* was recorded in four (3.7%) cattle with Big 5 hlabisa having the highest percentage (5.8%) of animals' infected. Among goats and sheep, no co-infection was observed. The distribution of mixed infection varied between hosts and municipalities

Table 4.4. Name of the municipalities and nested PCR results obtained with species specific primers

Name of the municipality	Total number of samples	<i>Babesia bigemina</i>		<i>Babesia bovis</i>		Mixed infection ^{a)}	
		<i>n</i> ^{b)}	Percentage	<i>N</i>	Percentage	<i>n</i>	Percentage
Big 5 hlabisa	69	24	34.8% (± 11.37) ^{c)}	17	24.6% (± 10.19)	4	5.8% (± 5.49)
Mtubatuba	20	6	30% (± 19.99)	4	20% (± 17.44)	0	0% (± 0.0)
uMhlabuyalingana	20	3	15% (± 15.64)	3	15% (± 15.64)	0	0% (± 0.0)
Total	109	33	30.3% (± 8.60)	24	22.2% (± 7.45)	4	3.7% (± 3.53)

a) Animals infected with both *B. bigemina* and *B. bovis*. b) *n*=total number of samples positive for *B. bigemina* and *B. bovis*. c) 95% confidence intervals.

4.2.4. Comparative analysis

To confirm if PCR fragments amplified with the different primers corresponded with the targeted genes, samples positive for each gene were sent to Inqaba Biotechnical Industries, Pty, Ltd in Pretoria for sequencing. The determined nucleotide sequences were confirmed to correspond with *B. bovis*, *B. bigemina* and *T. ovis* sequences published in GenBank's NCBI database. The BLASTn homology search in GenBank showed that *B. bovis* *RAP-1* sequences determined in the present study exhibited 99% nucleotide identity with previously published sequences of *B. bovis* strains from cattle in china (accession no. KT318580.1), Philippines (JX860283.1), South Africa (KC894392.1) and USA (XM001610859.1) (Table 4.5). When one of the sequences from the study was aligned with a published sequence, only one gap and no mutations were observed (Figure 4.5). On the other hand, *B. bigemina* *SpeI-AvaI* sequences determined in this study exhibited identities of below 90% when compared to other published sequences from cattle in UK (XM012911573.1-LK055273.1) (Table 4.6). The alignment of our *B. bigemina* sequence with a published sequence from UK is shown in figure 4.6. The homology search showed that *T. ovis* 18S rRNA sequences generated in this study exhibited 99% identity with published sequences of *T. ovis* from ovine in Iran (JN412663.1), Algeria (MH327772.1), Sudan (KT894392.1), Turkey (KT851438.1), and Iraq (KR094869.1) (Table 4.7). The alignment of the *T. ovis* from the current study with one of the published sequences is shown in figure 4.7.

Table 4.5. BLASTn results for *RAP-1 Babesia bovis* sequences

Target gene	BLAST description	Query cover	E-value	Identity	Accession number	Geographical distribution
<i>RAP-1</i>	<i>B. bovis</i> rap-1a gene	97%	4e-122	99%	KT318580.1	China
	<i>B. bovis</i> rap-1a gene	97%	4e-122	99%	XM001610859.1	USA
	<i>B. bovis</i> rap-1a gene	97%	4e-122	99%	KC894392.1	South Africa
	<i>B. bovis</i> rap-1a gene	97%	4e-122	99%	JX860283.1	Philippines
	<i>B. bovis</i> rap-1a gene	97%	4e-122	99%	KT318579.1	China

Table 4.6. BLASTn results for Spel-Aval *B. bigemina* sequences

Target gene	BLAST description		Query cover	E-value	Identity	Accession number	Origin
	<i>B. bigemina</i>	hypothetical protein	88%	1e-20	89%	XM012911573.1	UK
<i>Spel-Aval</i>	<i>B. bigemina</i>	genome assembly	88%	1e-20	89%	LK391707.1	UK
	<i>B. bigemina</i>	hypothetical protein	82%	8e-17	87%	XM012914873.1	UK
	<i>B. bigemina</i>	genome assembly	82%	8e-17	87%	LK054839.1	UK
	<i>B. bigemina</i>	hypothetical protein	85%	3e-16	86%	XM012915468.1	UK

Table 4.7. BLASTn results for 18S rRNA *T. ovis* sequences

Target gene	BLAST description	Query cover	E-value	Identity	Accession number	Origin
18S	<i>T. ovis</i> isolate a1-1yfs	94%	0.0	99%	JN412663.1	Iran
	<i>T. ovis</i> voucher 707	94%	0.0	99%	MH327772.1	Algeria
	<i>T. ovis</i> 18S	94%	0.0	99%	KT894392.1	Sudan
	<i>T. ovis</i> isolate orkun-TK96	94%	0.0	99%	KT851438.1	Turkey
	<i>T. ovis</i> isolate DRC X1	94%	0.0	99%	KR094869.1	Iraq

	Score	Expect	Identities	Gaps	Strand
	470 bits(254)	1e-128	257/258(99%)	1/258(0%)	Plus/Plus
KZN-Hlabisa- B9- bovine	TTATTTGACAGT*	AACAGTATGAACGCCAAGTTTTTCAACAGATT	CAGCTTCACTACAAA	61	
<i>B. bovis</i> isolate CQ (rap-1a) gene	TTATTTGACAGTAAACAGTATGAACGCCAAGTTTTTCAACAGATT	CAGCTTCACTACAAA	364		
KZN-Hlabisa- B9- bovine	GATATTCAGCCGTCGTATTAGGCAACATTGAGTGATATCATCAGGTGGAATGTTCTGA	121			
<i>B. bovis</i> isolate CQ (rap-1a) gene	GATATTCAGCCGTCGTATTAGGCAACATTGAGTGATATCATCAGGTGGAATGTTCTGA	424			
KZN-Hlabisa- B9- bovine	AGATTTTGAAGAAAGGAGCATCGAACGTATCACTCAACTTACTAGCAGCTACGAAGATTA	181			
<i>B. bovis</i> isolate CQ (rap-1a) gene	AGATTTTGAAGAAAGGAGCATCGAACGTATCACTCAACTTACTAGCAGCTACGAAGATTA	484			
KZN-Hlabisa- B9- bovine	CATGTTGACCCAGATTCCAACCTTTTCCAAGTTTGACGTCGTTATGCTGACATGGTGAA	241			
<i>B. bovis</i> isolate CQ (rap-1a) gene	CATGTTGACCCAGATTCCAACCTTTTCCAAGTTTGACGTCGTTATGCTGACATGGTGAA	544			
KZN-Hlabisa- B9- bovine	GAAGGTTCTGCTCGGTAG	259			
<i>B. bovis</i> isolate CQ (rap-1a) gene	GAAGGTTCTGCTCGGTAG	562			

Figure 4.5: BLASTn results showing the alignment of *B. bovis* RAP-1 gene sequence from this study which was from a cattle sample from Big 5 hlabisa local municipality. The subject sequence (*B. bovis* isolate CQ (Rap-1a) gene), accession no: KT318580.1 covered 91% of the query sequence (KZN_10MAY-B9 Bovine) and it had 99% identity with one gap. The red star indicates the gap between sequences.

Score	Expect	Identities	Gaps	Strand
109 bits(120)	1e-20	74/83(89%)	0/83(0%)	Plus/Minus
KZN_Hlabisa B9-Bovine				
B. bigemina hypothetical protein partial mRNA				
KZN_Hlabisa B9-Bovine				
B. bigemina hypothetical protein partial mRNA				

Figure 4.6: BLASTn results showing the alignment of *B. bigemina* isolate Spel-Aval gene sequence from this study which was from a cattle sample from Big 5 hlabisa local municipality. The subject sequence (*B. bigemina* hypothetical protein partial mRNA), accession no: XM012911573.1 covered 88% of the query sequence (KZN-Hlabisa B9 Bovine) and it had 89% identity with no gaps. The black stars indicate transitions and transversions that occurred between sequences.

	Score 682 bits(369)	Expect 0.0	Identities 373/375(99%)	Gaps 0/375(0%)	Strand Plus/Plus
KZN-Big 5-B1 ovine	ATGATGGGAATTTAAACCTCTTCCAGAATATCAATTGGAGGGCAAGTCTGGTGCCAGCAG		*		86
<i>T. Ovis</i> isolate a1-1yfs 18S rRNA gene	ATGATGGGAATTTAAACCTCTTCCAGAGTATCAATTGGAGGGCAAGTCTGGTGCCAGCAG				72
KZN-Big 5-B1 ovine	CCGCGGTAATTCAGCTCCAATAGCGTATATTTAAATTTGTTGCAGTTAAAAAGCTCGTAG				146
<i>T. Ovis</i> a1-1yfs 18S rRNA gene	CCGCGGTAATTCAGCTCCAATAGCGTATATTTAAATTTGTTGCAGTTAAAAAGCTCGTAG				132
KZN-Big 5-B1 ovine	TTGAATTTCTGCTGCATTGCTTTTCTCCTTTACGAGTCTTTGCATTGTGGCTTATTTTCG				206
<i>T. Ovis</i> a1-1yfs 18S rRNA gene	TTGAATTTCTGCTGCATTGCTTTTCTCCTTTACGAGTCTTTGCATTGTGGCTTATTTTCG				192
KZN-Big 5-B1 ovine	GACTTTGTTTTACAATGTCCGGATGTTTACTTTGAGAAAATTAGAGTGCTCAAAGCAGGC				266
<i>T. Ovis</i> a1-1yfs 18S rRNA gene	GACTTTGTTTTACAATGTCCGGATGTTTACTTTGAGAAAATTAGAGTGCTCAAAGCAGGC				252
KZN-Big 5-B1 ovine	TTTCGCCTTGAATAGTTTAGCATGGAATAATAAAGTAGGACTTTGGTTCTATTTTGTGG				326
<i>T. Ovis</i> a1-1yfs 18S rRNA gene	TTTCGCCTTGAATAGTTTAGCATGGAATAATAAAGTAGGACTTTGGTTCTATTTTGTGG				312
KZN-Big 5-B1 ovine	TTTATAGGTACCAAAGTAATGGTTAATAGGAACAGTTGGGGGCCTTCGTATTTAACTGTCA			*	386
<i>T. Ovis</i> a1-1yfs 18S rRNA gene	TTTATAGGTACCAAAGTAATGGTTAATAGGAACAGTTGGGGGCATTTCGTATTTAACTGTCA				372
KZN-Big 5-B1 ovine	TAGGTGAAATTCTTA				401
<i>T. Ovis</i> a1-1yfs 18S rRNA gene	TAGGTGAAATTCTTA				387

Figure 4.7: BLASTn results showing the alignment of *T. ovis* 18S rRNA sequences from this study which was from a sheep sample from Big 5 False Bay local municipality. The subject sequence (*Theileria ovis* isolate a1-lyfs), accession no: JN412663.1 covered 99% of the query sequence (KZN-Big 5-B1 ovine) and it had 99% identity with no gaps. The black stars indicate transitions and transversions that occurred between sequences.

4.2.5. Phylogenetic analysis

Two *B. bovis* *RAP-1* sequences obtained from cattle in this study were aligned by clustal Won MEGA 7 along with other published sequences. Alignment revealed significant difference in the overall nucleotide composition (Table 4.8). Pairwise distance to determine the number of base substitutions per site from between sequences is shown in table 4.9. Analysis involved 18 nucleotide sequences with 319 bp. 1st, 2nd, 3rd codon positions as well as the noncoding positions were included in the analysis, albeit positions containing gaps and missing data were eliminated from this analysis following the example of Tamura et al. (1992). Nucleotide polymorphisms were observed between sequences and nucleotide diversity was estimated. Multiple nucleotide polymorphisms were observed between sequences with conserved regions and similar segments of the alignments in the beginning, middle and end sites. Conserved regions in the alignment

are represented by dots which represent homologous nucleotides in the alignment of the 18 *B. bovis* sequences (Figure 4.8). Nucleotide diversity within *B. bovis* species in the study which represented the mean evolutionary diversity for the entire population was $d=0.139$ and the estimated transition/transversion bias (R) was 1.14. The substitution patterns and rates were estimated under the Tamura (1992) model. The nucleotide frequencies were A= 29.37%, T/U= 29.37, C= 20.63% and G= 20.63% (Table 4.10). The parameters on the MEGA 7 software were set as follows: bootstrap procedure with 10 000 replicates, 1st, 2nd and 3rd codon positions as well as non-coding positions were also included in the analysis however, gaps and missing data in the alignments were eliminated in this evolutionary analysis (Tamaru et al. 1992). The disparity index test was conducted for the probability of rejecting the null hypothesis that sequences have evolved with the same pattern of substitution, as judged from the extent of differences in base composition biases between sequences. A Monte Carlo test with 500 replicates was used to estimate the P-values and P-values lower than 0.05 were considered significant (Table 4.11).

With regard to *B. bigemina* *SpeI-AvaI* restriction fragment, alignment also revealed a significant difference in the overall nucleotide composition (Table 4.12). Pairwise distance to determine the number of base substitution per site from between sequences is shown in table 4.13. Analysis involved 10 nucleotide sequences with 91 bp. 1st, 2nd, 3rd codon positions as well as the noncoding positions were included in the analysis, even though the positions containing gaps and missing data were eliminated from this analysis. Nucleotide polymorphisms were observed between sequences and nucleotide diversity was estimated. Multiple nucleotide polymorphisms were observed between sequences with conserved regions and similar segments of the alignments in the middle and end sites. Conserved regions in the alignment are represented by dots which shows homologous nucleotides in the alignment of the 10 *B. bigemina* sequences (Figure 4.9). Nucleotide diversity within *B. bigemina* species in the study which represented the mean evolutionary diversity for the entire population was $d=1.56$ and the estimated transition/transversion bias (R) was 1.75. The substitution patterns and rates were estimated under the Tamura (1992) model. The nucleotide frequencies were A= 33.41%, T/U= 33.41%, C= 16.59% and G= 16.59% (Table 4.14). The parameters on the MEGA 7 software were set as follows: bootstrap procedure with 10000 replicates, 1st, 2nd and 3rd codon positions as well as non-coding positions were also included in the analysis

however, gaps and missing data in the alignments were eliminated in this evolutionary analysis (Tamaru et al. 1992). The disparity index test was conducted for the probability of rejecting the null hypothesis that sequences have evolved with the same pattern of substitution, as judged from the extent of differences in base composition biases between sequences. A Monte Carlo test with 500 replicates was used to estimate the P-values and P-values lower than 0.05 were considered significant (Table 4.15).

