Genetic diversity of *Anaplasma marginale* in cattle and in putative novel *Anaplasma* species from wildlife in Mpumalanga, South Africa

by

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Submitted in partial fulfilment of the requirements for the degree of **Philosophiae Doctor** in the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria

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DECLARATION

I declare that this thesis, which I hereby submit for the degree of **Philosophiae Doctor** at the University of Pretoria, is my own work and has not been previously submitted by me for a degree at any other tertiary institution.

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DEDICATION

This body of work is dedicated to the memory of my late deputy mom, ***Nape Evelyn Mojapelo****, you believed in me and my dreams wholeheartedly. I love you and miss you every day!*

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LIST OF ABBREVIATIONS AND ACRONYMS

16S rRNA	16S ribosomal ribonucleic acid
µl	Microlitre
µM	Micromolar
BLAST	Basic local alignment search tool
bp	Base pair
CAT	Capillary agglutination test
CCS	Circular consensus sequencing
cELISA	Competitive enzyme-linked immunosorbent assay
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FMD	Foot and mouth disease
Gb	Gigabase pair
HDSS	Health and demographic surveillance study
HHWRS	Hans Hoheisan Wildlife Research Station
HKY85	Hasegawa-Kishino-Yano, 85
IA@UP	Innovation Africa @ University of Pretoria
ICNP	International Code of Nomenclature of Prokaryotes
IFA	Immunofluorescent assay
IFAT	Indirect fluorescent antibody test
Kb	Kilobase pair
KNP	Kruger National Park
KZN	KwaZulu-Natal
LPSN	List of Prokaryotic Names with Standing in Nomenclature

Mb	Megabase pair
MEGA	Molecular evolutionary genetics analysis
min	Minute
ml	Millilitre
mm	Millimetre
mM	Millimolar
MSP	Major surface protein
NCBI `	National Center for Biotechnology Information
NGS	Next-generation sequencing
nPCR	Nested PCR
ºC	Degrees Celsius
OMP	Outer membrane protein
PacBio	Pacific Biosciences
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pH	Potential of hydrogen
qPCR	Quantitative polymerase chain reaction
QV	Quality value
RDP	Ribosomal Database Project
RLB	Reverse line blot
RNA	Ribonucleic acid
rpm	Revolutions per minute
SANParks	South African National Parks
Sec	Second
SMRT	Single-molecule real-time
TBD	Tick-borne disease

tRNA	Transfer RNA
USA	United States of America
WGA	Whole genome amplification

THESIS SUMMARY

Bovine anaplasmosis is amongst the three most important tick-borne diseases (TBDs) of ruminants in southern Africa and results in major economic losses in food and animal production not only in southern Africa but also on a global scale. This disease is mainly caused by the obligate intracellular rickettsia, *Anaplasma marginale*, which is currently widespread in South Africa. *Anaplasma centrale* can also cause disease in cattle although this is rare. Other *Anaplasma* species have been identified in cattle in South Africa, but it is not known if they cause disease. The economic impact of bovine anaplasmosis in South Africa has been estimated at approximately R115 million ($US9.6 million) per year due to mortalities and the cost of controlling the disease, as well reduced production. In other parts of the world, costs arising from anaplasmosis have been estimated from $US300 to $US800 million. Clinical signs caused by infection with *A. marginale* are characterized by fever, progressive anaemia, weight loss, abortion in pregnant cows and lowered milk production, as well as icterus that may result in mortality. Animals under one year of age are usually asymptomatic to infection with *A. marginale*. However, older animals are more likely to react severely and fatally upon challenge. Several other species of *Anaplasma* which infect cattle have been reported in South Africa: these include *A. centrale*, *A. bovis*, *A. platys* and *Anaplasma* sp. (Omatjenne). This study centres around assessing the diversity of *Anaplasma* species harboured by African wildlife and the possible impact thereof in humans and livestock situated at the wildlife-livestock interface.

The rapid advancement of high-throughput sequencing technologies in the 21st century has resulted in the discovery of a plethora of genetic material ascribed to the genus *Anaplasma* worldwide, with the proposal of over 20 new species with unique 16S rRNA sequences from various hosts since the last formal organization of the genus. The relationship of these newly detected agents to known pathogens and their ability to serve as a source of cross-reaction in detection assays, have not been well assessed. Third-generation sequencing and bioinformatics tools were used to profile *Anaplasma* populations in wildlife species roaming in the Kruger National Park (KNP) and surrounding game reserves, Mpumalanga Province, South Africa, situated adjacent to the resource-poor rural area, the Mnisi community in Mpumalanga Province, thus resulting in the wildlife-livestock-human interface in the area. In a comprehensive screening of 343 wildlife samples using an *Anaplasma* genus-specific real-time

PCR assay, *Anaplasma* species were detected in 70.0% (21/30) of African buffalo (*Syncerus caffer*), 86.7% (26/30) of impala (*Aepyceros melampus*), 36.7% (11/30) of greater kudu (*Tragelaphus strepsiceros*), 3.2% (1/31) of African wild dog (*Lycaon pictus*), 40.6% (13/32) of Burchell's zebra (*Equus quagga burchelli*), 43.3% (13/30) of warthog (*Phacochoerus africanus*), 22.6% (7/31) of spotted hyena (*Crocuta crocuta*), 40.0% (12/30) of leopard (*Panthera pardus*), 17.6% (6/34) of lion (*Panthera leo*), 16.7% (5/30) of African elephant (*Loxodonta africana*) and 8.6% (3/35) of white rhinoceros (*Ceratotherium simum*) samples. Microbiome sequencing data from the *Anaplasma*-positive samples revealed four genotypes that phylogenetically group with known and previously published *Anaplasma* 16S rRNA sequences, as well as nine novel *Anaplasma* 16S rRNA sequences. Our findings reveal a greater genetic diversity of *Anaplasma* sequences and potentially novel species circulating in wildlife hosts in South Africa than are currently classified within the genus *Anaplasma* which might be transmitted to livestock or companion animals. Furthermore, these putative species are phylogenetically similar to known *Anaplasma* spp. and may possibly serve as a source of cross-reaction in the current detection assays. Our findings further highlight the need for additional genetic data and genome sequencing of these putative species for correct *Anaplasma* species classification and further assessment of their occurrence in livestock and companion animals.

Data collected previously in the study area of the Mnisi Community in the Mpumalanga Province indicated the presence of *A. marginale*, with occasional bovine anaplasmosis cases reported at villages close to the wildlife-livestock interface. In an attempt to understand the clinical cases of bovine anaplasmosis in the study area, the infection dynamics and *A. marginale* strain diversity during a 12-month period were examined in ten calves in a peri-urban area and at a wildlife-livestock interface. The composition of *Anaplasma* species circulating in these calves was also assessed. *Anaplasma marginale* was detected in all five calves in the peri-urban area from the first month, but in only two calves at the wildlife-livestock interface and only after six months. *Msp1α* genotype analysis revealed 42 *A. marginale* genotypes in calves in the peri-urban area and ten genotypes in calves at the wildlife-livestock interface, with superinfections evident in calves from both areas. The 16S microbiome sequencing data revealed the presence of four *Anaplasma* species circulating in the ten calves. Of the total number of *Anaplasma* 16S rRNA sequences detected, 87% were identified in calves in the peri-urban area and 13% in calves at the wildlife-livestock interface. The 16S rRNA sequencing data consisted mostly of *A. platys*-like 16S rRNA sequences (83.3%), followed by *A. marginale* (16.6%) and *A. boleense* (<0.1%). Our findings therefore

suggest that the occasional bovine anaplasmosis cases observed at the wildlife-livestock interface in the Mnisi communal area might be attributed to a localised lack of endemic stability since calves at the wildlife-livestock interface are not continually infected with *A. marginale* in their first year when natural immunity is higher. Our findings further highlight complex *A. marginale* infection in infected cattle driven by co-infection and superinfection by distinct *A. marginale* strains in both areas within the 12-month study period, indicating continuous challenge with multiple strains that should lead to robust immunity in infected animals. Other *Anaplasma* species detected in the calves might be due to proximity with wildlife hosts and might confer cross-protection against *A. marginale* thus contributing to endemic stability, but this requires further investigation.

Considering the strain variation detected in *A. marginale* in the Mnisi community and other South African provinces (based on previous studies) and the difficulty of isolating and culturing *Anaplasma* species, alternative methods of genome sequencing of *Anaplasma* species from cattle and wildlife in South Africa are required. An attempt was therefore made to obtain *A. marginale* genome sequence data directly from infected carrier cattle. Cattle at the Innovation Africa @ University of Pretoria Experimental Farm were screened for the presence of *A. marginale* and other haemoparasites and the *msp1α* genotypes in *A. marginale*-positive animals were determined. Blood was drawn from cattle infected with a single *A. marginale* strain and no other haemoparasites, red blood cells were separated and washed seven times with phosphate-buffered saline. High molecular weight DNA was extracted directly from the washed red blood cells. Three rounds of microbial enrichment were conducted to deplete the host DNA in the sample, followed by whole genome amplification. The resulting DNA sample was sent for whole genome sequencing on a Pacific Biosciences (PacBio) sequencing platform. A total of 298 058 raw PacBio reads were retrieved from the PacBio single-molecule real-time (SMRT) analysis 2.3.0 software, which were mainly bovine host reads. *Anaplasma* reads mapped to the *A. marginale* St Maries and *A. marginale* Florida reference genomes resulted in two different incomplete *A. marginale* assemblies, each informed by the reference sequence. Further sequencing data is thus needed for full closure of the genome sequence. Advances in molecular techniques for microbial DNA enrichment and sequencing, and assortment of contigs into species-specific bins and assembly of binned data could be incorporated in this study to complete the *A. marginale* genome. Such a technique could then be used to obtain the whole genome sequences of the different *Anaplasma* spp. circulating in livestock, wildlife and companion animals without the need to culture. Therefore, there is a need for molecular

techniques for microbial DNA enrichment and metagenomics to generate more genome sequences of *A. marginale* and the different *Anaplasma* spp. circulating in livestock, wildlife and companion animals. This will allow for correct classification in the *Anaplasma* taxonomy and to study the natural rate of variation between the different *Anaplasma* species and their specific genotypes and to fully understand their evolution and diversity. This will further assist with the identification of species-specific targets for the development of more specific serological and nucleic-acid-based detection methods suitable for examining the epidemiology of all *Anaplasma* spp. from various hosts.

CHAPTER 1

Introduction

Major limitations to livestock farming throughout the world but particularly in Africa are ticks and tick-borne diseases (TBDs) (Smith & Parker, 2010). Ticks and TBDs together with gut associated helminth infections arguably represent the single biggest burden for livestock farming in South Africa, especially for the resource-constrained smallholder farmers, who represent the majority of farmers in the country. Bovine anaplasmosis (or gall sickness as popularly known) is a TBD of cattle caused by infection with the obligate intracellular bacterium, *Anaplasma marginale* (Theiler, 1910; Aubry & Geale, 2011). The causative agent of bovine anaplasmosis was first characterised and isolated by Sir Arnold Theiler between 1907 and 1910 in South Africa. Theiler noted the 'marginal points' of *A. marginale*, in blood smears from tick infested cattle imported from England to South Africa (Theiler, 1910, 1911, 1912).

Anaplasma marginale is the most prevalent tick-borne pathogen in tropical and subtropical areas of the world, causing bovine anaplasmosis in Africa, the United States of America (USA), Central and South America, Asia, southern Europe, and Australia (Kahn & Line, 2010), with symptoms ranging from weight loss, fever, abortion to lower milk yields and mortality in up to 36% of clinical cases (Kocan et al., 2004; Aubry & Geale, 2011; Hammac et al., 2013).

Bovine anaplasmosis ranks amongt the top three most economically important TBDs affecting cattle in southern Africa resulting in major economic losses. These include expensive control measures, decreased meat and milk production, morbidity and even death (Uilenberg, 1995; de Waal, 2000; Makala et al., 2003; Mtshali, de Waal & Mbati, 2004). Bovine anaplasmosis is prevalent and endemic in most cattle-farming regions in South Africa (de Waal, 2000; Mtshali, de Waal & Mbati, 2004; Mutshembele et al., 2014; Hove et al., 2018), with an approximate annual cost of R115 million ($US9.6 million) from cattle mortalities (Hove, 2018). Further economic impacts are associated with the costs of treatment (tetracycline compounds) and control (vaccination or tick control with acaricides).

The most important route of transmission of bovine anaplasmosis from infected to naïve cattle is through tick bites; however, infected erythrocytes can also be transmitted mechanically by

biting flies or veterinary instruments. In addition, transplacental transmission has been reported (Aubry & Geale, 2011). *Rhipicephalus decoloratus* and *R. microplus* are the two most important tick species assumed to be playing a key role in the transmission of anaplasmosis in South Africa (Potgieter & Stoltsz, 2004; Nyangiwe, Harrison & Horak, 2013; Nyangiwe et al., 2017).

Control of bovine anaplasmosis worldwide involves the use of acaricides to control tick vector infestation, immunization with the less virulent *A. centrale* formulated as a live blood-borne vaccine, intentional exposure of calves to fields known to contain infercted ticks and treatment with tetracycline compounds including chlortetracycline, oxytetracycline and tetracycline (Blouin et al., 2002; Kuttler, 1980). Immunization with *A. centrale* does not prevent infection with *A. marginale*, but does prevent acute disease (Bigalke, 1980). Despite these recommended means of controlling anaplasmosis, more than 99% of the cattle population in South Africa is at risk of being infected, resulting in cattle mortalities (de Waal, 2000).

1.1. Problem Statement

Bovine anaplasmosis is currently prevalent and endemic in most cattle-farming regions in South Africa, except for the Northern Cape Province, where the tick vector mostly does not occur (de Waal, 2000; Mtshali, de Waal & Mbati, 2004; Mutshembele et al., 2014; Hove et al., 2018). Data collected in the study area of the Mnisi Community, Mpumalanga Province indicate the presence of *A. marginale* in cattle, with sporadic bovine anaplasmosis cases reported at villages close to the wildlife-livestock interface (Choopa, 2015).

The live, blood-borne *A. centrale* vaccine is available for bovine anaplasmosis in South Africa, but it is costly to produce due to the requirements of live animals and maintaining a strict cold chain. Furthermore, there is potential risk of introducing unintended blood-borne pathogens (de Waal, 2000). In some areas, enzootic stability contributes to disease resistance (Dreyer et al., 1998), but the mechanisms underlying enzootic stability and the causes of disease outbreaks have not been well studied. In order to control ticks and TBDs in an affordable and sustainable way, it is of crucial importance to improve our comprehension of the pathogen ecology and transmission along with development of improved tools for pathogen detection and identification, and development of safer, more effective vaccines.

The most accurate and recommended serological test available for the detection of *A. marginale* is a competitive enzyme-linked immunosorbent assay (cELISA) that uses a monoclonal antibody (MAb) ANAF16C1 specific for major surface protein (MSP5) of *Anaplasma* spp. (Knowles et al., 1996; Torioni de Echaide et al., 1998; Strik et al., 2007). The assay is known to cross-react with other *Anaplasma* species (Dreher et al., 2005), and will therefore probably cross-react with *Anaplasma* sp. (Omatjenne) and other novel *Anaplasma* sp. occuring in South Africa. Therefore, the existing tests cannot be used to distinguish between *A. marginale* and other *Anaplasma* spp. in mixed infections in the host, because they all express the *Anaplasma* specific MSP5 antigen and induce antibodies recognized by the MSP5-specific Mab (Visser et al., 1992). In South Africa various species of *Anaplasma* have been shown to be present in cattle, often as co-infections. These include *A. marginale*, *A. centrale*, *A. bovis* and *Anaplasma* sp. (Omatjenne) (de Kock et al., 1937; Zweygarth et al., 2006; Harrison et al., 2013; Khumalo et al., 2016; Hove et al., 2018). A more specific serological test, which could accurately identify *A. marginale* and distinguish it from other *Anaplasma* spp., would assist in the unambiguous detection of the pathogen and eventually in the application of appropriate control measures. While there is evidence that multiple *Anaplasma* spp. are present in wildlife in South Africa, the full range of *Anaplasma* spp. remains unknown, and their impact on the epidemiology of anaplasmosis in domestic animals is currently unclear. Therefore, this study was aimed at assessing the range of *Anaplasma* spp. in wildlife, by analyzing the 16S microbiome to identify additional *Anaplasma* spp. that could contribute to erroneous testing with existing serological tests. Furthermore, in the first steps towards developing an *A. marginale*-specific serological diagnostic assay, we plan on devising methods to sequence South African strains of *A. marginale* and other *Anaplasma* species from carrier animals in order to identify genes and proteins that are specific for each *Anaplasma* species.

1.2. OVERALL AIM

Bovine anaplasmosis caused by *A. marginale* is currently widespread and endemic throughout the cattle-farming areas in South Africa, with sporadic cases being reported continuously. Furthermore, there is limited understanding about the range and genetic diversity of *Anaplasma* species occurring in South African wildlife hosts with potential for transmission to companion animals, livestock and humans and which could further contribute to erroneous testing with existing serological assays. Therefore, to address these questions, this study was aimed at

conducting genomic and 16S microbiome analysis of South African *Anaplasma* spp. to identify and examine other *Anaplasma* spp. circulating in South Africa. Genome sequences generated from other *Anaplasma* species identified in wildlife and cattle could be compared to existing *Anaplasma* genome sequences to identify more specific targets for serological and nucleic-acid based tests for specific detection of *A. marginale*.

1.3. SPECIFIC OBJECTIVES:

- **Identification of *Anaplasma* species in wildlife hosts in the Kruger National Park and surrounding game reserves using a bacterial microbiome approach.**
  Use a targeted 16S microbiome analysis to identify *Anaplasma* spp. in the blood of wildlife species to improve our understanding of the epidemiology of organisms causing anaplasmosis.

- **Assess the temporal dynamics of *Anaplasma marginale* infections and the composition of *Anaplasma* spp. in calves in the Mnisi communal area, Mpumalanga, South Africa.**
  Investigate the occurrence of *Anaplasma* spp. infections in ten calves in two areas (peri-urban and wildlife-livestock interface) of the Mnisi community over a one-year period, identify *A. marginale* genotypes circulating in each village, determine *A. marginale* strain infections and superinfections in the calves.

- **Genome sequence analysis of an *Anaplasma marginale* strain from South Africa.**
  Explore a method for obtaining the genome sequence of a South African strain of *A. marginale* from blood collected from a carrier animal without infecting splenectomized cattle or initiating *in vitro* cultures.

1.4. References

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CHAPTER 2

Literature Review

2.1. *Anaplasma* species

Historically, *Anaplasma* spp. have been difficult to classify due to their intracellular nature and the consequent difficulties associated with producing cultures. The taxonomy of rickettsial pathogens began with the discovery of *A. marginale* over a century ago. Since then, the classification of *Anaplasma* spp. has ranged from viruses to bacteria to protozoans (Brayton et al., 2009). The classification of *Anaplasma* species within the order Rickettsiales was only established in the seventh edition of Bergey's manual in 1957 (Ristic & Kreier, 1974). *Anaplasma marginale* was initially recognized and described by sir Arnold Theiler in South Africa (Theiler, 1910). Theiler named the organism *Anaplasma marginale* referring to the lack of stained cytoplasm (hence "*Anaplasma"*) and the location of the parasite at the margins of bovine erythrocytes (hence "*marginale*"). In 1911, Theiler discovered and characterized a parasite with similar features to *A. marginale* and named it *A. marginale* variety *centrale* (Theiler, 1911). Subsequently additional *Anaplasma* species were identified, including *A. bovis* (Dumler et al., 2001) (formerly known as *Ehrlichia bovis*) (Donatien & Lestoquard, 1936), *A. ovis* (Bevan, 1912), *A. platys* (Dumler et al., 2001) (formerly known as *E. platys*) (Ristic et al., 1981) and *A. phagocytophilum* (Dumler et al., 2001) (formerly known as *E. phagocytophila*) (Tyzzer, 1938).

In 2001, species in the order Rickettsiales were reorganized and reclassified into two families: *Rickettsiaceae*, which grow freely in the cytoplasm of the eukaryotic host cell and *Anaplasmataceae*, which replicate within a vacuole in the eukaryotic host cell (Dumler et al., 2001). The reorganization of the species was based on phylogenetic analyses of the 16S rRNA and *groEL* genes. The family Anaplasmataceae now comprises the following genera: *Anaplasma*, *Ehrlichia*, *Neorickettsia* and *Wolbachia.* Dumler et al. (2001) further reorganized the genus *Anaplasma* to include species which were previously classified within the genus *Ehrlichia*. These included *Anaplasma platys, Anaplasma phagocytophilum* and *Anaplasma bovis.* Species within the genus *Anaplasma* can be divided into two clades, the ruminant clade and the *A. platys*-like clade, often referred to as the zoonotic clade, as highlighted previously Caudill & Brayton (2022). The ruminant clade comprises *Anaplasma* spp. infecting

erythrocytes of livestock, such as *A. marginale*, *A. centrale* and *A. ovis*, while the *A. platys*-like clade consists of organisms in the genus *Anaplasma* infecting leukocytes and platelets, and includes *A. platys*, *A. bovis* and *A. phagocytophilum* (Caudill & Brayton, 2022) (Figure 2.1).

Although Dumler et al. (2001) clarified the taxomomic status of many of the *Anaplasma* species, the species status of *A. centrale* remained uncertain, due to insufficient molecular evidence to confirm the taxonomic status of *A. marginale* and *A. centrale*. Recent studies using phylogenetic analyses of the *groEL, msp4* and 16S rRNA gene sequences from several field samples of *A. marginale* and *A. centrale* in South Africa (Khumalo et al., 2018), as well as variation in *Msp1a*/*Msp1aS* gene structure (Khumalo et al., 2016), and overall genomic structure (Brayton, Dark & Palmer, 2009; Herndon et al., 2010), elucidated that *A. centrale* is indeed distinct from *A. marginale*.

Anaplasma sp. (Omatjenne) [formerly *Ehrlichia* sp. (Omatjenne)] (Allsopp et al., 1997; Zweygarth et al., 2006), *A. odocoilei* (Tate et al., 2013) and the zoonotic "*A. capra"* (Li et al., 2015; Yang et al., 2017) are additional species described in the genus *Anaplasma* based on morphology and sequence analysis; however, they are not formally recognized in the taxonomic literature. In fact, according to the most recent List of Prokaryotic names with Standing in Nomenclature (LPSN) (Parte et al., 2020), there are only five validly published *Anaplasma* species: *A. marginale, A. ovis*, *A. centrale*, *A. phagocytophilum*, and *A. caudatum* (even though experts in the field consider this last "species" to be a tailed strain of *A. marginale*). On the other hand, *A. bovis*, *A. platys*, *A. odocoilei* and *A. capra* are considered to have been "effectively published" and are generally accepted as *Anaplasma* species in the literature, but they are not considered to be validly published according to the International Code of Nomenclature of Prokaryotes (ICNP) (Parker et al., 2019).

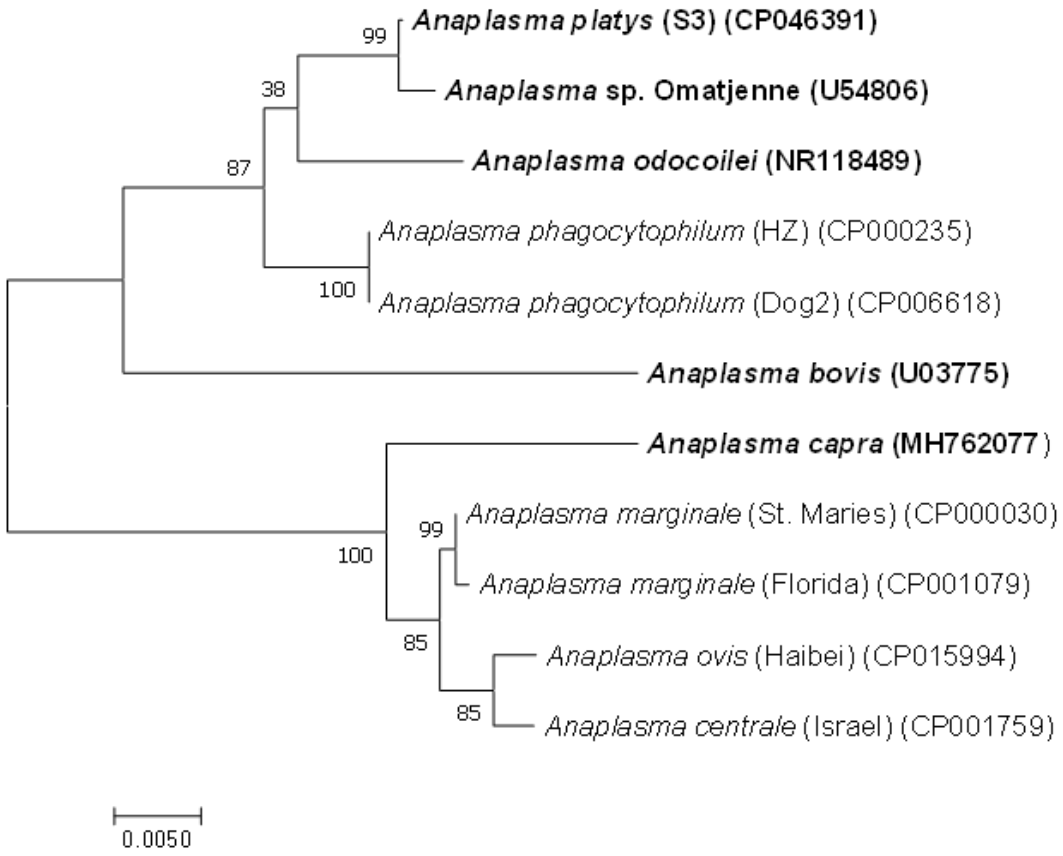

Figure 2.1: Maximum likelihood phylogenetic tree, modified from Caudill & Brayton et al. (2022), based on 16S rRNA gene sequences from the validly and effectively published species in the genus *Anaplasma*, as well as additional species described in the genus. Sequences were approximately 1427 bp in length. Species printed in bold are "effectively published" and are generally accepted as *Anaplasma* species in the literature, but they are not considered to be validly published according to the International Code of Nomenclature of Prokaryotes (ICNP). The evolutionary history inferred by using the maximum likelihood method based on the HKY85 (Hasegawa, Kishino & Yano, 1985) evolutionary model in MEGA 7 (Kumar, Stecher & Tamura, 2016). The numbers at the internal nodes represent the percentage of 1000 replicates (bootstrap). The accession numbers of each sequence are indicated next to the sequence name. Branch lengths are proportional to the estimated genetic distance between the taxa. Scale bar refers to a phylogenetic distance of 0.005 nucleotide substitutions per site.

The new age of high-throughput sequencing technologies has allowed for an explosion of molecular, taxonomic and metagenomic analyses. These efforts have led to the identification of numerous sequences ascribed to the genus *Anaplasma* globally since the genus was formally organized. These studies have resulted in the proposal of over 20 new *Anaplasma* species with distinct 16S rRNA sequences (Caudill & Brayton, 2022). Putative *Anaplasma* spp. have recently been reported from a variety of hosts such as human, livestock, wildlife and/or from vectors across the world (Caudill & Brayton, 2022). *Anaplasma capra*, is a novel zoonotic pathogen, found in humans, domestic and wildlife hosts and dogs in China, and was identified

based on the 16S rRNA, *gltA*, *groEL*, *mps2* and *msp4* genes (Li et al., 2015; Sun et al., 2015). A novel *Anaplasma* species, "*Candidatus* Anaplasma camelii", was identified in camels in Saudi Arabia (Bastos et al., 2015) and Iran (Sharifiyazdi et al., 2017) by sequence analysis of the 16S rRNA and *groEL* genes. "*Candidatus* Anaplasma corsicanum" and "*Candidatus* Anaplasma mediterraneum" were identified in sheep in France (Dahmani et al., 2017), while "*Candidatus* Anaplasma africae" was found in sheep, cattle and goats in Senegal by sequence analysis of the 23S rRNA, *rpoB,* and *groEL* genes (Dahmani et al., 2019). "*Candidatus* Anaplasma boleense" was identified from cattle and mosquitoes in China by sequence analysis of the *groEL, gltA* and 16S rRNA genes (Guo et al., 2016) and has further been identified in cattle in Mozambique (Fernandes et al., 2019) and South Africa (Kolo et al., 2020), as well in *R. microplus* ticks and goats from Argentina by sequence analysis of the 16S rRNA gene (Sebastian et al., 2023). *Anaplasma* sp. Hadesa, *Anaplasma* sp. Saso and *Anaplasma* sp. Dedessa that were initially reported in cattle in Ethiopia (Hailemariam et al., 2017), have also been identified in South Africa (Kolo et al., 2020). "*Candidatus* Anaplasma rodmosense" was found in mosquitoes in China (Guo et al., 2016), while "*Candidatus* Anaplasma ivorensis" was identified in *Amblyomma variegatum* in Côte d'Ivoire based on 23S rRNA gene sequencing (Ehounoud et al., 2016). The 16S rRNA and *gltA* gene sequence analysis revealed a novel *Anaplasma* sp. (*Anaplasma* sp. SA dog or *Anaplasma* sp. ZAM dog) in dogs (Inokuma et al., 2005; Vlahakis et al., 2018; Kolo et al., 2020) which has also been identified in cattle, as well as in *R. sanguineus* ticks (Kolo et al., 2020), in South Africa and Zambia. *Anaplasma* sp. Mymensingh was identified in cattle in Bangladesh by sequence analysis of the 16S rRNA and *groEL* genes (Roy et al., 2018) and in South Africa by 16S rRNA microbiome analysis (Kolo et al., 2020). *Anaplasma* sp. Mongolia is another putative *Anaplasma* sp. that was recently reported in cattle, sheep and *Dermacentor nuttalli* ticks in Mongolia (Fischer et al., 2020). Furthermore, novel 16S rRNA *Anaplasma* genotypes have also been reported in cattle in Uganda (Ikwap et al., 2010; Muhanguzi et al., 2010).

Putative *Anaplasma* spp. recently identified from wildlife hosts include "*Candidatus* Anaplasma sphenisci" detected from erythrocytes of an African penguin (*Spheniscus demersus*) in South Africa by sequence analysis of the *groEL* and 16S rRNA genes (Vanstreels et al., 2018). "*Candidatus* Anaplasma pangolin" was identified from pangolins in Malaysia (Koh et al., 2016), while *Anaplasma* sp. strain AnAj360 was identified from *Amblyomma javanense* ticks collected from pangolins in Thailand (Parola et al., 2003). The 16S rRNA gene sequence analysis was used to identify "*Candidatus* Anaplasma testudinis" from tortoises that

presented with anemia and intracytoplasmic vacuoles containing bacteria within erythrocytes in Florida, USA (Crosby et al., 2021). "*Candidatus* Anaplasma brasiliensis" from anteaters and "*Candidatus* Anaplasma amazonensis" from sloths in Brazil were identified using sequence analysis of the 16S rRNA gene and 23S–5S intergenic region (Calchi et al., 2020). Recently, a novel *Anaplasma* spp. was identified, by *gltA* and 16S rRNA gene sequence analysis, in *Argas walkerae* and *Ornithodoros moubata* ticks collected from African warthog burrows in Zambia (Qiu et al., 2021). In the absence of genome sequences for these putative agents, there is a need for more sequence data, both from individual genes and genomes. The consistent use of a combination of different *Anaplasma* genes such the *groEL*, 16S rRNA, *msp4* and *gltA* genes would assist in the correct classification of putative *Anaplasma* species, although it may not always be possible to amplify all of the genes from all samples, if gene sequences are not conserved between *Anaplasma* species or if the organisms are present at very low levels in the samples.

2.2. *Anaplasma* species of cattle in South Africa

2.2.1 *Anaplasma marginale*

Anaplasma marginale was fully described by Sir Arnold Theiler in 1910. He observed "marginal points" (inclusion bodies) in infected erythrocytes of calves and concluded that this was the causative agent of gall sickness in South Africa (Theiler, 1910).

Anaplasma marginale is the most globally prevalent tick-borne pathogen of ruminants; it is an obligate intracellular rickettsial pathogen and is the main causative agent of anaplasmosis in ruminants, predominantly cattle (Kocan et al., 2003; Brayton, Dark & Palmer, 2009). This pathogen is known to be highly pathogenic and responsible for almost all outbreaks of clinical anaplasmosis (Brayton, Dark & Palmer, 2009). Although cattle of all breeds can be infected by *A. marginale*, the severity of anaplasmosis depends on age, nutritional status, and herd management (Aubry & Geale, 2011).

Bovine anaplasmosis caused by *A. marginale* is widespread globally, occurring in tropical and subtropical regions and is a major constraint to cattle production (Kocan et al., 2003). Anaplasmosis is widely distributed in the USA, with seasonal outbreaks recorded frequently during summer and autumn due to an increased number *A. marginale* transmitting ticks and blood-sucking flies (Kocan et al., 2003, 2010). It is considered to be one of the three most

important TBDs affecting cattle in South Africa. It is prevalent in all of the cattle-farming areas in South Africa except the Northern Cape Province where the tick vectors do not occur (de Waal, 2000). It was proposed that more than 99% of the cattle population in South Africa is at risk of being infected with *A. marginale* and that infection results in 3% of the country's cattle mortalities (de Waal, 2000).

Studies conducted in Limpopo (Rikhotso et al., 2005), North West (Ndou et al., 2010) and Free State Provinces (Dreyer et al., 1998; Mbati et al., 2003), provided serological evidence of *A. marginale* in the South African cattle population. Mutshembele et al. (2014) reported the presence and molecular prevalence of *A. marginale* in South African cattle. In their study, *A. marginale* was prevalent in all South African provinces except the Northern Cape, with the highest prevalence in Mpumalanga, Gauteng and Eastern Cape Provinces (Mutshembele et al., 2014). Hove et al. (2018) corroborated these findings, by identifying *A. marginale* in a larger sample size of cattle in all South African provinces except the Northern Cape, where the cattle poplution is relatively small and *A. marginale*-transmitting tick vectors are mostly absent. However, Hove et al. (2018) found that the highest prevalence of *A. marginale* was in Mpumalanga, Western Cape and KwaZulu-Natal (KZN) Provinces. These results are all consistent with the distribution of *A. marginale* in South Africa as reported by de Waal (2000) (Figure 2.2).