The maximum likelihood trees constructed with *RAP-1* and *SpeI-aval* gene sequences of *B. bovis* and *B. bigemina* are shown in figure 4.10 and 4.11, respectively. Closely related sequences of both the *Babesia* sp. genes retrieved from GenBank were also included in the phylogeny. When comparing *RAP-1* nucleotide sequences from KwaZulu-Natal *B. bovis* isolates with other published *B. bovis* strains of countries apart from South Africa, they exhibited a high level of sequence identity. The *RAP-1* nucleotide sequence of *B. bovis* obtained from two randomly selected samples (named 21 Bov Hlabisa and 23 Bov Hlabisa) shared 99% identity when compared with one another. These sequences were also compared with other published sequences, thereby revealing 82% identity to *B. bovis* isolates from South Africa, China and Brazil.

With regard to the analysis of *SpeI-Aval* nucleotide sequences of Kwa-Zulu Natal *B. bigemina* field isolate, multiple sequence alignment indicated a high degree of polymorphism between the sequences of the present study and those of *B. bigemina* strains published in GenBank. The Maximum likelihood tree constructed with the *SpeI-Aval* sequences of *B. bigemina* provided branches that were supported by high bootstrap values. Nonetheless, the phylogeny showed a distinct division among the KwaZulu-Natal *B. bigemina* field isolate and other strains of South Africa. To our surprise, KZN *B. bigemina* isolates designated B. big-Hlabisa grouped with *B. bigemina* strain of India published in GenBank.

On the other hand, *T. ovis* 18S rRNA sequences obtained in the present study were clustered into a single clade with *T. ovis* sequences from different countries including South Africa (Figure 4.12).

Table 4.8. Nucleotide composition of *B. bovis* RAP-1 gene sequences

Sample no	T(U)	C	A	G	Total	T-1	C-1	A-1	G-1	Pos #1	T-2	C-2	A-2	G-2	Pos #2	T-3	C-3	A-3	G-3	Pos #3
KC894397.1 <i>Babesia bovis</i> South Africa	27,9	20,7	30,7	20,7	319,0	21	14,2	46,2	18,9	106,0	33	17,9	31,1	17,9	106,0	30	29,9	15,0	25,2	107,0
KC894396.1 <i>Babesia bovis</i> South Africa	27,9	20,7	30,7	20,7	319,0	21	14,2	46,2	18,9	106,0	33	17,9	31,1	17,9	106,0	30	29,9	15,0	25,2	107,0
KC894395.1 <i>Babesia bovis</i> South Africa	27,9	20,7	30,7	20,7	319,0	21	14,2	46,2	18,9	106,0	33	17,9	31,1	17,9	106,0	30	29,9	15,0	25,2	107,0
KC894394.1 <i>Babesia bovis</i> South Africa	27,9	20,7	30,7	20,7	319,0	21	14,2	46,2	18,9	106,0	33	17,9	31,1	17,9	106,0	30	29,9	15,0	25,2	107,0
KC894392.1 <i>Babesia bovis</i> South Africa	27,9	20,7	30,7	20,7	319,0	21	14,2	46,2	18,9	106,0	33	17,9	31,1	17,9	106,0	30	29,9	15,0	25,2	107,0
JX993940.2 <i>Babesia orientalis</i>	31,3	21,6	28,5	18,5	319,0	25	13,2	39,6	22,6	106,0	35	20,8	34,0	10,4	106,0	35	30,8	12,1	22,4	107,0
KT318580.1 <i>Babesia bovis</i> China	27,9	20,7	30,7	20,7	319,0	21	14,2	46,2	18,9	106,0	33	17,9	31,1	17,9	106,0	30	29,9	15,0	25,2	107,0
KT318579.1 <i>Babesia bovis</i> China	27,9	20,7	30,7	20,7	319,0	21	14,2	46,2	18,9	106,0	33	17,9	31,1	17,9	106,0	30	29,9	15,0	25,2	107,0
FJ588013.1 <i>Babesia bovis</i> Brazil	27,9	20,7	30,7	20,7	319,0	21	14,2	46,2	18,9	106,0	33	17,9	31,1	17,9	106,0	30	29,9	15,0	25,2	107,0
FJ588012.1 <i>Babesia bovis</i> Brazil	27,9	20,7	30,7	20,7	319,0	21	14,2	46,2	18,9	106,0	33	17,9	31,1	17,9	106,0	30	29,9	15,0	25,2	107,0
FJ588011.1 <i>Babesia bovis</i> Brazil	27,9	20,7	31,0	20,4	319,0	21	14,2	46,2	18,9	106,0	33	17,9	32,1	17,0	106,0	30	29,9	15,0	25,2	107,0
FJ588010.1 <i>Babesia bovis</i> Brazil	27,9	20,7	31,0	20,4	319,0	21	14,2	46,2	18,9	106,0	33	17,9	32,1	17,0	106,0	30	29,9	15,0	25,2	107,0
FJ588009.1 <i>Babesia bovis</i> Brazil	27,9	20,7	31,0	20,4	319,0	21	14,2	46,2	18,9	106,0	33	17,9	32,1	17,0	106,0	30	29,9	15,0	25,2	107,0
KM504166.1 <i>Babesia bovis</i> Israel	26,6	21,6	31,3	20,4	319,0	20	14,2	47,2	18,9	106,0	32	18,9	31,1	17,9	106,0	28	31,8	15,9	24,3	107,0
KM504165.1 <i>Babesia bovis</i> Israel	26,6	21,6	31,0	20,7	319,0	20	14,2	47,2	18,9	106,0	32	18,9	30,2	18,9	106,0	28	31,8	15,9	24,3	107,0
KM504164.1 <i>Babesia bovis</i> Israel	26,6	21,6	31,3	20,4	319,0	20	14,2	47,2	18,9	106,0	32	18,9	31,1	17,9	106,0	28	31,8	15,9	24,3	107,0
21 Bov-Hlabisa	28,7	20,2	31,5	19,6	321,0	22	15,0	45,8	16,8	107,0	34	16,8	31,8	17,8	107,0	30	29,0	16,8	24,3	107,0
23 Bov-Hlabisa	27,6	21,0	32,0	19,4	319,0	19	16,0	47,2	17,9	106,0	32	17,9	31,1	18,9	106,0	32	29,0	17,8	21,5	107,0
Average.	27,9	20,9	30,8	20,4	319,1	21	14,2	46,0	18,9	106,1	33	18,2	31,4	17,4	106,1	30	30,2	15,2	24,7	107,0

Table 4.9. Estimates of evolutionary divergence between the *RAP-1* sequences.

1. KC894397.1 Babesia bovis South Africa																	
2. KC894396.1 Babesia bovis South Africa	0.000																
3. KC894395.1 Babesia bovis South Africa	0.000	0.000															
4. KC894394.1 Babesia bovis South Africa	0.000	0.000	0.000														
5. KC894392.1 Babesia bovis South Africa	0.000	0.000	0.000	0.000													
6. JX993940.2 Babesia orientalis	0.842	0.842	0.842	0.842	0.842												
7. KT318580.1 Babesia bovis China	0.000	0.000	0.000	0.000	0.000	0.842											
8. KT318579.1 Babesia bovis China	0.000	0.000	0.000	0.000	0.000	0.842	0.000										
9. FJ588013.1 Babesia bovis Brazil	0.000	0.000	0.000	0.000	0.000	0.842	0.000	0.000									
10. FJ588012.1 Babesia bovis Brazil	0.000	0.000	0.000	0.000	0.000	0.842	0.000	0.000	0.000								
11. FJ588011.1 Babesia bovis Brazil	0.009	0.009	0.009	0.009	0.009	0.809	0.009	0.009	0.009	0.009							
12. FJ588010.1 Babesia bovis Brazil	0.009	0.009	0.009	0.009	0.009	0.809	0.009	0.009	0.009	0.009	0.009						
13. FJ588009.1 Babesia bovis Brazil	0.009	0.009	0.009	0.009	0.009	0.809	0.009	0.009	0.009	0.009	0.009	0.000					
14. KM504166.1 Babesia bovis Israel	0.102	0.102	0.102	0.102	0.102	0.909	0.102	0.102	0.102	0.102	0.098	0.098	0.098				
15. KM504165.1 Babesia bovis Israel	0.106	0.106	0.106	0.106	0.106	0.925	0.106	0.106	0.106	0.106	0.102	0.102	0.102	0.003			
16. KM504164.1 Babesia bovis Israel	0.102	0.102	0.102	0.102	0.102	0.909	0.102	0.102	0.102	0.102	0.098	0.098	0.098	0.000	0.003		
17. 21 Bov-Hlabisa	0.058	0.058	0.058	0.058	0.058	1.022	0.058	0.058	0.058	0.058	0.069	0.069	0.069	0.178	0.183	0.178	
18. 23 Bov-Hlabisa	0.050	0.050	0.050	0.050	0.050	1.048	0.050	0.050	0.050	0.050	0.061	0.061	0.061	0.164	0.168	0.164	0.037

Table 4.10. Maximum Likelihood Estimate of Substitution Matrix.

	A	T/U	C	G
A	-	<i>6.75</i>	<i>4.74</i>	11.15
T/U	<i>6.75</i>	-	11.15	<i>4.74</i>
C	<i>6.75</i>	15.88	-	<i>4.74</i>
G	15.88	<i>6.75</i>	<i>4.74</i>	-

Rates of different transitional substitutions are shown in bold and those of transversional substitutions are shown in italics.

Table 4.11. Test of homogeneity of substitution patterns between *B. bovis* sequences. P-values are shown below the diagonal and the disparity index per site are shown for each sequence above the diagonal.

1. KC894397.1 Babesia bovis South Africa		0.000	0.000	0.000	0.000	0.009	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.006
2. KC894396.1 Babesia bovis South Africa	1.000		0.000	0.000	0.000	0.009	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.006
3. KC894395.1 Babesia bovis South Africa	1.000	1.000		0.000	0.000	0.009	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.006
4. KC894394.1 Babesia bovis South Africa	1.000	1.000	1.000		0.000	0.009	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.006
5. KC894392.1 Babesia bovis South Africa	1.000	1.000	1.000	1.000		0.009	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.006
6. JX993940.2 Babesia orientalis	0.368	0.434	0.380	0.376	0.350		0.009	0.009	0.009	0.009	0.019	0.019	0.019	0.179	0.169	0.179	0.000	0.066
7. KT318580.1 Babesia bovis China	1.000	1.000	1.000	1.000	1.000	0.372		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.006
8. KT318579.1 Babesia bovis China	1.000	1.000	1.000	1.000	1.000	0.382	1.000		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.006
9. FJ588013.1 Babesia bovis Brazil	1.000	1.000	1.000	1.000	1.000	0.410	1.000	1.000		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.006
10. FJ588012.1 Babesia bovis Brazil	1.000	1.000	1.000	1.000	1.000	0.380	1.000	1.000	1.000		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.006
11. FJ588011.1 Babesia bovis Brazil	1.000	1.000	1.000	1.000	1.000	0.366	1.000	1.000	1.000	1.000		0.000	0.000	0.000	0.000	0.000	0.000	0.000
12. FJ588010.1 Babesia bovis Brazil	1.000	1.000	1.000	1.000	1.000	0.382	1.000	1.000	1.000	1.000	1.000		0.000	0.000	0.000	0.000	0.000	0.000
13. FJ588009.1 Babesia bovis Brazil	1.000	1.000	1.000	1.000	1.000	0.382	1.000	1.000	1.000	1.000	1.000	1.000		0.000	0.000	0.000	0.000	0.000
14. KM504166.1 Babesia bovis Israel	1.000	1.000	1.000	1.000	1.000	0.218	1.000	1.000	1.000	1.000	1.000	1.000	1.000		0.000	0.000	0.000	0.000
15. KM504165.1 Babesia bovis Israel	1.000	1.000	1.000	1.000	1.000	0.212	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000		0.000	0.000	0.000
16. KM504164.1 Babesia bovis Israel	1.000	1.000	1.000	1.000	1.000	0.200	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000		0.000	0.000
17. 21 Bov-Hlabisa	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000		0.000
18. 23 Bov-Hlabisa	0.332	0.376	0.352	0.346	0.348	0.344	0.392	0.336	0.356	0.384	1.000	1.000	1.000	1.000	1.000	1.000	1.000	

Table 4.12. Nucleotide composition from SpeI-aval restriction fragment between *B. bigemina* strains from the study and GenBank

	T(U)	C	A	G	Total	T- 1	C-1	A-1	G-1	Pos #1	T- 2	C-2	A-2	G-2	Pos #2	T- 3	C-3	A-3	G-3	Pos #3
AB922127.1 <i>Babesia bigemina</i> India	44,0	16,5	27,5	12,1	91,0	52	32,3	16,1	,0	31,0	37	10,0	43,3	10,0	30,0	43	6,7	23,3	26,7	30,0
B. big-Hlabisa	43,6	17,0	23,4	16,0	94,0	44	31,3	12,5	12,5	32,0	29	19,4	35,5	16,1	31,0	58	,0	22,6	19,4	31,0
KC894399.1 <i>Babesia bigemina</i> South Africa	33,3	17,2	28,0	21,5	93,0	38	25,0	18,8	18,8	32,0	26	16,1	32,3	25,8	31,0	37	10,0	33,3	20,0	30,0
KC894404.1 <i>Babesia bigemina</i> South Africa	36,6	15,1	28,0	20,4	93,0	44	21,9	15,6	18,8	32,0	26	12,9	35,5	25,8	31,0	40	10,0	33,3	16,7	30,0
AB112336.2 <i>Babesia microti</i>	43,6	5,3	37,2	13,8	94,0	41	6,3	37,5	15,6	32,0	52	9,7	32,3	6,5	31,0	39	,0	41,9	19,4	31,0
KC894409.1 <i>Babesia bigemina</i> South Africa	36,6	15,1	28,0	20,4	93,0	44	21,9	15,6	18,8	32,0	26	12,9	35,5	25,8	31,0	40	10,0	33,3	16,7	30,0
KC894410.1 <i>Babesia bigemina</i> South Africa	33,3	17,2	28,0	21,5	93,0	38	25,0	18,8	18,8	32,0	26	16,1	32,3	25,8	31,0	37	10,0	33,3	20,0	30,0
KC894414.1 <i>Babesia bigemina</i> South Africa	37,6	15,1	24,7	22,6	93,0	41	25,0	12,5	21,9	32,0	29	12,9	29,0	29,0	31,0	43	6,7	33,3	16,7	30,0
KC894415.1 <i>Babesia bigemina</i> South Africa	37,6	15,1	24,7	22,6	93,0	41	25,0	12,5	21,9	32,0	29	12,9	29,0	29,0	31,0	43	6,7	33,3	16,7	30,0
KC894416.1 <i>Babesia bigemina</i> South Africa	37,6	15,1	24,7	22,6	93,0	41	25,0	12,5	21,9	32,0	29	12,9	29,0	29,0	31,0	43	6,7	33,3	16,7	30,0
Average	38,4	14,8	27,4	19,4	93,0	42	23,8	17,2	16,9	31,9	31	13,6	33,3	22,3	30,9	42	6,6	32,1	18,9	30,2

Table 4.13. Estimates of evolutionary divergence between the *B. bigemina* *SpeI*-*AvaI* restriction fragment sequences

1. AB922127.1 Babesia bigemina India									
2. B. big-Hlabisa	0.678								
3. KC894399.1 Babesia bigemina South Africa	2.123	2.606							
4. KC894404.1 Babesia bigemina South Africa	2.256	2.759	0.047						
5. AB112336.2 Babesia microti	2.137	2.423	3.384	4.109					
6. KC894409.1 Babesia bigemina South Africa	2.256	2.759	0.047	0.000	4.109				
7. KC894410.1 Babesia bigemina South Africa	2.123	2.606	0.000	0.047	3.384	0.047			
8. KC894414.1 Babesia bigemina South Africa	2.219	2.905	0.287	0.258	3.844	0.258	0.287		
9. KC894415.1 Babesia bigemina South Africa	2.219	2.905	0.287	0.258	3.844	0.258	0.287	0.000	
10. KC894416.1 Babesia bigemina South Africa	2.219	2.905	0.287	0.258	3.844	0.258	0.287	0.000	0.000

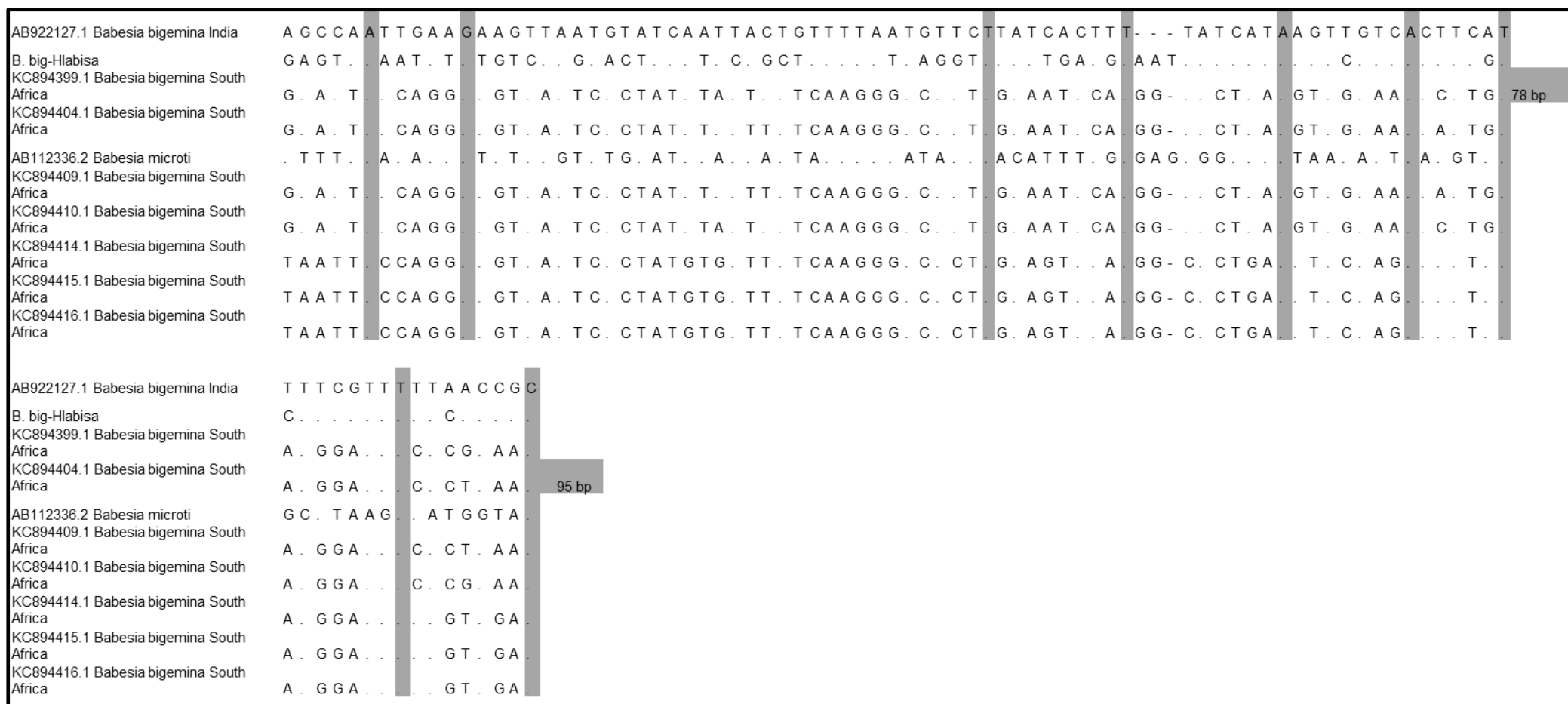


Figure 4.9: Alignment of *B. bigemina* *SpeI-AvaI* nucleotide sequences (98 bp). The gray shaded area represent conserved regions

Table 4.14. Maximum Likelihood Estimate of Substitution matrix

	A	T/U	C	G
A	-	<i>5.62</i>	<i>2.79</i>	11.01
T/U	<i>5.62</i>	-	11.01	<i>2.79</i>
C	<i>5.62</i>	22.16	-	<i>2.79</i>
G	22.16	<i>5.62</i>	<i>2.79</i>	-

Rates of different transitional substitutions are shown in bold and those of transversional substitutions are shown in italics.

Table 4.15. Test of homogeneity of substitution patterns between *B. bigemina* sequences. P-values are shown below the diagonal and the disparity index per site are shown for each sequence above the diagonal. P-values less than 0.05 are highlighted

1. AB922127.1 Babesia bigemina India		0.000	0.132	0.000	0.462	0.000	0.132	0.000	0.000	0.000
2. B. big-Hlabisa	1.000		0.077	0.000	1.220	0.000	0.077	0.000	0.000	0.000
3. KC894399.1 Babesia bigemina South Africa	0.312	0.326		0.033	1.154	0.033	0.000	0.000	0.000	0.000
4. KC894404.1 Babesia bigemina South Africa	1.000	1.000	0.246		0.560	0.000	0.033	0.000	0.000	0.000
5. AB112336.2 Babesia microti	0.162	0.040	0.050	0.140		0.560	1.154	0.967	0.967	0.967
6. KC894409.1 Babesia bigemina South Africa	1.000	1.000	0.234	1.000	0.186		0.033	0.000	0.000	0.000
7. KC894410.1 Babesia bigemina South Africa	0.302	0.334	1.000	0.246	0.046	0.236		0.000	0.000	0.000
8. KC894414.1 Babesia bigemina South Africa	1.000	1.000	1.000	1.000	0.076	1.000	1.000		0.000	0.000
9. KC894415.1 Babesia bigemina South Africa	1.000	1.000	1.000	1.000	0.060	1.000	1.000	1.000		0.000
10. KC894416.1 Babesia bigemina South Africa	1.000	1.000	1.000	1.000	0.070	1.000	1.000	1.000	1.000	

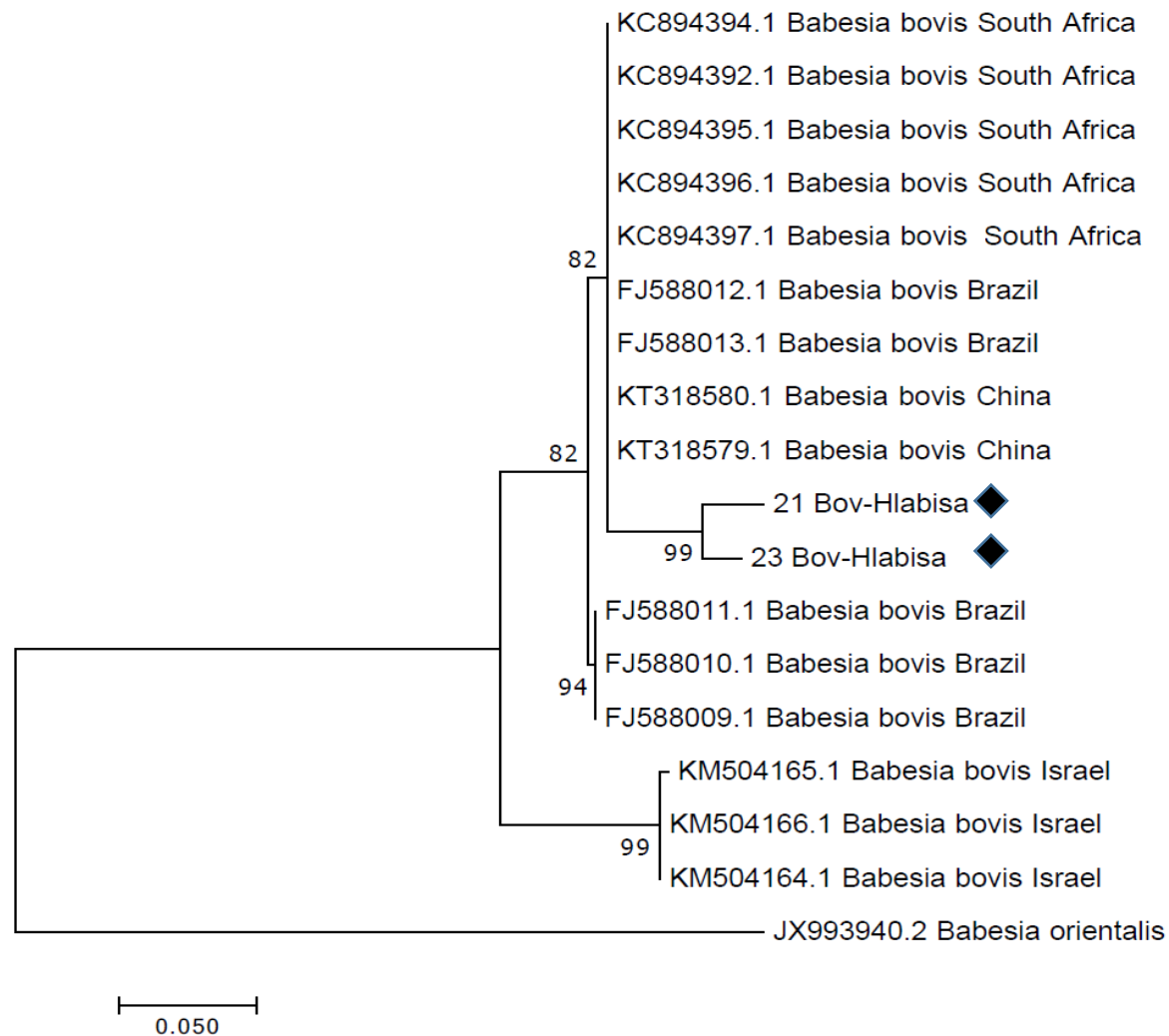


Figure 4.10: Phylogenetic tree based on *rap-1* gene sequences of *B. bovis* isolates identified in this study (Indicated with bullets) and those of strains whose sequences were retrieved from GenBank. The tree was constructed using maximum likelihood method, with bootstrap values (expressed as percentages of 10000 replications) superimposed at branching points. The horizontal bar represents the number of substitutions per sites. *Babesia orientalis* was used as an outgroup