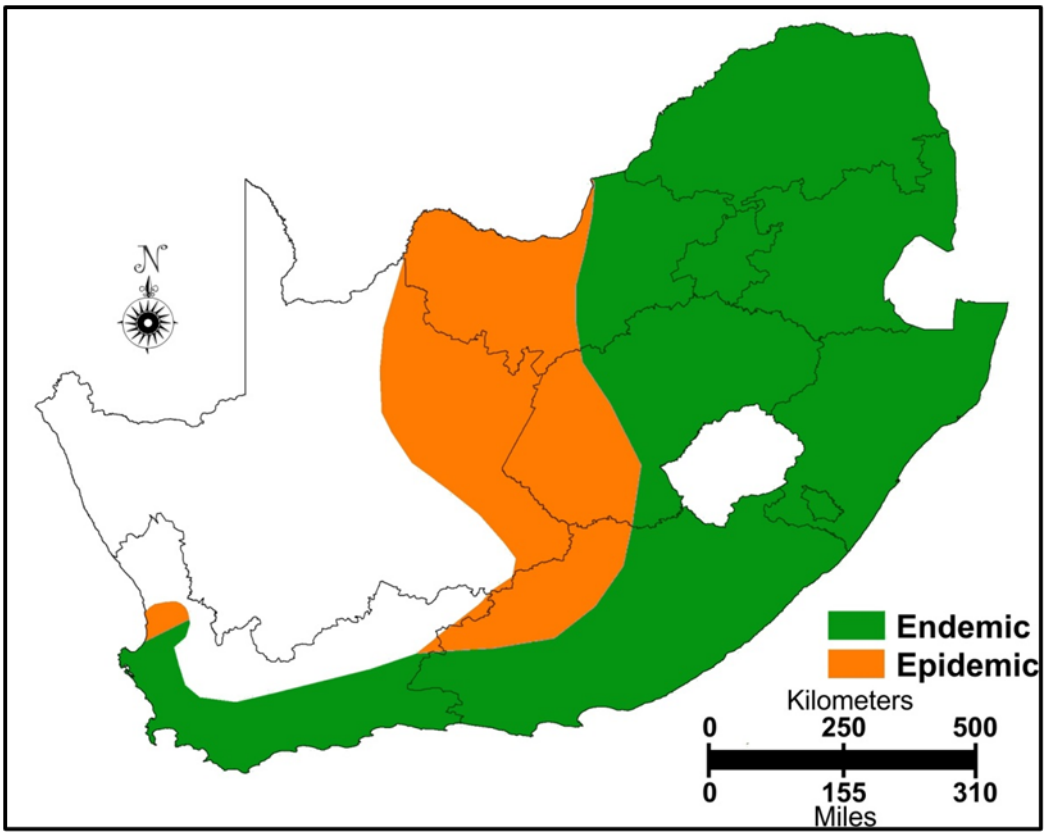

Figure 2.2: The distribution of *Anaplasma marginale* in South Africa. Endemic areas are shown in green and epidemic areas in orange (de Waal, 2000).

2.2.1.1. Transmission of *A. marginale*

Anaplasma marginale can be transmitted biologically by ticks and mechanically by blood-contaminated veterinary instruments or biting flies (Kocan, Blouin & Barbet, 2000). The efficiency of transmission of *A. marginale* by biting flies has been reported to be very low (Scoles et al., 2005), and it is thought that biological transmission by ticks is the most important means of transmission (Potgieter, 1979; Eriks, Stiller & Palmer, 1993; Kocan, Blouin & Barbet, 2000). *Anaplasma marginale* can also be transmitted transplacentally (Aubry & Geale, 2011; Costa et al., 2016). Although the prevalence of transplacental transmission of *Anaplasma* infections in new-born calves from infected cows has been poorly studied, transplacental transmission was demonstrated by experimental studies (Potgieter & van Rensburg, 1987a; Pypers, Holm & Williams, 2011; Grau et al., 2013; Da Silva & Da Fonseca, 2014), and is mainly associated with acute infection of the dam between the second and third trimesters of pregnancy (Fowler & Swift, 1975; Swift & Paumer, 1976; Potgieter & van Rensburg, 1987a; Kahn & Line, 2010). Although the incubation period of *A. marginale* varies from days to months, in a recent study (Makgabo, 2019), calves that were only a few days old

located at the wildlife-livestock interface in Mpumalanga Province, South Africa tested positive for *A. marginale* DNA using a real-time PCR assay; this could indicate either that transplacental transmission of *A. marginale* occurred or that these calves were exposed from birth to vectors that transmit *A. marginale*.

Biological transmission of pathogens by ticks can occur in three different ways; transstadially,when a tick remains infected from one life stage to the next before transmiting the pathogen to the host, intrastadially, when a tick in the same life stage transmits a pathogen between two hosts, and transovarially, when the pathogen is passed from parent female ticks to offspring and the resulting larvae transmit it to the host (Stich et al., 1989; Kocan et al., 2010; Fournière et al., 2022). The principal tick vector of *A. marginale* are Ixodid ticks (Eriks, Stiller & Palmer, 1993; Potgieter & Stoltsz, 2004). In the north-western USA, *Dermacentor andersoni* is a three-host tick which transmits for the *A. marginale* intrastadially and transstadially, while in the eastern USA *Dermacentor variabilis* transmits *A. marginale* (Kocan, Blouin & Barbet, 2000).

In South Africa, experimental tick transmission studies revealed that *A. marginale* can be transmitted intrastadially by adult *Rhipicephalus microplus*, *R.decoloratus* , *R. simus, R. evertsi evertsi* and *Hyalomma rufipes* ticks. Furthermore, *R. decoloratus*, *R. microplus*, and *R. simus* ticks also transmit *A. marginale* transstadially (Potgieter, 1981; de Waal, 2000; Potgieter & Stoltsz, 2004). Transovarial transmission of *A. marginale* is thought not to occur (Eriks, Stiller & Palmer, 1993; Kocan, Blouin & Barbet, 2000), although it has been recently reported under natural conditions and experimentally in *R. microplus* ticks (Fournière et al., 2022). Studies on the distribution of *R. microplus* in South Africa highlighted that the tick vector is currently spreading within the country and therefore playing an increasing role in the transmission of various pathogens, including *A. marginale* (Nyangiwe, Harrison & Horak, 2013; Nyangiwe et al., 2017).

Male ticks, in particular, are considered important for the spread of anaplasmosis, as they may feed on more than one bovine host, while adult female ticks generally feed on a single bovine host. It has been previously shown that male ticks can quickly become infected with *A. marginale* from an infected bovine host, with colonies forming in the tick midgut after the acquisition feed (Leverich et al., 2008; Noh et al., 2011). The male ticks feed on a susceptible bovine host for a period of four to eight days, they then detach and move to another host in

search of a female tick and this results in intrastadial transmission of the parasite between cattle hosts (Kocan et al., 1992; Eriks, Stiller & Palmer, 1993; Kocan, Blouin & Barbet, 2000; Dumler et al., 2001).

2.2.1.2. Life cycle of *A. marginale*

The life cycle of *A. marginale* in ticks is complex, as illustrated in Figure 2.3. The development of *A. marginale* in the tick occurs when the tick ingests infected erythrocytes from infected bovine host during a blood meal, resulting in propagation of *A. marginale* in the midgut tissues and salivary gland cells. *Anaplasma marginale* in infected salivary gland cells are infective for cattle during tick feeding (Kocan, 1986; Kocan et al., 1992).

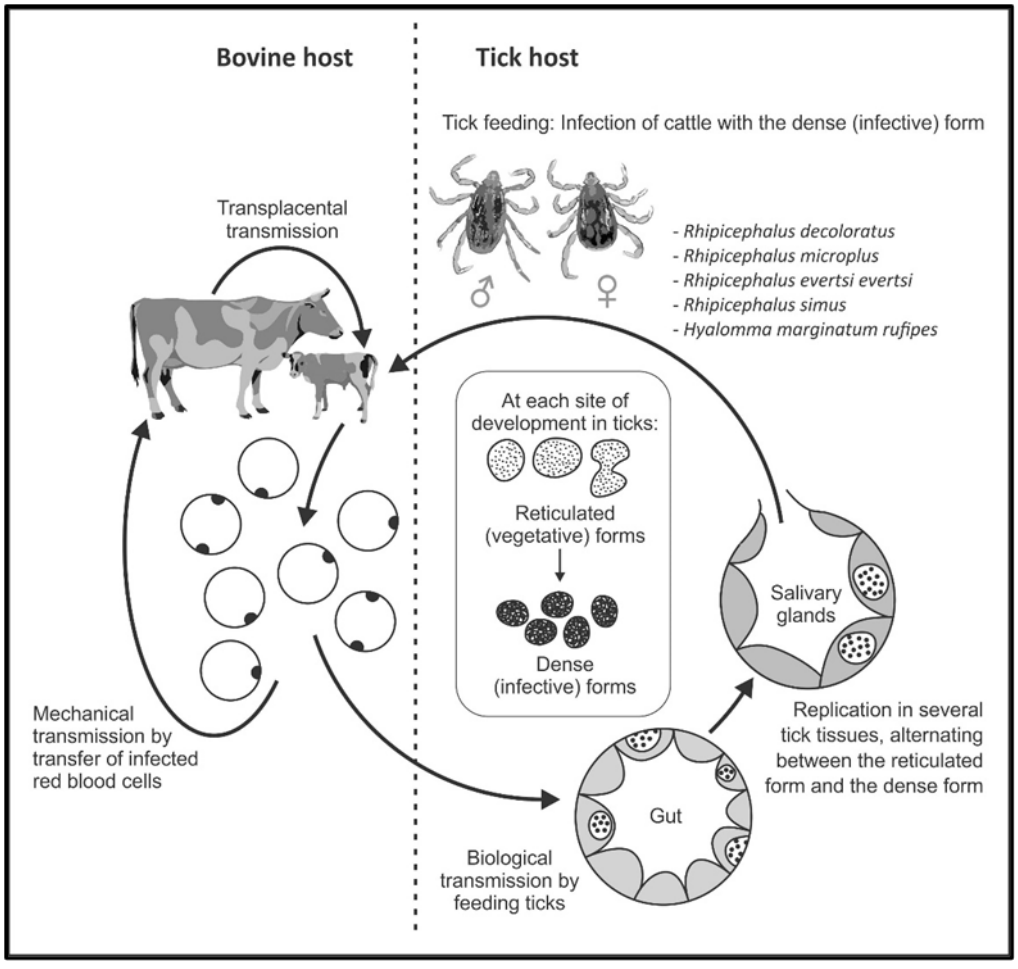

Figure 2.3: The developmental cycle of *Anaplasma marginale* in South Africa modified from Kocan et al. (2003). The life cycle was modified to include mechanical transmission, transplacental transmission and tick vectors transmitting the pathogen in South Africa.

The first form of *A. marginale* seen within colonies in tick cells is the reticulated (vegetative) form that divides by binary fission, forming large colonies that may contain hundreds of organisms. The reticulated form then changes into the dense form, which is the infective form and can survive outside host cells for a limited period of time (Kocan et al., 2010). Although it is known that *A. marginale* only infects erythrocytes of cattle (Kocan et al., 2010), *A. marginale* infection and maintenance has been reported in a bovine endothelial cell line (Munderloh et al., 2004). Failed attempts to demonstrate infection of endothelial cells *in vivo* suggests that endothelial cells are not an important component of the *A. marginale* life-cycle (Wamsley et al., 2011).

Cattle become infected with *A. marginale* when the dense form is transmitted during tick feeding via the salivary glands (Kocan et al., 2003). Once in the bovine erythrocytes, it undergoes cycles of replication and subsequent reinvasion of erythrocytes within the ruminant, as the average lifespan of a bovine red blood cell is 160 days and infected erythrocytes are removed by the reticuloendothelial system (Aubry & Geale, 2011). The severity of bovine anaplasmosis depends on the susceptibility of the host and the strain of *A. marginale.* Acute infections are characterised by 10-20% rickettsemia (Aubry & Geale, 2011). This acute phase of anaplasmosis is characterized by the following clinical signs: hemolytic anemia, with marked weight loss, abortion and, in 36% of clinical cases, death (Losos, 1986). However, cattle surviving the acute phase of anaplasmosis remain persistently infected with the pathogen, with microscopically undetectable levels of rickettsemia, thus playing an active role in the biological transmission of the pathogen via ticks to naïve cattle (Losos, 1986; Palmer et al., 1999).

2.2.1.3. Genetic diversity of *Anaplasma marginale*

Anaplasma marginale is known to be genetically diverse with each diverse strain defined by its *msp1α* genotype (Allred et al., 1990; Kocan et al., 2010). *Msp1α* is a single-copy gene encoding major surface protein 1a (Msp1a), which is one of the six major surface proteins that have been described in the *A. marginale* genome. Msp1a is a large protein containing tandem repeats of 23–31 amino acid repeats near the amino-terminus that vary both in sequence and number (Allred et al., 1990) (Figure 2.4). *Anaplasma marginale* strain differentiation studies worldwide based on the *mspIα* gene have revealed a large number of Msp1a repeats which are

named alphanumerically (Catanese, Brayton & Gebremedhin, 2016) and the presence of a neutralization-sensitive epitope in every tandem repeat (Palmer et al., 1987; Allred et al., 1990).

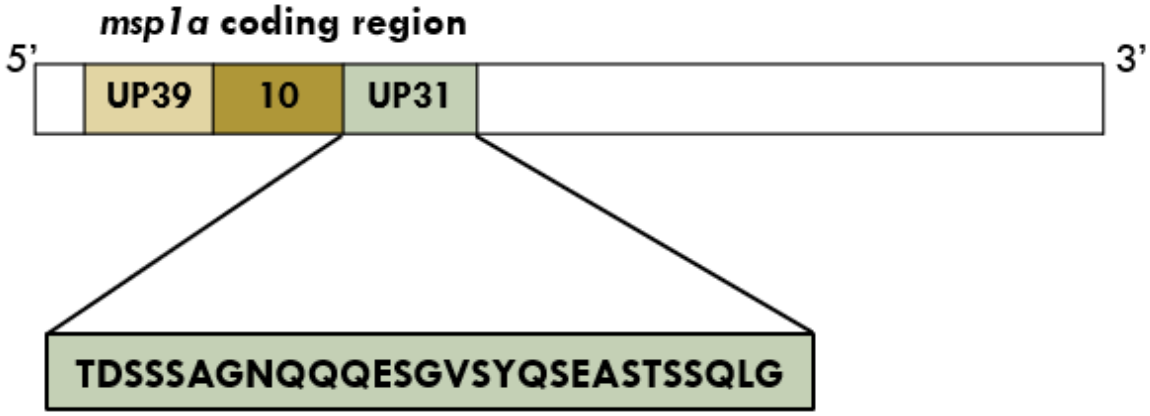

Figure 2.4: A diagrammatic illustration of the *msp1α* gene highlighting the *msp1α* coding region. The tandem repeats, named alphanumerically, are shown in the different coloured boxes with each shade representing a different repeat. The repeats range in size from 23 to 31 amino acids. An example of the amino acid sequence of one of the repeats, UP31, is shown in the expanded box. The combination of repeats, known as the *mspIα* genotype, differs between strains and thus provides a genetic strain identity marker. In this example, the *mspIα* genotype is UP39/10/UP31.

Anaplasma marginale strains are characterized by the *mspIα* genotype; for example, the first fully sequenced strain, St. Maries, the *A. marginale* reference strain, has an *mspIα* genotype of J/B/B (Brayton et al., 2005) and the Florida strain has an *mspIα* genotype of A/B/B/B/B/B/B (Rodríguez et al., 2005).

Studies characterizing the *A. marginale* strains worldwide have revealed genotypic variation of *A. marginale* strains that vary in geographic location and phenotypic traits (Palmer, Rurangirwa & McElwain, 2001; Blouin et al., 2002; Lew et al., 2002; Palmer et al., 2004; Espinoza et al., 2006; De La Fuente et al., 2007; Mtshali et al., 2007; Mutshembele et al., 2014; Baêta et al., 2015; Machado et al., 2015; Yang et al., 2017; Hove et al., 2018). Studies conducted in South Africa (Mtshali et al., 2007; Mutshembele et al., 2014; Hove et al., 2018) demonstrated genetic diversity in the South African strains of *A. marginale*, highlighting some of the Msp1a tandem repeats that are shared between South African strains and those from other geographical regions of the world, such as South America, North America and Europe. Although many of the individual Msp1a repeat sequences have been found in many different

countries including South Africa, only two of the South African *msp1α* genotypes (consisting of specific combinations of Msp1a repeat sequences) were found elsewhere in the world (Hove et al., 2018). Currently, a total of 99 Msp1a repeats making up 190 *msp1a* genotypes have been identified and described in cattle in South Africa (Mutshembele et al., 2014; Hove et al., 2018). In South African *A. marginale* strains, six Msp1a repeats (3, 4, 13, 27, 34, and 37) were found to be the most common, with genotypes SW12: 42 43 25 31, SW32: 34 13 13 37 and NW-C1-160312: 34 13 3 36 38 being the most commonly identified in cattle in the different provinces of the country (Hove et al., 2018).

2.2.2. *Anaplasma centrale*

Anaplasma centrale is a mildly pathogenic rickettsia of the genus *Anaplasma* that was first identified in South Africa (Theiler, 1911). Theiler initially named this organism *Anaplasma marginale* variety *centrale* based on its similarity to *A. marginale*. However, he noted that *A. centrale* was located in the center of the erythrocyte in contrast to *A. marginale* and exhibited reduced virulence in cattle. Additionally, Theiler recognized the potential of *A. centrale* to confer protection against bovine anaplasmosis. To this day, the *A. centrale* live blood vaccine remains in use in numerous countries, including South Africa (Theiler, 1911; Aubry & Geale, 2011).

Anaplasma marginale variety *centrale* was erroneously classified as a different species from *A. marginale* (Ristic, 1984), based on the assertion by Ristic (1968) that Theiler had regarded the organism as a distinct species. Although it was observed that *A. centrale* and *A. marginale* were closely related based on the morphological (Theiler, 1911) and phylogenetic (Dumler et al., 2001) similarities between the two species, the phylogenetic classification of *A. centrale* has been unclear for over a century. Recent studies (Khumalo et al., 2016; 2018) have finally shed light on the phylogenetic classification of *A. centrale*. Phylogenetic analyses of *groEL*, *msp4* and 16S rRNA gene sequences from numerous field samples of *A. marginale* and *A. centrale* in South Africa (Khumalo et al., 2018), as well as genetic variation in the Msp1a/Msp1aS structure (Khumalo et al., 2016), and overall genomic stucture (Brayton et al., 2005; Herndon et al., 2010) finally revealed that *A. centrale* should be regarded as a separate species from *A. marginale*.

The life cycle and worldwide distribution of *A. centrale* is poorly understood. This is mainly because the distribution and occurrence of *A. centrale* in cattle is assumed to be due to vaccination against *A. marginale*; it is thus considered as a coinfection due to vaccination and rarely further investigated (Georges et al., 2001). This is partly because *A. centrale* is not commonly associated with massive disease outbreaks, does not have major implications for national or international trade and is not considered to be an important zoonosis. However, a case of bovine anaplasmosis caused by *A. centrale* was reported in Europe (Carelli et al., 2007), highlighting the need for more studies on the occurrence of the organism and the presence of different strains. *Anaplasma centrale* was also recently detected in cattle in all South African provinces, except for the Eastern Cape and Northern Cape Provinces (Hove et al., 2018). Detection of this pathogen as single or mixed infections in wildlife species such as blue wildebeest (*Connochaetes taurinus*) and black wildebeest (*Connochaetes gnou*), African buffalo, eland (*Taurotragus oryx*) and waterbuck (*Kobus ellipsiprymnus*) in several national parks in South Africa and Botswana, suggest that wildlife species are possible reservoirs for *A. centrale* (Debeila, 2012; Eygelaar et al., 2015; Khumalo et al., 2016). Blesbok (*Damaliscus albifrons*) have also been shown to be susceptible to *A. centrale* infection (Potgieter & Stoltsz, 2004).

2.2.2.1. Transmission of *Anaplasma centrale*

The role played by ticks in the transmission and life cycle of *A. centrale* both in South Africa and worldwide has not been elucidated. It was thought that *A. centrale* could not be transmitted by ticks because, although this pathogen was shown to infect various tick species including *Rhipicephalus sanguineus, R. annulatus* and *Hyalomma excavatum*, transmission to splenectomized calves had not been achieved (Shkap et al., 2009). Tick transmission experiments revealed that, similar to *A. marginale*, *A. centrale* can colonize and replicate to comparable levels in the midgut and salivary glands of *D. andersoni*. Nonetheless, this alone was insufficient for transmission (Ueti et al., 2007). It was only possible to achieve transmission of *A. centrale* by increasing the number of *D. andersoni* ticks. This is because *A. centrale* is secreted into the saliva at a much lower rate than *A. marginale*. The increased number of ticks compensated for the reduced pathogen load in the saliva, thus facilitating in transmission (Ueti et al., 2009). Experimental transmission studies showed that *R. simus* is able to transmit this pathogen to cattle (Potgieter & van Rensburg, 1987b). A recent study which

involved screening for *A. centrale* infection in ticks collected in South Africa, suggests that *R. appendiculatus* is a possible vector for *A. centrale* (Khumalo, 2017).

2.2.3. *Anaplasma* species in wildlife hosts

The substantial growth of the wildlife industry in South Africa in recent years has led to an expansion of wildlife species in both game reserves and farming areas and thus increase in wildlife-livestock interfaces in many regions of the country (Parker & Bernard, 2005; Smith & Parker, 2010; Jori et al., 2011; Horak et al., 2015). This, in turn, has increased TBD transmission opportunities between wildlife, livestock and humans by facilitating the spread of ticks (Yusufmia et al., 2010; Caron et al., 2013; Mbizeni et al., 2013).

Various infections and diseases can occur in livestock, domestic animals and wildlife; however, the severity of these diseases is most well studied in livestock and domestic animals (Ryser-Degiorgis, 2013). Amongst the wildlife species with an increased range are eland, African buffalo, roan antelope (*Hippotragus equinus*), sable antelope (*Hippotragus niger*), white and black rhinoceros (*Diceros bicornis* and *Ceratotherium simum*), (Horak et al., 2015). These wildlife species are known to harbor rickettsial (*Anaplasma* and *Ehrlichia*) and protozoal (*Babesia* and *Theileria*) agents of veterinary importance (Tonetti et al., 2009; Pfitzer et al., 2011; Debeila, 2012; Berggoetz et al., 2014; Eygelaar et al., 2015; Khumalo et al., 2016).

Although little is known about the prevalence and impact of *Anaplasma* species in wildlife, members of the genus are known to be multi-host pathogens that can infect several ruminant species, including buffalo (Potgieter, 1979; Kocan et al., 2010), as well as other wild animals such as black and blue wildebeest (Neitz, 1935; Kuttler, 1965, 1984; Smith et al., in press), blesbok (Kuttler, 1984), American bison (*Bison bison*), white-tailed deer (*Odocoileus virginianus*), black-tailed deer (*O. hemionus culumbianus*) (Kuttler, 1984), mule deer (*O. h. hemionus*) and other species of the deer family (Kocan et al., 2010).

Anaplasma species known to infect wildlife in South Africa include *A. marginale* which has been detected in buffalo (Debeila, 2012; Henrichs et al., 2016; Khumalo et al., 2016; Sisson et al., 2017), eland, blue wildebeest, black wildebeest and waterbuck (Khumalo et al., 2016) in various game reserves around the country. Detection of *A. centrale* as single or mixed infections in wildlife species such as blue and black wildebeest, African buffalo, eland and

waterbuck in national parks in South Africa and Botswana (Debeila, 2012; Eygelaar et al., 2015; Khumalo et al., 2016), suggests that wildlife species are possible reservoirs for *A. centrale*. Blesbok have also been shown to be susceptible to *A. centrale* infection (Potgieter & Stoltsz, 2004). *Anaplasma* (formerly *Ehrlichia*) sp. Omatjenne is a novel non-pathogenic parasite that mostly occur as mixed infection with other hemoparasites (Pfitzer et al., 2011). Although not much is known about this organism, it has been isolated in culture (Zweygarth et al., 2006) and has been detected in various wildlife hosts such as African buffalo and nyala (*Tragelaphus angasii*), and in livestock such as cattle and goats in South Africa, Turkey and other African countries, such as Mozambique, Botswana, Uganda and Ethiopia (Bekker et al., 2002; Aktas, Altay & Dumanli, 2011; Pfitzer et al., 2011; Aktas et al., 2012; Eygelaar et al., 2015; Byaruhanga et al., 2016; Hailemariam et al., 2017).

While the presence and abundance of wildlife species in South Africa is likely to play a significant role in the spread and epidemiology of anaplasmosis, there is limited information available on the susceptibility of wild ruminants to infection by *Anaplasma* species and their role in the epidemiology of bovine anaplasmosis. It is well known that *Anaplasma* spp. infection in cattle may result in severe clinical signs; however, the limited information available for African buffalo indicates a minimal response by this host to the same infection (Debeila, 2012; Henrichs et al., 2016). The presence of *A. marginale* and other *Anaplasma* spp. in African buffalo and other wildlife hosts raises a question as to the role of wildlife species as reservoirs for *Anaplasma* spp. Wildlife species are commonly implicated as potential reservoir hosts of pathogenic organisms, as they are frequently not negatively affected by the infection; however, they can potentially contribute to the spread of various organisms at the wildlife/livestock interface (Kuttler, 1984; Ngeranwa et al., 2008). Although these organisms might not cause disease, their occurrence in livestock and domestic animals could confound species-specific detection methods.

2.3. Diagnosis

Diagnosis of anaplasmosis during the acute stage of the disease is usually made on the basis of clinical signs and hematological changes. Diagnostic methods such as microscopic examination of blood smears stained with Giemsa, serology (complement fixation (CF) test, rapid card agglutination, indirect immunofluorescent antibody (IFA) test, capillary agglutination test (CAT), enzyme-linked immunosorbent assays (ELISA), latex agglutination

and radioimmunassays) and nucleic acid-based methods have proved to be effective in the diagnosis of anaplasmosis (de Waal, 2000; Potgieter & Stoltsz, 2004; Aubry & Geale, 2011)

Under a light microscope, the Giemsa-stained blood smears from the acute stage of anaplasmosis appear as circular, deep purple, intra-erythrocytic bodies with a diameter ranging of 0.3 to 1.0 µm. These inclusion bodies are mostly situated on the edges of erythrocytes infected by *A. marginale*, while being more central for *A. centrale* (Potgieter & Stoltsz, 2004; Kahn & Line, 2010). As anaplasmosis persists for long period after the acute phase, it is not advisable to perform the microscopy method as a means of diagnosis in asymptomatic or carrier animals as they are characterized by low levels of rickettsemia. In such cases, anaplasmosis is mainly diagnosed serologically and confirmed by nucleic-acid based methods (Aubry & Geale, 2011).

The serological assays currently used for the diagnosis of anaplasmosis include the competitive ELISA (cELISA), IFA test, CAT and CF test. However, only the cELISA and CAT are the recommended assays for diagnosing the disease (Kocan et al., 1992; de la Fuente et al., 2005; OIE, 2008). The commercially available genus-specific cELISA kit developed by Knowles et al. (1996), using recombinant major surface protein 5 (Msp5) as antigen, is now used routinely for diagnosis of anaplasmosis. However, this test cross-reacts with other *Anaplasma* spp. (Munodzana et al., 1998) and *Ehrlichia* spp. (Al-Adhami et al., 2011). Furthermore, the test cannot distinguish mixed *Anaplasma* infections, as Msp5 is present in all known *Anaplasma* species, and the Msp5 epitope defined by monoclonal antibody ANAF16C1 is broadly conserved among *Anaplasma* spp. (Knowles et al., 1996; Munodzana et al., 1998; de la Fuente et al., 2004; Hofmann-Lehmann et al., 2004; Dreher et al., 2005; Strik et al., 2007). Scoles et al. (2008) and Mason et al. (2017) extensively validated the cELISA kit for detection of *A. ovis* in sheep and to identify *Anaplasma* spp. in wild ruminants (Scoles et al., 2008). This test is extensively used in many countries such as the USA where cross-reactions are not considered a problem as, in those countries, *A. marginale* is the only *Anaplasma* spp. in cattle while *A. ovis* is the only *Anaplasma* in sheep. However, this is not the case in South Africa where multiple *Anaplasma* species are known to infect cattle, often as co-infections: these include *A. marginale*, *A. centrale*, *A. bovis* and *Anaplasma* sp. (Omatjenne) (de Kock et al., 1937; Zweygarth et al., 2006; Harrison et al., 2013; Khumalo et al., 2016; Hove et al., 2018). Development of a more specific serological test is thus important for accurate identification of *A. marginale* and distinguishing it from other *Anaplasma* spp., thereby leading to a better

understanding of the epidemiology of *Anaplasma* spp. in South Africa, as well as informing the application of appropriate control measures.

Assays involving hybridisation of nucleic acid such as polymerase chain reaction (PCR), nested PCR (nPCR), quantitative real-time PCR (qPCR), and the reverse line blot (RLB) hybridization assay have been demonstrated to be effective in detecting various species of the genus *Anaplasma* (Bekker et al., 2002; Lew et al., 2002; Molad et al., 2006; Carelli et al., 2007; Decaro et al., 2008). In a recent study (Chaisi et al., 2017), the efficacy of three nucleic acid-based assays; RLB hybridization, nPCR and a duplex qPCR assay was evaluated and compared in the detection of *A. centrale* and *A. marginale* positive samples from South Africa and concluded that the duplex qPCR is more sensitive than the other two methods. Although the conventional PCR, nPCR and RLB assays are considered to be less sensitive than the qPCR assay, they can all detect low levels of rickettsemia which cannot be detected in thin blood smears. However, even the most sensitive of the nucleic-acid based tests cannot always detect *Anaplasma* DNA in samples from animals with a very low rickettsemia.

2.4. Control of anaplasmosis

In South Africa, the long-term approach to managing and controlling ticks and TBDs, including bovine anaplasmosis, relies mainly on the use of acaricides and application of vaccines (de Waal, 2000). It is generally accepted that viability of cattle farming in tick-infestation areas around the world has been greatly improved by the application of acaricides on cattle. However, mortalities as a result of bovine anaplasmosis in South Africa correlate with high levels of acaricide treatment, supporting the well-known concept that adequate exposure to ticks bestows a degree of immunity to anaplasmosis, and prevents outbreaks (de Waal, 2000). The other major concern associated with effective acaricide treatment is the emergence of acaricide resistance by ticks, a phenomenon that varies depending on the tick species and the country (Dolan, 1999). Besides the use of acaricide treatment, outbreaks of anaplasmosis can be controlled naturally if bovine hosts are continuously infected from a young age (Kahn & Line, 2010). After recovery from the acute phase of infection, cattle become persistently infected but immune to further clinical disease due to anaplasmosis; however, persistently infected cattle may relapse when immunocompromised (Kahn & Line, 2010).

The use of Imizo® (imidocarb dipropionate) formulations and tetracycline compounds is widely recommended for treating anaplasmosis; the latter involves the use of tetracyclines such as tetracycline, oxytetracycline (mainly used in South Africa), chlortetracycline, minocycline to treat acute infections (de Waal, 2000; Blouin et al., 2002; Potgieter & Stoltsz, 2004; Kahn & Line, 2010; Kuttler, 1980). However, there are some disadvantages, including cost, the need for continuous administration and the risk of development and spread of acaricide resistant strains that could present a constraint to livestock farming and development (Kocan, Blouin & Barbet, 2000).

Immunization by vaccination is one other strategic approach that has been successfully applied to control bovine anaplasmosis and this has been practiced in South Africa since 1912 (Theiler, 1912; Kocan, Blouin & Barbet, 2000). A live vaccine developed from *A. centrale* has been in use for over a century since Sir Arnold Theiler first observed that *A. centrale* can confer cross-protection against *A. marginale*, thus affording protection against bovine anaplasmosis (Theiler, 1911). This vaccine is currently used in Australia, Israel, South Africa and South America (Kuttler, 1984; Kahn & Line, 2010). Although the live *A. centrale* vaccine is recommended and has been shown to be the most effective method of controlling bovine anaplasmosis, there are several disadvantages. This vaccine is costly due to the requirements of live cattle and strict maintenance of a cold chain. Furthermore, there is a risk of introducing additional unknown blood-borne pathogens, it does not provide immunity against all *A. marginale* field strains and can also produce severe reaction in some cattle (Bigalke, 1980; Palmer et al., 1989; Palmer & McElwain, 1995; Kahn & Line, 2010). Therefore, the use of this live vaccine is currently prohibited in countries such as the USA because of the potential for introducing *A. centrale* into the country or spreading emerging pathogens. Where the live vaccine is prohibited, countries currently rely on the use of attenuated strains of *A. marginale* (Kahn & Line, 2010). However, vaccination with *A. centrale* attenuated strains and killed whole-organism is reported to be less efficacious than live vaccines, although they have been used extensively to induce protection in cattle in endemic regions and have shown to induce immunity against anaplasmosis by significantly reducing the rickettsemia following vaccination (Losos, 1986; Palmer et al., 1989; Kahn & Line, 2010).

Development of a safe vaccine which reduces the disease burden caused by anaplasmosis would improve animal health and thus the agricultural productivity, as well as economic well-

being of both commercial and resource-constrained farmers in South Africa and other parts of the world.

2.5. The development of a safe recombinant vaccine for bovine anaplasmosis

It was reported that there are 25 proteins shown to be involved in the process of inducing protection against *A. marginale* (Lopez et al., 2005); however, not all of them were surface-exposed. Surface-expressed proteins are preferred candidates for the development of a safe vaccine as they are involved in either inducing protective immunity in the mammalian host or preventing colonization of the tick vector (Noh et al., 2008). The development and application of genomic and proteomic techniques in recent years have allowed the identification of several outer membrane protein (OMP) vaccine candidates that are critical targets of the protective immune response. Eight OMPs, Am779, Am854, Omp7, Omp8, Omp9, Omp11, Omp13 and Omp14, have been identified as being important vaccine candidates. These proteins are highly conserved and expressed at high levels in bovine erythrocytes, thus inducing protection against severe disease in nearly all animals tested and protection from infection (sterile immunity) in approximately 40% of animals (Lopez et al., 2005; Noh et al., 2006, 2008, 2010; Agnes et al., 2011).

The list of OMP vaccine candidates was further analyzed and reduced to five; Am779, Am854, Omp7, Omp8 and Omp9 (Palmer et al., 2012). This was based on a comparative genomic study (Dark, Al-Khedery & Barbet, 2011) that showed that the OMPs have a broadly conserved B-cell epitope (Am779, Am854, Omp7/9), thus classified as immunologically 'subdominant' antigens. Another study (Lopez et al., 2008) further identified specific $CD4^+$ T-cell responses targeted against Am779, Am854, and Omp7/9 in vaccinates and this is consistent with the need for both class switching to IgG2 and affinity maturation associated with MHC class II-dependent CD4+ T-lymphocyte help, and specific CD4+ T-cell responses (Palmer et al., 1999).

In South Africa, Am779 and Am854 have been shown to be highly conserved in field samples from *A. marginale*-positive cattle in eight of nine provinces, with sequences of both OMPs exhibiting minimal variation in the tested samples (Hove et al., 2020). There was no variation at all in South African Am854 amino acid sequences, whereas OMP Am779 variants 1 and 4 detected in the study were found to have 99.9% amino acid sequence identity with the Florida and St. Maries strains of *A. marginale* from USA (Hove et al., 2020). South African OMP

Am854 has 100% identity with OMP Am854 from the Florida strains and St. Maries of *A. marginale* from USA (Hove et al., 2020). Although Omp7, Omp8 and Omp9 were less conserved in the overall sequences, some areas of the protein sequences were highly conserved including a T-cell epitope (Hove et al., 2020). Therefore, this data highlights the presence of these proteins in South African strains of *A. marginale* and suggest that they may be useful vaccine candidates.

2.6. *Anaplasma* genome analyses

Although anaplasmosis has a history that spans more than a century since it was discovered, it is still one of the most challenging and understudied TBDs of human and veterinary health due to the obligate intracellular nature of the disease-causing organisms. To date, the genus *Anaplasma* is characterized by relatively few genome sequences with only two species, *A. marginale* and *A. phagocytophilum*, having more than a single strain that have been sequenced and published, while *A. centrale*, *A. ovis* and *A. platys* are represented by a single strain each.

A new dawn in *Anaplasma* research came with the announcement of the complete genome sequence of the St. Maries strain of *A. marginale* isolated from a severely infected animal (Brayton et al., 2005). The complete circular genome of *A. marginale* is approximately 1.2 Mb (Megabase pairs) in length (Brayton et al., 2005) and is predicted to encode 949 protein-coding genes of which 62 are OMPs. The genome is further characterized by three ribosomal ribonucleic acid (rRNA) genes, 37 transfer ribonucleic acid (tRNA) genes and 14 functional pseudogenes, (Brayton et al., 2005). Complete genome sequences obtained from the Florida, Gypsy Plains and Dawn strain of *A. marginale* and other *A. marginale sensu stricto* strains were subsequently published (Dark et al., 2009; Dark, Al-Khedery & Barbet, 2011; Pierlé et al., 2014). The genome of the Florida strain of *A. marginale* is about the same size as the St. Maries strain (1.2 Mb) and is completely syntenic with the exception of a 15 Kb inversion. The coding content is essentially the same between the two strains, with variation in gene number due to differences in annotation (Dark et al., 2009).

Herndon et al. (2010) reported the complete circular genome sequence of the *A. centrale* Israel vaccine strain (Herndon et al., 2010). Similar in size to that of *A. marginale*, the genome sequence of *A. centrale* is 1,206,806 bp in length. It has three rRNA genes, 37 tRNA genes, 19 pseudogenes and 984 predicted coding sequences(Herndon et al., 2010). The genome sequence

of the *A. marginale* St. Maries strain has 18 distinct genes that are absent in *A. centrale* while the *A. centrale* genome contains 10 putative genes that are not present in *A. marginale* senso stricto strains (Herndon et al., 2010).

Since immunity against severe morbidity and mortality caused by *A. marginale* can be induced by immunization with *A. centrale* (Theiler, 1911; Pipano, 1995; Bock & De Vos, 2001), the epitopes critical for protective immunity are likely to be broadly conserved. A comparative genomics approach, comparing the *A. marginale* sequences and the *A. centrale* vaccine strain or other closely related species could thus aid in the identification of well conserved OMPs between the two genomes. Members of the Msp1 superfamily are not well conserved between *A. centrale* and *A. marginale*, and are therefore likely to be poor vaccine candidates. However, sequence similarity between six vaccine candidate genes (Msp2 superfamily genes: *msp4*, *Omp1*, *Omp7*, and *OpAG2*; and two non-superfamily members: *Am854/ACIS486* and *Am779/ACIS557*) between *A. marginale* and the vaccine strain ranged from 63% to 88% (Herndon et al., 2010), suggesting that these might be good vaccine candidates.

Other complete circular genome sequences from other species in the genus *Anaplasma* include those of *A. ovis* (Liu et al., 2019) and *A. platys* (Llanes & Rajeev, 2020) which are about the same size as *A. marginale* and *A. centrale* (1.2 Mb), as well as several different strains of *A. phagocytophilum* which are approximately 1.4 Mb in length (Dunning et al., 2006). The lack of genome sequences of organisms in the genus *Anaplasma* has impeded progress in elucidating their biology, correct classification of reported “putative” species within the genus, and in developing vaccines, as well as specific and sensitive detection methods.

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CHAPTER 3

Unravelling the diversity of *Anaplasma* species circulating in selected African wildlife hosts by targeted 16S microbiome analysis[1]

3.1. Abstract

Organisms in the genus *Anaplasma* are obligate intracellular alpha-proteobacteria. Bovine anaplasmosis, predominantly caused by *Anaplasma marginale*, is the most prevalent tick-borne disease (TBD) of cattle worldwide. Other *Anaplasma* species are known to cause disease; these include *A. ovis* in sheep, *A. platys* in dogs, *A. capra* in goats and humans, and *A. phagocytophilum* in humans. The rapid advancement of next-generation sequencing technologies has led to the discovery of many novel sequences ascribed to the genus *Anaplasma*, with over 20 putative new species being proposed since the last formal organization of the genus. Most 16S rRNA gene surveys for *Anaplasma* were conducted on cattle and to a lesser extent on rodents, dogs, and ticks. Little is known about the occurrence, diversity, or impact of *Anaplasma* species circulating in wildlife species. Therefore, we conducted a 16S rRNA gene survey with the goal of identifying *Anaplasma* species in a variety of wildlife species in the Kruger National Park and neighbouring game reserves, using an unbiased 16S rRNA gene microbiome approach. An *Anaplasma*/*Ehrlichia*-group specific quantitative real-time PCR (qPCR) assay revealed the presence of *Anaplasma* and/or *Ehrlichia* species in 70.0% (21/30) of African buffalo, 86.7% (26/30) of impala, 36.7% (11/30) of greater kudu, 3.2% (1/31) of African wild dog, 40.6% (13/32) of Burchell's zebra, 43.3% (13/30) of warthog, 22.6% (7/31) of spotted hyena, 40.0% (12/30) of leopard, 17.6% (6/34) of lion, 16.7% (5/30) of African elephant and 8.6% (3/35) of white rhinoceros samples. Microbiome sequencing data from the qPCR positive samples revealed four 16S rRNA sequences identical to previously published *Anaplasma* sequences, as well as nine novel *Anaplasma* 16S genotypes. Our results reveal a greater diversity of putative *Anaplasma* species circulating in wildlife than currently classified within the genus. Our findings highlight a potential expansion of the *Anaplasma* host range and the need for more genetic information from other important

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genes or genome sequencing of putative novel species for correct classification and further assessment of their occurrence in wildlife, livestock and companion animals.

3.2. Introduction

Bovine anaplasmosis is among the three most important tick-borne diseases (TBDs) of ruminants and results in major economic losses in food animal production globally (Uilenberg, 1995). This disease is mainly caused by the obligate intracellular rickettsial pathogen, *Anaplasma marginale*, which is currently widespread in cattle in South Africa (Hove et al., 2018; Makgabo et al., 2023). Several other species of *Anaplasma* have been reported to infect cattle in South Africa: these include *A. centrale*, *A. bovis*, *A. platys* and *Anaplasma* sp. (Omatjenne) (de Kock et al., 1937; Zweygarth et al., 2006; Harrison, Bown & Horak, 2011; Harrison et al., 2013; Khumalo et al., 2016; Kolo et al., 2020). Of the seven species included in the most recent reorganization of the genus *Anaplasma* (Dumler et al., 2001), four species, *A. marginale*, *A. bovis*, *A. centrale* and *A. phagocytophilum*, are known to cause anaplasmosis in cattle (Aktas and Özübek, 2017; Hove et al., 2018; Jurković et al., 2020; M'Ghirbi et al., 2016). Of these, *A. marginale* is the most important pathogen in cattle (Kocan et al., 2010). Although *A. bovis*, *A. centrale*, and *A. phagocytophilum* are widely known to cause subclinical disease in cattle, a clinical case of bovine anaplasmosis caused by *A. centrale* was reported in Europe in 2008 (Carelli et al., 2008). *Anaplasma ovis* mainly causes a subclinical disease characterized by fever in sheep and goats (Kuttler, 1984). *Anaplasma phagocytophilum* causes human granulocytic anaplasmosis (HGA) in humans (Bakken et al., 1994), equine granulocytic anaplasmosis (EGA) in horses (M'ghirbi et al., 2012) and granulocytic anaplasmosis (GA) in dogs (Granick et al., 2009), while *A. capra* infects both goats and humans (Li et al., 2015). *Anaplasma platys* is a pathogen that mostly infects platelets in dogs causing infectious cyclic thrombocytopenia (Abarca et al., 2007).

The past few decades have seen the global occurrence of several new, emerging and re-emerging tick-borne rickettsial pathogens of major public and veterinary health concern (Walker & Dumler, 1996; Dumler et al., 2001; Paddock & Childs, 2003; Li et al., 2015). A significant increase in the wildlife industry in South Africa over the past two decades has resulted in an increase in land use dedicated to wildlife and thus an increase in wildlife species in both game reserves and farming areas, thus resulting in an increase in wildlife-livestock

interfaces in many parts of the country (Parker & Bernard, 2005; Smith & Parker, 2010; Jori et al., 2011; Horak et al., 2015). This, in turn, increases potential TBD transmission opportunities between wildlife, livestock and the humans who maintain them, through increased opportunities for ticks to move between them (Yusufmia et al., 2010; Caron et al., 2013; Mbizeni et al., 2013). Very little is known about the role played by wildlife hosts in the distribution and epidemiology of anaplasmosis in domestic animals, livestock and possibly in humans. *Anaplasma marginale*, *A. centrale* and/or *A. ovis* have been identified in several wild ruminant species in Africa, including African buffalo, black wildebeest, blue wildebeest, blesbok, grey duiker (*Sylvicapra grimmi*), nyala (*Tragelaphus angasii*), eland and giraffe (*Giraffa camelopardalis*) (Neitz, 1935; Peirce, 1972; Augustyn, 1974; Kuttler, 1984; Smith et al., 1982; Ngeranwa et al., 1998; Potgieter & Stoltsz, 2004; Eygelaar et al., 2015; Khumalo et al., 2016). *Anaplasma bovis* has been identified in rock sengis (*Elephantulus myurus*) (Harrison, Bown & Horak, 2011; Harrison et al., 2013) and nyala (Pfitzer et al., 2011), and a sequence with 99% identity to *A. bovis* was identified in a *Rhipicephalus evertsi evertsi* tick collected on a gemsbok from the Sandveld nature reserve (Tonetti et al., 2009). *Anaplasma* sp. (Omatjenne) was identified in 33% of nyalas examined from four game ranches in northern KwaZulu-Natal (Pfitzer et al., 2011). It is clear that African wildlife harbor several *Anaplasma* spp., but the full range of *Anaplasma* spp. present in wildlife hosts is not known, and the importance of wildlife as a disease reservoir is unclear.

The rapid advancement of high-throughput sequencing technologies has enabled a massive increase in molecular, metagenomic, microbiome and taxonomic analyses, which have resulted in the discovery of a plethora of sequences ascribed to the genus *Anaplasma* worldwide. Over 20 putative *Anaplasma* species with unique 16S rRNA sequences have been identified from various hosts since the last formal organization of the genus (Dumler et al., 2001; Caudill & Brayton, 2022). These putative *Anaplasma* spp. have been reported from a variety of hosts including human, livestock and wildlife and/or tick and mosquito vectors from across the world (a list of the putative *Anaplasma* spp. is shown in Table A1 (Appendix 1), modified from Caudill & Brayton (2022)). Several novel *Anaplasma* 16S rRNA gene sequences have been reported in cattle, including a putative novel *Anaplasma* species from Uganda (Ikwap et al., 2010; Muhanguzi et al., 2010), "*Candidatus* Anaplasma boleense" (Guo et al., 2016; Fernandes et al., 2019; Kolo et al., 2020), *Anaplasma* sp. Saso, *Anaplasma* sp. Hadesa and *Anaplasma* sp. Dedessa (Hailemariam et al., 2017; Kolo et al., 2020), *Anaplasma* sp. Mymensingh (Roy et al., 2018; Kolo et al., 2020), and "*Candidatus* Anaplasma africae" (Dahmani et al., 2019). The

phylogenetic relationships of these newly detected agents to known pathogens and their ability to serve as a source of cross-reaction in detection assays have not been well assessed. The present study was aimed at using next-generation sequencing and bioinformatics to profile *Anaplasma* populations in selected wildlife species, to better understand the range and genetic diversity of *Anaplasma* species with potential for transmission to humans, livestock and companion animals.

3.3. Materials and Methods

3.3.1. Ethics approval

The study was performed in accordance with the conditions of the Animal Ethics Committee of the Faculty of Veterinary Science, University of Pretoria (REC 252-19) (Appendix 6). Research and Material Transfer Agreements were obtained from the Scientific Services Committee of the South African National Parks (SANParks), Kruger National Park (KNP) (BMTA 005/20) and the Hans Hoheisan Wildlife Research Station (HHWRS). Permission to conduct research under Section 20 of the Animal Disease Act 35 of 1984 was granted by the Department of Agriculture, Land Reform and Rural Development (12/11/1/1/6 (1734 LH)) (Appendix 7).

3.3.2. Field samples

A total of 343 frozen EDTA blood samples collected from 11 free roaming wildlife species in the KNP and surrounding game reserves including the Timbavati Game Reserve, Klaserie Private Nature Reserve and Manyeleti Game Reserve were made available by the Veterinary Wildlife Services, KNP (SANParks) and HHWRS biobanks (Table 3.1). These were collected from African elephant, African lion, African wild dog, Burchell's zebra, African buffalo, common warthog, greater kudu, impala, leopard, spotted hyena and white rhinoceros from 2012 to 2020.

Table 3.1: Origin and number of blood samples collected from wildlife hosts.

Wildlife host	Sample type	Biobank	Origin	Year	Number of samples
Buffalo	EDTA-blood	SANParks	Kruger National Park	2019	30
Impala	EDTA-blood	SANParks	Kruger National Park	2020	30
Kudu	EDTA-blood	SANParks	Kruger National Park	2018/19	30
Wild dog	EDTA-blood	SANParks	Kruger National Park	2017/18	30
	EDTA-blood	HHWRS	Timbavati Game Reserve	2020	1
Zebra	EDTA-blood	SANParks	Kruger National Park	2018/19	30
	EDTA-blood	HHWRS	Private Owner	2020	2
Warthog	EDTA-blood	SANParks	Kruger National Park	2017/18/19	30
Hyena	EDTA-blood	SANParks	Kruger National Park	2019/20	30
	EDTA-blood	HHWRS	Timbavati Game Reserve	2020	1
Leopard	EDTA-blood	SANParks	Kruger National Park	2012-2019	30
Lion	EDTA-blood	SANParks	Kruger National Park	2018/19/20	29
	EDTA-blood	HHWRS	Timbavati Game Reserve	2019	5
Elephant	EDTA-blood	SANParks	Kruger National Park	2019/20	30
Rhinoceros	EDTA-blood	SANParks	Kruger National Park	2020	29
	EDTA-blood	HHWRS	Klaserie Nature Reserve	2020	4
	EDTA-blood	HHWRS	Manyeleti Game Reserve	2020	2
Total					**343**

3.3.3. DNA extractions

DNA was extracted from 1 ml of blood from the SANParks and HHWRS biobanked samples using the Gentra Puregene Kit® (Qiagen) according to the manufacturer's instructions.

3.3.4. *Anaplasma/Ehrlichia* group-specific quantitative real-time PCR (qPCR) assay

A multiple sequence alignment of 16S rRNA reference sequences of all known species of *Anaplasma* and closely related species in the genera *Ehrlichia* and *Rickettsia* was created using CLC Genomics Workbench 20 (Qiagen, Aarhus, Denmark). Primers, Ma16SF: (5'-ACA GAA GAA GTC CCG GCA AA-3'), Ma16SR: (5'-TTG CCC CCT CCG TAT TAC C-3') (Inqaba Biotech, South Africa) and a TaqMan MGB^{TM} probe, Ma16SP: (FAM-5'-CCG TGC CAG CAG C-3'-MGB) (Thermo Fisher Scientific, South Africa) were designed to target a 64 bp fragment in the V3 hypervariable region that is conserved between *Anaplasma* and *Ehrlichia* species. Reactions, performed in a final volume of 20 µl, contained 2 X TaqMan® Universal PCR Master Mix (Thermo Fisher Scientific, South Africa), 0.5 µM of each forward and reverse primer, 0.25 µM of TaqMan MGB^{TM} probe and 2 µl of target DNA. The quantitative real-time PCR (qPCR) assays were performed on the StepOnePlus^{TM} Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Cycling conditions included UNG incubation at 50ºC for

2 min, followed by AmpliTaq Gold pre-activation at 95ºC for 10 min, 45 cycles of denaturation at 95ºC for 20 sec and annealing at 60ºC for 1 min. DNA extracted from the *A. centrale* and *E. ruminantium* vaccine strain (Onderstepoort Biological Products, Pretoria, South Africa), *A. marginale* and *A. platys* field samples (confirmed by sequence analysis) collected, respectively, from the Innovation Africa @ University of Pretoria (IA@UP) Experimental Farm and the Mnisi community, Mpumalanga province, were used as positive controls and molecular grade water as a negative control. Data was analyzed using the StepOnePlus™ software version 2.2. The analytical specificity of the qPCR assay was determined by testing DNA samples from tick-borne haemoparasites of ruminants including *Rickettsia africae*, *Babesia bigemina*, *B. bovis* and *T. parva*. All DNA samples extracted from the wildlife blood samples included in this study were screened for the presence of *Anaplasma* (and *Ehrlichia*) species using the *Anaplasma*/*Ehrlichia* group-specific qPCR assay. Since this assay was developed for screening purposes, no Ct value cut-off for true positives was used.

3.3.5. 16S rRNA gene amplification and PacBio sequencing

The full-length 16S rRNA gene (V1-V9 variable regions) was amplified in triplicate from all *Anaplasma* and/or *Ehrlichia*-positive wildlife DNA samples using modified barcoded 16S rRNA gene specific primers, 27F: (5'-AGR GTT YGA TYM TGG CTC AG-3') and 1492R: (5'-RGY TAC CTT GTT ACG ACT T-3') as recommended for the PacBio Sequel II platform (Pacific Biosciences, Menlo Park, California, USA) (Lane, 1991; Turner et al., 1999) (a list of the barcoded primers is shown in Table A2 in Appendix 2). Reactions were performed in triplicate in a final volume of 25 µl containing 1 X Phusion Flash® High Fidelity Master Mix (Thermo Fisher Scientific, South Africa), 0.15 µM of each forward and reverse primer and 5 µl of target DNA. To prevent contamination, master mixes were prepared in a dedicated master mix preparation laboratory where no DNA or PCR products are allowed. PCRs for each wildlife species were prepared on separate days. DNA extracted from the *A. centrale* vaccine strain (Onderstepoort Biological Products, South Africa) was used as a positive control and molecular grade water as a negative control. Cycling conditions included 98ºC for 30 sec, followed by 35 cycles of 98ºC for 10 sec, 60ºC for 30 sec and 72ºC for 30 sec and a final extension at 72ºC for 10 min. Amplicons were visualized under UV light after electrophoresis on a 1.5% agarose gel stained with ethidium bromide. Amplicons were purified using the QIAquick® PCR purification kit (Qiagen) according to the manufacturer's instructions and submitted to the Genomic Sequencing Core of Washington State University, Pullman, USA for

circular consensus sequencing (CCS) on the PacBio (Pacific Biosciences, Menlo Park, California, USA) platform. Two sequencing libraries were prepared using 500 ng of pooled amplicons in each library and the SMRT Bell library 2.0 express kit. Samples were sequenced following standard annealing and loading conditions detailed in SMRT Link software 8.0 (Pacific Biosciences, Menlo Park, California, USA).

3.3.6. 16S Microbiome sequence analysis

The 16S rRNA amplicon sequence data were demultiplexed using SMRT Link software 8.0 according to a minimum barcode score of 70. Sequences were then trimmed and filtered using SMRT Link software 8.0, with the following filtering parameters: Quality value (QV) minimum at 0.9999 (QV40), min read length 500 bp, max read length 3000 bp and 4 passes. Final Fasta and Fastq data sets were analyzed using the Ribosomal Database Project (RDP) 16S classifier (Cole et al., 2009; Gall et al., 2016) for *Anaplasma* genus level classification of the sequences with a 95% confidence interval. Sequences classified in the genus *Anaplasma* were further used in a BLAST search against a local NCBI BLASTn customized database created from all known and published *Anaplasma* spp. sequences downloaded from GenBank using the command line application to establish the correct identity of the sequences. Sequences were further filtered and excluded based on sequence length (minimum of 1275 bp), quality and sequence identity in Microsoft Excel (Gall et al., 2016; Caudill & Brayton, 2022). Since some distinct *Anaplasma* spp. are known to have more than 98.7% shared sequence identity, and *A. platys*, *Anaplasma* sp. Mymensingh, "*Candidatus* Anaplasma camelii" and *Anaplasma* sp. Omatjenne share more than 99.5% 16S rRNA gene sequence identity, it is clear that 16S rRNA gene sequences cannot be used to resolve these organisms to species level (Caudill & Brayton, 2022). Thus, only 16S rRNA sequences that were identical to previously published sequences were classified to species level. The *Anaplasma* species classification was further examined using a newly developed single-nucleotide polymorphism method of identifying and classifying *Anaplasma* spp. (Caudill & Brayton, 2022).

3.3.7. Terminology

It is difficult to formally name *Anaplasma* species due to their obligate intracellular nature and the requirement to deposit viable cultures in two type collections in different countries, and many of the newly identified putative species have been molecularly detected from samples but not isolated in culture. While the new sequences may well represent putative novel species,

additional sequence data is required for confirmation; we will therefore refer to the newly detected novel 16S rRNA sequences as "sequence type" (ST). Where we refer to the organisms represented by the newly detected sequence types, we will refer to them as putative *Anaplasma* species.

3.3.8. Sequence and phylogenetic analysis

The16S rRNA gene sequences classified as *Anaplasma* were aligned with reference sequences from GenBank and the extent of sequence variation was analysed using CLC Genomics Workbench 20 (Qiagen, Aarhus, Denmark). Alignments were trimmed using CLC Genomics Workbench. The HKY85 (Hasegawa, Kishino & Yano, 1985) evolutionary model (Guindon & Gascuel, 2003; Anisimova & Gascuel, 2006; Chevenet et al., 2006) was the best-fit model for the 16S rRNA gene sequences as determined by Jmodel test 1.3 (Darriba et al., 2012). Phylogenetic trees for the 16S rRNA gene sequences were constructed using the maximum likelihood (ML) method in MEGA 7 with a HKY85 substitution model, an estimated proportion of invariant sites and four gamma-distributed rate categories (Kumar, Stecher & Tamura, 2016).

All of the sequence data generated from this study have been registered in GenBank under the BioProject accession number: PRJNA965916. The raw microbiome sequence reads from *Anaplasma*-positive wildlife hosts are available at the Sequence Read Archive (SRA) under accession numbers SRX20180660 to SRX20180741. The near-full length *Anaplasma* 16S rRNA nucleotide sequences were deposited under GenBank accession numbers OQ909436 to OQ909508.

3.4. Results

3.4.1. The presence of *Anaplasma*/*Ehrlichia* species in African wildlife hosts.

The *Anaplasma*/*Ehrlichia* group-specific qPCR assay based on the 16S rRNA gene revealed the presence of *Anaplasma*/*Ehrlichia* spp. in all eleven wildlife species examined (Figure 3.1) (qPCR results for individual samples are shown in Table A3 in Appendix 3).

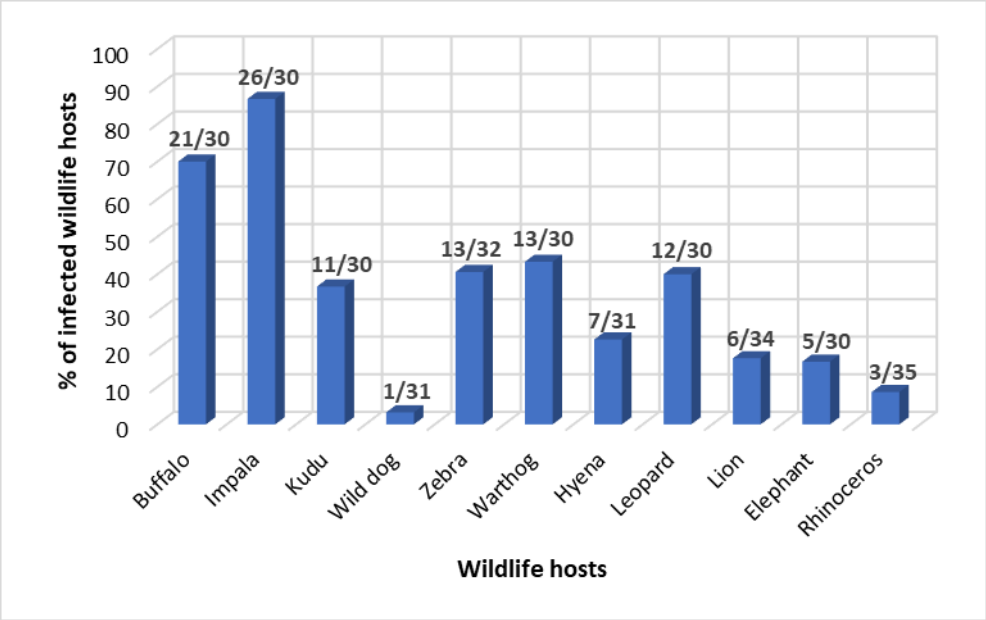

Figure 3.1: Percentage of *Anaplasma*/*Ehrlichia*-positive samples from 11 wildlife species detected using the *Anaplasma*/*Ehrlichia* group-specific qPCR assay. The numbers above each bar indicate the number of *Anaplasma*/*Ehrlichia*-positive samples out of the total for each wildlife species included in the study.

3.4.2. 16S rRNA gene amplification and PacBio CCS sequence analysis

Of the samples that tested positive using the *Anaplasma/Ehrlichia* group-specific qPCR assay, a visible 16S rRNA PCR product was obtained from 21 impala, 13 buffalo, 6 kudu, 6 zebra, 8 warthog, 3 spotted hyena, 9 leopard, 6 lion and 2 African elephant samples. No amplicon could be generated from the *Anaplasma/Ehrlichia*-positive wild dog or rhinoceros samples. PacBio CCS sequencing of the 16S rRNA gene amplicons revealed the presence of a total of 40,589 *Anaplasma* 16S nucleotide sequences. Further examination of the genus *Anaplasma* using the RDP 16S classifier and the customized 16S *Anaplasma* NCBI BLASTn databases resulted in the classification of 40,063 of these 16S rRNA nucleotide sequences to the *Anaplasma* species level. A total of 526 sequences were excluded based on sequence quality, length and sequence identity (Table 3.2).

Table 3.2: PacBio CCS sequencing data of the 16S rRNA gene of *Anaplasma* spp. from wildlife hosts

Wildlife species	Number of samples that yielded a visible 16S rRNA amplicon	Number of sequences classified as *Anaplasma*		Excluded sequences
		Genus (16S RDP)	Species (NCBI BLASTn)	
Impala	21	2786	2744	42
Buffalo	13	5458	5393	65
Kudu	6	1351	980	371
Zebra	6	2514	2513	1
Warthog	8	3498	3495	3
Hyena	3	31	31	0
Leopard	9	20729	20693	36
Lion	6	3715	3707	8
Elephant	2	507	507	0
Total	**74**	**40589**	**40063**	**526**

3.4.3. Identification of *Anaplasma* species in wildlife hosts

From the 40,063 16S rRNA nucleotide sequences classified as *Anaplasma*, 13 distinct 16S rRNA sequences were identified. Sequences with 100% identity to the 16S rRNA gene of known species were designated with the formal species name; novel 16S rRNA sequences were designated *Anaplasma* sequence type (ST) KNP-1 to KNP-9. *Anaplasma* spp. identified comprised 11,449 (28.6%) sequences of *Anaplasma* ST KNP-1, followed by 8107 (20.2%) of *Anaplasma* ST SA dog, 6347 (15.8%) of *A. marginale*, 4361 (10.9%) of *Anaplasma* ST KNP-8, 4163 (10.4%) of *Anaplasma* ST KNP-6, 2597 (6.5%) of *Anaplasma* ST KNP-2, 2482 (6.2%) of *A. centrale*, 271 (0.7%) of *Anaplasma* ST KNP-4, 206 (0.5%) of *Anaplasma* ST KNP-5, 55 (0.1%) of *Anaplasma* ST KNP-7, and less than 0.1% of *Anaplasma bovis* (14 sequences), *Anaplasma* ST KNP-9 (9 sequences), as well as *Anaplasma* ST KNP-3 (2 sequences).

Table 3.3 highlights the presence of the 13 *Anaplasma* 16S rRNA sequences in the different wildlife hosts. *Anaplasma* ST KNP-1, *Anaplasma* ST SA dog and *Anaplasma* ST KNP-2 were detected in seven of the wildlife hosts, followed by *A. marginale* and *A. centrale* detected in four of the wildlife hosts. The remaining *Anaplasma* spp. and STs were detected in either three or two of the wildlife hosts.

Table 3.3: Percentage of each *Anaplasma* 16S rRNA sequence type identified in each wildlife host.

***Anaplasma* 16S rRNA sequence type**	**Percentage of each *Anaplasma* 16S rRNA sequence type**								
	Impala (n=21)	Buffalo (n=13)	Kudu (n=6)	Zebra (n=6)	Warthog (n=8)	Hyena (n=3)	Leopard (n=9)	Lion (n=6)	Elephant (n=2)
A. bovis	0	0	0.71	0	0	0	0.03	0	0
A. centrale	0	24.29	0	0.03	0.37	0	0	31.24	0
A. marginale	0	73.11	0	64.27	0	0	0.03	21.09	0
A. ST SA dog	3.68	0.06	0	26.70	0.06	100	32.14	17.51	0
A. ST KNP-1	85.94	1.48	0.71	0.32	0	0	43.36	0.22	3.16
A. ST KNP-2	10.20	0.98	93.28	0.28	0	0	3.93	1.21	95.66
A. ST KNP-3	0	0.02	0	0.04	0	0	0	0	0
A. ST KNP-4	0	0	0.61	0	0	0	1.28	0	0
A. ST KNP-5	0	0	0	3.98	0	0	0.51	0	0
A. ST KNP-6	0	0	0	4.38	0	0	18.70	4.96	0
A. ST KNP-7	0	0.06	4.69	0	0	0	0	0	1.18
A. ST KNP-8	0	0	0	0	99.57	0	0	23.77	0
A. ST KNP-9	0.18	0	0	0	0	0	0.02	0	0

3.4.4. Phylogenetic analyses of 16S rRNA gene sequences from wildlife hosts

The relationships between the near full-length *Anaplasma* 16S rRNA gene sequences identified in the wildlife hosts were revealed by phylogenetic analyses. Maximum likelihood phylogenetic trees of the 16S rRNA gene sequences resulted in two clades (Figures 3.2 and 3.3), as highlighted in previous studies (Kolo et al., 2020; Caudill & Brayton, 2022). The first clade, which will be referred to as clade-1, includes *Anaplasma* spp. known to infect erythrocytes of livestock, such as *A. marginale*, *A. centrale* and *A. ovis*, while the second clade, which will be referred to as clade-2, includes *Anaplasma* spp. known to infect leukocytes and platelets, namely *A. platys*, *A. bovis* and *A. phagocytophilum* (Kolo et al., 2020; Caudill & Brayton, 2022) (Figure 3.2).

Only four of the 13 sequences amplified belong to previously identified and described species; these were *A. bovis*, *A. centrale*, *A. marginale* and *Anaplasma* ST SA Dog (Figure 3.2). *Anaplasma bovis* 16S rRNA gene sequences obtained from kudu and leopard samples were identical to the 16S rRNA gene sequence of *A. bovis* (GenBank accession no: U03775) from South Africa. *Anaplasma marginale* 16S rRNA gene sequences obtained from buffalo, zebra, leopard and lion samples were conserved and identical to *A. marginale* sequences previously reported from South Africa (GenBank accession no: AF414873) (Lew et al., 2003) and only varied by one nucleotide from the 16S rRNA gene of *A. marginale* St. Maries strain (GenBank accession no: CP000030) (Brayton et al., 2005). The *Anaplasma* sp. SA dog sequences were identical to the *Anaplasma* sp. 16S rRNA sequences previously identified and described in

dogs in Zambia and South Africa (GenBank accession no: LC269823 and MK814441, respectively) (Vlahakis et al., 2018; Kolo et al., 2020). The *A. centrale* sequences obtained from buffalo, zebra, warthog and lion samples were conserved and identical to the *A. centrale* vaccine strain from Israel (GenBank accession no: CP015994) (Herndon et al., 2010) and only varied by one nucleotide from the 16S rRNA sequence of the *A. centrale* vaccine strain from Australia (AF414868) (Lew et al., 2003).