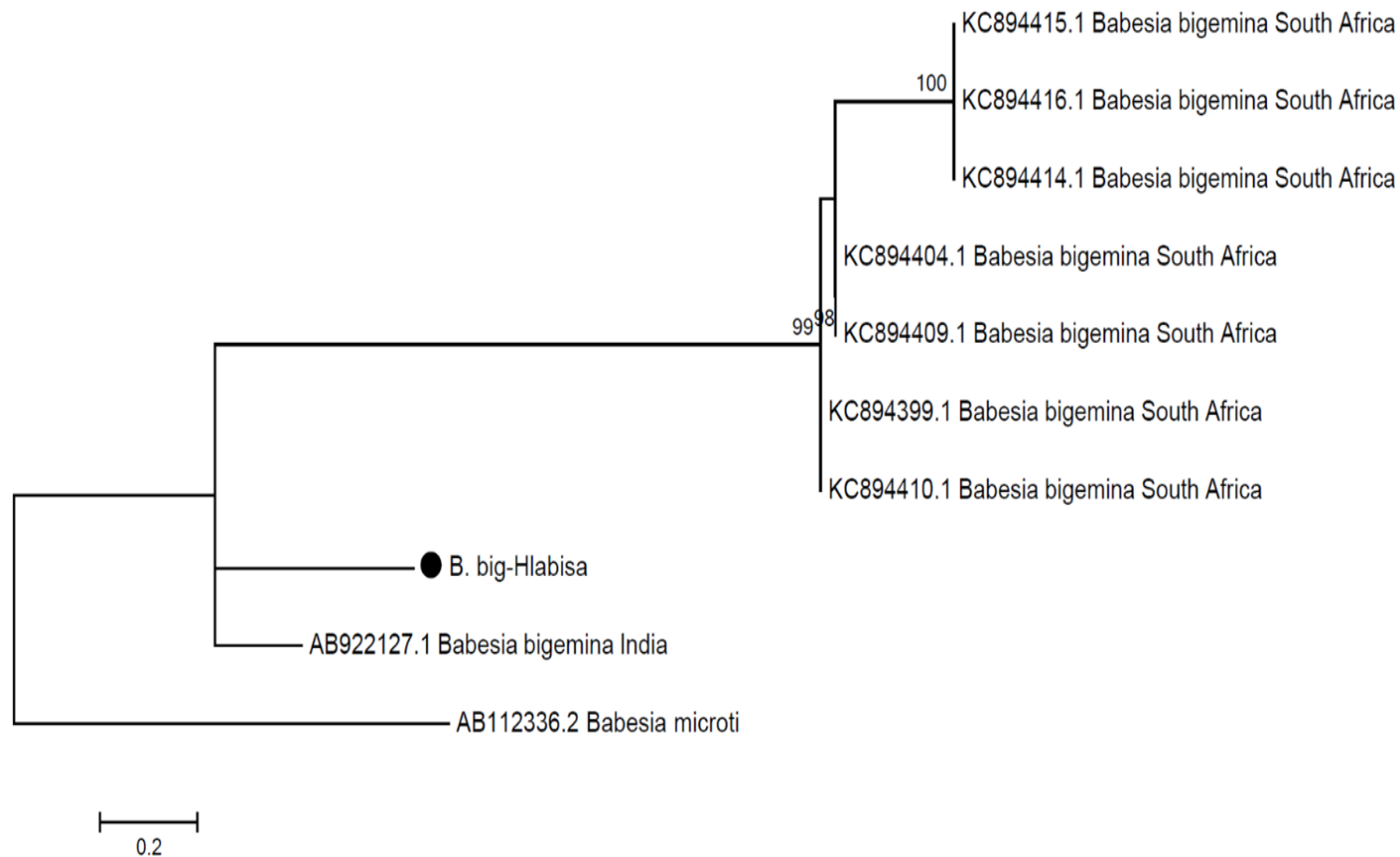


Figure 4.11: Phylogenetic tree based on *Spel-Aval* gene sequences of *B. bigemina* isolates identified in this study (Indicated with bullets) and those of strains whose sequences were retrieved from GenBank. The tree was constructed using maximum likelihood method, with bootstrap values (expressed as percentages of 10000 replications) superimposed at branching points. Only values above 50% are shown. The horizontal bar represents the number of mutations per sites. *Babesia microti* was used as an outgroup.

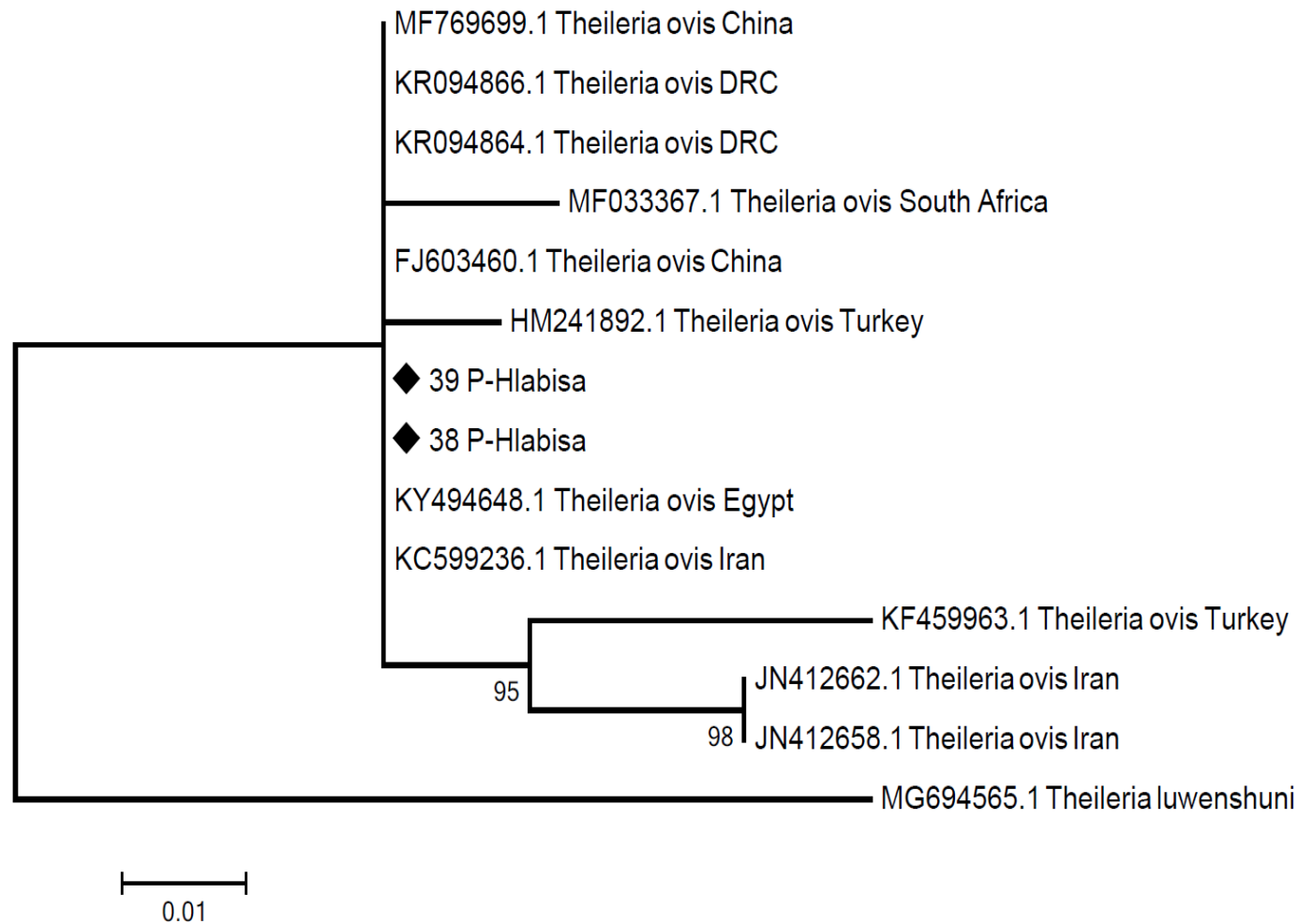


Figure 4.12: Phylogenetic tree analysis of *Theileria ovis* based on 18S rRNA gene, the tree was constructed with maximum likelihood method, with bootstrap values (expressed as percentages of 10000 replications) superimposed at branching points. The sequences produced in this study are shown with bullet points. The evolutionary distances were computed using the p-distance method (Kumar et al 2016). *Theileria luwenshuni* was used as an outgroup

4.3. Infection rate of *Toxoplasma gondii*

Out of 208 samples screened for *T. gondii*, only 5 samples were PCR positive. The PCR amplification that were positive with semi nested PCR primers produced single DNA bands of approximately 190 bp for *Toxoplasma gondii* B1 gene (Figure 4.13). From the PCR detection, it was noted that *T. gondii* parasite was positive in the blood samples of cattle only. The *T. gondii* was not detected in either sheep, goats or dogs. The infection rate of *Toxoplasma* was 4.6% in cattle, 0.0% in sheep, goats and dogs, respectively. The cattle found to contain the DNA *T. gondii* (5/69, 7.2% CI=±6.08) originated from the Big 5 hlabisa municipality. None of the blood samples collected from the other three municipalities possessed *T. gondii*.

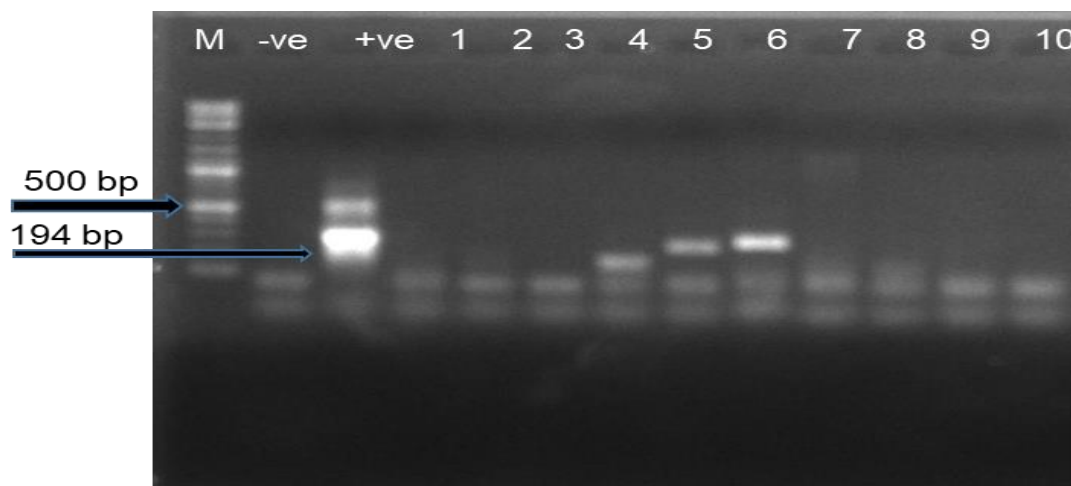


Figure 4.13: Agarose gel showing PCR amplification of *Toxoplasma gondii* DNA from cattle using B1 gene nested primers. M is the molecular marker, -ve is for the no template negative control, +ve is for the positive control. Lane 4, 5 & 6 shows positive samples for *T. gondii* at approximately

4.3.1. Comparative sequence analysis

To confirm if PCR fragments amplified with the nested primers corresponded with the targeted gene (B1 gene); positive samples were sent to Inqaba biotec for sequencing. The determined nucleotide sequences were confirmed to correspond with *T. gondii* sequences published in GenBank. The BLASTn homology search in GenBank showed that *T. gondii* B1 gene sequences determined in the present study exhibited 98%

nucleotide identity with previously published sequences of *B. bovis* strains from cattle in Mexico (accession no. KX270384.1-KX270388.1) and USA (accession no: AF179871.1) (Table 4.16). when One of the sequences from the study was aligned with a published sequence from GenBank, it showed one gap and there were mutations between the two sequences (Figure 4.14).

4.3.2. Phylogenetic analysis

Three *T. gondii* B1 sequences obtained from cattle in the study were aligned by Clustal-W on MEGA 7 along with other published sequences. Alignment revealed significant difference in the overall nucleotide composition (Table 4.17). Pairwise distance to determine the number of base substitutions per site from between sequences is shown in table 4.18. Analysis involved 13 nucleotide sequences with 76 positions in the final data set. 1st, 2nd, 3rd codon positions as well as the noncoding positions were included in the analysis, albeit positions containing gaps and missing data were eliminated from this analysis following the example of Tamaru et al (2011). Nucleotide polymorphisms were observed between sequences and nucleotide diversity was estimated. Multiple nucleotide polymorphisms were observed between sequences with conserved regions and similar segments of the alignments in the beginning, middle and end sites. Conserved regions in the alignment are represented by dots which represent homologous nucleotides in the alignment of the 13 *T. gondii* sequences (Figure 4.15). Nucleotide diversity within *T. gondii* species in the study which represented the mean evolutionary diversity for the entire population was $d=1.09$ and the estimated transition/transversion bias (R) was 0.87. The substitution patterns and rates were estimated under the Tamura (1992) model. The nucleotide frequencies were A= 25.00%, T/U= 25.00%, C= 25.00% and G= 25.00% (Table 4.19). The parameters on the MEGA 7 software were set as follows: bootstrap procedure with 10000 replicates, 1st, 2nd and 3rd codon positions as well as non-coding positions were also included in the analysis however, gaps and missing data in the alignments were eliminated in this evolutionary analysis (Tamaru *et al.*, 1992). The disparity index test was conducted for the probability of rejecting the null hypothesis that sequences have evolved with the same pattern of substitution, as judged from the extent of differences in base composition biases between sequences. A Monte Carlo

test with 500 replicates was used to estimate the P-values and P-values lower than 0.05 were considered significant (Table 4.20).

The maximum likelihood tree constructed with *B1* gene sequences of *T. gondii* is shown in figure 4.16. Closely related sequences of the *T. gondii* genes retrieved from GenBank were also included in the phylogeny. When comparing *B1* nucleotide sequences from KwaZulu-Natal with other published *T. gondii* strains of countries apart from South Africa, they exhibited a high level of sequence identity. The *B1* nucleotide sequence of *T. gondii* obtained from the study, shared 100% identity when compared with one another. These sequences were also compared with other published sequences, thereby revealing 88% identity to *T. gondii* isolates from Mexico, Italy and Iran

Table 4.16. BLASTn results of *T. gondii* B1 gene

Target gene	Description		Query cover	E-value	Identity	Accession number	Origin
B1 gene	<i>T. gondii</i> Coalatilla	isolate	100%	3e-83	98%	KX370388.1	Mexico
	<i>T. gondii</i> Tecuanillo	isolate	100%	3e-83	98%	KX270387.1	Mexico
	<i>T. gondi</i> Tecuanillo	isolate	100%	3e-83	98%	KX270385.1	Mexico
	<i>T. gondi</i> Tecuanillo	isolate	100%	3e-83	98%	KX270384.1	Mexico
	<i>T. gondii</i> sequence	partial	100%	3e-83	98%	AF179871.1	USA

	Score	Expect	Identities	Gaps	Strand
	316 bits(350)	1e-82	182/185(98%)	1/185(0%)	Plus/Minus
kzn-hlabisa Cattle.					
<i>T. gondii</i> isolate B1 gene					
kzn-hlabisa Cattle.					
<i>T. gondii</i> isolate B1 gene					
kzn-hlabisa Cattle.					
<i>T. gondii</i> isolate B1 gene					
kzn-hlabisa Cattle.					
<i>T. gondii</i> isolate B1 gene					
kzn-hlabisa Cattle.					
<i>T. gondii</i> isolate B1 gene					

	ATCTGGCGGATACACCAAAGTTGCACAGATACTCATGAATTTCACTTTTCGCCAGCAGAG	60
	ATCTG-CGAATACACCAAAGTTGCACAGATACTCATGAATTTCACTTTTCGCCAGCAGAG	616
	GGGAGCTCGTCAGTGACTGCAACCTATGCAAACACATTAGCGTCTCTTTCATTCCCACAT	120
	GGGAGCTCGTCAGTGACTGCAACCTATGCAAACACATTAGCGTCTCTTTCATTCCCACAT	556
	ttttttCTTATACTCATGAACGGATGCAGTTCATTCTGATCTACTCGACAATACGCTGC	180
	TTTTTCTTATACTCATGAACGGATGCAGTTCCTTCTGATCTACTCGACAATACGCTGC	496
	TTGAA	185
	TTGAA	491

Figure 4.14: BLASTn results showing the alignment of *T. gondii* isolate with one of the sequences from the study which was from cattle in Hlabisa municipality. The subject sequence (*T. gondii* isolate B1 gene) accession no: KX270388.1 covered 100% of the query cover and it had 98% identity with one gap. Black stars indicate nucleotide polymorphisms that occurred between the sequences. A gap is indicated by a red star.