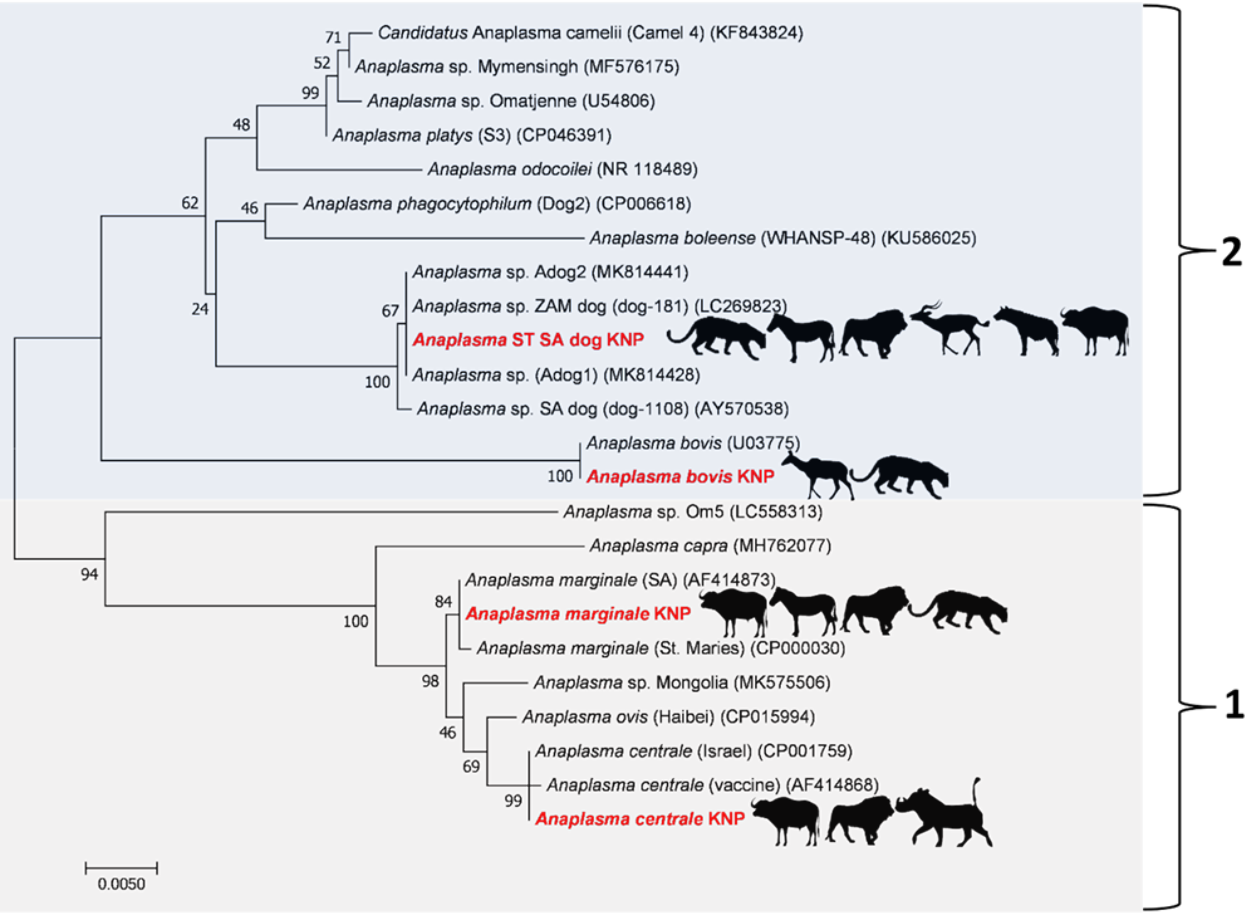

Figure 3.2: Maximum likelihood phylogenetic tree based on 16S rRNA sequence data showing the relationships between *Anaplasma* species and highlighting sequences obtained (shown in red) that were identical to known *Anaplasma* species. The tree is separated into the two prominent *Anaplasma* clades; clade-1, shaded in grey and clade 2, shaded in blue. Wildlife silhouettes indicate the hosts in which the sequences were identified. Near full-length 16S rRNA gene sequences of approximately 1328 bp in length were used to construct the tree. The numbers associated with each node indicate the percentage of 1000 bootstrap replications supporting the node. Phylogenetic analyses were conducted in MEGA7 with an HKY85 evolutionary model.

Phylogenetic relationships between the 16S rRNA sequences of known *Anaplasma* species and the newly detected 16S rRNA sequences are shown in Figure 3.3. Except for *Anaplasma* ST

KNP-2, which is made up of several similar *A. platys*-like sequences, the unknown *Anaplasma* sequences identified formed monophyletic clades distinct from other validated reference *Anaplasma* spp. (Figure 3.3). *Anaplasma* ST KNP-1 and *Anaplasma* ST KNP-3 grouped within clade-1. Two variants of *Anaplasma* ST KNP-1 were identified which formed a sister clade to the *A. ovis* group which includes *Anaplasma* sp. clone Mongolia. *Anaplasma* ST KNP-1a and *Anaplasma* ST KNP-1b had 99.9% sequence identity and had, respectively 99.5% and 99.6% shared sequence identity with *A. ovis* (GenBank accession no: CP015994) and 99.1% and 99.2% identity to *Anaplasma* sp. Mongolia (GenBank accession no: MK575506). *Anaplasma* ST KNP-1 was detected primarily in impala, but was also identified in buffalo, kudu, zebra, leopard, lion and African elephant samples. *Anaplasma* ST KNP-3 was obtained from buffalo and zebra samples and was closely related to *A. centrale*, with 99.6% identity. The two novel sequences, *Anaplasma* ST KNP-7 and *Anaplasma* ST KNP-8, were closely related, with 99.4% identity. The two sequences had, respectively, 99.4% and 99.6% shared sequence identity with *Anaplasma* sp. Om5 (GenBank accession no: LC558313) that was recently detected in *Ornithodoros moubata* ticks collected from African warthog burrows in Zambia (Qiu et al., 2021). *Anaplasma* ST KNP-7, *Anaplasma* ST KNP-8 and *Anaplasma* sp. Om5 formed a distinct sister group within clade-1 with less than 96.0% sequence identity to a multitude of sequences within the two prominent clades of *Anaplasma*. *Anaplasma* ST KNP-7 was detected in buffalo, kudu and leopard samples, while *Anaplasma* ST KNP-8 was obtained from warthog and lion samples and could be a variant of the putative novel *Anaplasma* sp. recently identified in *O. moubata* ticks (LC558313).

The remaining unknown *Anaplasma* sequences grouped in clade-2. *Anaplasma* ST KNP-6, identified in zebra, leopard and lion samples, grouped with *Anaplasma* sp. SA dog (GenBank accession no: AY570538) and *Anaplasma* sp. ZAM dog (GenBank accession no: LC269823) 16S rRNA sequences with 99.6% and 99.8% identity, respectively, suggesting it might be a variant of these. *Anaplasma* ST KNP-9, found only in impala and leopard samples, grouped in a distinct clade with *Anaplasma* sp. boleense (GenBank accession no: KU586025) with 99.0% identity. *Anaplasma* ST KNP-4 and seven variants of *Anaplasma* ST KNP-2 all grouped with *A. platys* (GenBank accession no: CP046391) and have more than 99.5% shared 16S rRNA sequence identity. Similarly, Caudill and Brayton (2022) reported that *Anaplasma* sp. Mymensingh (GenBank accession no: MF576175), "*Candidatus* Anaplasma camelii" (GenBank accession no: 843824) and *Anaplasma* sp. Omatjenne (GenBank accession no: U54806) all group with *A. platys* with more than 99.5% shared 16S rRNA sequence identity.

Although *Anaplasma* ST KNP-5 also groups with *A. platys*, *Anaplasma* sp. Mymensingh, "*Candidatus* Anaplasma camelii" and *Anaplasma* sp. Omatjenne, it is less similar with 98.7%-99.1% identity between these sequences. *Anaplasma* ST KNP-4 sequences were obtained from kudu and leopard samples, while *Anaplasma* ST KNP-5 sequences were found in zebra and leopard. *Anaplasma* ST KNP-2 was identified from a variety of wildlife hosts, including impala, buffalo, kudu, zebra, leopard, lion and elephant.

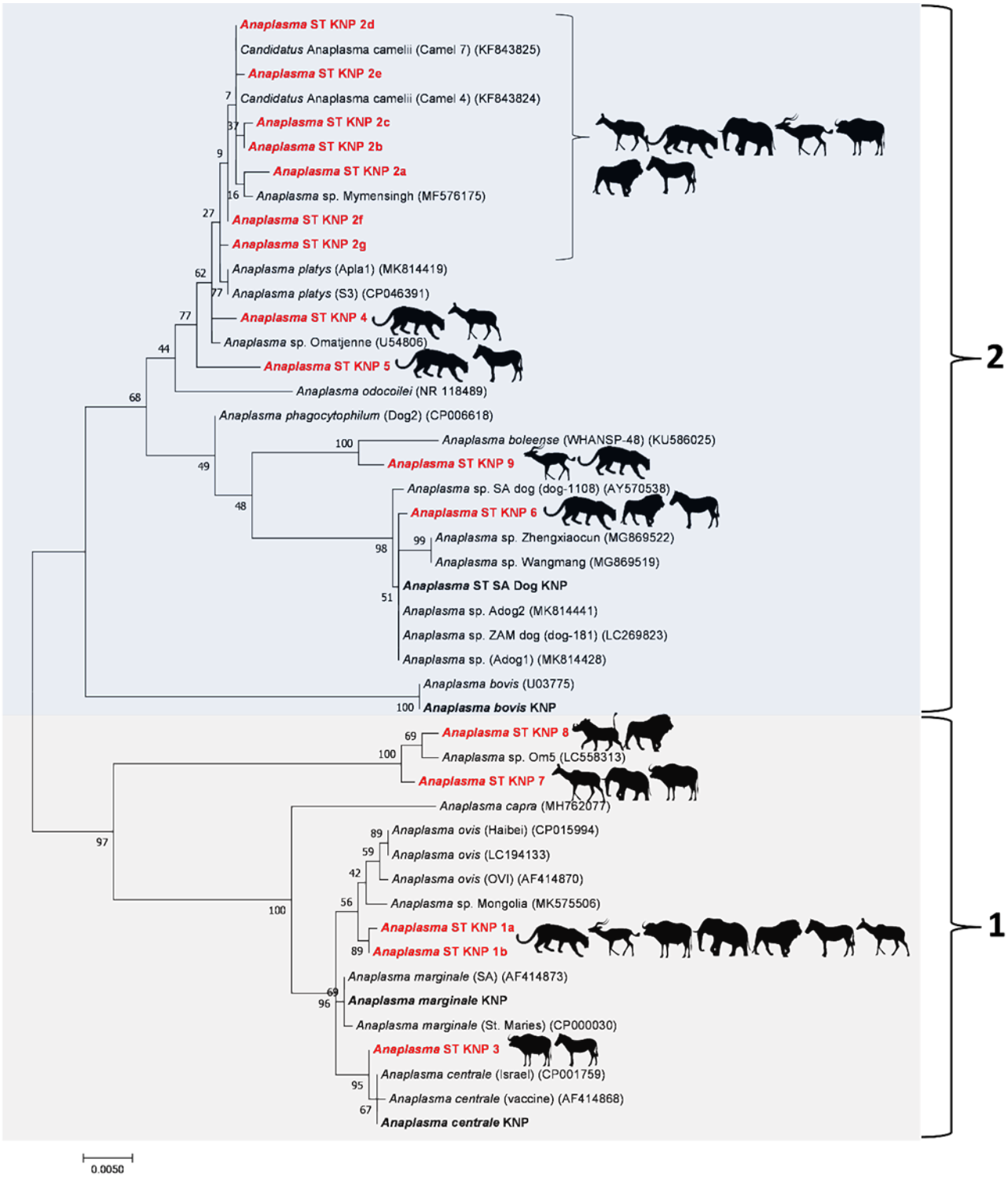

Figure 3.3: Maximum likelihood phylogenetic tree based on 16S rRNA sequence data showing the phylogenetic relationships between the novel *Anaplasma* 16S rRNA sequence types identified (shown in red) and previously described *Anaplasma* species. The tree is separated into the two prominent *Anaplasma* clades; clade-1, shaded in grey and clade 2, shaded in blue. Wildlife silhouettes indicate the hosts in which the novel *Anaplasma* 16S rRNA sequences were identified. Near full-length 16S rRNA gene sequences of approximately 1328 bp in length were used to construct this tree. The numbers associated with each node indicate the percentage of 1000 bootstrap replications supporting the node. Phylogenetic analyses were conducted in MEGA7 with an HKY85 evolutionary model.

3.4.5. Analysis of *Anaplasma* 16S rRNA sequences detected in wildlife hosts using an *Anaplasma* species-discriminating single-nucleotide polymorphism method

The *Anaplasma* species-discriminating bases technique of identifying and classifying *Anaplasma* spp. as proposed by Caudill & Brayton et al. (2022) identified six species-specific bases that differentiate *Anaplasma* species within clade-1. The two 16S rRNA gene sequence variants of *Anaplasma* ST KNP-1 (*Anaplasma* ST KNP-1a and *Anaplasma* ST KNP-1b) varied by a single nucleotide which was not one of the six species-discriminating bases. The *Anaplasma* ST KNP-1 sequences varied from known species in two to four of the species-discriminating bases (Table 3.4). Similarly, *Anaplasma* ST KNP-7 and *Anaplasma* ST KNP-8 varied from the other *Anaplasma* sequences in clade-1 by two or three species-discriminating bases (Table 3.4). The six species-discriminating bases of *Anaplasma* ST KNP-3 differed from *A. centrale*, *A. ovis* and *Anaplasma* sp. Mongolia by one to five bases but were identical to those of *A. marginale*. However, *Anaplasma* ST KNP-3 grouped most closely with *A. centrale* sequences on phylogenetic analysis. Should further analysis of these putative novel species indicate that they are distinct from the known *Anaplasma* species, a new typing scheme will become necessary.

Table 3.4: Species-discriminating bases of clade-1 of the genus *Anaplasma*.

Species	Base Number[d]					
	144	156	220	265	274	1250
A. marginale	A	G	T	T	G	T
A. centrale	A	A	T	T	G	T
A. ovis	G	R[f]	Y[f]	C	T	T
Anaplasma sp. Mongolia	G	A	C	C	G	C
Anaplasma ST KNP-1a & b[e]	G	A	C	T	A	T
Anaplasma ST KNP-3	A	G	T	T	G	T
Anaplasma ST KNP-7	G	G	C	T	G	T
Anaplasma ST KNP-8	G	G	C	T	G	T

[d] Numbering and sequence alignment based on the *Anaplasma marginale* St. Maries 16S rRNA gene sequence. Differences between the six species-discriminating bases in *A. marginale* and the other *Anaplasma* spp. are highlighted by white text on a black background.
[e] Two variants with identical species-differentiating bases but which differ elsewhere in the sequence.
[f] The degenerate position R denotes either A or G, while Y denotes either C or T.

Caudill & Brayton (2022) proposed 14 bases to differentiate between species within clade-2. One of these 14 bases in the *Anaplasma* ST. KNP-4 16S rRNA sequence differed from the *A. platys* sequence (Table 3.5), and there were other nucleotide differences elsewhere in the sequence. The *Anaplasma* ST KNP-5 sequence varied from *A. platys* and closely related

species by two to eight *Anaplasma* species-discriminating bases, and *Anaplasma* ST KNP-6 varied from *A. platys* and closely related species by four to seven *Anaplasma* species-discriminating bases. *Anaplasma* ST KNP-9 varied from *A. platys* and closely related species by four to eight *Anaplasma* species-discriminating bases.

Table 3.5: Species-differentiating bases of clade-2 of the genus *Anaplasma*.

Species	**Base Number[d]**													
	213	**224**	**262**	**289**	**693**	**696**	**878**	**879**	**885**	**886**	**890**	**1052**	**1309**	**1358**
A. platys	A	T	T	T	N[e]	T	R[e]	C	G	T	T	R[e]	Y[e]	C
A. sp. Mymensingh	A	T	T	T	C	T	A	C	G	T	T	A	C	C
A. sp. Omatjenne	A	C	T	T	C	T	R[e]	C	G	T	T	G	C	T
"*Candidatus* A. camelii"	A	T	T	T	C	T	A	C	G	T	T	A	T	C
A. odocoilei	G	A	G	C	A	A	G	T	A	C	C	G	C	C
A ST KNP-4	A	T	T	T	C	T	G	T	G	T	T	G	T	C
A ST KNP-5	A	T	T	T	A	A	G	C	G	C	T	A	C	C
A ST KNP-6	A	T	T	C	C	T	A	T	A	T	C	G	C	C
A ST KNP-9	A	T	T	T	A	A	A	T	A	T	T	G	C	T

[d] Numbering and sequence alignment based on the 16S rRNA gene from the *Anaplasma platys* strain S3 genome sequence. Differences between the 14 species-discriminating bases in *A. platys* and the other *Anaplasma* spp. are highlighted by white text on a black background.
[e] The degenerate position N denotes any possible nucleotide, while R denotes either A or G and Y denotes either C or T.

Seven variants of *Anaplasma* ST KNP-2 (a-g) 16S rRNA sequences were detected which group in clade-2. Although all of the *Anaplasma* ST KNP-2 variants, except for *Anaplasma* ST KNP-2g, were identical to *A. platys* according to the single-nucleotide polymorphism method of classifying *Anaplasma* spp. (Table 3.6), the sequences differed from *A. platys* elsewhere in the full length 16S rRNA sequence. Similarly, three of the *Anaplasma* ST KNP-2 sequences (2b, 2d and 2e) were identical to *"Candidatus* Anaplasma camelii" according to the species-discriminating nucleotides, however, the full length 16S rRNA sequences differed elsewhere.

Table 3.6: Species differentiating bases of clade-2 of the genus *Anaplasma*.

Species	Base Number[d]													
	213	**224**	**262**	**289**	**693**	**696**	**878**	**879**	**885**	**886**	**890**	**1052**	**1309**	**1358**
A. platys	A	T	T	T	N[e]	T	R[e]	C	G	T	T	R[e]	Y[e]	C
A sp. Mymensingh	A	T	T	T	C	T	A	C	G	T	T	A	C	C
A sp. Omatjenne	A	C	T	T	C	T	R[e]	C	G	T	T	G	C	T
"*Candidatus* A. camelii"	A	T	T	T	C	T	A	C	G	T	T	A	T	C
A. odocoilei	G	A	G	C	A	A	G	T	A	C	C	G	C	C
A ST KNP-2a	A	T	T	T	C	T	A	C	G	T	T	A	C	C
A ST KNP-2b, d & e[f]	A	T	T	T	C	T	A	C	G	T	T	A	T	C
A STKNP-2c & f[f]	A	T	T	T	C	T	A	C	G	T	T	G	T	C
A ST KNP-2g	A	C	T	T	C	T	A	C	G	T	T	G	C	C

[d] Numbering and sequence alignment based on the 16S rRNA gene from the *Anaplasma platys* strain S3 genome sequence.
[f] Two or three variants with identical species-differentiating bases which but differ elsewhere in the sequence.
[e] The degenerate position N denotes any possible nucleotide, while R denotes either A or G and Y denotes either C or T

3.5. Discussion

This study provides insight into the diversity of *Anaplasma* species circulating in wildlife hosts in the KNP and surrounding game reserves. The most recent reorganization of the genus *Anaplasma* included seven species: *A. marginale*, *A. centrale*, *A. ovis*, *A. bovis*, *A. platys, A. phagocytophilum* and *A. caudatum* (Dumler et al., 2001). *Anaplasma capra* has been effectively published in the literature but not formally recognized (Li, Zheng, et al., 2015; Yang et al., 2017). More than 20 other putative *Anaplasma* species have been identified, mostly by 16S rRNA gene sequence analysis (Caudill & Brayton, 2022). We identified four previously known and nine novel *Anaplasma* genotypes in nine wildlife hosts, namely, African buffalo, impala, kudu, zebra, warthog, hyena, leopard, lion and elephant. The four known *Anaplasma* sequences identified were *A. marginale*, *A. centrale*, *A. bovis* and *Anaplasma* ST SA dog. The nine novel *Anaplasma* genotypes were genetically distinct from but closely related to known *Anaplasma* spp. based on the 16S rRNA gene sequence analysis. Although not much is known about the pathogenicity of *Anaplasma* species in wildlife hosts, subclinical infections of known *Anaplasma* species have been reported and wildlife are thus usually regarded as reservoir hosts (Neitz, 1935; Peirce, 1972; Augustyn, 1974; Kuttler, 1984; Smith et al., 1982; Ngeranwa et al., 1998; Potgieter & Stoltsz, 2004; Eygelaar et al., 2015; Khumalo et al., 2016; Sisson et al., 2023). *Anaplasma marginale* infections in cattle can cause disease with varying levels of severity, from icterus and anaemia, to abortions and death, while *A. centrale*, *A. bovis* and *A.*

platys are regarded as non-pathogenic in cattle and usually cause subclinical infection in these animals.

As expected, our results indicate that *A. marginale* and *A. centrale* are widespread in the African buffalo population in the KNP. These two tick-borne haemoparasites have previously been identified in African buffalo, as well as black wildebeest, blue wildebeest, eland and waterbuck (Henrichs et al., 2016; Khumalo et al., 2016; Sisson et al., 2017; 2023). Interestingly, we detected 16S rRNA sequences identical to *A. marginale* in zebra, leopard and lion, while 16S rRNA sequences identical to *A. centrale* were also found in zebra and warthog. These data may suggest an expansion of the potential host range for *A. marginale* and *A. centrale*, as they are regarded as ruminant-specific *Anaplasma* species. However, it should be noted that low numbers of *A. marginale* and *A. centrale* 16S rRNA sequences were detected in a minority of feline samples. Nevertheless, more work needs to be done in a larger population of felines to determine whether the detection of *A. marginale* and *A. centrale* was incidental or if felines are reservoir hosts.

Anaplasma sp. dog strain was initially detected and identified in three dogs at the Veterinary Teaching Hospital of the Medical University of South Africa (Inokuma et al., 2005). The 16S rRNA gene sequence, designated *Anaplasma* sp. SA dog (GenBank accession no: AY570539 and AY570540), has 99.8% sequence identity to the 16S rRNA sequence from an *Anaplasma* sp. identified in dogs in Zambia, designated *Anaplasma* sp. ZAM dog (GenBank accession no: LC269823). The *Anaplasma* sp. ZAM dog 16S rRNA sequence was detected in domestic dogs in Lusaka, Zambia (Vlahakis et al., 2018) and recently in dogs and *Rhipicephalus sanguineus* ticks in the Mnisi community, Mpumalanga, South Africa (Kolo et al., 2020). Kolo et al. (2022) suggested that the *Anaplasma* 16S rRNA sequences identified in dogs represent variants of a single novel organism and proposed that it be referred to as *Anaplasma* sp. SA dog (for *Anaplasma* sp. Southern Africa dog) until type material can be deposited. Little is known about this putative *Anaplasma* species. It groups closely with *A. phagocytophilum* and other *Anaplasma* 16S rRNA sequences identified in dogs in clade-2. In our study, a 16S rRNA gene sequence identical to the *Anaplasma* sp. ZAM dog 16S rRNA sequence (GenBank accession no: LC269823) was widespread in the wildlife species examined; it was detected mainly in leopard, zebra and lion and to a lesser extent in impala, spotted hyena, buffalo and warthog. Although this *Anaplasma* species has thus far only been detected in dogs and associated ticks, our findings suggest additional wildlife hosts as possible reservoir hosts. Interestingly,

Anaplasma ST KNP-6, detected in leopard, lion and zebra, also grouped in the *Anaplasma* sp. SA dog clade with 99.6% shared 16S rRNA sequence identity. Our data could therefore suggest that *Anaplasma* ST KNP-6, *Anaplasma* sp. ZAM dog and *Anaplasma* sp. SA dog represent variants of the same species, however, additional genomic data is required to resolve this question, and, given their close relationship to *A. phagocytophilum* further work is required to determine their zoonotic potential.

We detected 16S rRNA sequences that are 100% identical to the *A. bovis* sequence (GenBank accession no: U03775) in kudu and leopard samples. Although *A. bovis* infection is mainly reported in cattle (Noaman & Shayan, 2010; Belkahia et al., 2015), little is known about the epidemiology of this agent. However, it is closely related to *A. phagocytophilum* and is regarded as a zoonotic agent that infects monocytes, it is usually associated with subclinical infection, and *Hyalomma* spp. are considered to be vectors of the organism (Donatien & Lestoquard, 1936). *Anaplasma bovis* was also detected in a population of eastern rock sengis (*Elephantulus myurus*) in South Africa, suggesting that sengis may be natural reservoir hosts of the organism (Harrison et al., 2013). It is thus possible that other rodent species are reservoir hosts of *A. bovis* and possibly other *Anaplasma* spp.

Of the nine novel *Anaplasma* 16S rRNA sequence types identified, *Anaplasma* ST KNP-1, *Anaplasma* ST KNP-3, *Anaplasma* ST KNP-7 and *Anaplasma* ST KNP-8 are found in clade-1, which is commonly referred to as the livestock clade, while *Anaplasma* ST KNP-2, *Anaplasma* ST KNP-4, *Anaplasma* ST KNP-5, *Anaplasma* ST KNP-6 and *Anaplasma* ST KNP-9 are found in clade-2, commonly referred to as the zoonotic clade. It should be noted that, since similarities above 98.70% occur between 16S rRNA sequences of known *Anaplasma* species (Caudill & Brayton, 2022), it is difficult to distinguish between *Anaplasma* species based on 16S rRNA sequences alone. Furthermore, it has previously been shown that the 16S rRNA gene sequences of *A. platys*, *Anaplasma* sp. Mymensingh, "*Candidatus* Anaplasma camelii" and *Anaplasma* sp. Omatjenne share more than 99.5% sequence identity and do not resolve these organisms to species level, although a high degree of intraspecies variance is evident based on the single-nucleotide polymorphisms used to distinguish the species within this clade (Caudill and Brayton, 2022). Many of the novel 16S rRNA sequences identified in our study are highly similar to previously reported *Anaplasma* sequences, and it is therefore not clear whether they represent novel species or variants of known or previously reported putative *Anaplasma* species. This will require further investigation, including the

sequencing and phylogenetic analysis of other genes, or whole genome sequence analyses. It is evident that there is an urgent need to identify an alternative gene or genes for the discrimination of species within the genus *Anaplasma*.

Although *Anaplasma* species and strains infecting one host species might not necessarily infect and cause disease in other host species, we have noted the presence of some of the novel 16S rRNA sequences in a variety of wildlife hosts, suggesting that they may be able to infect multiple host species. Therefore, the ability of these newly identified agents to infect cattle, other domestic animals and possibly even humans should be assessed. Novel 16S rRNA sequences have already been identified in cattle in South Africa. These include "*Candidatus* Anaplasma boleense", *Anaplasma* sp. Mymensingh and *Anaplasma* sp. SA dog (Kolo et al., 2020) which were identified in cattle in the Mnisi community, which borders on the Manyeleti and Timbavati Game Reserves and the Kruger National Park. If the putative *Anaplasma* species identified in our study are found to infect livestock, they could affect the specificity of existing tests for detection of *A. marginale*. Furthermore, the presence of the novel 16S rRNA sequences in wildlife could impact on the use of existing tests for the detection of known *Anaplasma* species in wildlife.

Seroprevalence studies are often used to determine the prevalence of *A. marginale.* Current serological tests used to diagnose anaplasmosis include the competitive ELISA (cELISA), complement fixation test, card agglutination test (CAT) and IFA test, with only cELISA and CAT recommended for the diagnosis of anaplasmosis (Kocan et al., 1992; De la Fuente et al., 2005). The commercially available *Anaplasma* genus-specific cELISA kit (Knowles et al., 1996) uses recombinant major surface protein 5 (Msp5) as antigen. Since the Msp5 epitope is widely conserved between *Anaplasma* species (Munodzana et al., 1998; Hofmann-Lehmann et al., 2004; Dreher et al., 2005; Strik et al., 2007), the cELISA cannot be used for the specific detection of *A. marginale* if multiple species of *Anaplasma* are known to occur in cattle, frequently as co-infections (Zweygarth et al., 2006; Khumalo et al., 2016; Hove et al., 2018; Makgabo et al., 2023). Indeed, in many parts of the world assumptions are generally made by host species: if the test is positive in cattle, it is likely that it is detecting *A. marginale*, whilst a positive result from sheep or goats should indicate *A. ovis* infection. However, these assumptions can lead people astray (Da Silva et al., 2018), due to the broad cross-reactivity among known *Anaplasma* species from both clades (Munodzana et al., 1998; Hofmann-Lehmann et al., 2004; Dreher et al., 2005; Strik et al., 2007). We suspect the cELISA will also

recognise the putative *Anaplasma* species identified in wildlife, although more work will be required to confirm this.

Based on our analysis, several primers and probes from nucleic acid-based assays targeting the 16S rRNA gene, such as the *A. platys* (Inokuma et al., 2001) and *A. phagocytophilum* (Kawahara et al., 2006) specific assays, as well the reverse line blot hybridization (RLB) assay (Georges et al., 2001; Bekker et al., 2002), would cross-react with some of the *Anaplasma* ST detected, ST KNP-2, ST KNP-4, ST KNP-5 as well as other previously described putative *Anaplasma* species, including *Anaplasma* sp. Omatjenne and *Anaplasma* sp. Mymensingh, while the *A. phagocytophilum* assay (Kawahara et al., 2006) would also cross-react with *Anaplasma* ST SA dog and *Anaplasma* ST KNP-6. The RLB *Anaplasma* sp. (Omatjenne) probe (Bekker et al., 2002) would cross-react with *Anaplasma* sp. Mymensingh, *Anaplasma* ST KNP-2 (all variants), *Anaplasma* ST KNP-4, as well as *Anaplasma* ST KNP-5. The *A. centrale*-specific probe (Georges et al., 2001) would cross-react with the two variants of *Anaplasma* ST KNP-1. The use of these assays to determine the presence of known *Anaplasma* species in wildlife should therefore be interpreted with caution. This highlights the necessity for more specific assays to be developed to assess the epidemiology of *Anaplasma* species more accurately.

The widely used duplex real-time assay to detect *A. marginale* and *A. centrale* infections in cattle (Decaro et al., 2008; Byaruhanga et al., 2016; Hove et al., 2018) is not based on the 16S rRNA gene but on the *A. marginale msp1β* gene and the *A. centrale groEL* gene (Decaro et al., 2008; Chaisi et al., 2017). While these assays have been used to detect *A. marginale* and *A. centrale* in wildlife (Khumalo et al., 2016), it remains to be seen whether these assays will cross-react with the putative novel *Anaplasma* species since nothing is known about their gene complement.

3.6. Conclusion

Our results revealed a greater genetic diversity of *Anaplasma* species circulating in wildlife hosts than currently classified within the genus *Anaplasma* and suggest potential for transmission to livestock or companion animals. Furthermore, these novel genotypes are phylogenetically similar to known *Anaplasma* spp. and may serve as a source of cross-reaction

in the current detection assays. Although this data, including that of single-nucleotide polymorphisms used to distinguish between the different *Anaplasma* species within the two clades, may provide sufficient genetic divergence between these organisms to potentially suggest classification as separate species within the clade, there is a need for additional genetic data and genome sequencing of these putative species for correct *Anaplasma* species classification and to further determine their occurrence in livestock and companion animals.

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CHAPTER 4

Temporal dynamics of *Anaplasma marginale* infections and the composition of *Anaplasma* spp. in calves in the Mnisi communal area, Mpumalanga, South Africa[2]

4.1. Abstract

Bovine anaplasmosis, caused by *Anaplasma marginale*, is one of the most important tick-borne diseases of cattle. *A. marginale* is known to be present in the Mnisi community, Mpumalanga Province, with frequent cases of anaplasmosis reported. This study investigated the infection dynamics in calves (n=10) in two habitats in the study area over 12 months. A duplex real-time PCR assay targeting the *msp1β* gene of *A. marginale* and the *groEL* gene of *A. centrale* confirmed the presence of *A. marginale* in five calves in a peri-urban area from the first month, but in only two calves at the wildlife–livestock interface and only after six months. These results were confirmed by 16S rRNA microbiome analysis. Over 50 *A. marginale msp1α* genotypes were detected in the calves along with five novel Msp1a repeats. Calves in the peri-urban area were more likely to be infected with *A. marginale* than calves in the wildlife–livestock interface. Cattle management, acaricide treatment, and cattle density could explain differences in infection prevalence in the two areas. Our results revealed that most calves were superinfected by distinct *A. marginale* strains within the study period, indicating continuous challenge with multiple strains that should lead to robust immunity in the calves and endemic stability in the area.

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4.2. Introduction

Bovine anaplasmosis is a tick-borne disease (TBD) caused by the obligate intracellular pathogen, *A. marginale* (Aubry & Geale, 2011). Bovine anaplasmosis occurs predominantly in cattle; however, infection can also occur in ruminants such as sheep, goats, African antelopes, Cape buffalo, and some species of deer (Aubry & Geale, 2011). Bovine anaplasmosis caused by *A. marginale* is prevalent throughout the world occurring in Africa, the Americas, Asia, Australia, the Caribbean, and Europe (Kocan et al., 2003). The disease is amongst the three most economically important TBDs of cattle resulting in mortality and morbidity, decreased milk and meat production, and expensive control measures (Uilenberg, 1995; de Waal, 2000; Makala et al., 2003; Mtshali, de Waal & Mbati, 2004). The economic impact of bovine anaplasmosis in South Africa has been estimated at approximately R115 million ($US9.6 million) per year due to mortalities (Hove, 2018), but this does not take into account costs associated with treatment and control. In other parts of the world, costs arising from anaplasmosis have been estimated from $US300 to $US800 million (Kocan et al., 2003). Clinical signs caused by infection with *A. marginale* are characterized by fever, progressive anaemia, weight loss and abortion, as well as icterus that may result in mortality. The closely related organism, *A. centrale,* usually causes asymptomatic infections and is currently used as a vaccine for cattle in South Africa (Kocan et al., 2004; Potgieter & Stoltsz, 2004; Palmer G.H., 2009). Animals under one year of age are usually asymptomatic to infection with *A. marginale* (Bock et al., 1997; Jonsson et al., 2012). However, older animals are more likely to react severely and fatally upon challenge (Aubry & Geale, 2011).

Biological transmission of *A. marginale* to naïve cattle occurs by feeding ticks, while mechanical transmission occurs by biting flies or blood-contaminated instruments (Kocan, Blouin & Barbet, 2000; de la Fuente et al., 2002). Transplacental transmission of *A. marginale* has also been reported (Aubry & Geale, 2011; Costa et al., 2016). Although the transmission of *A. marginale* in South Africa has not been extensively studied, five tick species, *R. decoloratus, R. microplus*, *R. evertsi evertsi*, *R. simus,* and *H. rufipes*, have been shown experimentally to transmit anaplasmosis and could therefore account for the widespread distribution of the disease (de Waal, 2000; Potgieter & Stoltsz, 2004). *Rhipicephalus decoloratus* has long been regarded as the main vector (Potgieter & Stoltsz, 2004), with *R. microplus* increasing in importance as a vector due to its recent spread into most South African provinces (Nyangiwe, Harrison & Horak, 2013; Nyangiwe et al., 2017).

The genetic diversity of *A. marginale* from many countries around the world has been characterized using a genotyping method based on sequence analysis of the single-copy *msp1α* gene that codes for the major surface protein 1a (Msp1a) (Palmer et al., 1999; de la Fuente et al., 2005, 2007). Msp1a is regarded as a determining marker for *A. marginale* transmission between cattle and ticks as it has been shown to be involved in the adhesion of the pathogen to tick cells and bovine erythrocytes (Aubry & Geale, 2011). The genotyping method uses differences in the number and sequence of tandem repeats located at the N-terminus of the Msp1a protein to differentiate between strains. The *msp1α* genotyping method was first described in 1990, and since then >300 genotypes have been reported worldwide (Allred et al., 1990; Catanese, Brayton & Gebremedhin, 2016). In South Africa, a diversity of *A. marginale* genotypes has also been identified (de la Fuente et al., 2005, 2007; Mtshali et al., 2007; Mutshembele et al., 2014; Hove et al., 2018).

The presence of single *msp1α* genotypes in infected cattle is a well-documented phenomenon (de la Fuente et al., 2001, 2002), but infection with multiple *A. marginale* strains (superinfection) has been less well studied. More recently, both co-infection and superinfection of cattle with multiple genetically distinct strains of *A. marginale* have been shown to be important drivers of *A. marginale* infection (Palmer et al., 2004; Futse et al., 2008; Castañeda-Ortiz et al., 2015). Co-infection and superinfection were recently shown to drive the development of complex infection with *A. marginale* under natural transmission conditions in Ghana (Koku et al., 2021).

In the South African context, bovine anaplasmosis is currently widespread and endemic throughout the cattle-farming areas in all South African provinces, except for the Northern Cape, where the vector is mostly absent (de Waal, 2000; Mtshali, de Waal & Mbati, 2004; Mutshembele et al., 2014; Hove et al., 2018). Data collected through the Health and Demographic Surveillance System in Livestock (HDSS) established in the study area of the Mnisi community, indicate the presence of *A. marginale* in cattle, with frequent bovine anaplasmosis cases reported at villages that abut provincial and private game reserves (the wildlife–livestock interface) (Choopa, 2015). The Mnisi community is a sprawling area that provides an opportunity to study natural *A. marginale* infection dynamics at both more densely populated peri-urban villages and at villages at the wildlife–livestock interface (Kolo et al., 2020). To understand *A. marginale* strain diversity, infection dynamics, and the nature of

clinical cases of anaplasmosis in the Mnisi community, ten calves were examined from birth for a period of 12-months in a peri-urban area and at a wildlife–livestock interface.

4.3. Materials and Methods

4.3.1 Ethical consideration

The study was carried out in strict accordance with the conditions and guidelines of the Animal Ethics Committee of the Faculty of Veterinary Science (reference number: V041-16) (Appendix 6)**.** Permission to perform the study under Section 20 of the Animal Disease Act of 1984 was granted by the South African Department of Agriculture, Forestry and Fisheries (currently Department of Agriculture, Land Reform and Rural Development) (reference number: 12/11/1/1/6) (Appendix 7).

4.3.2. Study area

The Mnisi community (24.8205° S, 31.1710° E) is situated in the north-eastern corner of the Bushbuckridge Municipality, Mpumalanga Province, South Africa (Figure 4.1). The community shares 75% of its boundary with adjacent wildlife areas, including the Andover and Manyeleti provincial game reserves and the Timbavati and Sabi Sand private game reserves. There are no fences between these reserves, including the Kruger National Park (KNP), such that game can freely roam between them. Livestock farming is the main agricultural activity in the area with more cattle than any other livestock species. The project was conducted in three villages, Eglington, Utha A, and Dixie. Eglington village is in a peri-urban area, while Utha A and Dixie are located at the wildlife–livestock interface close to the border with Manyeleti provincial game reserve.

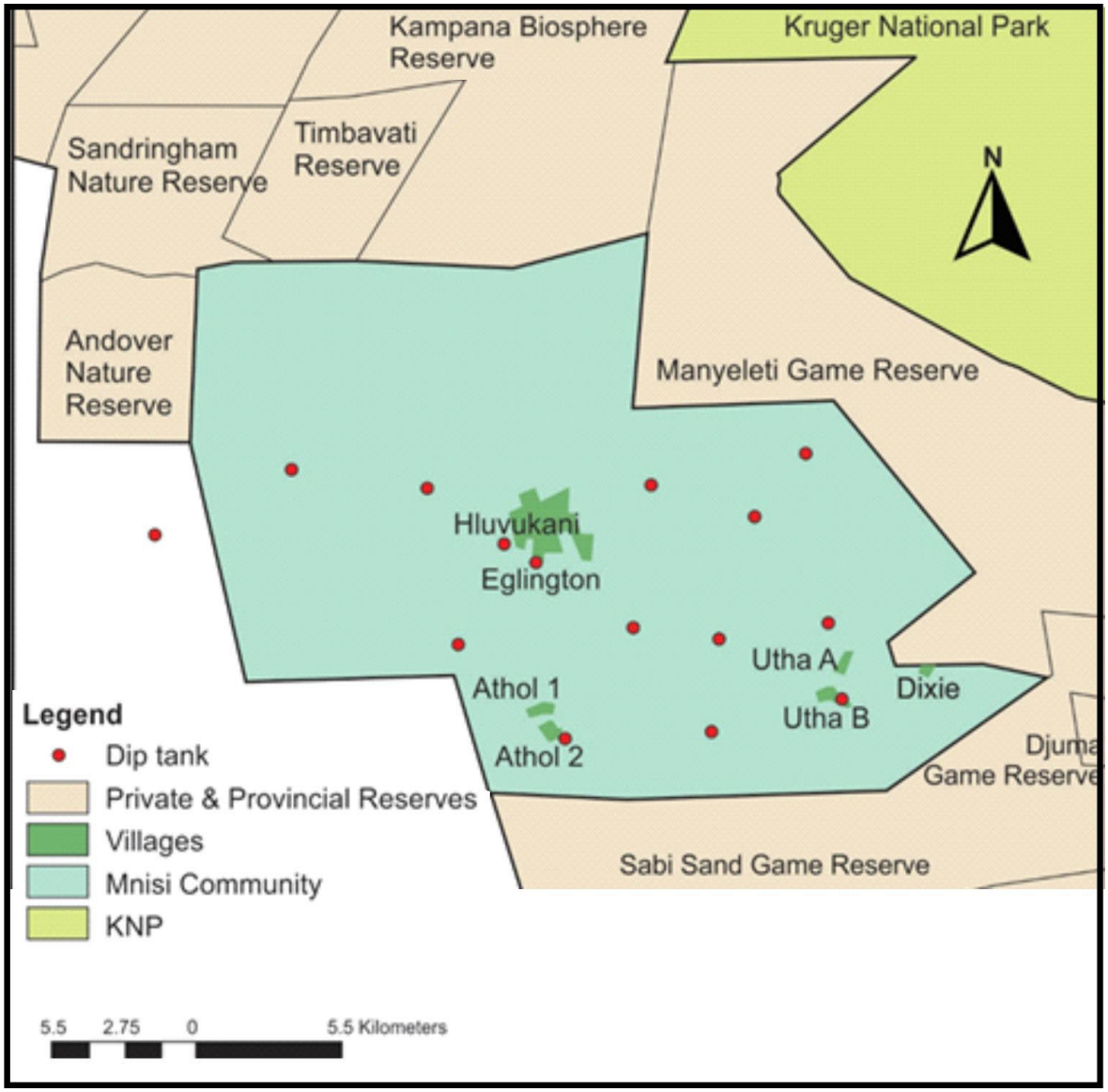

Figure 4.1: Map of the Mnisi communal area, showing the location of the three villages where the study was conducted, Eglington, Utha A, and Dixie, relative to various wildlife reserves and the Kruger National Park (KNP).

Over 40,000 people live in the Mnisi community, with livestock farming being the main agricultural activity and cattle as the most important species. Eglington village is a peri-urban area, situated approximately 11.5 km away from the Manyeleti Game Reserve, 12.1 km from the Andover Game Reserve, and 15.1 km from the Timbavati Game Reserve. Each day, cattle herders collect the cattle from the owners' homes where they are kept in kraals overnight, and they are taken to fully protected and fenced cattle grazing camp, where the chosen calves grazed during the study period. The Eglington cattle grazing camp is located approximately 16 km away from Manyeleti Game Reserve, 13 km from Andover Game Reserve, and 15.1 km from

Timbavati Game Reserve. Utha A and Dixie villages are only 2 km apart and are located close to the wildlife/livestock interface being, respectively, approximately 2.4 km and 0.5 km away from the Manyeleti Game Reserve. In this area, cattle grazing camps are located adjacent to the Manyeleti Game Reserve and cattle are often seen grazing alongside wildlife separated only by the game fence, which is the only barrier between livestock and the abundant wildlife populations in the game reserves. Due to the study area being in the foot and mouth disease (FMD) protection zone, animals can move between villages in the zone only with permission from the state vet, however, trading of livestock out of the zone is not permitted. There is therefore little animal cross-traffic between villages. The community is characterized by a human health centre, animal health clinic, and shopping complex in Hluvukani, where people from the different villages gather. There is human cross-traffic in the study area, with villagers, commuters, researchers, and veterinarians travelling freely between villages.

Due to the study area being in the FMD protection zone, and the proximity of wildlife species, which harbour and facilitate the spread of ticks and tick-borne diseases between wildlife, livestock and humans, comprehensive disease surveillance measures are implemented in the area by local veterinary services, mainly in the form of cattle dip tanks built throughout the region, which every cattle herd must visit for dipping and FMD inspection once a week. The dip consists of the Delete® X5 acaricide which is used on cattle, sheep, and goats, for the prevention and treatment of ectoparasite infestation. The farmers in the Mnisi community do not vaccinate their cattle against bovine anaplasmosis.

4.3.3. Animals

Ten local mixed breed *Bos taurus* calves (0–1 months of age, 6 males and 4 females) were monitored for a period of one year. Three of the ten calves were situated in Utha A (with a total of 715 cattle and a cattle density of 128 cattle/km^2) and two were in Dixie (with a total of 137 cattle and cattle density of 27 cattle/km^2); these two villages are located approximately 2.4 km and 0.5 km away, respectively to the wildlife–livestock interface. The remaining five cattle were based in Eglington village (with a total of 1009 cattle and a cattle density of 194 cattle/km^2); this is a peri-urban area, located 11.5 km away from the border with Manyeleti Game Reserve. The local veterinary services used the following different methods of acaricide treatment in the two areas: the plunge method of dipping cattle was used at Eglington (the peri-urban area), as well as Utha A (wildlife-livestock interface), while the hand spraying method

was used at Dixie village (wildlife-livestock interface) due to water-shortages. The study required farmers with a relatively small herd of cattle who do not dip their cattle privately.

4.3.4. Study design and sample collection

This longitudinal study was conducted between November 2016 (when the calves were 0–1-month-old) and October 2017. Whole blood samples were collected in 10 mL Vacutainer® ethylenediaminetetraacetic acid (EDTA) tubes from the ten calves once a month for 12 months according to the 12 time-point collection timeline (Figure 4.2).

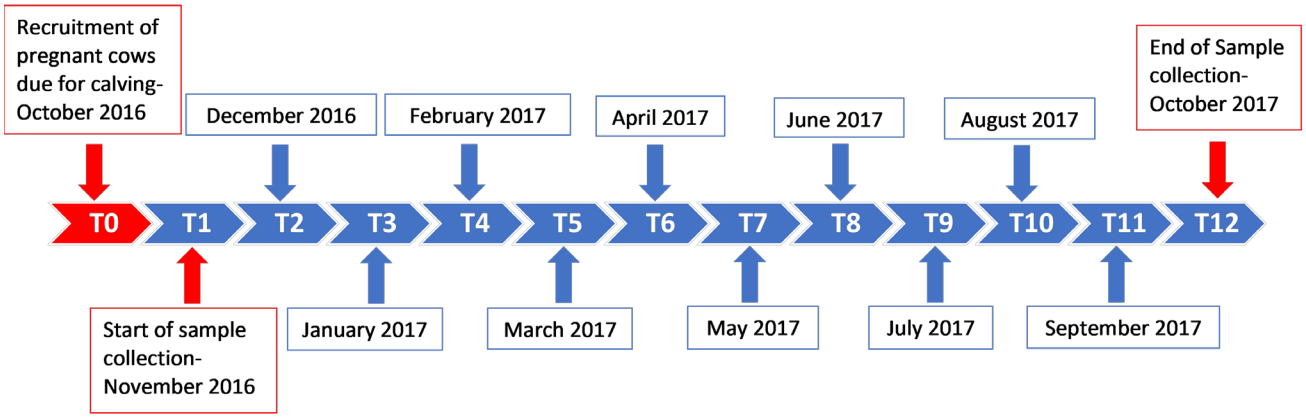

Figure 4.2: Sample collection timeline for the study. Samples were collected monthly from the ten calves for a period of one year (T1- November 2016, T2- December 2016 and T12- October 2017), from the ages of 0–1 month old (0-1M) to 11–12 months old (11-12M). T(x) = time point (month number).

4.3.5. Genomic DNA extraction and quantitative Real-Time PCR (qPCR) assay

Genomic DNA was extracted from the samples collected from all time-points using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA was eluted in 100 µL elution buffer and stored at –20°C. Genomic DNA samples were screened for the presence of *A. marginale* and *A. centrale* using a duplex qPCR assay targeting the *msp1β* gene of *A. marginale* and the *groEL* gene of *A. centrale* [34]. Primers, AM-For (5′-TTG GCA AGG CAG CAG CTT-3′), AM- Rev (5′-TTC CGC GAG CAT GTG CAT-3′) and a probe, AM-Pb (6-FAM-TCG GTC TAA CAT CTC CAG GCT TTC AT-BHQ1) were used to amplify and detect a 95 bp fragment of the *msp1β* gene of *A. marginale*

while primers, AC-For (5′-CTA TAC ACG CTT GCA TCT C-3′), AC-Rev (5′-CGC TTT ATG ATG TTG ATG C-3′) and probe AC-Pb (LC610-ATC ATC ATT CTT CCC CTT CCC CTT TAC CTC GT-BHQ2) were used to amplify a 77 bp fragment of the *groEL* gene of *A. centrale*. Reactions were performed in a 20 µL final reaction volume comprising 4 µL FreshStart Taqman mix (Roche Diagnostics, South Africa), 0.5 µL UDG, 0.6 µM of the *A. marginale*-specific primers, 0.9 µM of the *A. centrale*-specific primers, 0.2 µM of each probe, and 2.5 µL of template DNA (approximately 200 ng). The duplex assay was performed on a LightCycler v2 (Roche Diagnostics, Mannheim, Germany), using the thermal cycling conditions described previously (Chaisi et al., 2017). Positive control for the *A. centrale* assay was DNA extracted from the *A. centrale* vaccine strain obtained from Onderstepoort Biological Products (OBP), Pretoria, South Africa. Field sample C14 (obtained from cattle in the Mnisi Community area, Mpumalanga) was used as the positive control for the *A. marginale* assay. Nuclease-free water was used as a negative control for the assay. Results were analyzed using the LightCycler Software version 4.0 (Roche Diagnostics, Mannheim, Germany). Samples were run in triplicate per time-point to ensure reproducibility and repeatability of the results. A published linear range of detection and assay efficiency (Chaisi et al., 2017; Hove et al., 2018) were used to quantify the level of *A. marginale* rickettsaemia which was expressed as infected red blood cells (iRBC) per mL of blood.

4.3.6. Amplification, cloning and sequencing of the *Anaplasma marginale msp1α* gene

The repeat-containing variable region of the *A. marginale msp1α* gene was amplified using primers 1733F (5′-TGT GCT TAT GGC AGA CAT TTC C-3′) and 2957R (5′-AAA CCT TGT AGC CCC AAC TTA TCC-3′) (Lew et al., 2002). Amplifications were performed in a 25 µL final reaction volume and consisted of 1x Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific), 0.5 µM of each primer, and 2.5 µL of template DNA (approximately 200 ng). The thermal cycling parameters used were modified from those previously reported (Lew et al., 2002) and comprised a pre-PCR denaturation at 94°C for 3 min and *Taq* activation at 98°C for 10 s, followed by 30 cycles of 98°C for 1 sec, 69.1°C for 5 sec, and 72°C for 18 sec, and a final extension at 72°C for 1 min. PCR products were analysed by electrophoresis on a 1.5% agarose gel (1 × TAE buffer, pH 8.0), stained with ethidium bromide and viewed under UV light. All positive PCR products were purified using the Omega Bio-tek® DNA purification kit (Whitehead Scientific, South Africa) according to the manufacturer's instructions. Purified PCR products were cloned into pJET 1.2 (Thermo Fisher

Scientific, South Africa). Recombinant clones were screened by colony PCR using vector specific primers, pJET1.2F and pJET1.2R; clones which yielded a product of 610 bp or greater were selected for sequencing. Fifteen recombinant clones per calf per time-point were sequenced bidirectionally on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, California, USA) at Inqaba Biotechnical Industries, Pretoria or at the Central Analytical Facility, Stellenbosch University. *Anaplasma marginale msp1α* nucleotide sequences generated in this study were named according to a naming to a proposed naming scheme (Catanese, Brayton & Gebremedhin, 2016) and deposited in GenBank under accession numbers OQ384772–OQ384912 and are also available under BioProject accession number PRJNA929355.

4.3.7. Characterization of *Anaplasma marginale* Msp1a repeats and *msp1α* genotypes

Msp1a sequences were trimmed, assembled, edited, and aligned using CLC Genomics Workbench 20 (Qiagen, Aarhus, Denmark). The RepeatAnalyzer command line software tool (Catanese, Brayton & Gebremedhin, 2016) was used to identify, store, curate, and analyse Msp1a repeats and *A. marginale msp1α* genotypes. Novel repeats that were not recognized by RepeatAnalyzer were designated UP37 to UP42. The Msp1a repeat structure determines the *msp1α* genotype of a strain.

4.3.8. 16S rRNA gene amplification and PacBio sequencing

In order to determine the composition and diversity of *Anaplasma* species present in the ten calves by T12, the full-length 16S rRNA gene (V1-V9 variable regions) was amplified in triplicate from the ten DNA samples collected at T12 using modified barcoded 16S rRNA gene specific primers, 27F: (5′-AGR GTT YGA TYM TGG CTC AG-3′) and 1492R: (5′-RGY TAC CTT GTT ACG ACT T-3′) recommended for the PacBio Sequel II sequencing instrument (Pacific Biosciences, Menlo Park, California, USA) (Lane, 1991; Turner et al., 1999). Reactions were performed in triplicate in a final volume of 25 µL containing 1 X Phusion Flash® High Fidelity Master Mix (Thermo Fisher Scientific, South Africa), 0.15 µM of each forward and reverse primer, and 5 µL of DNA (approximately 400 ng). DNA extracted from the *A. centrale* vaccine strain (Onderstepoort Biological Products, South Africa) was used as a positive control and molecular grade water as a negative control. Cycling conditions included 98°C for 30 sec, followed by 35 cycles of 98°C for 10 sec, 60°C for 30 sec, and 72°C for 30 sec and a final extension at 72°C for 10 min. Amplicons were visualized under UV light after

electrophoresis on a 1.5% agarose gel stained with ethidium bromide. Amplicons were purified using the QIAquick® PCR purification kit (Qiagen) according to the manufacturer's instructions and submitted to the Genomics Sequencing Core at Washington State University, Pullman, USA for circular consensus sequencing (CCS) on the PacBio (Pacific Biosciences, Menlo Park, California, USA) platform. The raw microbiome data from the ten calves is available at the Sequence Read Archive (SRA) with BioProject accession number PRJNA929355.

4.3.9. Analysis of *Anaplasma* 16S rRNA sequences identified by microbiome sequencing

The 16S rRNA amplicon sequence data was curated and filtered using SMRT Link software 8.0 according to a minimum barcode score of 70 and 99% precision. Final Fasta and Fastaq data sets were analyzed using the Ribosomal Database Project (RDP) 16S classifier (Cole et al., 2009; Gall et al., 2016) for *Anaplasma* genus level classification of the sequences with a 95% confidence interval. Sequences were further classified to the *Anaplasma* species level using a customized NCBI BLASTn database of all known and published *Anaplasma* spp. sequences downloaded from GenBank using the command line application. Sequences were further filtered and excluded based on sequence length (minimum of 1275 bp), quality, and sequence identity in Microsoft Excel (Gall et al., 2016; Caudill & Brayton, 2022). Since some distinct *Anaplasma* species are known to have more than 98.7% shared sequence identity, and *A. platys*, *Anaplasma* sp. Mymensingh, "*Candidatus* Anaplasma camelii" and *Anaplasma* sp. Omatjenne share more than 99.5% 16S rRNA gene sequence identity, it is not possible to resolve these organisms to species level (Caudill & Brayton, 2022). Thus, only 16S rRNA sequences that were identical to previously published sequences were classified to species level; the remainder were reported as putative novel *Anaplasma* species and/or genotypes. The *Anaplasma* 16S rRNA nucleotide identified in this study were deposited in GenBank under accession numbers OQ348128-OQ348132, with BioProject accession number PRJNA929355.

4.3.10. Sequence and phylogenetic analysis

The *Anaplasma* 16S rRNA gene sequences identified by microbiome sequencing were aligned with reference sequences from GenBank. *Anaplasma* 16S rRNA sequences from representative genome sequences as well as the most closely related sequences from cattle and other ruminants in South Africa and worldwide, as identified by BLAST analysis, were selected to construct the phylogenetic tree. *Anaplasma* 16S rRNA sequences from wildlife were included

(Makgabo et al., 2023). The extent of sequence variation was analysed using CLC Genomics Workbench 20 (Qiagen, Aarhus, Denmark). Alignments were further trimmed using Bioedit 7 (Hall, 1999). Jmodel test 1.3 (Darriba et al., 2012) predicted the HKY85 (Hasegawa–Kishino–Yano, 85) evolutionary model (Guindon & Gascuel, 2003; Anisimova & Gascuel, 2006; Chevenet et al., 2006) as the best fit model for the 16S rRNA gene sequences. Phylogenetic trees for the 16S rRNA gene were constructed using the neighbor-joining and maximum likelihood (ML) method in MEGA 7 with bootstrap analysis using 1000 replicates to estimate the confidence levels of the tree branches (Kumar, Stecher & Tamura, 2016), as well as the Bayesian inferences in Mr Bayes 3.2 (Ronquist et al., 2012).

4.4. Results

Anaplasma marginale was detected in seven of the 10 (70%) calves recruited to the study using the *A. marginale* and *A. centrale* duplex qPCR. Of the seven calves that tested positive for *A. marginale*, five were in the peri-urban area (Eglington village), while only two were located at the wildlife–livestock interface, both in Utha A village.

Four of the calves in the peri-urban area were already infected with *A. marginale* at the first time point (T1), and by the second time-point (T2), all five calves in this area tested positive (Figure 4.3A). The two calves that tested positive for *A. marginale* at the wildlife–livestock interface became infected only at T7 and T8, while the remaining three calves at the wildlife–livestock interface were either infected at levels below the detection limit of the assay (250 copies per reaction) or were not infected with *A. marginale* at (Figure 4.3B). *Anaplasma centrale* was not detected in any of the calves.

The levels of *A. marginale* infection fluctuated over the course of the 12-month study period, exhibiting the cyclic rickettsaemia known to occur in persistently infected animals (Kieser, Eriks & Palmer, 1990; Kocan et al., 2003). In calves in the peri-urban area, the rickettsaemia ranged from 4 x 10^6 to 3 x 10^9 iRBC/mL from time of infection to a year. The levels of rickettsaemia in the two calves at the wildlife–livestock interface ranged from 2 × 10^6 to 2 × 10^7 iRBC/mL in the five and six months of infection.

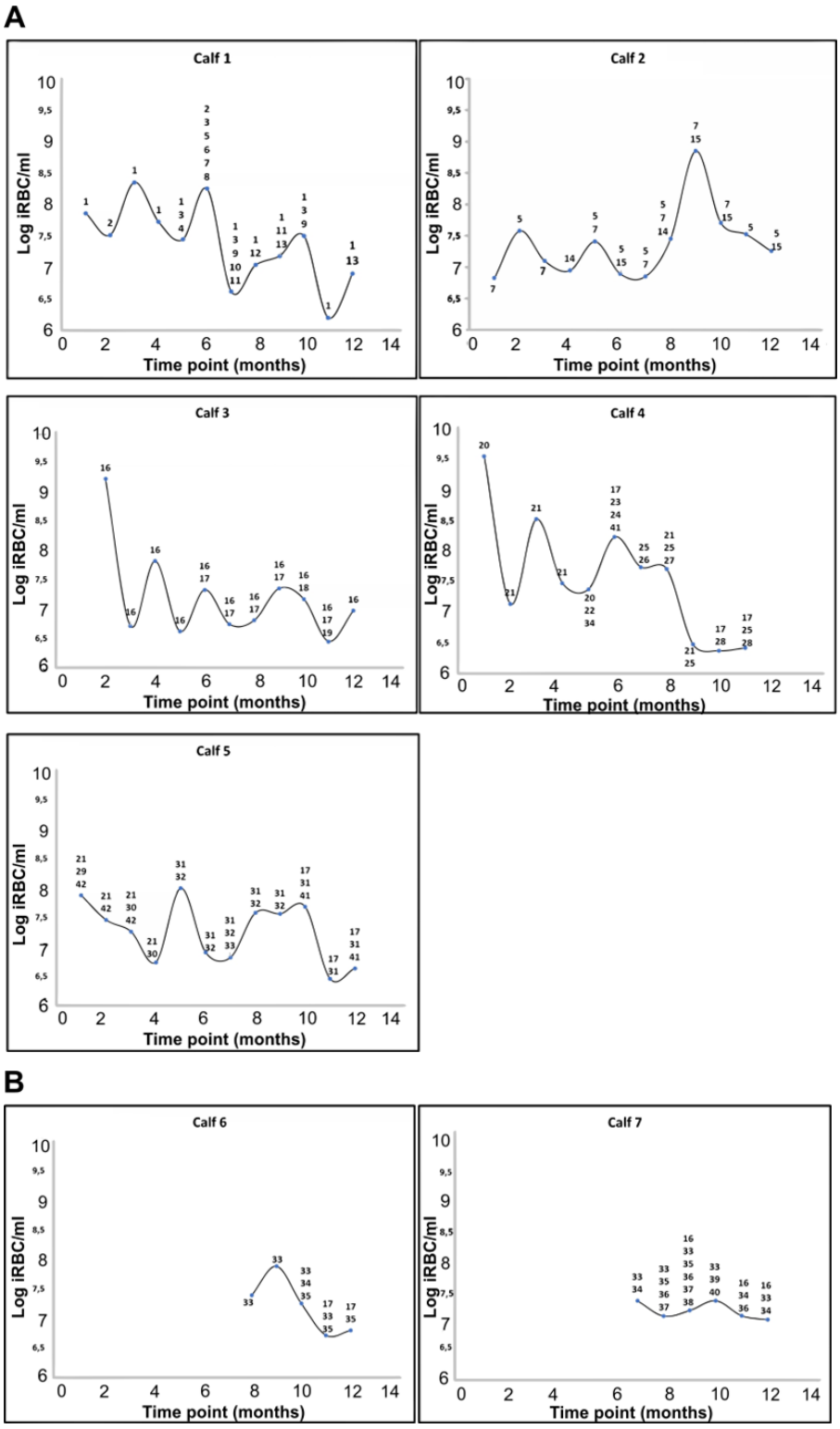

Figure 4.3: Cyclic *Anaplasma marginale* rickettsemia in calves from the Mnisi community as determined by qPCR (Chaisi et al., 2017). The level of infection is expressed as the log of infected red blood cells (RBC) per mL (iRBC/mL) of blood. A) Calves infected with *A. marginale* at the peri-urban area. B) Calves infected with *A. marginale* at the wildlife-livestock interface. The number and type of *A. marginale msp1α* genotypes detected at each time-point are indicated for each calf; genotypes were assigned numbers as shown in Table 4.1.

4.4.1. *Anaplasma marginale msp1α* genotype analysis in the calves for a period of a year

A total of 406 *msp1α* nucleotide sequences were generated from the seven *A. marginale*-positive calves and, in total, 42 unique *msp1α* genotypes were generated from the seven calves over the 12-month study period; however, several of the genotypes occurred in more than one animal (Table 4.1). Of the total number of *A. marginale* genotypes generated from the seven calves, 76.4% were identified in the five calves at the peri-urban area and 23.6% were identified in the two calves at the wildlife–livestock interface. Calves were infected with four to 13 genotypes (Table 4.1). Of the 42 *msp1α* genotypes identified, only four occurred in both areas (Table 4.1).

The *A. marginale msp1α* genotypes identified in the seven calves were made up of a total of 56 Msp1a repeats; 50 of these have been reported previously while six sequences are novel Msp1a repeats detected for the first time in this study (Figure 4.4). While only three of the *msp1α* genotypes occurred in both areas, 47.4% of the Msp1a repeats were common to both areas; a further 47.4% of the Msp1a repeats were identified only in calves at the peri-urban area while 5.2% were unique to calves at the wildlife–livestock interface. The six novel Msp1a repeats (named UP37-UP42) were all identified in calves from the peri-urban area.

Table 4.1: *Anaplasma marginale msp1α* genotypes identified from infected calves over the 12-month study period.

Calf no.[a]	No. of genotypes	Size (bp)	No. of Msp1a repeats	Genotype	Number allocated to genotype	Genotype #, if previously detected in study
1 (EG1	13	949	5	171-2;UP3[b] 172-2;UP4 61 172-2;UP4 172-2;UP4	1	
		700	2	τ 10	2	
		697	2	171-2;UP3 172-2;UP4	3	
		866	3	171-2;UP3 172-2;UP4 61	4	
		836	6	UP37[c] UP31 UP31 UP31 UP31 UP31	5	
		781	2	τ UP31	6	
		787	3	τ 10 22-2	7	
		610	1	UP38[c]	8	
		893	5	61 172-2;UP4 61 172-2;UP4 172-2;UP4	9	
		781	3	61 172-2;UP4 169-2	10	
		781	3	61 172-2;UP4 172-2;UP4	11	
		781	3	171-2;UP3 172-2;UP4 172-2;UP4	12	
		697	2	172-2;UP4 172-2;UP4	13	
2 (EG2)	4	784	3	UP39[c] 10 UP31	14	5, 7
		781	3	179-2 169-2 172-2;UP4	15	
3 (Eg3)	4	787	3	84 172-2;UP4 172-2;UP4	16[d]	
		959	5	34 3 36 36 38	17[d]	
		958	5	13 27 36 3 38	18	
		700	2	13 27	19	
4 (Eg4)	12	1040	6	34 36 36 3 36 38	20	17[d]
		1037	6	UP40[c] β β β β F	21	
		959	5	34 36 36 27 18	22	
		954	5	MZ2 3 UP41[c] 36 38	23	
		880	4	3 β 36 3	24	
		962	5	42 43 43 25 31	25	
		1131	7	34 3 UP1 43 43 25 31	26	
		1026	6	UP40[c] β β β Is9;78 31	27	
		965	5	84 172-2;UP4 172-2;UP4 172-2;UP4 172-2;UP4	28	
		870	4	τ 22-2 13 18	34[b]	
		705	2	34 3	41	
5 (EG5)	9	689	2	UP40[c] β	29	17[d], 21, 41
		1001	5	UP40[c] β β β F	30	
		875	4	42 43 25 31	31	
		790	3	42 UP42[c] 27	32	
		791	3	H M 27	33[c]	
		602	1	UP40[c]	42	
6 (UT1)	4	919	5	UP5 UP6 25 31 31	35	17[d],33[d],34[d]
7 (UT2)	9	1075	7	UP5 UP6 25 31 UP6 27 18	36	16[d], 33[d], 34[d], 35
		787	3	84 61 31	37	
		863	4	UP5 UP6 25 31	38	
		1202	8	UP5 UP6 25 31 UP6 25 31 31	39	
		955	4	84 Is9;78 31 31	40	

[a] Calves 1–5 were in the peri-urban area, Eglington; Calves 6–7 were in the wildlife–livestock interface, Utha A.
[b] Msp1a repeats denoted with a semicolon (e.g., 171-2;UP3) have been given two names in the literature.
[c] indicates a novel Msp1a repeat (red).
[d] *msp1α* genotypes that occur in both areas.

Figure 4.4: Novel Msp1a repeat sequences detected in this study. Six novel Msp1a repeats (UP37-UP42) were identified in the five calves located in the peri-urban area of the Mnisi community, Mpumalanga. Msp1a sequences were aligned using BioEdit. Conserved amino acid residues in the alignment are highlighted by white text on a black background, while variable residues are shown by black text on a white background.

4.4.2. Occurrence of *Anaplasma marginale* multi-strain infections in the calves

The complexity of *A. marginale* infection in the calves was determined by single/co-infection or superinfection events over the 12-month period (Figure 4.3). Detection of one or multiple genotypes at the initial time-point was defined as either single or co-infection, respectively. Detection of additional genetically distinct genotypes in the calves over time was defined as superinfection.

Animals in the peri-urban area acquired four to thirteen *msp1α* genotypes over the 12-month period. At the initial time-point, four of the five calves in the peri-urban area were infected with a single *A. marginale* genotype (or other genotypes were below the level of detection) and one was co-infected with more than two genotypes (Figure 4.3A). Superinfection with distinct *msp1α* genotypes occurred in all five calves during the study period. The same trend of infection was observed in the two calves that eventually became infected with *A. marginale* at the wildlife–livestock interface (Figure 4.3B). Although they only became positive for *A. marginale* from time-point T6 and T7, they were either singly infected or co-infected at the beginning but became superinfected with distinct *msp1α* genotypes over time (Figure 4.3B).

4.4.3. The composition of *Anaplasma* spp. in the ten calves

PacBio CCS sequencing of 16S rRNA gene amplicons from the final sample taken from each of the ten calves generated a total of 57,683 raw nucleotide sequences that were classified in

the genus *Anaplasma* using the RDP 16S classifier. Of these, 55,079 sequences were classified to *Anaplasma* species level using a customized 16S *Anaplasma* NCBI BLASTn database.