Table 4.17. Nucleotide composition of B1 *T. gondii* sequences

	T(U)	C	A	G	Total	T-1	C-1	A-1	G-1	Pos #1	T-2	C-2	A-2	G-2	Pos #2	T-3	C-3	A-3	G-3	Pos #3
KR559682.1 <i>Toxoplasma gondii</i> Europe	30,1	25,5	26,0	18,4	196,0	26	24,6	29,2	20,0	65,0	35	18,2	28,8	18,2	66,0	29	33,8	20,0	16,9	65,0
AB703302.1 <i>Toxoplasma gondii</i> Iran	21,0	20,5	31,0	27,5	200,0	19	23,9	22,4	34,3	67,0	18	16,4	31,3	34,3	67,0	26	21,2	39,4	13,6	66,0
AB703300.1 <i>Toxoplasma gondii</i> Iran	21,5	18,0	31,0	29,5	200,0	19	20,9	22,4	37,3	67,0	19	14,9	31,3	34,3	67,0	26	18,2	39,4	16,7	66,0
KY706491.1 <i>Toxoplasma gondii</i> Italy	25,4	20,3	28,8	25,4	118,0	23	22,5	22,5	32,5	40,0	23	25,6	28,2	23,1	39,0	31	12,8	35,9	20,5	39,0
KU288755.1 <i>Toxoplasma gondii</i> Brazil	28,3	27,0	27,0	17,6	159,0	23	26,4	32,1	18,9	53,0	30	20,8	28,3	20,8	53,0	32	34,0	20,8	13,2	53,0
KX270388.1 <i>Toxoplasma gondii</i> Mexico	21,4	18,8	31,8	28,1	192,0	19	21,9	23,4	35,9	64,0	19	15,6	31,3	34,4	64,0	27	18,8	40,6	14,1	64,0
KX270387.1 <i>Toxoplasma gondii</i> Mexico	21,4	18,8	31,8	28,1	192,0	19	21,9	23,4	35,9	64,0	19	15,6	31,3	34,4	64,0	27	18,8	40,6	14,1	64,0
KX270386.1 <i>Toxoplasma gondii</i> Mexico	21,4	18,8	31,8	28,1	192,0	19	21,9	23,4	35,9	64,0	19	15,6	31,3	34,4	64,0	27	18,8	40,6	14,1	64,0
KP835555.1 <i>Besnoitia besnoiti</i>	33,3	13,6	28,3	24,7	198,0	36	13,4	28,4	22,4	67,0	25	16,4	29,9	28,4	67,0	39	10,9	26,6	23,4	64,0
B1 TOXOPLASMA12 Hlabisa	28,7	27,2	25,6	18,5	195,0	26	26,2	27,7	20,0	65,0	32	18,5	30,8	18,5	65,0	28	36,9	18,5	16,9	65,0
KX270379.1 <i>Toxoplasma gondii</i> Mexico	21,4	18,8	31,8	28,1	192,0	19	21,9	23,4	35,9	64,0	19	15,6	31,3	34,4	64,0	27	18,8	40,6	14,1	64,0
B1 TOXOPLASMA1 2 Hlabisa	29,6	25,5	26,5	18,4	196,0	26	24,6	29,2	20,0	65,0	33	18,2	30,3	18,2	66,0	29	33,8	20,0	16,9	65,0
B1 TOXOPLASMA Hlabisa	29,6	25,5	26,5	18,4	196,0	26	24,6	29,2	20,0	65,0	33	18,2	30,3	18,2	66,0	29	33,8	20,0	16,9	65,0
Average.	25,6	21,4	29,1	23,9	186,6	23	22,6	25,9	28,4	62,3	25	17,4	30,4	27,2	62,5	29	24,1	31,0	16,2	61,8

Table 4.18. Estimates of evolutionary divergence between the B1 gene sequences

1. KR559682.1 <i>Toxoplasma gondii</i> Europe													
2. AB703302.1 <i>Toxoplasma gondii</i> Iran	1.881												
3. AB703300.1 <i>Toxoplasma gondii</i> Iran	1.769	0.027											
4. KY706491.1 <i>Toxoplasma gondii</i> Italy	1.881	0.000	0.027										
5. KU288755.1 <i>Toxoplasma gondii</i> Brazil	0.000	1.881	1.769	1.881									
6. KX270388.1 <i>Toxoplasma gondii</i> Mexico	1.881	0.000	0.027	0.000	1.881								
7. KX270387.1 <i>Toxoplasma gondii</i> Mexico	1.881	0.000	0.027	0.000	1.881	0.000							
8. KX270386.1 <i>Toxoplasma gondii</i> Mexico	1.881	0.000	0.027	0.000	1.881	0.000	0.000						
9. KP835555.1 <i>Besnoitia besnoiti</i>	1.478	1.758	1.661	1.758	1.478	1.758	1.758	1.758					
10. B1 TOXOPLASMA12 Hlabisa	0.000	1.881	1.769	1.881	0.000	1.881	1.881	1.881	1.478				
11. KX270379.1 <i>Toxoplasma gondii</i> Mexico	1.881	0.000	0.027	0.000	1.881	0.000	0.000	0.000	1.758	1.881			
12. B1 TOXOPLASMA1 2 Hlabisa	0.000	1.881	1.769	1.881	0.000	1.881	1.881	1.881	1.478	0.000	1.881		
13. B1 TOXOPLASMA Hlabisa	0.000	1.881	1.769	1.881	0.000	1.881	1.881	1.881	1.478	0.000	1.881	0.000	

Table 4.19. Maximum Likelihood Estimate of Substitution Matrix

	A	T/U	C	G
A	-	<i>8.33</i>	<i>8.33</i>	8.33
T/U	<i>8.33</i>	-	8.33	<i>8.33</i>
C	<i>8.33</i>	8.33	-	<i>8.33</i>
G	8.33	<i>8.33</i>	<i>8.33</i>	-

Rates of different transitional substitutions are shown in bold and those of transversional substitutions are shown in italics.

Table 4.20. Test of homogeneity of substitution patterns between *T. gondii* sequences. P-values are shown below the diagonal and the disparity index per site are shown for each sequence above the diagonal. P-values less than 0.05 are highlighted.

1. KR559682.1 <i>Toxoplasma gondii</i> Europe		0.000	0.026	0.000	0.000	0.000	0.000	0.000	0.000	0.618	0.000	0.000	0.000	0.000
2. AB703302.1 <i>Toxoplasma gondii</i> Iran	1.000		0.026	0.000	0.000	0.000	0.000	0.000	0.000	0.053	0.000	0.000	0.000	0.000
3. AB703300.1 <i>Toxoplasma gondii</i> Iran	0.396	0.090		0.026	0.026	0.026	0.026	0.026	0.026	0.118	0.026	0.026	0.026	0.026
4. KY706491.1 <i>Toxoplasma gondii</i> Italy	1.000	1.000	0.098		0.000	0.000	0.000	0.000	0.000	0.053	0.000	0.000	0.000	0.000
5. KU288755.1 <i>Toxoplasma gondii</i> Brazil	1.000	1.000	0.376	1.000		0.000	0.000	0.000	0.000	0.618	0.000	0.000	0.000	0.000
6. KX270388.1 <i>Toxoplasma gondii</i> Mexico	1.000	1.000	0.084	1.000	1.000		0.000	0.000	0.000	0.053	0.000	0.000	0.000	0.000
7. KX270387.1 <i>Toxoplasma gondii</i> Mexico	1.000	1.000	0.086	1.000	1.000	1.000		0.000	0.053	0.000	0.000	0.000	0.000	0.000
8. KX270386.1 <i>Toxoplasma gondii</i> Mexico	1.000	1.000	0.104	1.000	1.000	1.000	1.000		0.053	0.000	0.000	0.000	0.000	0.000
9. KP835555.1 <i>Besnoitia besnoiti</i>	0.110	0.320	0.310	0.366	0.124	0.340	0.334	0.382		0.618	0.053	0.618	0.618	0.618
10. B1 TOXOPLASMA12 Hlabisa	1.000	1.000	0.432	1.000	1.000	1.000	1.000	1.000	0.110		0.000	0.000	0.000	0.000
11. KX270379.1 <i>Toxoplasma gondii</i> Mexico	1.000	1.000	0.100	1.000	1.000	1.000	1.000	1.000	0.350	1.000		0.000	0.000	0.000
12. B1 TOXOPLASMA1 2 Hlabisa	1.000	1.000	0.392	1.000	1.000	1.000	1.000	1.000	0.096	1.000	1.000			0.000
13. B1 TOXOPLASMA Hlabisa	1.000	1.000	0.376	1.000	1.000	1.000	1.000	1.000	0.106	1.000	1.000	1.000		

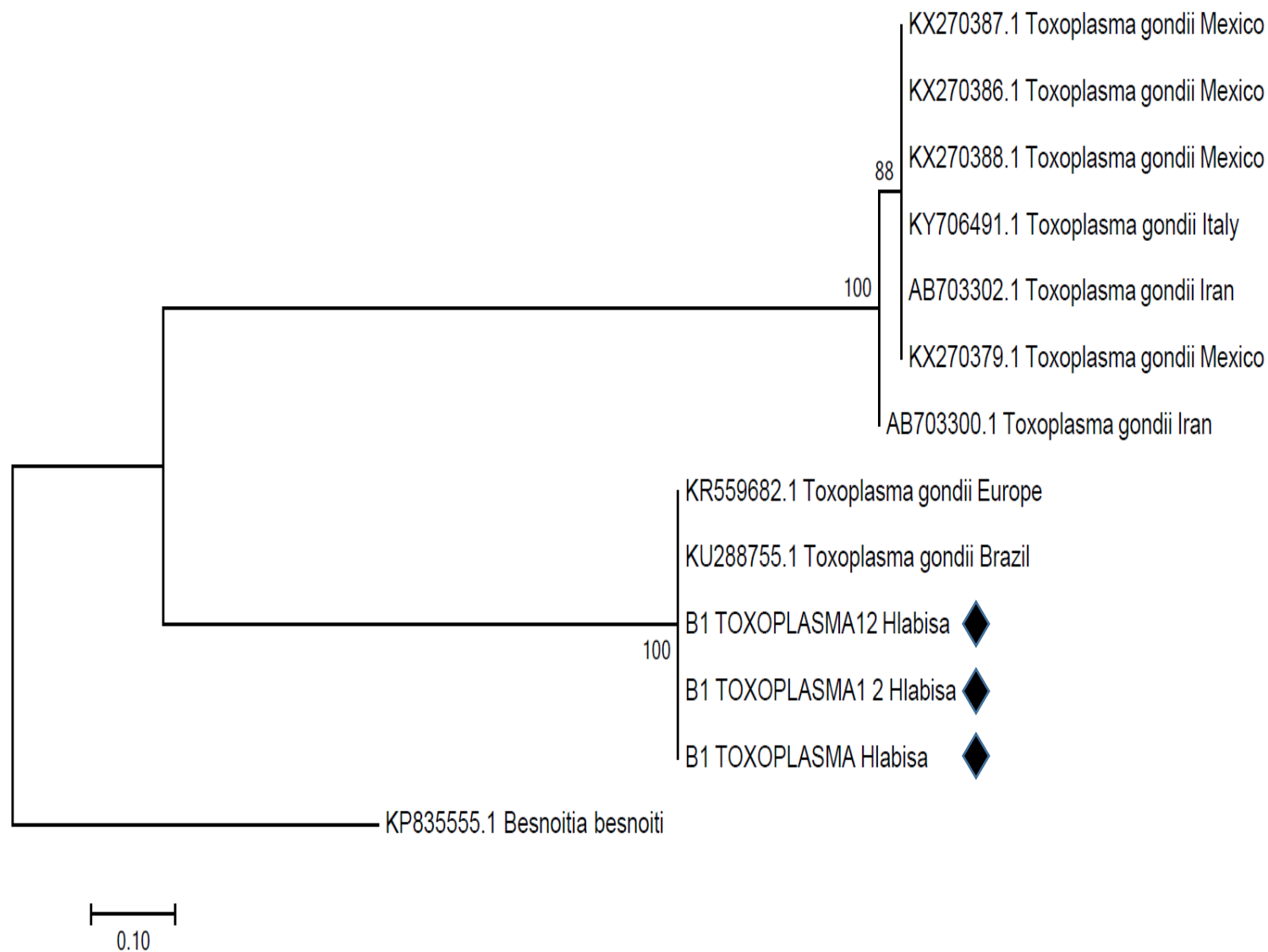


Figure 4.16: Maximum likelihood tree created from B1 nucleotide sequences of *T. gondii* determined in this study (with bullets) and those retrieved from Gen Bank with accession numbers. The numbers at the branching points are bootstrap values expressed as percentages of 10, 000 replications. The horizontal scale bar indicates the number of nucleotide substitutions per site. The origin of published sequences is indicated after the isolate name. *Besnoitia besnoiti* was used as an outgroup

4.4. Infection rate of *Trypanosoma* spp.

In the present study, *Trypanosoma* parasites were only documented in bovine samples as they were the only domestic animals that tested positive by PCR. From the 109 tested bovine samples only 20 were PCR positive. No *Trypanosoma* species were found in dogs, sheep and goats. The overall prevalence of *Trypanosoma* sp. was 18.3% in cattle, 0.0% in dogs, sheep and goats, respectively. The trypanosomes were detected from all the four municipality with Hlabisa having the highest prevalence.

4.5. Infection rate of *Ehrlichia canis*

From the PCR detection results, it was observed that the dogs infected with *Ehrlichia canis* were distributed across all the three municipalities sampled. The highest occurrence was recorded in uMhlabauyalingana municipality in which 55.6% (95% CI=±) of animals were infected, respectively. The Big 5 hlabisa municipality had the lowest infection rate (30%) of *E. canis*.

4.5.1. Comparative analysis

To confirm if PCR fragments amplified with the 16S primers corresponded with the targeted gene, positive were sent to Inqaba biotec Southern Africa for sequencing. The determined nucleotide sequences were confirmed to correspond with *E. canis* sequences published in GenBank. The BLASTn homology search in GenBank showed that *E. canis* 16S gene sequences determined in the present study exhibited 89% nucleotide identity with previously published sequences of *E. canis* strains from dogs and ticks in Vietman (accession no. MH686051.1- MH686052.1), Malaysia (MH457244.1- MH457245.1), and Turkey (142921.1) (Table 4.21). When one of the sequences from the study was aligned with a published sequence, only one gap and eight transversions and transitions were observed (Figure 4.17).

Table 4.21. BLASTn results of 16S rRNA *Ehrlichia canis* sequences

Target gene	Description	Query cover	E-value	Identity	Accession number	Origin
16S rRNA	<i>E. canis</i> clone M	93%	8e-25	89%	MH686052.1	Vietman
	<i>E. canis</i> clone L	93%	8e-25	89%	MH686051.1	Vietman
	<i>E. canis</i> isolate B20AEC	93%	8e-25	89%	MH457245.1	Malaysia
	<i>E. canis</i> isolate BH2EC	93%	8e-25	89%	MH457244.1	Malaysia
	<i>E. canis</i> isolate mkk2	93%	8-e25	89%	MH142921.1	Turkey

	Score	Expect	Identities	Gaps	Strand
	107 bits(118)	1e-24	75/84(89%)	1/84(1%)	Plus/Minus
			* * * * *		
KZN-dog- Hlabisa	CTCTC-GACCAGTTACGGATCGTCGCCTTGGTGAGCCATTACCTCACCAACTAGCTAATC	64			
<i>E. Canis</i> clone M 16S rRNA gene	CTCTCAGACCAGCTATAGATCATAGCCTTGGTAAGCCATTACCTCACCAACTAGCTAATC	154			
	**				
KZN-dog- Hlabisa	CGACGTAGGCTCATCTAATAGCGA	88			
<i>E. Canis</i> clone M 16S rRNA gene	TAACGTAGGCTCATCTAATAGCGA	130			

Figure 4.17: BLAST results showing the alignment of *E. canis* isolate and one of the sequences from this study which was from a dog sample from Big 5 hlabisa local municipality. The subject sequence (*E. canis* clone M), accession no: MH686052.1 covered 93% of the query sequence (KZN-dog-Hlabisa) and it had 89% identity with one gap. The black stars indicate transitions and transversions that occurred between sequences

4.5.2. Phylogenetic analysis

Evolutionary statistics were performed to find the best model for evolutionary distances and phylogeny. Three *E. canis* 16S sequences obtained from dogs in the study were aligned by clustal W on MEGA 7 along with other published sequences. Alignment revealed significant difference in the overall nucleotide composition (Table 4.22). Pairwise distance to determine the number of base substitutions per site from between sequences is shown in table 4.23. The number of base substitutions per site between sequences revealed reliable differences between the strains studied. Analysis involved 13 nucleotide sequences with 76 positions in the final data set. 1st, 2nd, 3rd codon positions as well as the noncoding positions were included in the analysis, albeit positions containing gaps and missing data were eliminated from this analysis following the example of Tamaru et al (2011). Nucleotide polymorphisms were observed between sequences and nucleotide diversity was estimated. Multiple nucleotide polymorphisms were observed between sequences with conserved regions and similar segments of the alignments in the beginning, middle and end sites. Conserved regions in the alignment are represented by dots which represent homologous nucleotides in the alignment of the 11 *E. canis* sequences (Figure 4.18). Nucleotide diversity within *E. canis* species in the study which represented the mean evolutionary diversity for the entire population was $d=0.328$ and the estimated transition/transversion bias (R) was 1.38, giving the probability of substitution from one base to another. The substitution patterns and rates were estimated under the Tamura (1992) model. The nucleotide frequencies were A= 26.10%, T/U= 22.78%, C= 21.41% and G= 29.72% (Table 4.24). The parameters on the MEGA 7 software were set as follows: bootstrap procedure with 10000 replicates, 1st, 2nd and 3rd codon positions as well as non-coding positions were also included in the analysis however, gaps and missing data in the alignments were eliminated in this evolutionary analysis (Tamaru et al. 1992). As a result, the substitution pattern test of homogeneity among sequences were performed to choose the best test to compute evolutionary distances between pathogens studied. The disparity index test was conducted for the probability of rejecting the null hypothesis that sequences have evolved with the same pattern of substitution, as judged from the extent of differences in base composition biases between sequences. A Monte Carlo test with 500 replicates was used to estimate the P-values and P-values lower than 0.05 were considered significant (Table 4.25).

The maximum likelihood tree constructed with 16S gene sequences of *E. canis* is shown in figure 4.19. Closely related sequences of the *E. canis* genes retrieved from GenBank were also included in the phylogeny. When comparing 16S nucleotide sequences from KwaZulu-Natal with other published *E. canis* strains of countries apart from South Africa, they exhibited a high level of sequence identity. The 16S nucleotide sequence of *E. canis* obtained from the study, shared 99% identity when compared with one another. These sequences were also compared with other published sequences, thereby revealing 67% identity to *E. canis* isolates from Nigeria, India, Malaysia and USA

Table 4.22. Nucleotide composition of 16S rRNA *E. canis* sequences

	T(U)	C	A	G	Total	T-1	C-1	A-1	G-1	Pos #1	T-2	C-2	A-2	G-2	Pos #2	T-3	C-3	A-3	G-3	Pos #3
KY434113.1 <i>Ehrlichia canis</i> Nigeria	25,4	20,6	27,0	27,0	126,0	26	16,7	26,2	31,0	42,0	24	26,2	11,9	38,1	42,0	26	19,0	42,9	11,9	42,0
KY434112.1 <i>Ehrlichia canis</i> Nigeria	25,4	20,6	27,0	27,0	126,0	26	16,7	26,2	31,0	42,0	24	26,2	11,9	38,1	42,0	26	19,0	42,9	11,9	42,0
KP662544.1 <i>Ehrlichia canis</i> India	25,4	20,6	27,0	27,0	126,0	26	16,7	26,2	31,0	42,0	24	26,2	11,9	38,1	42,0	26	19,0	42,9	11,9	42,0
KP662543.1 <i>Ehrlichia canis</i> India	25,4	20,6	27,0	27,0	126,0	26	16,7	26,2	31,0	42,0	24	26,2	11,9	38,1	42,0	26	19,0	42,9	11,9	42,0
KC453992.1 <i>Babesia rossi</i>	21,4	19,8	26,2	32,5	126,0	19	14,3	23,8	42,9	42,0	21	33,3	19,0	26,2	42,0	24	11,9	35,7	28,6	42,0
MF059353.1 <i>Ehrlichia canis</i> USA	24,8	21,6	26,4	27,2	125,0	24	19,0	26,2	31,0	42,0	24	26,8	9,8	39,0	41,0	26	19,0	42,9	11,9	42,0
JQ976640.1 <i>Ehrlichia canis</i> Nigeria	22,9	20,8	27,1	29,2	96,0	28	21,9	18,8	31,3	32,0	19	28,1	12,5	40,6	32,0	22	12,5	50,0	15,6	32,0
MH457245.1 <i>Ehrlichia canis</i> Malaysia	25,4	20,6	27,0	27,0	126,0	26	16,7	26,2	31,0	42,0	24	26,2	11,9	38,1	42,0	26	19,0	42,9	11,9	42,0
MG564256.1 <i>Ehrlichia canis</i> India	25,4	20,6	27,0	27,0	126,0	26	16,7	26,2	31,0	42,0	24	26,2	11,9	38,1	42,0	26	19,0	42,9	11,9	42,0
E canis 2 Assembly of 2 reads: 4 EC-R (reversed) 4 EC-F	23,6	22,0	26,0	28,5	123,0	27	17,1	22,0	34,1	41,0	22	26,8	9,8	41,5	41,0	22	22,0	46,3	9,8	41,0
E canis 3 Assembly of 2 reads: 4 EC-R (reversed) 4 EC-F	23,6	22,0	26,0	28,5	123,0	27	17,1	22,0	34,1	41,0	22	26,8	9,8	41,5	41,0	22	22,0	46,3	9,8	41,0
Average.	24,5	20,9	26,7	27,9	122,6	26	17,1	24,7	32,7	40,9	23	27,2	12,0	37,9	40,8	25	18,4	43,3	13,3	40,9

Table 4.23. Estimates of evolutionary divergence between the 16S *rRNA* gene sequences

	1	2	3	4	5	6	7	8	9	10	11
1. KY434113.1 Ehrlichia canis Nigeria											
2. KY434112.1 Ehrlichia canis Nigeria	0.000										
3. KP662544.1 Ehrlichia canis India	0.000	0.000									
4. KP662543.1 Ehrlichia canis India	0.000	0.000	0.000								
5. KC453992.1 Babesia rossi	1.588	1.588	1.588	1.588							
6. MF059353.1 Ehrlichia canis USA	0.011	0.011	0.011	0.011	1.588						
7. JQ976640.1 Ehrlichia canis Nigeria	0.034	0.034	0.034	0.034	1.501	0.045					
8. MH457245.1 Ehrlichia canis Malaysia	0.000	0.000	0.000	0.000	1.588	0.011	0.034				
9. MG564256.1 Ehrlichia canis India	0.000	0.000	0.000	0.000	1.588	0.011	0.034	0.000			
10. E canis 2 Assembly of 2 reads: 4 EC-R (reversed) 4 EC-F	0.092	0.092	0.092	0.092	1.801	0.104	0.105	0.092	0.092		
11. E canis 3 Assembly of 2 reads: 4 EC-R (reversed) 4 EC-F	0.092	0.092	0.092	0.092	1.801	0.104	0.105	0.092	0.092	0.000	

KY434113.1 Ehrlichia canis Nigeria	ATCGCTATTAGATGAGCCTACGTTAGATTAGCTAGTTGGTGAGGTAA TG GT TACCAAGGCTATGATCTATAGCTGGT
KY434112.1 Ehrlichia canis Nigeria	
KP662544.1 Ehrlichia canis India	
KP662543.1 Ehrlichia canis India	
KC453992.1 Babesia rossi	T. GA. CCC. TC. G. . . . T. GA. AG. A. . C. ATGG. . . CTG. G. . G. G. A T(G. CG. GAA. CT. . A. . GAAT. [78]
MF059353.1 Ehrlichia canis USA	
JQ976640.1 Ehrlichia canis Nigeria	 AT.
MH457245.1 Ehrlichia canis Malaysia	
MG564256.1 Ehrlichia canis India	
E canis 2 Assembly of 2 reads: 4 EC-R (reversed) 4 EC-F	T. CG. W. . . . C. . . . G. C. . . CG. . A. . . .
E canis 3 Assembly of 2 reads: 4 EC-R (reversed) 4 EC-F	T. CG. W. . . . C. . . . G. C. . . CG. . A. . . .
KY434113.1 Ehrlichia canis Nigeria	CTGAGAGGACGATCAGCCACACTGGAACCTGAGATACGGTCCAGACTC C
KY434112.1 Ehrlichia canis Nigeria	
KP662544.1 Ehrlichia canis India	
KP662543.1 Ehrlichia canis India	
KC453992.1 Babesia rossi	GACG. . A. GGC. C. . C. AGGCG. . . . G. CTGCGGCTTAATTT. . . . A
MF059353.1 Ehrlichia canis USA	 [126]
JQ976640.1 Ehrlichia canis Nigeria	 A
MH457245.1 Ehrlichia canis Malaysia	
MG564256.1 Ehrlichia canis India	
E canis 2 Assembly of 2 reads: 4 EC-R (reversed) 4 EC-F	. . ? K. . . . T. A
E canis 3 Assembly of 2 reads: 4 EC-R (reversed) 4 EC-F	. . ? K. . . . T. A

Figure 4.18: Alignment of 16S rRNA *E. canis* nucleotide sequences. The gray shaded area represent conserved regions

Table 4.24. Maximum composite likelihood estimate of the pattern of nucleotide substitution

	A	T	C	G
A	-	<i>3.82</i>	<i>3.59</i>	10.04
T	<i>4.38</i>	-	23.07	<i>4.98</i>
C	<i>4.38</i>	24.55	-	<i>4.98</i>
G	8.81	<i>3.82</i>	<i>3.59</i>	-

Rates of different transitional substitutions are shown in bold and those of transversional substitutions are shown in italics

Table 4.25. Test of homogeneity of substitution patterns between *E. canis* sequences. P-values are shown below the diagonal and the disparity index per site are shown for each sequence above the diagonal. P-values less than 0.05 are highlighted.