From the 55,079 16S rRNA sequences classified to *Anaplasma* species level, 87% of those were identified in calves at the peri-urban area and only 13% were identified in calves at the wildlife–livestock interface. The raw sequences were randomly sub-sampled to a total of 9950 sequences to equalize the sequencing depth, with 995 sequences analyzed per sample. A total of three *Anaplasma* species were identified in the 10 calves. They consisted mostly of *A. platys*-like 16S rRNA sequences (83.3%), followed by *A. marginale* (16.6%) and *Anaplasma boleense* (<0.1%) as highlighted in Table 4.2.

Table 4.2: The percentage of 16S rRNA sequences of each *Anaplasma* spp. identified in each calf.

Calf no.	*A. platys*-like	*A. marginale*	*A. boleense*
1 (Eg1)	67.3	32.3	0.4
2 (Eg2)	98.7	1.3	0
3 (Eg3)	56.0	43.9	0.1
4 (Eg4)	0	100	0
5 (Eg5)	68.5	31.3	0.2
6 (Ut1)	7.0	93.0	0
7 (Ut2)	95.3	4.7	0
8 (Di1)	0	0	0
9 (Di2)	0	0	0
10 (Di3)	100	0	0

A. marginale and *A. platys*-like 16S rRNA gene sequences were the most abundant sequences identified in the *Anaplasma* infected calves and frequently occurred as a co-infection. The *A. platys*-like sequence was detected in four of the five calves at the peri-urban area and in three of the calves at the wildlife–livestock interface. Of the three calves that tested negative for *A. marginale* at the wildlife–livestock interface, two were also negative for other *Anaplasma* spp., whilst the third was infected with the *A. platys*-like organism. In terms of *Anaplasma* spp. infections, calf-4 at the peri-urban area that died at T11, was only infected with *A. marginale*.

4.4.4. 16S rRNA phylogenetic analyses

The phylogenetic relationships between *Anaplasma* spp. 16S rRNA gene sequences identified in this study and other published sequences are shown in Figure 4.5. The phylogenetic tree topologies obtained using three tree algorithms were very similar, and the maximum likelihood

tree was chosen as a representative tree. The *A. marginale* sequences had 100% identity to *A. marginale* St Maries (Brayton et al., 2005) and had 99.9% sequence identity to *A. marginale* sequences identified in the various wildlife hosts in the Kruger National Park (KNP) (Makgabo et al., 2023). A minority of sequences had 99.8% identity with *A. boleense* (Guo et al., 2016), and 99.4% identity with *Anaplasma* sp. KNP9, a novel *Anaplasma* species recently identified in wildlife from KNP (Makgabo et al., 2023). The *A. platys*-like sequences were closely related to *Anaplasma* sp. Omatjenne (Allsopp et al., 1997) with 99.7% identity, *A. mymensingh* (Roy et al., 2018) with 99.9% identity, and "*Candidatus* Anaplasma camelii" (Bastos et al., 2015) with 99.6%. They had 99.7–99.9% identity to *Anaplasma* sp. KNP2, a novel *Anaplasma* species recently identified in wildlife from the Kruger National Park (Makgabo et al., 2023).

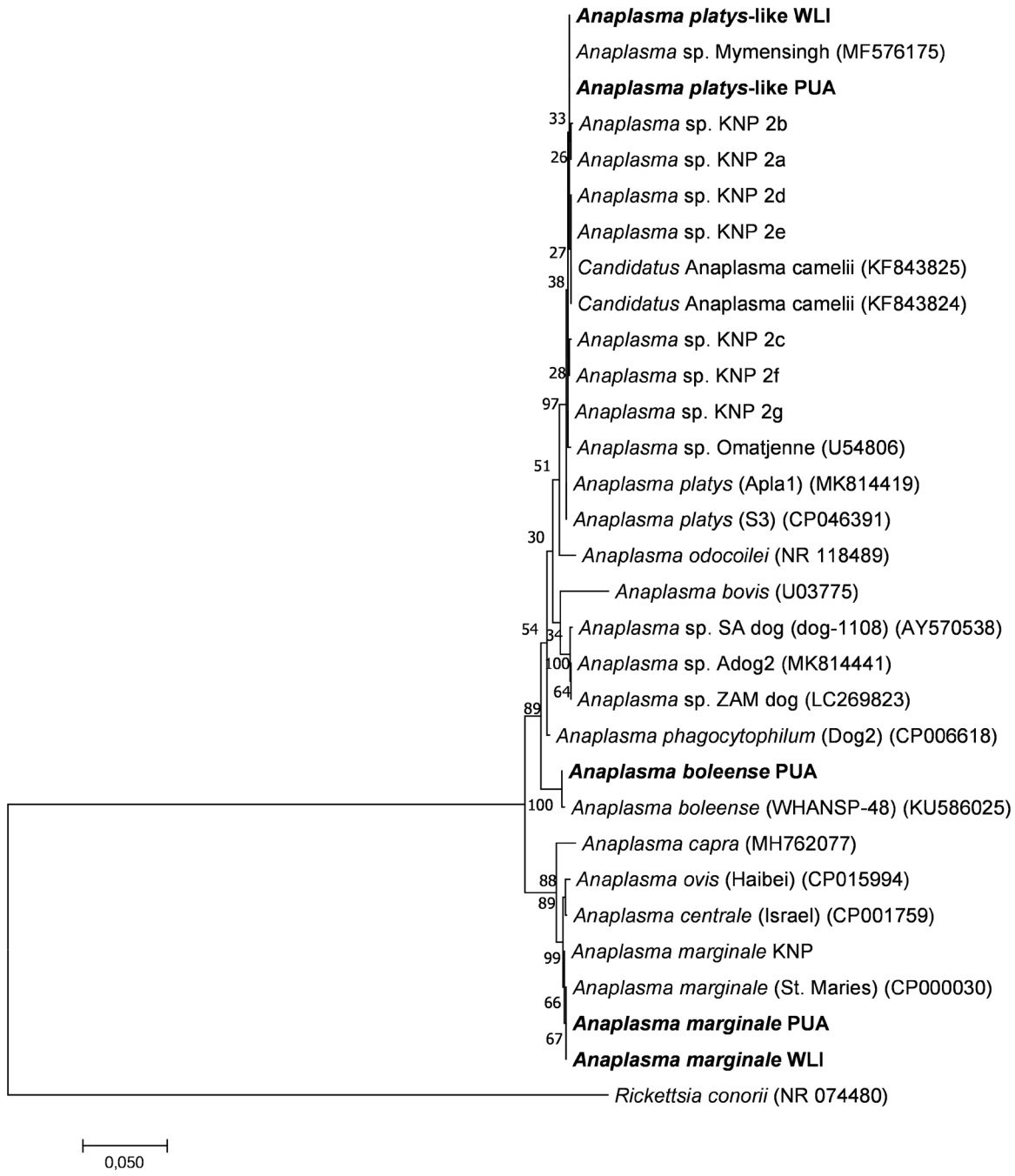

Figure 4.5: Maximum likelihood phylogenetic tree based on 16S rRNA sequences. The tree shows the phylogenetic relationship between *Anaplasma* 16S rRNA gene sequences obtained from the ten calves included in this study (in bold) and previously published *Anaplasma* 16S rRNA gene sequences. Near full-length 16S rRNA gene sequences of approximately 1328 bp in length were used to construct the tree. The numbers associated with each node indicate the percentage of 1000 bootstrap replications supporting the node. Phylogenetic analyses were conducted in MEGA7 with an HKY85 evolutionary model. Sequences with abbreviations PUA, WLI, and KNP highlight that the sequences were retrieved from animals in the peri-urban area, wildlife–livestock interface and Kruger National Park, respectively. *Rickettsia conorii* was used as the outgroup. The scale bar highlights a 5% nucleotide sequence divergence.

4.5. Discussion

The presence of *A. marginale* in cattle in the Mnisi community was expected, since the pathogen is currently widespread and endemic in cattle in eight of the nine South African provinces (de Waal, 2000; Mutshembele et al., 2014; Hove et al., 2018) and is known to occur in most cattle farming areas in the country (Potgieter, 1979; Potgieter & Stoltsz, 2004). However, *A. marginale* was detected in only seven of the ten calves in the 12-month study period. Our results further revealed that *A. marginale* infects calves early in their lives or during intra-uterine development (Aubry & Geale, 2011), since 50% of the calves were infected at T1 and T2, and they did not show clinical symptoms for the duration of the study. This agrees with previous findings (Bock et al., 1997; Jonsson et al., 2012), showing that calves up to 12 months of age are not clinically affected by anaplasmosis. The fact that three of the five calves at the wildlife-livestock interface were not infected was a surprising result. The bovine anaplasmosis cases observed at the wildlife-livestock interface in the Mnisi communal area might thus be attributed to a localised lack of endemic stability since calves at the wildlife–livestock interface are not continually infected with *A. marginale* in their first year when natural immunity is higher. The level of infection (number of infected red blood cells) in the calves that were infected in the two areas did not appear to be significantly different; however, our sample size is very limited and a larger study with more animals would be required to confirm these findings.

Although the Mnisi community is a non-anaplasmosis vaccinating area, absence of *A. centrale* infections was not expected, as *A. centrale* was previously detected in cattle in the study area (Kolo et al., 2020), furthermore the natural circulation *A. centrale* infection was previously observed in buffalo (*Syncerus caffer*), zebra (*Equus quagga burchelli*), warthog (*Phacochoerus africanus*), and lion (*Panthera leo*) in the KNP (Henrichs et al., 2016; Khumalo et al., 2016; Sisson et al., 2017). The absence of *A. centrale* in the calves might be due to our limited sample size or the absence of ticks transmitting *A. centrale*. A larger study including more animals would be appropriate to study the prevalence of *A. centrale* in livestock in the Mnisi community, as well as the diversity of *Anaplasma* spp. in ticks feeding on livestock in the area.

The calves that were infected with *A. marginale* from both areas of the Mnisi community displayed complex *A. marginale* infections driven by co-infection and superinfection, with four to thirteen *msp1α* genotypes detected per animal over the 12-month period, indicating

continuous challenge with multiple strains over time that should lead to robust immunity in these animals. Our results are similar to recent findings, where complex *A. marginale* infection with two to six strains per animal was detected in 97% of naïve calves that were introduced into an *A. marginale* infected herd in southern Ghana (Koku et al., 2021). Another study from a high *A. marginale* prevalence region in Mexico, showed that up to six *A. marginale* genotypes could be detected per animal using *A. marginale msp1α* genotyping for strain characterization (Castañeda-Ortiz et al., 2015).

Although our small sample size might have skewed the results, our findings highlight differences in temporal *A. marginale* infection dynamics between the villages, with all five of the calves at Eglington village (a peri-urban area) being infected at T1 and T2, but only two of the three calves at Utha A (at the wildlife livestock–interface) infected at T5 and T6, and no infection detected in the remaining three calves (one at Utha A and two at Dixie at the wildlife–livestock interface). Factors such as cattle density and management, which differ at the three villages, may drive the dynamics of *A. marginale* infection, with a lack of early *A. marginale* infection at the wildlife–livestock interface resulting in sporadic clinical cases in the area. The rapid migration of *R. microplus* ticks (larvae and adult ticks) from infested to un-infested cattle has been implicated in the interstadial transmission of *A. marginale* (Mason & Norval, 1981); furthermore, attachment of three infected *R. microplus* ticks is sufficient for transmission of *A. marginale* from infected to naïve cattle (Aguirre et al., 1994), while a single *Dermacentor andersoni* infected tick is sufficient for transmission (Scoles et al., 2005). Therefore, transmission of *A. marginale* is more likely to occur in areas where cattle density is higher, due to increased opportunities for migration of vector ticks from *A. marginale*-infected to uninfected cattle, thus increasing the chances of transmission in the area. Additionally, different methods of acaricide treatments are used in the two study areas, and this might have had an effect on the disease transmission dynamics observed. The frequency of cattle dipping in the Mnisi communal area is greatly affected by water shortages. Cattle in the peri-urban site, Eglington village, as well as Utha A, at the wildlife–livestock interface are dipped using the plunge method of dipping cattle, while a hand spraying method is used at Dixie village (at the wildlife–livestock interface). Several factors, such as the inability to clean and empty the dip tank resulting in a heavily silted dip tank, and incorrect mixing ratios of water and the acaricide, have been shown to be the prime causes of tick control failure at communal plunge dip tanks (Jonsson, 1997; Sungirai et al., 2016) such as the ones used at Eglington and Utha A. Thus, the hand spraying method, as is used at Dixie village, may be more effective in controlling tick

infestation and thus preventing disease transmission, than the plunge method of cattle dipping where the concentration of the acaricide in the dip tank might not be consistent.

Our findings further indicate the presence of other *Anaplasma* species circulating in the calves, which mainly comprised an *A. platys*-like organism that is closely related to a novel *Anaplasma* species recently identified in wildlife in the Kruger National Park (Makgabo et al., 2023). Very low levels of *A. boleense* 16S rRNA sequences were also detected in the calves, which were also previously detected in cattle in the area (Kolo et al., 2020). The high levels the *A. platys*-like organism present in the calves suggest the presence of ticks responsible for the transmission of this organism in the area. It has been postulated that exposure to closely related non-pathogenic organisms might provide some cross-protection against the pathogenic species in cattle and thus decrease the pathogenicity of the infection (Woolhouse et al., 2015). It is thus possible that infection with the *A. platys*-like organism might confer heterologous protection against *A. marginale* in cattle in the area, thus contributing to endemic stability of *A. marginale*. An experimental study conducted in Kenya (Löhr & Meyer, 1973) showed that cattle are highly susceptible to infection by less pathogenic *Anaplasma* species from wildlife hosts. Cattle having recovered from anaplasmosis caused by *Anaplasma* species from wildlife showed slight protection against subsequent infection with *A. marginale*. Future studies should be aimed at confirming these observations and further determining the mechanisms underlying heterologous protection against bovine anaplasmosis by closely related non-pathogenic species.

4.6. Conclusions

Complex *A. marginale* infection in the Mnisi community is driven by co-infection and superinfection. Factors such as cattle density and management, which differ at the three villages, may drive the temporal dynamics of the infection. A localized lack of endemic stability at the wildlife–livestock interface could result in clinical cases caused by challenge with *A. marginale* at a later point in life. Our findings suggest that cattle in the Mnisi community are exposed to other *Anaplasma* spp. which might confer cross-protection against the pathogenic *A. marginale* infection and might suggest that other, previously unrecognized *Anaplasma* species could contribute to the control of bovine anaplasmosis in South Africa. While our results suggest that there are differences in the time-course of infection in calves in

different areas of the Mnisi community, it should be noted that only five calves were examined from each area. A future in-depth longitudinal study in more villages of the Mnisi community with a larger sample size is recommended to confirm and further analyze the dynamics of *A. marginale* infections in the Mnisi communal area, especially at the wildlife–livestock interface.

4.7. References

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CHAPTER 5

Exploring a method for *Anaplasma* whole genome sequencing that does not require infection of splenectomized cattle or initiation of *in vitro* cultures

5.1. Abstract

Despite a research history that spans over a century, *Anaplasma* spp. are amongst the most understudied bacteria due to their obligate intracellular nature, with relatively few genomes available in the public databases. The past few years have seen the proposal of more than 20 *Anaplasma* spp. using a range of methods, from clinical cases to 16S rRNA gene analysis to multi-locus sequence typing to comprehensive analysis such as establishment of the organism in culture and sequencing. With scant information available for any isolate, it is difficult to know if a newly identified isolate with a similar single gene sequence to an existing isolate belongs to the same species. This issue is magnified for obligate intracellular organisms where isolates tend not to be maintained or cultivated and cannot be used as reference. The availability of whole genome sequences would be helpful in sorting out relationships among similar organisms. Therefore, this study was aimed at exploring a method of sequencing the genome of a South African strain of *Anaplasma marginale* from a carrier animal without infecting splenectomized cattle or initiating *in vitro* cultures. Genomic DNA was extracted from a cow from the Innovation Africa @ University of Pretoria (IA@UP) Experimental Farm which was shown to be infected with a single strain of *A. marginale* and no other hemoparasites. Three rounds of microbial enrichment were used to deplete the host DNA in the sample, followed by whole genome amplification. Whole genome sequencing was performed on a Pacific Biosciences (PacBio) sequencing platform. A total of 298 058 raw PacBio reads were retrieved from the PacBio single-molecule real-time (SMRT) analysis 2.3.0 software, which were mainly bovine host reads. *Anaplasma* reads mapped to the *A. marginale* St Maries and *A. marginale* Florida reference genomes resulted in two different, incomplete *A. marginale* assemblies. Further sequencing data is thus needed for full closure of the genome assembly. Advances in molecular techniques for microbial DNA enrichment and sequencing, and assortment of contigs into species-specific bins and assembly of binned data could be incorporated in this

study to complete the *A. marginale* sequence and to obtain whole genome sequences of the different *Anaplasma* spp. circulating in livestock, wildlife and companion animals.

5.2. Introduction

The genus *Anaplasma* comprises tick-transmitted obligate intracellular bacteria that cause anaplasmosis in livestock, wildlife and humans (Aubry & Geale, 2011). *Anaplasma* research has a history that spans more than a century since the first *Anaplasma* species was discovered, yet they remain amongst the most challenging and understudied tick-borne agents in veterinary and human health. There are only five validly published and formally described species, that include *A. marginale, A. ovis*, *A. centrale*, *A. phagocytophilum*, and *A. caudatum*, according to the most recent List of Prokaryotic names with Standing in Nomenclature (LPSN) (Parte et al., 2020). Additionally, *A. bovis*, *A. platys*, *A. odocoilei* and *A. capra* are considered to have been "effectively published" and are generally accepted as *Anaplasma* species in the literature, but are not considered to be validly published according to the International Code of Nomenclature of Prokaryotes (ICNP) (Parker et al., 2019). The advent of high throughput sequencing technologies has revealed a greater genetic diversity within the genus worldwide (Caudill & Brayton, 2022), with the proposal of over 20 new *Anaplasma* spp. from various hosts, although these novel species do not have any formal standing in nomenclature as per the rules of the ICNP. Clear species definitions in bacterial taxonomy are often difficult, if not impossible, without the presentation of whole genome sequences of the proposed novel species (Wayne et al., 1987). This highlights the urgent need for genome sequencing of more *Anaplasma* spp., to fully explore the natural rate of variation within the genus and fully elucidate the evolution and epidemiology of related species.

To date, the genus *Anaplasma* is characterized by relatively few genome sequences with only two species, *A. marginale* (Brayton et al., 2005; Dark et al., 2009; Dark, Al-Khedery & Barbet, 2011; Pierlé et al., 2014) and *A. phagocytophilum* (Dunning et al., 2006), having more than a single strain that have been sequenced and published. The genome sequences of *A. centrale* (Herndon et al., 2010), *A. ovis* (Liu et al., 2019) and *A. platys* (Llanes & Rajeev, 2020) are represented by a single strain each.

Even in the modern era of high-throughput sequencing technologies, the generation of genome sequence data requires large amounts of pure DNA template. The difficulties of working with intracellular bacteria pose a major limitation to the generation of high-quality genetic material suitable for genomic and proteomic analyses. In order to generate sufficiently large amounts of DNA, either splenectomized animals must be infected with *Anaplasma* isolates, or *Anaplasma* isolates must be grown in *in vitro* culture. *Anaplasma* organisms are difficult to grow in culture as they are fastidious and can only replicate in either mammalian or arthropod cells (Munderloh et al., 1996). While some *Anaplasma* species have been successfully cultured *in vitro* (Blouin et al., 2000, 2002; Zweygarth et al., 2006; Baêta et al., 2015; Park et al., 2023), the success rate is usually low, e.g. a 5% success rate of *Anaplasma* spp. culture was reported by Baêta et al. (2015). Although successful *in vitro* cell culture has been previously documented from blood samples with 10% rickettsemia (Baêta et al., 2015), it is recommended to use blood samples with 30% to 40% rickettsemia to achieve a successful establishment and propagation of *A. marginale* in tick cell culture (Blouin et al., 2000; Bell-Sakyi et al., 2007; Felsheim et al., 2010). Many of the *Anaplasma* species that have been identified and proposed in recent years are present at low levels in apparently healthy wildlife hosts, making it very difficult to impossible to initiate *in vitro* cultures. Even once sufficient numbers of organisms have been obtained, it is difficult to separate *Anaplasma* DNA from host nuclear DNA due to the intracellular lifestyle of the bacteria. Alternative ways of sequencing the whole genome of *Anaplasma* species are urgently needed. This study was aimed at exploring an alternative means of obtaining the genome sequence of a South African strain of *A. marginale* from blood collected from a carrier animal without the need for infection of splenectomized cattle or initiation of *in vitro* cultures.

5.3. Materials and Methods

5.3.1. Ethics approval

The study was conducted in accordance with the conditions of the Animal Ethics Committee of the Faculty of Veterinary Science, University of Pretoria (REC 252-19) (Appendix 6). Furthermore, permission to conduct research under Section 20 of the *Animal Disease Act* of 1984 was granted by the South African Department of Agriculture, Land Reform and Rural Development (DALRRD) (12/11/1/1/6 (1461 AC) (1) (Appendix 7)

5.3.2. Origin of cattle

Cattle samples were collected at the IA@UP Experimental Farm, Future Africa Campus, University of Pretoria (GPS Coordinates: -25.749697929810253, 28.260559709824367), Gauteng, South Africa. Approximately 370 Holstein (*Bos taurus taurus*) dairy cattle are kept on the farm, mainly used for breeding purposes. This is a closed herd of cattle, they are born and bred on the farm, with a monthly dipping schedule to control tick infestation.

5.3.3. Sample collection and DNA extraction

Whole blood samples were collected in 10 ml Vacutainer® ethylenediaminetetraacetic acid (EDTA) tubes from 92 cattle using 18G needles. DNA was extracted from a 200 µl aliquot of each blood sample using the Invitrogen Purelink™ Genomic DNA Mini Kit (Thermo Fisher Scientific), according to the manufacturer's instructions.

5.3.4. Pathogen detection

DNA samples were screened for the presence of *A. marginale* and *A. centrale* using a duplex quantitative real-time polymerase chain reaction (qPCR) assay for the specific detection of *A. marginale* (targeting the *msp1β* gene) and *A. centrale* (targeting the *groEL* gene) as previously described (Chaisi et al., 2017) with modification of the *A. centrale* probe, AC-Pb (NED- ATC ATC ATT CTT CCC CTT TAC CTC GT- NFQ-MGB) (Thermo Fisher Scientific, South Africa) for use on an Applied Biosystem StepOnePlus™ Real-time PCR System 2.3 (Applied Biosystems, Life Technologies, South Africa). Reactions were prepared in a 25 µl final reaction volume comprising 12.5 µl TaqMan® Universal PCR Master Mix (LTC Tech South Africa (Pty) Ltd), 0.6 µM of the *A. marginale*-specific primers, 0.9 µM of the *A. centrale*-specific primers, 0.2 µM of each probe and 2.5 µl of target DNA (approximately 200 ng). Positive control for the *A. centrale* assay was DNA extracted from the *A. centrale* vaccine strain obtained from Onderstepoort Biological Products (OBP), Pretoria, South Africa. Positive control for the *A. marginale* assay was DNA extracted from field sample C14 (previously obtained from cattle in the Mnisi Community area, Mpumalanga) and confirmed by sequencing of the *A. marginale msp1α* gene. Nuclease-free water was used as a negative control for the assay. Cycling conditions were as follows: UNG incubation at 50°C for 2 min and activation of AmpliTaq Gold at 95°C for 10 min, followed by 40 cycles of 1 min at 90°C, 1 min at 60°C. Data was analyzed with the StepOnePlus™ Real-time PCR System Software version 2.3.

Anaplasma marginale- and *A. centrale*-positive samples with high levels of rickettsemia, determined using the *A. marginale* and *A. centrale* duplex qPCR Ct values (Chaisi et al., 2017; Hove et al., 2018), were subjected to the reverse-line blot (RLB) hybridization assay as described previously (Gubbels et al., 1999; Bekker et al., 2002; Nijhof et al., 2003, 2005) to screen for the presence of other tick-borne hemoparasites of the genera *Ehrlichia*, *Babesia* and *Theileria*.

5.3.5. Characterization of *Anaplasma marginale* Msp1a repeats and *msp1α* genotypes

In order to assess the *A. marginale* strain composition in samples that tested positive only for *A. marginale*, the repeat-containing variable region of the *A. marginale msp1α* gene was amplified using primers 1733F (5-TGT GCT TAT GGC AGA CAT TTC C- 3) and 2957R (5-AAA CCT TGT AGC CCC AAC TTA TCC- 3) (Lew et al., 2002). Amplifications were performed in a 25 µl final reaction volume and consisted of 1x Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific, South Africa), 0.5 µM of each primer and 2.5 µl of target DNA (approximately 10–100 ng). The thermal cycling conditions were modified from those previously reported (Lew et al., 2002) and were as follows: 98°C for 10 sec, followed by 30 cycles of 98°C for 1 sec, 63°C for 5 sec and 72°C for 18 sec, and a final extension at 72°C for 1 min. PCR products were analyzed by gel-electrophoresis on a 1.5% agarose gel (1 × TAE buffer, pH 8.0), stained with ethidium bromide and viewed under UV light. PCR products were purified using the QIAquick® PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Purified PCR products were cloned into pJET 1.2 (Thermo Fisher Scientific, South Africa). Recombinant clones and amplicons were sequenced bi-directionally on an ABI Prism 3100 Genetic Analyzer at the DNA Sequencing Unit at Stellenbosch University. *Anaplasma marginale msp1α* nucleotide sequences were trimmed, assembled, edited aligned and translated to amino acid sequences using CLC Genomics Workbench 20 (Qiagen, Aarhus, Denmark). Msp1a amino acid sequences containing the repeats were aligned using Bioedit 7 (Hall, 1999). The RepeatAnalyzer command line software tool (Catanese, Brayton & Gebremedhin, 2016) was used to identify and analyze Msp1a repeats and *A. marginale* genotypes present in the samples.

5.3.6. Pathogen purification

Four cattle that were positive only for *A. marginale* and that were shown to be infected with a single *A. marginale* strain were selected for further blood collection. A total of 50 ml of whole

blood was collected in five 10 ml Vacutainer® EDTA tubes from each of the four animals. The red blood cells were separated from plasma and buffy coat by centrifugation of 50 ml of blood at 3000 rpm for 10 minutes in a clinical centrifuge. The plasma and buffy coat were removed and discarded. The remaining red blood cells were washed seven times with phosphate-buffered saline (PBS, pH 7.4) (Thermo Fisher Scientific, South Africa), by dilution 1:1 with PBS and centrifugation at 3000 rpm for 10 minutes, removing the supernatant containing PBS and excess buffy coat after each spin. The washed red blood cells were suspended in PBS and stored at -80°C.

5.3.7. Genomic DNA preparation

In order to extract high molecular weight genomic DNA, the washed red blood cells suspended in PBS from cow 1708 were thawed in a 37°C water bath and genomic DNA was then extracted using a Genomic-tip 20/G (Qiagen, Hilden, Germany), according to manufacturer's instructions. The kit uses the unique QIAGEN anion-exchange technology that results in a high-molecular-weight DNA extract, and operates by gravity flow as opposed to centrifugation to maintain the integrity of the DNA. The DNA concentration was determined using a Qubit™ dsDNA BR (Broad Range) Assay Kit (Thermo Fisher Scientific, South Africa) on a Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, South Africa) and the purity of the DNA (A260/A280 and A260/A230 ratio) was assessed using a spectrophotometer (Xpose, Trinean, Belgium).

5.3.8. Microbial DNA enrichment and genome amplification

In order to deplete the bovine DNA in the sample, bacterial DNA was enriched three times and purified using an NEBNext Microbiome DNA enrichment kit (New England BioLabs, Inc., Ipswich, MA, USA) according to manufacturer's instructions. The kit facilitates enrichment of microbial DNA from complex samples containing host DNA, by selective binding and removal of the CpG-methylated host DNA. An input of 2 µg of DNA was used for microbial DNA enrichment and the DNA was purified using the ethanol precipitation method to remove any residual beads, as recommended by the manufacturer. The host DNA capture method commenced by resuspending and washing the NEBNext Protein-A Magnetic Beads supplied by the kit. The NEBNext Protein-A Magnetic Beads were resuspend by gently pipetting up and down until the solution was homogeneous. In one tube, 16 µl of MBD2-Fc protein and 160 µl of Protein-A Magnetic Beads were added for a total input of 1 µg DNA and mixed by gently pipetting up and down until the beads were completely homogeneous. The bead-protein

mixture was further mixed with a rotating mixer for 10 minutes at room temperature. The mixture was briefly spun and placed on a magnetic rack until the solution was clear and the beads were collected to the side of the tube. The supernatant was carefully removed without disturbing the beads and the beads were washed by gently pipetting up and down until they were completely homogeneous using 1 ml of 1X Bind/wash Buffer, that was prepared on ice by diluting 1-part NEBNext Bind/wash Buffer (5X) with 4 parts DNase-free water. The beads were then placed on a rotating mixer for 3 minutes at room temperature, briefly spun and placed on the magnetic rack until the solution was clear and the beads were collected to the side of the tube. The supernatant was carefully removed without disturbing the beads and the wash step was repeated. In order to resuspend the washed beads, the tube containing the beads was removed from the rack and 160 µl of 1X Bind/wash Buffer (kept on ice) was added and mixed by gently pipetting up and down a few times. The methylated host DNA was captured by adding 1 µg (in up to 200 µl) of input DNA to the tube containing the 160 µl of MBD2-Fc-bound magnetic beads. Undiluted Bind/wash Buffer (5X) was added to the solution for a final concentration of 1X. The solution was mixed until it was clear and placed on a rotating mixer for 15 minutes at room temperature. The enriched microbial DNA was collected by briefly spinning the tube and placing it on the magnetic rack until the solution was clear and all the beads had collected to the side of the tube. The supernatant containing the target microbial DNA was carefully removed with a pipette without disturbing the beads and transferred to a clean microcentrifuge. The DNA was purified by ethanol precipitation method: 2.5 volumes of 100% ethanol were added, incubated for 10 minutes on ice and centrifuged at 16,000 rcf for 30 minutes. Ethanol was removed and the pellet was allowed to air dry, and then resuspended in 50 µl of TE buffer for further analysis.

Whole genome amplification (WGA) from the microbially enriched sample was conducted in triplicate using the GenomiPhi V3 Ready-To-Go DNA Amplification Kit (Cytiva, Marlborough, Massachusetts, USA) according to the manufacturer's instructions. Briefly, 10 µl of 2X denaturation buffer supplied with the kit was mixed with 1 µl (containing 10 ng) of microbially enriched DNA, followed by the addition of 9 µl of PCR-grade water. The solution was incubated at 95°C for 3 minutes to denature the DNA, and was placed immediately on ice. Amplification of DNA then proceeded by reconstituting the Ready-To-Go GenomiPhi V3 cake with the denatured DNA and incubated at 30°C for 1.5 hours. The Phi29 DNA polymerase enzyme was inactivated by incubation at 65°C for 10 minutes and placed on ice. The integrity of the amplified DNA was analyzed by agarose gel electrophoresis and the concentration was

determined using the Qubit™ dsDNA BR (Broad Range) Assay Kit (Thermo Fisher Scientific, South Africa).

5.3.9. Host DNA analysis

In order to assess the amount of host DNA in our samples, a qPCR assay targeting mammalian DNA (specifically the hosts of interest, artiodactyls and carnivores) was developed. A multiple sequence alignment of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene sequences of the artiodactyl and carnivore species shown in Table 5.1 was created using CLC Genomics Workbench 20 (Qiagen, Aarhus, Denmark). Primers, MaGAP_F (5'-TGA YCC CTT CRT TGA CCT TCA-3'), MaGAP_R (5'-TGC CGT GGG TGG AAT CAT-3') (Inqaba Biotech, South Africa) and a TaqMan MGB^{TM} probe, MaGAP_P (VIC-5'-CAT GGT CTA CAT GTT CCA G-3'-MGB) (Thermo Fisher Scientific, South Africa) were designed to target a 62 bp fragment that is conserved between the artiodactyl and carnivore species included. Reactions, performed in a final volume of 20 µl, contained 2X TaqMan® Universal PCR Master Mix (Thermo Fisher Scientific, South Africa), 0.5 µM of each forward and reverse primer, 0.25 µM of TaqMan MGB^{TM} probe and 2 µl (approximately 10-100 ng) of template DNA. The qPCR assay was performed on the StepOnePlus^{TM} Real-Time PCR System 2.3 (Applied Biosystems, Foster City, CA, USA). Cycling conditions included UNG incubation at 50ºC for 2 min, followed by AmpliTaq Gold pre-activation at 95ºC for 10 min, 45 cycles of denaturation at 95ºC for 20 sec and annealing at 58.5ºC for 1 min. DNA extracted from a hemoparasite-free bovine (*Bos taurus*) obtained from pre-infection studies conducted previously (Hove, 2018) was used as positive control and molecular grade water as a negative control. Data was analyzed using the StepOnePlus^{TM} software version 2.3.

Table 5.1: The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene sequences from mammalian species used in the multiple sequence alignment for the design of primers and a probe for the qPCR assay.

Common name	Scientific name	NCBI Accession number
Cow	*Bos taurus*	AJ786261; BC102589
Zebu cattle	*Bos indicus*	XM_027541122; XM_019960295
Domestic yak	*Bos grunniens*	EU195062
Wild yak	*Bos mutus*	XM_014482068
Water buffalo	*Bubalus bubalis*	XM_006065800
African buffalo	*Syncerus caffer*	MF133531
American bison	*Bison bison*	XM_010844969
Wild mouflon sheep	*Ovis aries musimon*	XM_01216646
Sheep	*Ovis aries*	XM_027961471; NM_001190390
Goat	*Capra hircus*	XR_001918676; XR_001295477
White-tailed deer	*Odocoileus virginianus*	XM_020878738; XM_020902047
Wild boar	*Sus scrofa*	NM_001206359
Common bottlenose dolphin	*Tursiops truncates*	XM_019925987
Pacific white-sided dolphin	*Lagenorhynchus obliquidens*	XM_027093223
California sea lion	*Zalophus californianus*	XM_027594890
Steller sea lion	*Eumetopias jubatus*	XM_028121022
Beluga whale	*Delphinapterus leucas*	XM_022577390
Narwhal	*Monodon monoceros*	XM_029204840
Northern fur seal	*Callorhinus ursinus*	XM_025851873
Cat	*Felis catus*	XM_006933438
Lion	*Panthera leo*	XM_042945628
Leopard	*Panthera pardus*	XR_002085836
Tiger	*Panthera tigris*	XM_042992481
Striped hyena	*Hyaena hyaena*	XM_039218398

5.3.10. *Anaplasma marginale* genome sequencing

The enriched, amplified DNA sample from cow 1708 was submitted to the Genomic Sequencing Core of Washington State University, Pullman, USA for whole genome sequencing on the PacBio RSII sequencing platform (Pacific Biosciences, Menlo Park, California, USA). A 20 Kb genomic DNA library was prepared suitable for P6/C4 chemistry using the SMRTbell template preparation kit 1.0 according to the manufacturer's protocol. The DNA was sequenced using the PacBio single-molecule real-time (SMRT) sequencing technology using two SMRT cells on the PacBio RSII sequencing platform (Pacific Biosciences, Menlo Park, CA, USA).

5.3.11. Sequencing data analysis

The raw PacBio Circular Consensus Sequencing (CCS) reads were analyzed, filtered and assembled using the PacBio SMRT Analysis 2.3.0 software and CLC Genomics Workbench

(Qiagen, Aarhus, Denmark). Sequencing reads were mapped against the *Bos taurus* whole genome sequence (NCBI BioSample: SAMEA7051353 and NCBI BioProject: PRJEB41519) to identify host reads, and then *A. marginale* St Maries (Brayton et al., 2005) and Florida (Dark et al., 2009) strains were used as reference genomes to identify and map the *A. marginale* reads. Assemblies were further analyzed and aligned using ACT: the Artemis comparison tool, which allows visualisation of comparisons between genome sequences and associated annotations (Carver et al., 2005). The average nucleotide identity (ANI) between the two assemblies and the two reference genome sequences was calculated using the ANI calculator which uses the OrthoANIu algorithm to compare two prokaryotic genome sequences (Yoon et al., 2017).

5.4. Results

5.4.1. Pathogen detection

Anaplasma marginale was detected in 57.6% (53/92) of the cattle samples from the IA@UP Experimental Farm, while *A. centrale* was detected in 1.1% (1/92) of the samples. All *A. marginale* - and *A. centrale*-positive samples contained single *Anaplasma* infections according to the qPCR assay. This data is presented in Table A3 (Appendix 4).

The repeat-containing variable region of the *A. marginale msp1α* gene was amplified from 24 of the *A. marginale*-positive samples, each of which had a relatively high rickettsemia. A single PCR product was obtained from all 24 samples, with amplicons of ~771 bp and ~850 bp (Figure 5.1). Characterization of *A. marginale* Msp1a repeats and *msp1α* genotypes revealed only two genotypes in all of the *A. marginale*-positive samples: genotype A with Msp1a repeats: 27 4 4 37 and genotype B with Msp1a repeats: 27 13 18 (Figure 5.2). Genotype A was found to be the most dominant and was present in 22 of the positive samples, while genotype B was only present in two samples. The *A. marginale*-positive samples with single infection and high rickettsemia (n=15) were further screened for other tick-borne hemoparasites and one sample with genotype B tested positive for the *Theileria* and *Babesia* group-specific probe (*T*/*B* catch all), as well as the *Theileria* genus-specific probe (*T.* catch all) as determined by RLB (Appendix 4).

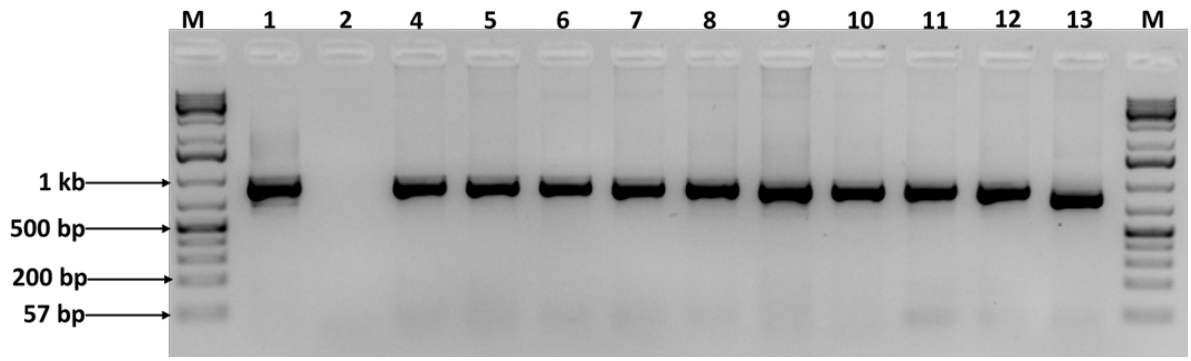

Figure 5.1: PCR products for the *Anaplasma marginale* repeat containing *msp1α* gene amplification. Lane M: 1kb plus DNA ladder (Thermo Fisher Scientific, South Africa), Lane 1: positive control, Lane 2: negative control, lane 4-13: representative sample set from A. marginale-positive cattle, with lane 8 representing cow 1708. Lane 4-12: genotype A, Lane 13: genotype B.

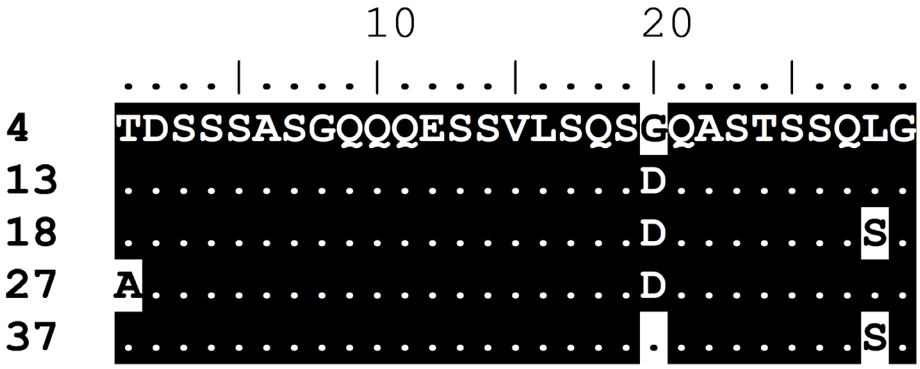

Figure 5.2: Amino acid sequence alignment of the Msp1a repeats from the two *msp1α* genotypes detected in this study. Msp1a amino acids were aligned using BioEdit. Conserved amino acid residues in the alignment are highlighted by white text on a black background, while variable residues are shown by black text on a white background.

5.4.2. Preparation of DNA

A total of 50 ml of whole blood was collected from four *A. marginale*-positive cattle, 1627, 1909, 20002 and 1708, that were infected with genotype A and were negative for other tick-borne hemoparasites. DNA extracted from a 250 µl aliquot of blood from each animal was tested using the *A. marginale*-specific qPCR. DNA extracted from Cow 1708, with the lowest Ct value, was chosen to proceed with the experiment. Red blood cells from 50 ml of blood from cow 1708 were separated from buffy coat and plasma, washed seven times with PBS, and DNA was extracted from the washed red blood cells. The concentration of DNA obtained was 225 ng/µl, with an A260/280 ratio of 1.8 and A260/230 ratio of 2.5 (Table 5.2). The *A. marginale* strain in this animal was named AmUP1708.

5.4.3. Microbial enrichment and whole genome amplification

The Ct values obtained using the *A. marginale*-specific qPCR and the mammalian (artiodactyl and carnivore) GAPDH qPCR were used to estimate the relative levels of *A. marginale* and bovine DNA in the DNA extract from cow 1708. Ct values of 26.89 for *A. marginale* and 18.5 for bovine DNA were obtained, indicating that the bovine DNA in the sample was more concentrated than the *A. marginale* DNA. The DNA sample was taken through two rounds of microbial enrichment which resulted in a sample with qPCR Ct values of 25.2 for *A. marginale* and 26.7 for bovine DNA. Whole genome amplification of the DNA sample was performed in triplicate resulting in the qPCR Ct values, concentrations and purity presented in Table 5.2, and the integrity of the amplified DNA samples is shown in Figure 5.3.

Table 5.2: The concentration and purity of DNA preparations from cow 1708 infected with a single strain of *Anaplasma marginale*, AmUP1708.

Sample Name	*A. marginale* Ct value	Mammalian GAPDH Ct value	Concentration (ng/ul)	A260/A280 ratio	A260/A230 Ratio
AmUP1708[a]	26.9	18.5	225	1.8	2.5
AmUP1708-1[b]	26.0	23.2	3.2	ND[e]	ND
AmUP1708-2[c]	25.2	26.7	2.9	ND	ND
AmUP1708-2a[d]	22.9	24.6	139	1.6	2.7
AmUP1708-2b[d]	21.2	24.9	692	1.6	2.8
AmUP1708-2c[d]	20.9	23.4	758	1.6	2.8

[a] Original DNA sample extracted from red blood cells from cow 1708.
[b] DNA sample after the first round of microbial enrichment
[c] DNA sample after the second round of microbial enrichment, used as a template for whole genome amplification.
[d] DNA samples that resulted from the in triplicate preparation of the whole genome amplification of AmUP1708-2.
[e] ND: not done (the purity of the sample was not determined).

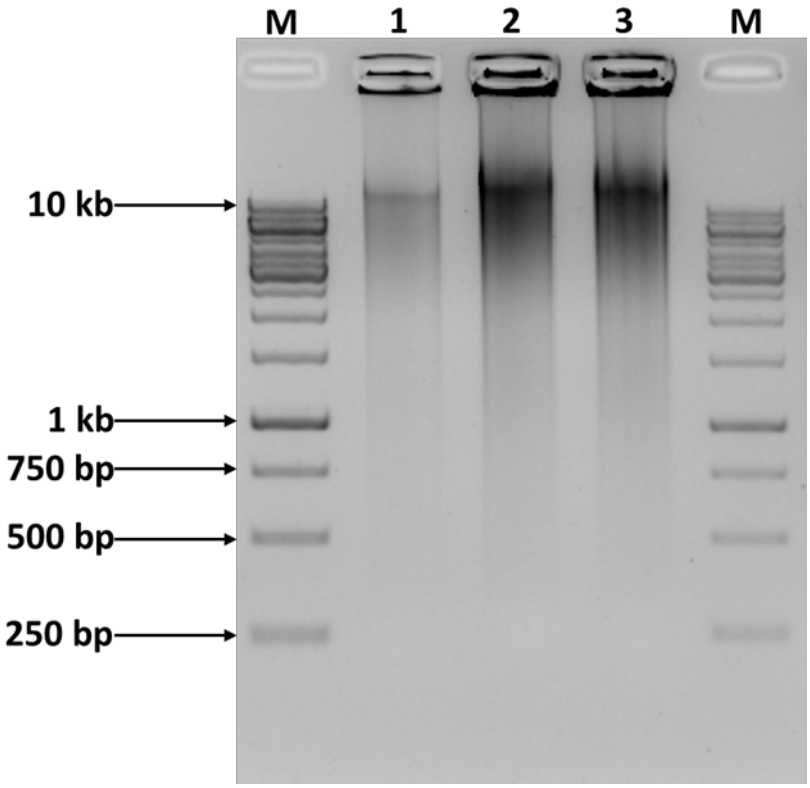

Figure 5.3: The integrity of the three enriched and amplified genomic DNA samples from AmUP1708-2 that was used for the *Anaplasma marginale* genome sequencing. Lane M: 1kb DNA ladder (Thermo Fisher Scientific, South Africa), Lane 1: 0.7 µg of DNA from AmUP1708-2a, Lane 2: 3.5 µg of DNA from AmUP1708-2b, Lane 3: 3.8 µg of DNA from AmUP1708-2c.

5.4.4. Genome sequencing data analysis

A total of 298 058 raw PacBio CCS reads with a total of 1 355 556 536 nucleotides were retrieved from the PacBio SMRT Analysis 2.3.0 software. In order to filter out host sequences, the raw CCS reads were mapped onto an approximately 2.8 Gb *Bos taurus* genome sequence (NCBI BioSample: SAMEA7051353 and NCBI BioProject: PRJEB41519) (Talenti et al., 2022), with a total of 32 chromosomes. The majority of the reads (more than 98%) mapped onto the reference *Bos taurus* genome sequence.

The 298 058 raw PacBio CCS reads were mapped onto the approximately 1.2 Mb *A. marginale* St Maries strain genome sequence (Accession number: CP000030). Only 3 327 reads with an average read length of 4.7 kb mapped to the *A. marginale* St Maries sequence, resulting in a fragmented genome assembly of 8 contigs (Figure 5.5) with a GC content of 49.8%, and an average nucleotide identity (ANI) of 98.6% to the reference genome. The coverage of each base was 11±8.9X. This will be referred to as Assembly-1.

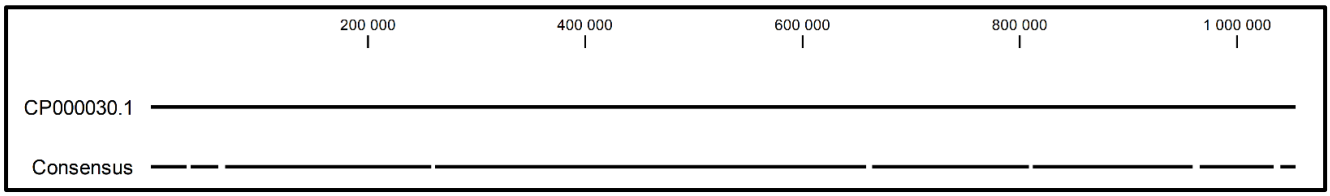

Figure 5.4: Schematic diagram showing genomic mapping of raw PacBio CCS reads generated from this study to the *Anaplasma marginale* St Maries reference genome.

The 298 058 raw PacBio CCS reads were also mapped against the approximately 1.2 Mb *A. marginale* Florida strain genome sequence (Accession number: CP001079). Only 3 361 reads with an average read length of approximately 4.7 kb mapped to the *A. marginale* Florida sequence, again, resulting in a fragmented genome assembly of 7 contigs (Figure 5.6) with a GC content of 49.8%, and an ANI of 98.6% to the reference genome. The coverage of each base was 11±9X. This will be referred to as Assembly-2.

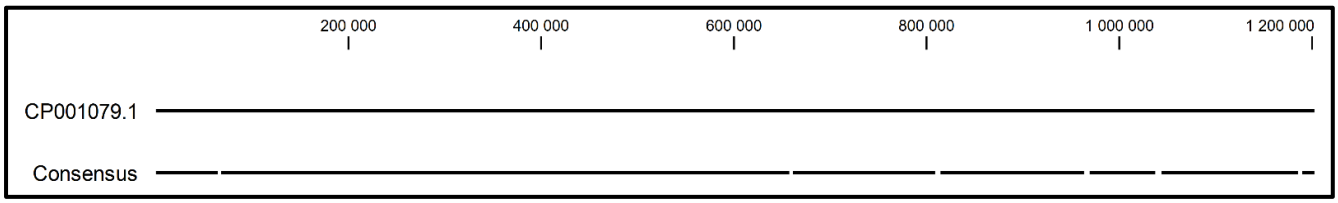

Figure 5.5: Schematic diagram showing genomic mapping of raw PacBio CCS reads generated from this study to the *Anaplasma marginale* Florida reference genome.

5.4.4.1. Genome sequence alignments

A genome sequence alignment of the *A. marginale* St Maries strain and Assembly-1 revealed that the two genomes were highly conserved in regions where sufficient sequencing reads were present in Assembly-1 (Figure 5.7). Assembly gaps were mostly found in repetitive regions of the *Anaplasma* genome, including regions where the pseudogenes, *msp2* and *msp3*, encoding Major Surface Protein 2 and 3 (Msp2 and Msp3), are found. Msp2 and Msp3 are antigenically variable surface proteins that serve to evade the host immune response by gene conversion (French, Brown & Palmer, 1999; Brown et al., 2003; Meeus et al., 2003). In *A. marginale*, *msp2* and *msp3* functional pseudogenes recombine by gene conversion into the single expression site resulting in immune escape variants (Brayton et al., 2005; Liu et al., 2019). The *msp2* and *msp3* gene sequences contain regions that are conserved between their specific

pseudogenes and can confound sequence assembly if reads are not long enough to span the repeats. Another large gap was found where the *Aaap* gene family is located. This gene family encodes the *Anaplasma* appendage associated protein (Stich et al., 2004), a highly repetitive region in the *A. marginale* genome, characterized by multiple copies of *Aaap*-like genes. The locus is known to be highly polymorphic among the different strains and species of *Anaplasma* and due to the repetitive nature, these sequences tend to be absent from genome assemblies (Dark et al., 2009). Interestingly, homologs of genes that encode outer membrane proteins, *omp9* and *omp10*, as well as a few *A. marginale* hypothetical proteins were also missing from this assembly, probably due to insufficient sequencing data.

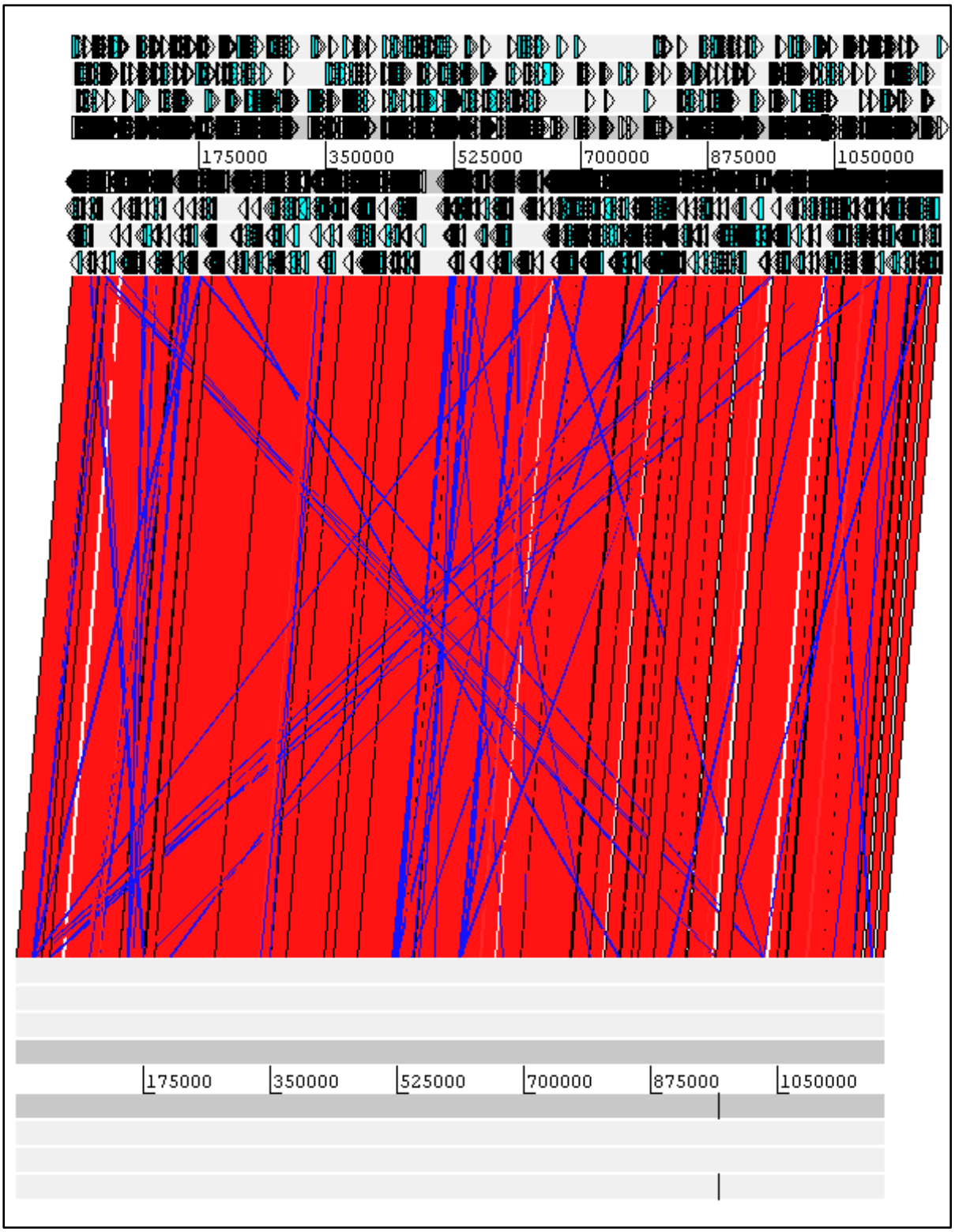

Figure 5.6: Genome sequence alignment between the *Anaplasma marginale* St Maries strain reference genome (above) and Assembly-1 generated in this study (below) showing the relationship between the two sequences. The red shaded areas represent regions that are conserved and in the same orientation, the blue shaded areas represent regions that are conserved but oriented in the opposite direction, while the white areas represent gaps.

The same pattern was also observed in the genome sequence alignment of the *A. marginale* Florida strain and Assembly-2 (Figure 5.8). The two assemblies were highly conserved in regions with sufficient sequencing reads, with gaps in regions with insufficient sequence data. Like in Assembly-1, all of the large assembly gaps were found in the most repetitive regions of the *Anaplasma* genome, including the regions where the *mps2* and *msp3* pseudogenes and *Aaap* gene family are located.

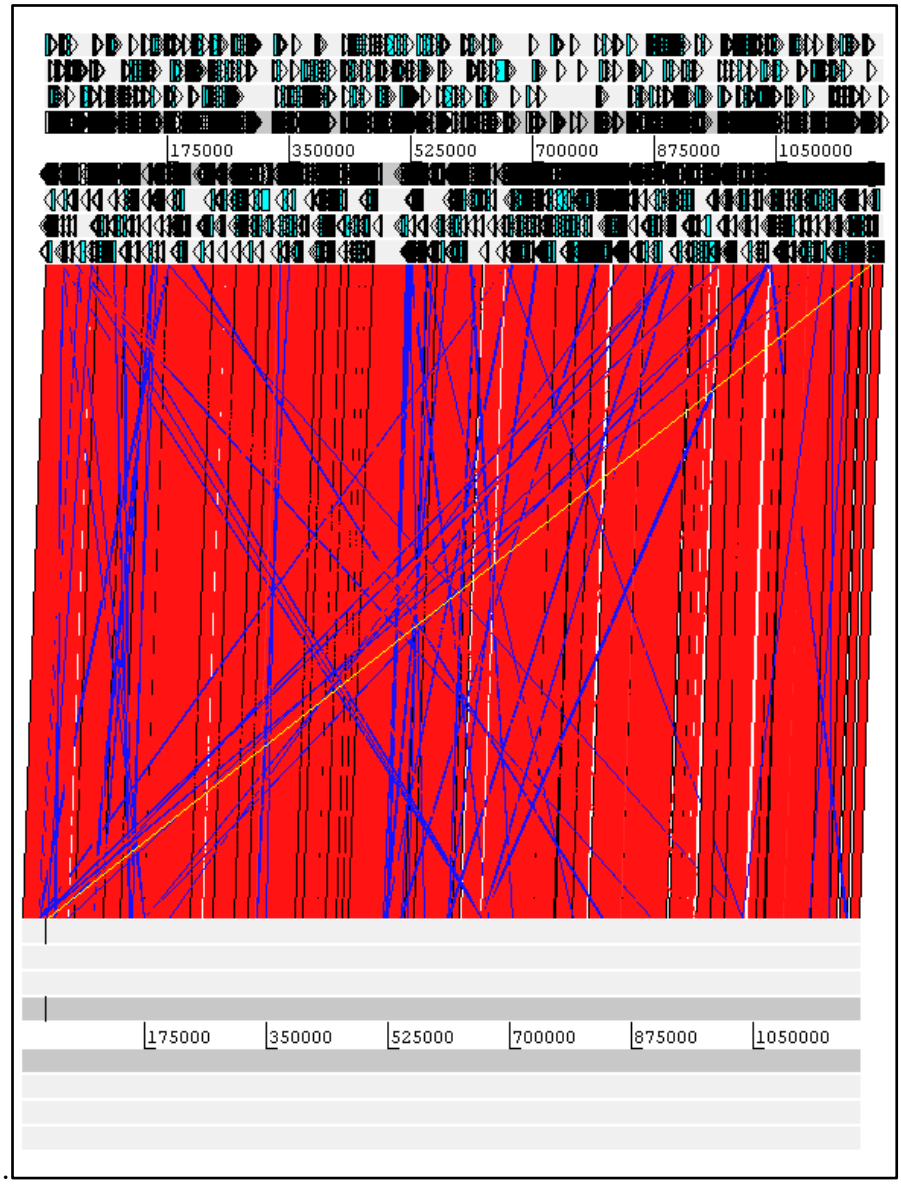

Figure 5.7: Genome sequence alignment between the *Anaplasma. marginale* Florida reference genome (above) and Assembly-2 generated in this study (below) showing the relationship between the two sequences. The red shaded areas represent regions that are conserved between the two sequences and in the same orientation, the blue shaded areas represent regions that are conserved but oriented in the opposite direction, while the white areas represent gaps in the genome.

A genome sequence alignment between Assembly-1 and Assembly-2 revealed that the two assemblies were mostly conserved, with an ANI of 99.93% and a similar genome architecture (Figure 5.9).

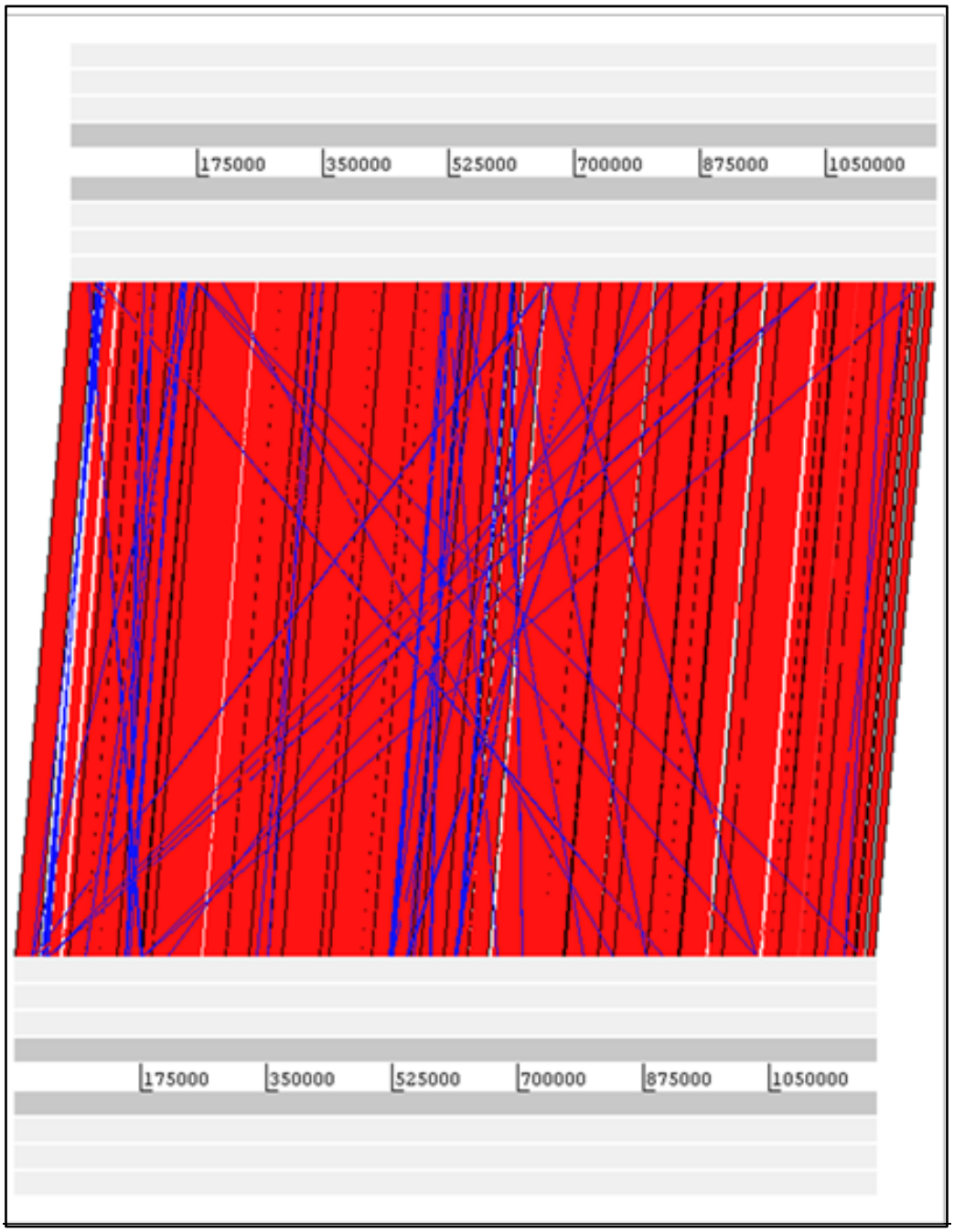

Figure 5.8: Genome sequence alignment between the two different *Anaplasma marginale* genome assemblies generated in this study (Assembly-1 above and Assembly-2 below) with an ANI of 99.93%. The red shaded areas represent regions that are conserved and in the same orientation, the blue shaded areas represent regions that are conserved but oriented in the opposite direction, while the white areas represent gaps.

The major differences between the two assemblies generated in this study were two inversions, approximately 9 and 5 kb long (Figure 5.10A and B). The two inversions are also present in the St Maries-Florida comparison (Figure 5.10C and D). This indicates that each assembly adopted the genome architecture of the reference sequence to which the reads were mapped. Assembly-1 and Assembly-2 therefore differed because they were informed by the reference genome, and neither is likely to be the correct assembly for AmUP1708.

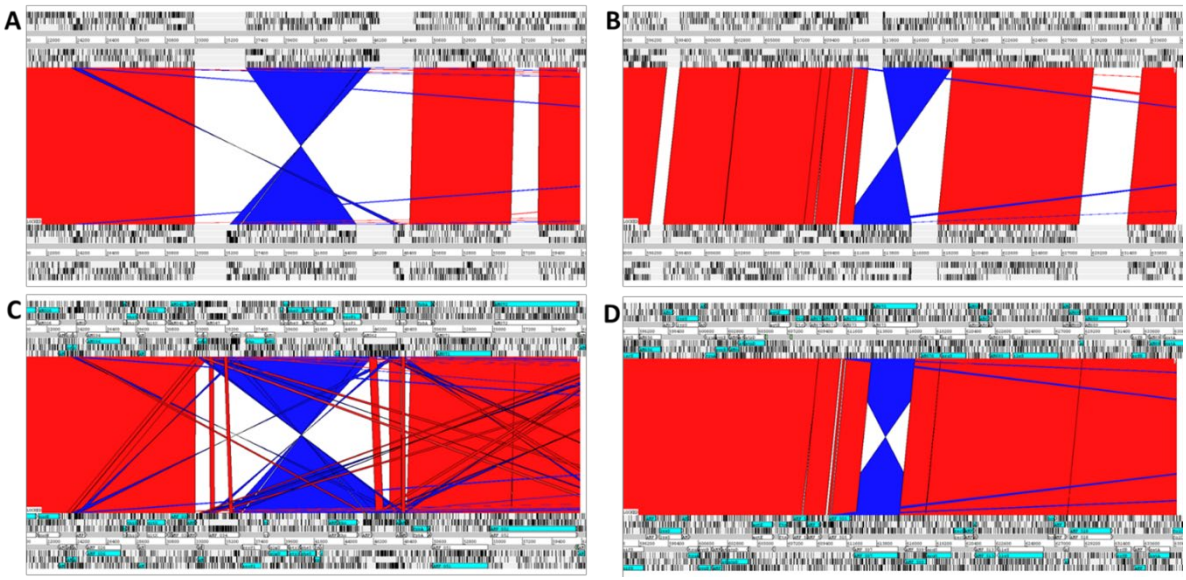

Figure 5.9: Detail of the genome sequence alignments highlighting the two genomic inversions observed between the two different *Anaplasma marginale* genome assemblies generated in this study (Panels A and B) in comparison to the same inversions observed in the St Maries-Florida alignment (Panels C and D). Panel A: ACT comparison of Assembly-1 and Assembly-2, showing Inversion 1, approximately 9 kb long. Panel B: ACT comparison of Assembly-1 and Assembly-2 showing Inversion 2, approximately 5 kb long. Panel C: ACT comparison of St Maries and Florida strains showing Inversion 1, approximately 13 kb long. Panel D: ACT comparison of St Maries and Florida strains showing Inversion 2, approximately 7 kb long.

Another example showing that the two assemblies generated in this study adopted the structure of the reference genome can be seen in the assembly of the *msp1α* gene. This gene, encoding major surface protein 1a (Msp1a), contains tandem repeats at its 5' end which vary in the reference genome sequences, St Maries and Florida. The *msp1α* gene sequence (and consequently the translated Msp1a amino acid sequence) was incorrectly assembled in the two assemblies generated in this study. *Anaplasma marginale* AmUP1708 strain is characterized by four Msp1a repeats 27 4 4 37, yet Assembly-1 only had three Msp1a repeats (Figure 5.11). Assembly-1 was mapped to the St Maries strain that is characterized by three repeats J B B

(Brayton et al., 2005). Assembly-2 was mapped to the Florida strain that is characterized by eight Msp1a repeats A B B B B B B B (Dark et al., 2009). The Msp1a amino acid sequence of Assembly-2 contained five Msp1a repeats, and it also contained a string of additional unspecified amino acids (X), which were translated from the string of Ns filled in by the assembly software to match the length of the Florida reference sequence in this region (Figure 5.11). The two different assemblies of the *msp1α* gene in Assembly-1 and Assembly-2 again highlight that each assembly was informed by the reference genome.