	1	2	3	4	5	6	7	8	9	10	11
1. KY434113.1 Ehrlichia canis Nigeria		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
2. KY434112.1 Ehrlichia canis Nigeria	1.000		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
3. KP662544.1 Ehrlichia canis India	1.000	1.000		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
4. KP662543.1 Ehrlichia canis India	1.000	1.000	1.000		0.000	0.000	0.000	0.000	0.000	0.000	0.000
5. KC453992.1 Babesia rossi	1.000	1.000	1.000	1.000		0.000	0.000	0.000	0.000	0.000	0.000
6. MF059353.1 Ehrlichia canis USA	1.000	1.000	1.000	1.000	1.000		0.000	0.000	0.000	0.000	0.000
7. JQ976640.1 Ehrlichia canis Nigeria	1.000	1.000	1.000	1.000	1.000	1.000		0.000	0.000	0.000	0.000
8. MH457245.1 Ehrlichia canis Malaysia	1.000	1.000	1.000	1.000	1.000	1.000	1.000		0.000	0.000	0.000
9. MG564256.1 Ehrlichia canis India	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000		0.000	0.000
10. E canis 2 Assembly of 2 reads: 4 EC-R (reversed) 4 EC-F	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000		0.000
11. E canis 3 Assembly of 2 reads: 4 EC-R (reversed) 4 EC-F	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	

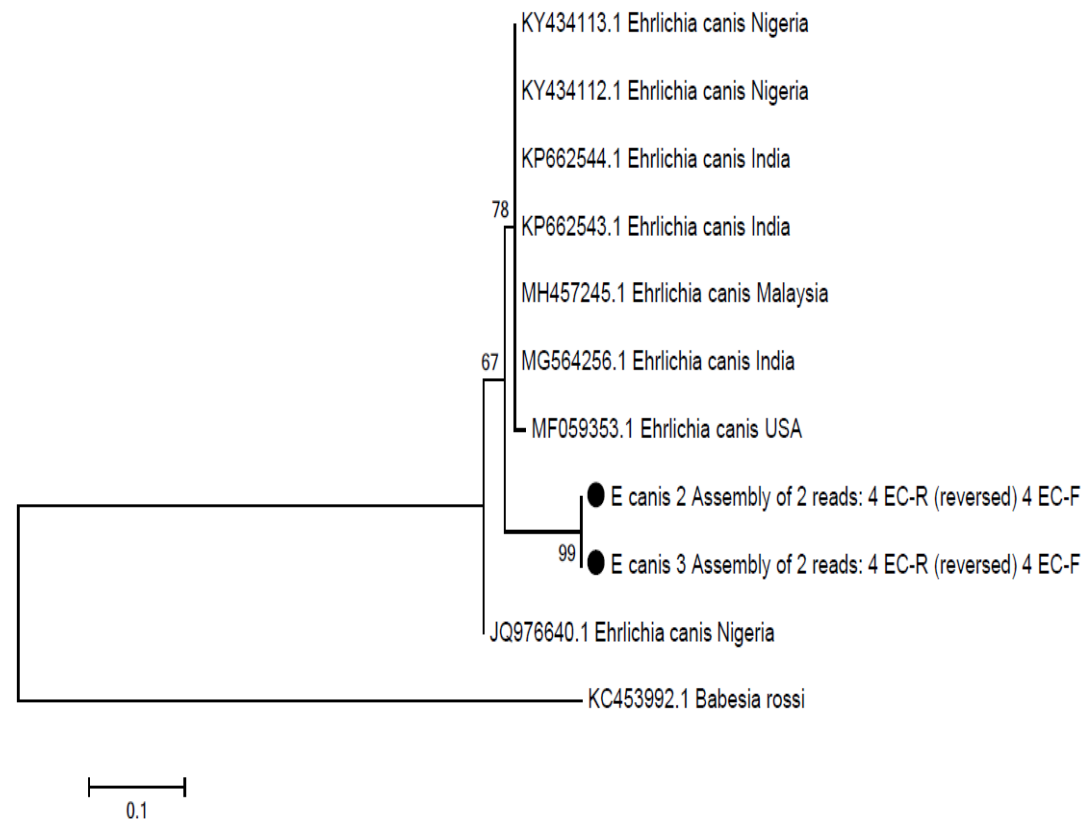


Figure 4.19: Maximum likelihood tree created from 16S rRNA nucleotide sequences of *E.canis* determined in this study (with bullets) and those retrieved from Gen Bank with accession numbers. The numbers at the branching points are bootstrap values expressed as percentages of 10, 000 replications. The horizontal scale bar indicates the number of nucleotide substitutions per site. The origin of published sequences is indicated after the isolate name. *Babesia rossi* was used as an outgroup.

CHAPTER 5

5.1. DISCUSSION, CONCLUSION AND RECOMMENDATIONS

Protozoan and ehrlichial diseases are veterinary, medically and economically important contagious diseases affecting the domestic animals in Sub-Saharan Africa, hence their prevalence and control is very important (Ademola and Onyiche 2013; Ringo et al. 2018). They have a global distribution, stretching from the polar circle to the equator. It is therefore important to document data on the occurrence of these diseases in South Africa. In the current study, molecular detection and characterization of protozoan parasites and *Ehrlichia* was performed from blood samples of domestic animals collected from KwaZulu-Natal (KZN) province of South Africa. Such information is essential for the control and prevention of infections caused by these pathogens that are responsible for the loss of livelihoods of livestock farmers (Moumouni et al. 2015).

Data produced in this study show overall protozoan parasite and *Ehrlichia* infection prevalence of 18.81% in cattle, 7.5% in sheep, 0.0% in goats and 13.61% dogs. All of the domestic animals sampled in this study appeared healthy, even though we were able to detect pathogenic protozoan and ehrlichial parasites in their blood. According to Bock et al. (2004) and Moumouni et al. (2015), a state of enzootic stability may be the reason for the absence of clinical disease in infected domestic animals. The detected pathogens included *Theileria* spp., *Babesia* spp., *Trypanosoma* spp., *Ehrlichia* spp. and *Toxoplasma gondii*.

Theileria ovis and *Babesia ovis* are specifically infect small ruminants such as sheep and goats (Oluwatayo et al. 2012) while *T. parva*, *B. bovis* and *B. bigemina* specific infect cattle. These estimates are lower than reported by Weny et al. (2017), who estimated a 30.0% and 16.4% prevalence of protozoan parasite infection in Ugandan cattle and goats, respectively. However, the protozoan parasite infection rate for cattle in the present study agrees with the work of Adua and Idahor (2017), who observed 20% protozoan infection rate in cattle from Nigeria. Be that as it may, the protozoan parasite infection rate for goats in the present study contradicts the same work of Adua and Idahor (2017), who reported a prevalence of 12.3% protozoan parasite infection rate in goats. Even though there are some differences in protozoan parasite infection among hosts in the current study and

previous studies, our study generally agrees with previous assessments that infection with protozoan parasites is persistent in domestic animals of South Africa. It was observed that cattle had the highest prevalence (18.81%) of protozoan parasite infections compared to other domestic animals in the study. This could be attributed to the accumulation of parasites by cattle due to extensive grazing and extended breeding for financial reasons such as calving and production of milk (Okorafor and Nzeako 2014). The low prevalence of protozoan parasites in the present study could be attributed to the fact that small scale farmers use chemoprophylaxis and acaricides on regular basis (Ademola & Onyiche 2013). However, the regular use of drugs and acaricides might lead to the development of drug resistance as well as presence of drug residue in meat if withdrawal period is not observed before slaughtering (Ademola & Onyiche 2013). Low prevalence of protozoan parasites could also be due to the improvement in the husbandry system, better veterinary care and climate change.

5.2. Bovine piroplasms

Babesia bigemina and *Babesia bovis* are the two economically significant species infecting cattle in Southern Africa, and they have shown to be present in all provinces of South Africa (Bock et al. 2004; Mtshali and Mtshali 2013). In the present study, the cattle infected with *B. bovis* and *B. bigemina* were found in all the three municipalities surveyed, but there were differences observed in the distribution of these protozoan parasites in cattle from some of the municipalities. The cattle found to contain DNA of both *B. bigemina* and *B. bovis* (5.8%) originated from only one of the municipalities sampled. Generally, the occurrence of both *B. bigemina* and *B. bovis* in the study area could be caused by the presence and distribution of their tick vectors (Mtshali & Mtshali 2013). In South Africa, the only vector of *B. bovis* is known to be *Rhipicephalus (Boophilus) decoloratus*, in contrast *B. bigemina* is transmitted by three tick vectors: *R. (B) microplus*, *Rhipicephalus (Boophilus) decoloratus* and *Rhipicephalus evertsi evertsi* (de Vos et al. 1994; Mtshali and Mtshali 2013). With regard to the geographical distribution of tick species in South Africa, all the three tick species that are known to transmit both *B. bigemina* and *B. bovis* are more prevalent in KZN, albeit *R. evertsi evertsi* is known to be found in all South African provinces (Baker 1989; Marufu et al. 2010). Uncontrolled movement of cattle that usually occurs within the province could also be one of the factors for the prevalence of bovine *Babesia* spp. in all municipalities sampled.

Nested PCR assays conducted in the study detected a high occurrence of *B. bigemina* as compared to *B. bovis*, these finding corresponds to a number of studies reported in KZN and elsewhere (Applewhaite et al. 1981; Miller et al. 1984; Jongejan et al. 1988; Mtshali & Mtshali 2013). The existence of *B. bigemina* and *B. bovis* in eight South African provinces was also demonstrated by Terkawi et al. (2011) using serology-based assays which showed high prevalence of *B. bovis* as compared to *B. bigemina* in KZN when tested with IFAT and ELISA (Terkawi et al. 2011).

The overall prevalence of *B. bigemina* and *B. bovis* in all samples was 30.3% and 22.2%, respectively. These prevalence values were indicative of an endemically unstable situation (Mtshali et al. 2013). By definition, an endemically unstable situation occurs when 21-60% of the cattle are infected with a particular *Babesia* Species. This low prevalence is unusual for an area that is considered to have a high prevalence of tick vectors transmitting bovine *Babesia* spp. By percentage of infection, the average prevalence of *B. bigemina* and *B. bovis* fell in the range of 1-20% (a minimal disease situation) and 21-60% (an endemically unstable situation). It was noted that uMhlabauyalingana had the lowest percentage (15.64%) of cattle infected by *B. bovis*, indicating a situation of minimal disease (Mtshali et al. 2013).

In the phylogenetic tree generated with *rap-1* gene sequences of *B. bovis*, it is clear that South African *B. bovis* isolates from this study are genetically identical to other *B. bovis* strains from South Africa, Brazil and China. The conservation observed between the *B. bovis rap-1* sequences has also been observed by Mtshali et al. (2013) and Ramos et al. (2012) in South Africa and Brazil, respectively. This conservation between geographically different isolates of *rap-1* gene sequences would be essential for the development of a diagnostic antigen in Africa. In light of the genetic diversity of this parasite in Africa, immune control strategies could be designed against the pathogen. Albeit, the isolates from the study formed a monophyletic grouping that is very distinct to that of other published *B. bovis* strains. According to Mtshali et al. (2013), this indicates the presence of micro-heterogeneities between the *rap-1* sequences within *B. bovis* strains.

With regard to the analysis of *SpeI-AvaI* restriction fragment sequence of *B. bigemina* isolates, multiple sequence alignment exhibited a high degree of polymorphism between the isolates of the present study and those of *B. bigemina* published in GenBank. Mtshali et al. (2013) also observed this high polymorphism with the analysis of a different gene

(*gp45*) sequences of *B. bigemina* in South Africa. The maximum likelihood tree constructed with the *SpeI-AvaI* restriction fragment and *gp45* sequences of *B. bigemina* yielded branches that were supported by high bootstrap values. Be that as it may, the phylogeny indicated a clear division between isolate from this study and published South African isolates of *B. bigemina*. However, isolate of this study clustered with *B. bigemina* strain from India. This could be due to the different genes used to construct a tree as there are no *SpeI-AvaI* sequences on GenBank especially from South Africa. The significant differences in the genotypes of *B. bigemina* isolates from the current study and those in GenBank permitted the discrimination among the isolates.

The *SpeI-AvaI* nPCR assay has been extensively used by a lot of researchers for detecting *B. bigemina* between susceptible cattle population, and it has proven to be highly sensitive (Figueroa et al. 1993; Oliveria-sequeira et al. 2005; Silva et al. 2009). On the other hand, the unknown identity of the target sequence and the possible disparity of primer BilBN towards the 3' end of the target region were identified as the two limitations of the *SpeI-AvaI* nPCR assay (Guerrero et al. 2007). It is also important to take notice to the fact that the *SpeI-AvaI* nested PCR assay specific for the detection of *B. bigemina* DNA also amplified a homologous fragment derived from *B. ovata* (Sivakumar et al. 2012). Nevertheless, this may not be the case in the present study, given the fact that the existence of *B. ovata* have not yet been reported in the country's cattle (Yoshinari et al. 2013; Mtshali et al. 2013). Up to the present time, only five countries (Japan, Korea, China, Mongolia and Thailand) have reported on the occurrence of *B. ovata* in cattle (Minami and Ishihara 1980; Suh 1987; Sivakumar et al. 2012; Yoshinari et al. 2013).

Theileria parva is considered the most significant theilerial species in sub-Saharan Africa and known to be the causative agent of a widespread mortality and morbidity in endemic areas. The absence of *T. parva* in the present study is comparable to results from a recent study in some parts of Nigeria where 0.0% prevalence of the pathogen was reported (Okorafor and Nzeako 2014). However, results of this study contradicts a study by Yusufmia et al. (2010), who reported a *T. parva* prevalence of 6.7% in cattle from South Africa. Observations of this study were not really surprising as it is known the Corridor disease is mainly restricted to buffaloes (Yusufmia et al. 2010) in South Africa due to strict preventative measures of the government which tries to make sure that the parasite is

not introduced to cattle. The specimens from this study were also obtained from apparently healthy cattle only. Additionally, South Africa is considered free of *T. parva*, except in designated Corridor disease-infected areas such as the Kruger National Park and Hluhluwe-iMfolozi Park which are housing wildlife.

5.3. Ovine piroplasms

Little attention has been given to ovine piroplasmosis compared to bovine piroplasmosis despite its widespread distribution through tropical and subtropical areas. According to Berggoetz et al. (2014), theileriosis in small ruminants can be caused by a number of well-known species such as *T. ovis*, *T. seperata* and *T. lestoquardi*. In the current study *T. ovis* was the only species detected (6.0%). Be that as it may, previous studies in South Africa including KZN have reported *T. ovis* with a higher infection rate in small stock whereby Ringo et al. (2018) reported an overall infection of 19.8% and Berggoetz et al. (2014) reported an overall infection of 10.9%. *Theileria ovis* which is known to be an agent of benign ovine and caprine theileriosis, and have low economic importance (Mtshali et al. 2015) was identified in 30% of sheep and 0.0% of goats. However, this study supports previous study in South Africa which could not detect *T. ovis* in goats (Berggoetz et al. 2014) although their prevalence in sheep was higher (50%). On the other hand, a recent study has detected *T. ovis* in 37.1% sheep samples but not in any of the goat samples tested using PCR assay in Sudan (Lee et al. 2018). Although the pathogen is less pathogenic it cannot be completely neglected.

The phylogenetic tree of 18S rRNA gene sequence constructed in the present study revealed that *T. ovis* from this study was placed in the same clade with other *T. ovis* sequences in this tree. The long branch length exhibited by our phylogenetic tree is an indication of increased degree of divergence among isolates of this study and other selected sequences most of which were from DRC, SA, and Iran, which suggest that geographical location could be the reason for this difference in divergence (Ringo et al. 2018).

The absence of ovine *Babesia* in this study supports previous reports by Aktas et al. (2007) and Ringo et al (2018) who could not detect this parasite in Turkey and South Africa, respectively. Similar results were also reported in Tunisia where *B. motasi* was not detected (Rjeibi et al. 2016). In Turkey and Iran, studies have demonstrated the presence

of only *B. ovis* and the absence of *B. motasi* in small ruminants (Altay et al. 2008; Esmailnejad et al. 2014). To the best of our knowledge, *B. ovis* have only been documented in northern African countries including Algeria and Tunisia (Aouadi et al. 2017; Rjeibi et al. 2014). Amongst the two pathogens, *B. ovis* is considered to be one of the most important tick-borne pathogens in small ruminants and its absence could also be an indication that the pathogen is not common in the study area. Soulby (1986) documented that small ruminants are endemically unstable for the parasite and animals that recovered from babesiosis become immune to re-infection. Further studies may be essential to confirm whether *B. ovis* and *B. motasi* are present or not in this district of KZN.

In the present study, a tick-borne pathogen (*Theileria ovis*) was only detected in sheep as compared to goats. Similar results were also observed in various countries such as China, Ethiopia, Tunisia, and Turkey, which indicated a higher occurrence of tick-borne pathogens in sheep than in goats (Aydin et al. 2015; Gebrekidan et al. 2014; Rjeibi et al. 2014). It was suggested that there are two possible reasons for the higher prevalence of Tick-borne pathogens on sheep as compared to goats: firstly, detection of ticks can be hampered by a lot of hair, which covers the sheep, resulting in persistence and low awareness of tick-borne pathogens (TBPs) in sheep. Secondly, differences in natural resistance against (TBPs) among sheep and goats could influence the prevalence of the parasites (Aydin et al. 2015; Gebrekidan et al. 2014; Rjeibi et al. 2014).

5.4. Canine piroplasms and *Hepatozoon canis*

Babesia and *Hepatozoon* species are causative agents of significant tick-borne protozoal diseases of dogs. Despite the fact that dogs are increasing in numbers due to their different purposes in the country, less attention has been given to them by researchers as compared to other animals (Abdel-Rhman et al. 2015). Canine babesiosis known as the infectious disease affecting dogs was investigated in this study. Uilenberg et al. (1989); Taboada and Mecharnt (1991); Lewis et al. (1996) documented *B. rossi* as the causative agent of canine babesiosis in South Africa due to the specificity of its vector. *Babesia* infections have been reported in numerous species of African carnivores including domestic dogs (Williams et al. 2014). Although none of the domestic dogs in our study were positive for *Babesia*, the infection is known to be common in domestic dogs in South Africa with a prevalence of approximately 10% and a low prevalence have also

been reported in Lusaka, Zambia (Collet 2000; Nalubamba et al. 2011). Failure to detect *Babesia* spp. in domestic dogs sampled in this study could be due to the fact that the sampled animals have little or no contact with wild dogs including the black backed jackal, a known natural host of *B. rossi*, or vectors, or the prevalence is too low to detect with our sample size. At present, in South Africa *B. vogelli* have only been detected from Free State and Onderstepoort Veterinary Academic Hospital, which is an indication that *B. vogeli* infection is not as widely spread as *B. rossi* (Matjila et al. 2004, 2008a).

The absence of *Hepatozoon canis* in the study is in agreement with studies by Criado-Fornelio et al. (2003) and Matjila et al. (2008a) who could not detect this pathogen in domestic dogs from Europe and South Africa, respectively. To the best of our knowledge, *H. canis* has not yet been reported in South African domestic dogs but only in wildlife. In South Africa, the lowest prevalence (0.7%) reported in African wild dogs was by (Matjila et al. 2008b). The high prevalence of the pathogen was reported in South African wildlife where Van Herden et al. (1995) found *Hepatozoon* in 89.7% of blood smears from wild dogs, and Penzhorn et al. (2018) also documented 21.9% of *Hepatozoon canis* in Black-backed jackals by the use of reverse line blot. Since *H. canis* is transmitted by ingestion of ticks, its absence in domestic dogs may be attributed to the fact that domestic dogs do not feed on live prey which reduce the probabilities of ingesting infected ticks (Christophers 1907). The parasite also seems to be present in high numbers in reticuloendothelial cells and lymphocytes, so blood samples are unlikely to harbour-infected cells (Christophers 1907).

5.5. *Toxoplasma gondii*

Infectious diseases, such as toxoplasmosis, have a substantial impact on animal productivity in sheep-farming regions of the world and some may remain undetected in flocks for prolonged periods, leading to unforeseen and unexplained abortions, foetal or new-born deaths and infertility. In some cases, these diseases lead to persistent or recurring infection in herds, resulting in poor reproductive output in the long term. Reproductive losses resulting from these diseases are a threat to the long-term economic viability of such flocks. Livestock serves as reservoirs for human infection, and this makes them important for public health.

Infection with *T. gondii* is relatively common in Domestic animals. The prevalence of the parasite differs significantly between countries and regions within the same country. The overall prevalence in the present study was 2.4% in cattle. A recent serology report by Lahmar et al. (2015), reported an overall seroprevalence of 36.8% in Tunisian domestic animals. There is no epidemiological data on livestock toxoplasmosis in this study area, and to the best of our knowledge, this is the first study that has detected *T. gondii* in cattle blood by PCR. This *T. gondii* infection has only being observed in Hlabisa Big 5 municipality local municipality, and this could be related to the fact that most samples were obtained from this area. Recent serology studies have also reported a seroprevalence of 16.9%, 10.1% and 13.81% in cattle using latex agglutination test, MAT and ELISA, respectively in Nigeria (Joshua and Akinwumi 2003; Okewole 2007; Onyiche and Ademola 2015). The low prevalence of *Toxoplasma* in cattle in the present study could be due to the fact that cattle are genetically resistant to *T. gondii*. However, its presence could be attributed to the fact that they are raised outdoors as grazing animals to meet their nutritional needs and as a result, they have more contacts with rodents and soil contaminated with oocyst (Onyiche and Ademola 2015). Differences in the levels of infection with *T. gondii* between cattle in the different studies could possibly reflect variations in exposure rates to the parasite, which could be attributed to the contamination rate of the environment. No *T. gondii* DNAs were detected in dogs, goats and sheep, this contradicts a reports from Ethiopia and Tunisia where *T. gondii* DNA prevalence was detected in 45.45% and 1.8% of goats and sheep, respectively (Gebremedhin et al. 2014; Gharbi et al. 2013). Previous studies have stated that the seroprevalence of *T. gondii* was highest in Sheep, followed by goats and then cattle (Chikweto et al. 2011, Lahmar et al. 2015). There are various risk factors such as age, sex, breed and climate conditions which may have contributed to the differences in prevalence in the current study and other studies across the world. The presence of *T. gondii* is an indication that there are stray cats in the area. It is also noteworthy that the uMkhanyakunye district is known for the frequent rains in summer, its biodiversity and traditional irrigation system. In this area, the humidity and vegetation cover protects the oocysts from desiccating thus promoting their survival and sporulation.

Genetic diversity is restricted within *T. gondii* and recombination is unusual, this has led to the suggestion that the parasite has a “clonal” population structure (Howe and Sibley 1995; Darde 1996). However, in the phylogenetic analysis of the present study, our

isolates showed a close relationship with *T. gondii* strains originating from Europe and Brazil, suggesting the high genetic similarity of our isolates with published strains. It has been documented that *T. gondii* has diverse genetic population consisting of typical clonal and atypical clonal lineages that have been grouped in 15 halo-groups in six clades. In our study the samples were not genotyped, therefore we could not differentiate or conclude that the *T. gondii* found in KZN cattle is a virulent type or not.

5.6. *Besnoitia besnoiti*

Bovine besnoitiosis has been reported to be responsible for major economic losses in various countries including South Africa (Taioe et al. 2017). In South Africa, the disease has been reported in seven provinces including KwaZulu-Natal. Álvarez-García et al. (2013) documented that prevalence studies in South Africa and Israel, were primarily depended on the detection of cysts in the vulva and conjunctiva of infected animals, and the effect of the parasite on the skin. This was mostly less than 10% as reported by Bigalke (1968). In the present study, no *B. besnoiti* DNA has been detected from sampled cattle. This is in agreement with Bwangamoi et al. (1989) and Njenga et al. (1993) who could not detect this pathogen in cattle from Kenya. In our study, the failure to detect *B. besnoiti* could be attributed to the fact that it has not been documented as a blood parasite. However, our findings contradict those of Malatji (2015) who detected an overall *B. besnoiti* prevalence of 5.3% in blood samples of cattle from KwaZulu-Natal, South Africa using PCR. Nevertheless, lack of molecular prevalence estimate studies on the pathogen makes it difficult to compare the results of our study with others.

5.7. *Trypanosoma* spp

In the present study, 18.35% of cattle showed the presence of *Trypanosoma* DNA in their blood. Boyt (1985), Van den Bossche (2006) and Mamabolo et al. (2009) documented that tsetse fly vectors prefers cattle as their hosts as compared to other animals. The presence of *Trypanosoma* spp. in cattle is an indication that the cattle from uMkhanyakude district partially have an encounter with tsetse flies and its low prevalence may be attributed to the low sample size. These findings are in agreement with a previous study by Mamabolo et al. (2009) who reported a *Trypanosoma* prevalence of 18.4% in KZN. No *Trypanosoma* DNA was detected from goats, sheep and dogs. In goats and sheep, the absence of *Trypanosoma* could be attributed to the fact that they are covered

with thick fur, which might make them least preferred hosts, by tsetse flies. Canine African Trypanosomosis (CAT) is scarce and seldom reported. In South Africa, to the best of our knowledge only two cases have been reported so far, the first case was documented by Gow et al. (2007) where a six year old dog was infected with *T. congolense* and Matjila et al. (2008b) reported a case of *T. congolense* in a dog sample from the northern parts KZN. Generally, CAT caused by *T. congolense* is fatal hence, it would not be easy to find dogs which are carriers.

5.8. *Ehrlichia canis*

Ehrlichia canis is a major pathogen in dogs throughout Africa. The high prevalence (40.8%) of *E. canis* in the blood samples of dogs from the present study is comparable to results from studies in South Africa, Zimbabwe and Namibia where 42%, 34% and 53.8% prevalence of *H. canis* was reported respectively (Pretorius and Kelly 1998; Kelly et al. 2004; Manyarara et al. 2015). Furthermore, prevalence of 19% for *E. canis* was reported by Mtshali et al. (2017) from *R. sanguineus* ticks in South Africa. It is worth noting that the dogs came from low-income area of KZN, which could have contributed to the high prevalence. In the phylogenetic tree constructed from the 16S rRNA gene data, the sequences obtained from the study formed a mono-phylogenetic grouping that is clearly different from that of other published *E. canis* sequences, and this is an indication of variation in the chemical structure of 16S rRNA sequences within *E. canis* species.

5.9. Conclusion

In conclusion, the result of this study clearly indicates that most of the domestic animals in the study area have the presence of various protozoan parasite and ehrlichial infections. Their owners may not have noticed the effects of the parasites on the animals because of the subclinical or chronic nature of the infection, which often do not result in mortality. However, their effects are usually manifested in production losses in the form of diminution of productive potential such as decreased growth rate in lambs and kids, late maturity, weight loss and increased susceptibility to other diseases. Additionally, the findings of this study has expanded our knowledge about the geographical distribution, genetic diversity and phylogenetic similarity between protozoan parasites and *Ehrlichia* isolates of South African origin. Taking into consideration the inadequate number of protozoan (*Toxoplasma*, *Babesia*) specific gene sequences available in GenBank,

especially those originating from South Africa, further studies from all provinces would be required. Without any doubt, this will allow for a better understanding of the epidemiology of protozoan parasites and *Ehrlichia* along with the degree of genetic heterogeneities among protozoan parasite isolates in South African domestic animals. To the best of our knowledge, this is the first study to comparatively investigate and characterize protozoan parasites and *Ehrlichia*, which occurs in domestic animals from uMkhanyakude district. Moreover, this is the first study to detect *T. gondii* infections in cattle using PCR in South Africa.

5.10. Recommendation

- Further studies covering larger geographical area including all South African provinces are required to estimate the prevalence, genetic diversity and economic importance of protozoan parasites.
- Future studies should evaluate the effectivity of current control studies of vector borne pathogens in rural areas of South Africa.
- A large-scale epidemiological study using serological survey is needed in order to determine the prevalence of toxoplasmosis in both domestic animals and humans.

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