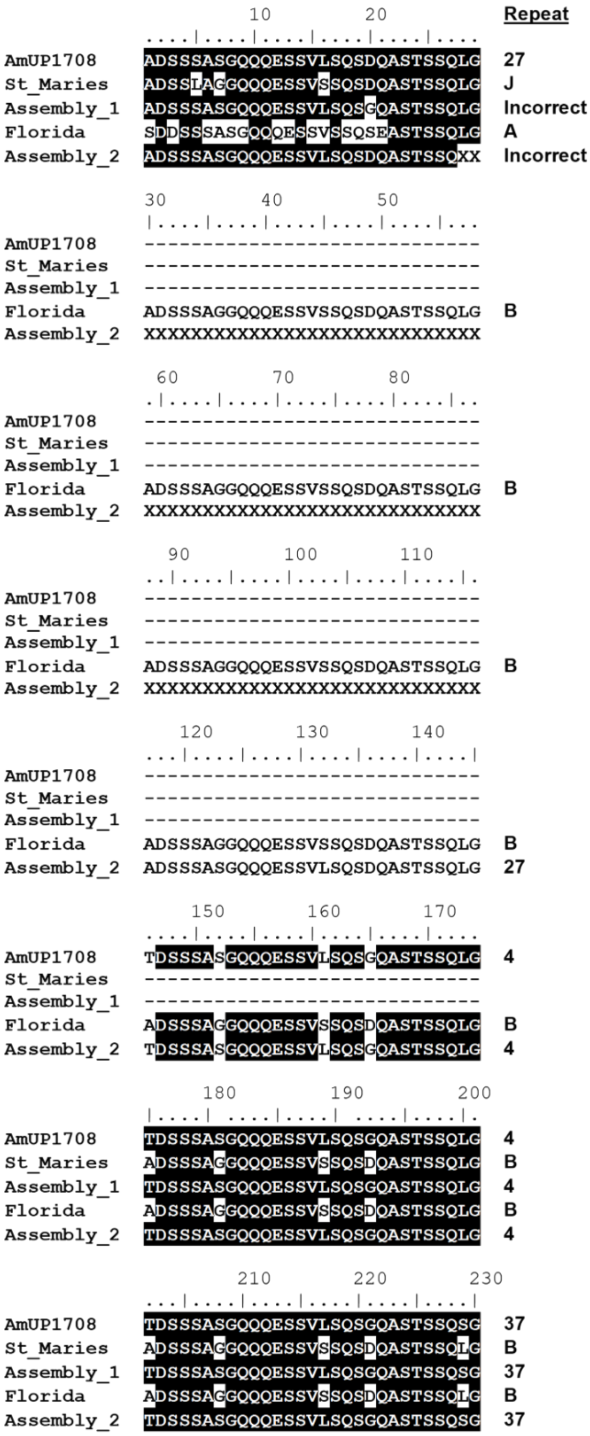

Figure 5.10: *Anaplasma marginale* Msp1a amino acid sequences of AmUP1708, St Maries strain and Florida strain in comparison to Assembly-1 and Assembly-2. Msp1a amino acids were aligned using BioEdit. Each block of amino acid sequences represents a repeat. Repeat names are shown on the right; for Assembly-1 and Assembly-2, repeats were only named if they were correct. Conserved amino acid residues in the alignment are highlighted by white text on a black background, while variable residues are shown by black text on a white background. X represents unspecified or unknown amino acids. A dash (-) represents a gap in the alignment.

Although the two assemblies of the AmUP1708 sequence data were mostly conserved with an ANI of 99.93%, the differences introduced by mapping of the data to the reference genomes cannot be resolved with the existing sequence data. Therefore, more sequencing data should be generated in order to conduct a de novo assembly to generate an accurate genome sequence of the South African strain of *A. marginale*.

5.5. Discussion

While we were able to generate an almost complete genome sequence of *A. marginale* from carrier cow 1708, with *msp1α* genotype 27 4 4 37, providing proof of concept for this approach, our study illustrates the challenges that must be overcome in order to sequence the whole genome of *A. marginale* strains and other *Anaplasma* species directly from their mammalian hosts. Strategies are needed to deplete host DNA, while safeguarding the integrity of the pathogen DNA.

In an earlier study, two *A. marginale msp1α* genotypes with Msp1a repeats 27 4 4 37 and 3 37 were identified in cattle on the IA@UP Experimental Farm (previously, Proefplaas, Hillcrest Campus, University of Pretoria) (Hove, 2018; Hove et al., 2018). We also identified two *msp1α* genotypes in cattle at the IA@UP Experimental Farm. Genotype A, with Msp1a repeats 27 4 4 37, was the most dominant genotype in the cattle we examined from the farm. Interestingly, in addition, a new genotype, genotype B, with Msp1a repeats 27 13 18, was identified in a minority of animals. Since the farm is run as a closed herd, it is not surprising that there is limited genetic diversity of the *A. marginale* strains present in the cattle.

We recommend the use of additional options for depleting host DNA, e.g. treating washed red blood cells with DNase prior to lysing/freezing, to destroy any excess host DNA present in the washed red blood cell preparation. However, this would only be possible for *Anaplasma* species known to infect erythrocytes such as *A. marginale*, *A. centrale*, as well as *A. ovis*. Some *Anaplasma* species infect other cell types, e.g. *A. phagocytophilum* infects neutrophils, *A. bovis* infects monocytes and *A. platys* infects platelets, so such a strategy would not be a possibility for these species. Not much is currently known for the majority of the recently identified *Anaplasma* species, including the host cell type that is infected, and for these species, an

alternative method for enrichment of the pathogen DNA in the presence of excess host DNA will still be required.

Unfortunately, pure *A. marginale* DNA was not available to generate a standard curve to determine the exact *A. marginale* DNA concentration in our DNA preparations. Therefore, the Ct values of the *A. marginale msp1β* and the mammalian GAPDH qPCRs were used to approximate the concentration of the *A. marginale* and bovine DNA present in the DNA samples. Although the Ct value of the *A. marginale msp1β* was lower than Ct value of the mammalian GAPDH in the final DNA generated after microbial enrichment and whole genome amplification (suggesting that the *A. marginale* DNA concentration was higher than the bovine DNA concentration), the sequencing data generated comprised approximately 98% *Bos taurus* reads, and only 1.13% *A. marginale*. Thus, the sequencing results revealed that the bovine DNA was still present at higher concentration than the *A. marginale* DNA. *Anaplasma marginale* has a genome sequence of approximately 1.2 Mb in length (Brayton et al., 2005), while the *Bos taurus* genome is 2.8 Gb in length (NCBI BioProject: PRJEB41519) (Talenti et al., 2022). Thus, every copy of the *msp1β* gene represents 1.2 Mb of *A. marginale* DNA, but every copy of the GAPDH gene represents 2.8 Gb of bovine DNA. Therefore, a larger difference in Ct values would have been required to ensure that the *A. marginale* DNA was present in higher concentration than the bovine DNA. Although another round of microbial enrichment might have increased the proportion of microbial-derived reads, increased rounds of enrichment are likely to sheer the DNA, thereby diminishing the quality of the DNA.

The low number of *A. marginale* reads resulted in a fragmented *A. marginale* genome assembly, which still requires more sequencing data to completely close the genome. While this might be achieved by increasing the number of microbial enrichment rounds of the host contaminated sample, this would probably result in further sheering of the DNA, thereby diminishing the power of the PacBio sequencing, which lies in its ability to generate long reads averaging 10–25 kb with accuracies greater than 99.5%. Such long reads are important in assemblies of genomes containing repetitive regions, as they span the areas containing the repeats, and result in accurate assemblies across repeats. The reads generated in this study, which ranged from 500 bp-16 kb with an average of approximately 4700 bp, were already not of optimal length to take advantage of the long reads generated by PacBio sequencing. However, sequencing the enriched, amplified DNA on a third and even a fourth PacBio cell might close the *A. marginale* genome, and would be more cost, time and labour efficient than

the conventional ways of sequencing the genome of intracellular bacteria like *A. marginale*, which include infection of splenectomized animals maintained in an insect-free environment, preparation of stabilates, establishing the isolate in tick or mammalian cell lines (which can take a very long time), purification of the pathogen from host cells and sequencing. We could further incorporate other molecular strategies that have been used recently to generate whole genome sequences from highly complex samples, such as the use of the SureSelect^{XT}Target enrichment method which uses a tailored RNA bait library to capture the target DNA (Hadfield et al., 2017; Seth-Smith et al., 2021) and the use of metagenomics by taxonomic binning for genome reconstruction (Gupta et al., 2016).

The whole genome sequences of the intracellular bacteria, *Chlamydia trachomatis* (Christiansen et al., 2014; Hadfield et al., 2017) and *Orientia tsutsugamushi* (Elliott et al., 2021) were recently sequenced from complex samples which included chigger or human DNA using the custom SureSelect^{XT}Target enrichment system method. This method was also successfully used to enrich and sequence the genomes of *Mycobacterium tuberculosis* (Brown et al., 2015), *Neisseria meningitidis* (Clark et al., 2018) and herpesviruses (Depledge et al., 2011) directly from clinical specimens. The SureSelect^{XT}Target enrichment method is based on a custom-made capture 120-mer RNA bait set, that captures all known diversity amongst intra-species genomes, synthesised by Agilent Technologies. This method can be incorporated in our study and modified to maintain the integrity of the genomic DNA for use on the PacBio sequencing platform to obtain the whole genome sequence. This method has an estimated genome sequencing success ratio of 45% in non-culture specimens, with ~25% of specimens yielding complete genomic data (Clark et al., 2018). However, further measures resulting in high quality DNA extracts, depletion of non-target DNA prior to target enrichment have previously resulted in an improved quality of the pathogen genome sequence obtained (Brown et al., 2015).

High-throughput sequencing technology or metagenomics has allowed the population study of natural microorganisms without the need to culture (Venter et al., 2004; DeLong et al., 2006; Zhou et al., 2015; White et al., 2016) with the goal of obtaining organism/species-specific, complete, genomic data from the complex mixture of sequence data generated from complex samples (Nelson, Tully & Mobberley, 2020). Bacterial genomes have also been successfully sequenced, reconstructed and reported from multiple metagenomes using the Binning-Assembly (BA) which is completely different and novel (Gupta et al., 2016; Nelson, Tully &

Mobberley, 2020). The accuracy of using the metagenomic technique for whole genome sequencing is difficult to measure as the technique relies on the availability of genome sequences in the dataset, abundance (number of reads) of the species in the metagenome, as well as efficiency of binning algorithms (Gupta et al., 2016; Nelson, Tully & Mobberley, 2020), however, techniques that have been developed to evaluate the accuracy of the binning process rely on conserved genes and consistency of nucleotide composition (Eren et al., 2015; Parks et al., 2015, 2017; Waterhouse et al., 2018; Chen et al., 2020). The lack of reference genome sequences in the public databases is regarded as a major limitation of using this approach for genomics, which might also be a limitation for genome sequencing of novel *Anaplasma* species due to the limited number of *Anaplasma* genome sequences available. However, this method has previously allowed for exploring and studying of diverse communities which are difficult to culture *in vivo* from complex environmental samples (Parks et al., 2017; Chen et al., 2020) and with modification, it is possible that it could also work for *Anaplasma* research.

5.6. Conclusions

The presence of host DNA remains a serious impediment in obtaining the genome sequences of *Anaplasma* species from carrier animals. Our sequencing data consisted mainly of bovine sequencing reads, and mapping of the *A. marginale* sequences to two different reference genomes resulted in two different incomplete *A. marginale* assemblies. This highlights the need for more sequencing data and further sequence analysis for a complete closed *A. marginale* genome assembly. Advances in molecular techniques for microbial DNA enrichment and in sequencing, assembly, and assortment of contigs into species-specific bins enabling the reconstruction of genomes from metagenomic data could be incorporated in this study to sequence the whole genome sequences of the different *Anaplasma* spp. circulating in livestock, wildlife and companion animals without the need to culture. Such a technique could be used in future for the genome sequencing of the different genotypes of *A. marginale* reported in cattle worldwide, as well other putative *Anaplasma* spp. reported in various hosts including wildlife for a clear species definition and to fully capture the diversity in the genus and for further studies of identifying alternative genetic markers for use in the development of species-specific diagnostic assays.

5.7. References

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CHAPTER 6

General Discussion, Conclusions and Recommendations

Bovine anaplasmosis is among the three most important tick-borne diseases (TBDs) of ruminants and results in major economic losses in food animal production globally (Uilenberg, 1995). This disease is mainly caused by the obligate intracellular rickettsia, *Anaplasma marginale*, which is currently widespread in South Africa (Hove et al., 2018). Bovine anaplasmosis is an economically important worldwide, with economic impact in South Africa estimated at approximately R115 million ($US9.6 million) per year due to mortalities (Hove, 2018), although this does not take into account costs associated with treatment and control. Bovine anaplasmosis is currently endemic throughout the cattle-farming areas in South Africa, and 3% of the total cattle deaths in the country are due to anaplasmosis (de Waal, 2000; Mtshali, de Waal & Mbati, 2004; Mutshembele et al., 2014; Hove et al., 2018).

The integrated strategic use of acaricides and vaccines has been suggested as the best strategy for the control of TBDs in South Africa (de Waal, 2000). However, acaricides are expensive and acaricide resistance is a concern, and the live, blood-borne *A. centrale* vaccine has some drawbacks. The vaccine is expensive to produce, it requires careful maintenance of a cold chain during storage and distribution, it does not protect against all field strains of *A. marginale* and, since it is produced in live animals, there is a risk of introducing additional blood-borne pathogens. The maintenance of endemic stability is important for the control of bovine anaplasmosis in many areas in South Africa, but the causes of clinical cases in endemic areas have not been well studied. A better understanding of pathogen ecology and transmission, the causes of disease outbreaks, as well as the development of improved tools for pathogen detection and development of safer, more effective vaccines, would lead to improved and appropriate control strategies against bovine anaplasmosis and other TBDs.

The current recommended cELISA kit (Knowles et al., 1996) for detection of *A. marginale* cross-reacts with other *Anaplasma* and *Ehrlichia* spp. (Al-Adhami et al., 2011). The test therefore cannot be used to distinguish between *A. marginale* and other *Anaplasma* spp. in mixed infections in the host. In the South African context, other *Anaplasma* species, including *Anaplasma centrale* and *Anaplasma* sp. (Omatjenne) are known to infect the same hosts as *A.*

marginale (domesticated and wild bovines), but cause much milder or no disease. While there is evidence that multiple *Anaplasma* spp. are present in wildlife in South Africa, the full range of *Anaplasma* spp. present is not known, and their impact on the epidemiology of anaplasmosis in domestic animals is currently unclear. Therefore, there is a need to assess the full range of *Anaplasma* spp. present in wild and domestic animals in South Africa and to develop more specific assays to accurately identify *A. marginale* and distinguish it from other *Anaplasma* spp. to better elucidate the epidemiology of this pathogen in South Africa.

6.1. Unravelling the diversity of *Anaplasma* species circulating in selected African wildlife hosts by targeted 16S microbiome analysis

Numerous *Anaplasma* spp. have been identified in several wild ruminant species, as well as in rodents in Africa, including *A. marginale*, *A. centrale*, *A. ovis, Anaplasma* sp. (Omatjenne) and *A. bovis* (Neitz, 1935; Peirce, 1972; Augustyn, 1974; Smith et al., 1982; Kuttler, 1984; Ngeranwa et al., 1998; Potgieter & Stoltsz, 2004; Harrison, Bown & Horak, 2011; Pfitzer et al., 2011; Harrison et al., 2013; Eygelaar et al., 2015; Khumalo et al., 2016). The full range of *Anaplasma* spp. present in wildlife hosts and their impact on the current diagnostic assays for bovine anaplasmosis is not known. Furthermore, the role of wildlife hosts in the distribution and epidemiology of anaplasmosis in domestic animals, livestock and possibly in humans is unclear. Our study highlights the importance of third-generation sequencing and bioinformatics to elucidate the presence and wide range of genetic diversity of *Anaplasma* spp. circulating in selected African wildlife hosts, with potential for transmission to humans, livestock and companion animals.

We have shown the presence of 13 *Anaplasma* sequences circulating in different African wildlife hosts in the Kruger National Park and surrounding game reserves in Mpumalanga, South Africa. Four of these are previously known *Anaplasma* species, *A. marginale*, *A. centrale*, *A. bovis* and *Anaplasma* sp. SA dog. *Anaplasma marginale* is endemic in cattle in South Africa (de Waal, 2000; Mtshali et al., 2007; Mutshembele et al., 2014; Hove et al., 2018) and is known to infect a range of wildlife species in South Africa and neighbouring countries (Tonetti et al., 2009; Pfitzer et al., 2011; Debeila, 2012; Berggoetz et al., 2014; Eygelaar et al., 2015; Khumalo et al., 2016). *Anaplasma centrale* is currently used in a live blood vaccine against bovine anaplasmosis in South Africa, and the vaccine strain as well as other strains of

A. centrale have been shown to circulate in wildlife hosts (Khumalo et al., 2016), though the occurrence and impact of other strains of *A. centrale* in livestock is currently unknown. *Anaplasma bovis* has been mainly reported in cattle (Noaman & Shayan, 2010; Belkahia et al., 2015) but has also been detected in eastern rock sengis, which have been implicated as natural reservoir hosts responsible for the increasing distribution of *A. bovis* in South Africa (Harrison et al., 2013). *Anaplasma* sp. SA dog is a novel *Anaplasma* spp. found in the zoonotic clade of the genus *Anaplasma* and was first detected in dogs in South African in 2005 (Inokuma et al., 2005; Kolo et al., 2020), however, not much else is known about this organism.

Nine of the 13 *Anaplasma* 16S rRNA sequences detected in this study were previously unknown. We showed that these sequences are phylogenetically similar to sequences from previously identified and described species in the two prominent phylogenetic clades of the genus *Anaplasma* and thus may serve as a source of cross-reaction in the current serological (Kocan et al., 1992; de la Fuente et al., 2005) and some molecular diagnostic assays (Georges et al., 2001; Inokuma et al., 2001; Bekker et al., 2002; Kawahara et al., 2006; Li et al., 2015; Agina et al., 2021; Kamani et al., 2022). The commercially available cELISA kit (Knowles et al., 1996) that is recommended for the diagnosis of bovine anaplasmosis, uses the recombinant major surface protein 5 (Msp5) as antigen. This test cross-reacts with other *Anaplasma* spp. (Munodzana et al., 1998) because Msp5 is present in all known *Anaplasma* spp. Furthermore, the epitope defined by monoclonal antibody ANAF16C1 is broadly conserved among *Anaplasma* spp., and it is thus highly likely that the putative *Anaplasma* agents will also cross-react with the serological tests. Primers and probes that target the 16S rRNA gene have been used to determine the epidemiology of anaplasmosis-causing organisms worldwide; these were designed based on the few previously identified and described species within the genus. The newly detected putative agents are highly likely to cross-react with these tests because *Anaplasma* spp. are closely related with 16S rRNA sequence identities of above 98.7% among known unique *Anaplasma* species (Caudill & Brayton, 2022). The 16S rRNA sequence alone is thus not recommended for species assignment for the genus *Anaplasma*. This highlights the necessity for more research focused on identifying and characterizing other genes (e.g. *groEL*, *gltA* and *msp4*) and antigens for development of more specific assays to assess the epidemiology of the different *Anaplasma* species.

Our study showed a greater genetic diversity of *Anaplasma* species circulating in wildlife hosts than currently classified within the genus *Anaplasma*, suggesting the significance of wildlife

hosts as reservoir hosts. This corroborates the findings from a recent analysis of 16S rRNA gene sequences from a wide range of putative *Anaplasma* species as well as formally named species in the genus (Caudill & Brayton, 2022), which indicated intra- and inter-species variation in the 16S rRNA sequences across the two recognised clades of the genus *Anaplasma*.

Anaplasma spp. are known to be multi-host parasites (Kocan et al., 2010), implicated as the cause of mortality and morbidity in cattle (de Waal, 2000). Thus, the presence of the putative species detected in this study may suggest potential for transmission to livestock or companion animals. Novel *Anaplasma* spp. that have previously been reported in cattle in South Africa and neighboring countries include "*Candidatus* Anaplasma boleense" (Fernandes et al., 2019; Kolo et al., 2020), *Anaplasma* sp. Saso, *Anaplasma* sp. Hadesa and *Anaplasma* sp. Dedessa, as well as *Anaplasma* sp. Mymensingh (Kolo et al., 2020). *Anaplasma* sp. ZAM dog 16S rRNA sequences were detected recently in dogs and the dog tick *Rhipicephalus sanguineus* in the Mnisi community, Mpumalanga, South Africa (Kolo et al., 2020). These findings highlight an urgent need for additional molecular data and genome sequencing for correct *Anaplasma* species classification and to study the natural rate of variation between *Anaplasma* species, to fully understand their evolution and epidemiology. There is a further need to assess the possible impact of these putative agents in heterologous protection against pathogenic species in livestock and companion animals, as well as determining species specific markers for more accurate diagnostics.

6.2. Temporal Dynamics of *Anaplasma marginale* Infections and the Composition of *Anaplasma* spp. in Calves in the Mnisi communal area, Mpumalanga, South Africa

Clinical cases of bovine anaplasmosis are frequently reported in cattle in the Mnisi community, located at a wildlife–livestock interface in the north-eastern corner of the Bushbuckridge Municipality, Mpumalanga Province, South Africa (Choopa, 2015). Our study highlights *A. marginale* strain diversity and infection dynamics in calves in a peri-urban area and at a wildlife–livestock interface within the Mnisi community, as well as the composition of *Anaplasma* spp. to which cattle are exposed in the area.

We have shown that calves in the study area are exposed to *A. marginale* infection either early in their lives or during intra-uterine development, since 50% of the calves were infected in the first or second month without any clinical symptoms for the duration of the study. Calves in the peri-urban area were all *A. marginale* positive by the second month, while only two calves at the wildlife-livestock interface were infected from the sixth month. It is well known that calves up to 12 months of age are not clinically affected by anaplasmosis (Bock et al., 1997; Jonsson et al., 2012). The lack of continuous infection with *A. marginale* in three of the five calves at the wildlife–livestock interface in their first year when natural immunity is higher, could explain the bovine anaplasmosis cases in adult cattle at the wildlife–livestock interface in the Mnisi communal area.

Our results showed that complex *A. marginale* infection detected in both areas of the Mnisi community is driven by co-infection and superinfection, with four to thirteen genetically distinct *msp1α* genotypes detected per animal over the 12-month period. Our results support recent findings where complex *A. marginale* infections were detected in 97% of naïve calves in southern Ghana (Koku et al., 2021); similar results were found in cattle in an *A. marginale*-prevalent region in Mexico (Castañeda-Ortiz et al., 2015).

Differences in the temporal *A. marginale* infection dynamics displayed in the two different areas may be attributed to factors such as cattle density and acaricide application which differed between the two areas. The higher density of cattle in the peri-urban area provides greater opportunity for rapid migration of *R. microplus* vector ticks (larvae and adult ticks) from infested to naïve cattle (Mason & Norval, 1981; Aguirre et al., 1994), thus increasing the likelihood of *A. marginale* transmission. Acaricide application practices differ in the two areas, mainly due to water shortages. Cattle in the peri-urban site, Eglington village, as well as in Utha A, at the wildlife–livestock interface, are dipped using the plunge method of dipping cattle, while a hand spraying method is used at Dixie village (also at the wildlife–livestock interface). Several factors, such as the inability to correctly manage the dip tank and incorrect mixing ratios of water and the acaricide, have been shown to be the prime causes of tick control failure at communal plunge dip tanks (Jonsson, 1997; Sungirai et al., 2016) such as the ones used at Eglington and Utha A. Thus, the hand spraying method, as is used at Dixie village, may be more effective in controlling tick infestation and thus preventing disease transmission, than the plunge method of cattle dipping where the concentration of the acaricide in the dip tank might not be consistent. Our sample size was very limited and a larger study with more animals

would be required to confirm the lack of *A. marginale* infection in cattle at the wildlife-livestock interface and the impact thereof.

We further highlighted the presence of other *Anaplasma* species in the calves, which mainly comprised an *A. platys*-like organism that is closely related to a novel *Anaplasma* species which we identified in wildlife in the Kruger National Park (Chapter 3). It is possible that this novel *Anaplasma* may have been introduced into cattle from wildlife, although the identical 16S rRNA sequence was not identified in the wildlife hosts examined (Chapter 3). This novel *Anaplasma* might provide some cross-protection against the pathogenic species and thus decrease the pathogenicity of anaplasmosis in cattle in the area (Woolhouse et al., 2015). We also detected the novel *A. boleense*, which has been identified in cattle in the Mnisi communal area previously (Kolo et al., 2020) and was also identified in cattle in Mozambique (Fernandes et al., 2019). Furthermore, several novel *Anaplasma* spp. have been reported in cattle elsewhere, including a novel *Anaplasma* from Uganda (Ikwap et al., 2010; Muhanguzi et al., 2010), *Anaplasma* sp. Saso, *Anaplasma* sp. Hadesa and *Anaplasma* sp. Dedessa in cattle in Ethiopia (Hailemariam et al., 2017) and in the Mnisi community in South Africa (Kolo et al., 2020), *Anaplasma* sp. Mymensingh from cattle in Bangladesh (Roy et al., 2018) and in the Mnisi community (Kolo et al., 2020), as well as "*Candidatus* Anaplasma africae" in Senegal (Dahmani et al., 2017). Our findings thus corroborate other studies in recent years that have detected a number of novel *Anaplasma* spp. in cattle. In the Mnisi communal area there are likely to be opportunities for natural transmission of *A. marginale* and other *Anaplasma* spp. from wildlife hosts to cattle due to the proximity of *Anaplasma* infected wildlife hosts in the surrounding game parks (Kolo et al., 2020). A future in-depth longitudinal analysis of the dynamics of *A. marginale* infections in livestock in the Mnisi communal study area is thus recommended with a larger sample size, to elucidate the possibility of there being a spill-over of *Anaplasma* species from wildlife hosts.

6.3. Exploring an alternative method for *Anaplasma* whole genome sequencing

Despite a research history that spans over a century (Theiler, 1910), *Anaplasma* spp. are amongst the most understudied bacteria due to their intracellular nature, with relatively few genome sequences available in the public databases. The advent of molecular sequencing

technologies, such as next- and third-generation sequencing, have revealed a greater genetic diversity of *Anaplasma* species worldwide; however, this diversity is mainly based on 16S rRNA gene studies (Kolo et al., 2020; Caudill & Brayton, 2022) with relatively few other genes used in characterization studies (Kolo et al., 2020). To date, relatively few genome sequences of species within the genus *Anaplasma* have been published. Genome sequences of two or more strains have been sequenced and published for only two *Anaplasma* species: *A. marginale* (Brayton et al., 2005; Dark et al., 2009; Dark, Al-Khedery & Barbet, 2011; Pierlé et al., 2014) and *A. phagocytophilum* (Dunning et al., 2006). *Anaplasma centrale* (Herndon et al., 2010), *A. ovis* (Liu et al., 2019) and *A. platys* (Llanes & Rajeev, 2020) are represented by a genome sequence of a single strain each. The low number of *Anaplasma* genome sequences in public databases can be attributed to their intracellular nature, which is a major constraint in the generation of sufficient, pure, high-quality genetic material for genome sequencing. In order to generate sufficiently large amounts of DNA, either splenectomised animals must be infected with *Anaplasma* isolates, or *Anaplasma* isolates must be grown in *in vitro* culture. Because *Anaplasma* organisms are obligate intracellular parasites, they must be grown in either mammalian or arthropod cells (Munderloh et al., 1996), and *Anaplasma* cultures are notoriously difficult to initiate. Furthermore, many of the *Anaplasma* species that have been identified in apparently healthy wildlife hosts in recent years were present at low levels, making it very difficult to isolate them, and impossible to initiate *in vitro* cultures. Therefore, this study was aimed at exploring an alternative means of obtaining genome sequences of South African strains of *A. marginale* directly from carrier cattle without the need for infection of splenectomised calves or *in vitro* culture.

Our findings illustrate the challenges posed by sequencing the whole genome of intracellular bacteria directly from a complex mixture including host DNA, and the need for strategies to deplete host DNA. Although *A. marginale* is characterized by a small genome sequence of approximately 1.2 Mb in length (Brayton et al., 2005; Dark et al., 2009; Herndon et al., 2010), sequencing the genome directly from carrier animals proved difficult because of the presence of overwhelming amounts of the host genome, which is approximately 2.8 Gb. The difficulty of separating *Anaplasma* DNA from host DNA thus poses a serious impediment in studying the genomics of *Anaplasma* species directly from carrier animals. Our sequencing results indicate that sequencing of the *A. marginale* genome from carrier cattle will require additional methods of depletion of bovine DNA, while safeguarding the integrity of the pathogen DNA.

One solution might be to increase the number of microbial enrichment rounds of the complex host contaminated sample; however, this might result in sheering of the DNA, thereby diminishing the power of PacBio sequencing which lies in its ability to sequence long reads. The method we used included separation of the red blood cells from the buffy coat, which will have removed much of the bovine host DNA. However, the inclusion of such a method would only be possible for *Anaplasma* species which infect erythrocytes, such as *A. marginale*, *A. centrale* and *A. ovis.* Little is known about the novel *Anaplasma* spp., including which cells they infect, although the novel *Anaplasma* spp. in the ruminant clade are likely to infect red blood cells since the known *Anaplasma* spp. in the ruminant clade are all intra-erythrocytic, but this would require further investigation. Those in the *A. platys*-like clade are likely to infect other blood cell types/components, including lymphocytes, neutrophils and platelets, so it would be impossible to separate them from host DNA. Host DNA depletion methods, or alternative methods, will be even more important for these novel *Anaplasma* spp. We can further incorporate other molecular strategies that were recently used to generate whole genome sequences from highly complex samples, such as the use of the SureSelect^{XT}Target enrichment method (Hadfield et al., 2017; Seth-Smith et al., 2021) and the use of metagenomics by taxonomic binning for genome reconstruction (Gupta et al., 2016).

6.4. Conclusions

In conclusion, this study highlights a greater genetic diversity of *Anaplasma* species circulating in wildlife hosts than currently classified within the genus *Anaplasma*. Furthermore, the putative species identified are phylogenetically similar to known *Anaplasma* spp. and may serve as a source of cross-reaction in the current detection assays, highlighting a need for additional genetic data and genome sequencing of these putative species for correct *Anaplasma* species classification and further assessment of their occurrence in livestock and companion animals. This study further highlights the occurrence of complex *A. marginale* infection in calves and therefore probably in adult cattle in the Mnisi community in South Africa, which was driven by co-infection and superinfection. A localized lack of endemic stability at the wildlife–livestock interface could result in clinical cases of bovine anaplasmosis. Furthermore, the presence of other *Anaplasma* spp. in cattle in the Mnisi community might confer cross-protection against infection with the pathogenic *A. marginale* and might contribute to the control of bovine anaplasmosis in South Africa. The study highlights a significant gap in the

knowledge and documentation of the presence of other *Anaplasma* spp. that infect wildlife hosts and cattle. These *Anaplasma* spp. could impact on the current detection methods. Therefore, there is a need for molecular techniques for microbial DNA enrichment and metagenomics to generate more genome sequences of *A. marginale* and the different *Anaplasma* spp. circulating in livestock, wildlife and companion animals, for correct classification in the *Anaplasma* taxonomy and to study the natural rate of variation between the different *Anaplasma* species and their specific genotypes, to fully understand their evolution and diversity. The genome sequence data of *Anaplasma* spp. will further assist with the identification of species-specific targets for the development of more specific serological nucleic-acid-based detection methods suitable for examining the epidemiology of all *Anaplasma* spp. from various hosts.

6.5. References

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APPENDICES

Appendix 1

Table A1: List of previously reported putative *Anaplasma* spp. modified from Caudill & Brayton (2022).

Putative *Anaplasma* sp.	Host	Origin	References
Anaplasma capra	Humans; domestic; wildlife hosts; dogs	China	Li, Zheng, et al., 2015; Sun et al., 2015
"Candidatus A. camelii"	Camels	Saudi Arabia; Iran	Bastos et al., 2015; Sharifiyazdi et al., 2017
"Candidatus A. corsicanum"	Sheep	France	Dahmani et al., 2017
"*Candidatus* A. mediterraneum"	Sheep	France	Dahmani et al., 2017
"*Candidatus* A. africae"	Cattle; sheep; goats	Senegal	Dahmani et al., 2019
"*Candidatus* A. boleense"	Cattle; mosquitoes	China; Mozambique; South Africa	Guo et al., 2016; Fernandes et al., 2019; Kolo et al., 2020
"*Candidatus* A. rodmosense"	Mosquitoes	China	Guo et al., 2016
"*Candidatus* A. ivorensis"	Ticks	Côte d'Ivoire	Ehounoud et al., 2016
Anaplasma sp. SA dog	Dogs; ticks; cattle	South Africa; Zambia	Inokuma et al., 2005; Vlahakis et al., 2018; Kolo et al., 2020
Anaplasma sp. Mymensingh	Cattle	Bangladesh; South Africa	Roy et al., 2018; Kolo et al., 2020
Novel *Anaplasma* sp.	Cattle	Uganda	Ikwap et al., 2010; Muhanguzi et al., 2010
"*Candidatus* A. sphenisci"	African penguin	South Africa	Vanstreels et al., 2018
"*Candidatus* A. pangolin"	Pangolins	Malaysia	Koh et al., 2016
Anaplasma sp. strain AnAj360	Ticks	Thailand	Parola et al., 2003
"*Candidatus* A. testudines"	Tortoises	United State of America	Crosby et al., 2021
"*Candidatus* A. brasiliensis"	Anteaters	Brazil	Calchi et al., 2020
"*Candidatus* A. amazonensis"	Sloths	Brazil	Calchi et al., 2020
Anaplasma sp. *O. moubata*	Ticks	Zambia	Qiu et al., 2021
Anaplasma sp. *Ar. walkerae*	Ticks	Zambia	Qiu et al., 2021
Anaplasma sp. Omatjenne	Wildlife hosts; livestock	South Africa; Botswana; Mozambique; Ethiopia; Uganda; Turkey	Allsopp et al., 1997 Bekker et al., 2002 Pfitzer et al., 2011
Anaplasma mesaeterum	Sheep; ticks	Europe	Uilenberg et al., 1979 Nakamura et al., 1993 Dantas-Torres & Otranto, 2017
''Candidatus A. boleense''	Cattle	Mozambique; China	Lu et al., 2017 Fernandes et al., 2019 Guo et al., 2019
Anaplasma sp. Hadesa	Cattle	Ethiopia; South Africa	Hailemariam et al., 2017 Kolo et al., 2020
Anaplasma sp. Saso	Cattle	Ethiopia; South Africa	Hailemariam et al., 2017 Kolo et al., 2020

Anaplasma sp. Dedessa	Cattle	Ethiopia; South Africa	Hailemariam et al., 2017 Kolo et al., 2020
Anaplasma sp. Mongolia	Cattle; sheep; ticks	Mongolia	Fischer et al., 2020

Appendix 2

Table A2: Barcoded primers used for the amplification of the 16S rRNA gene.

Primer name	Code	Multiplex identifier (MID) sequence[a]	Primer[b]
16S_For_bc1005	F1	GCATC**CACTCGACTCTCGCGT**AGRGTTYGATYMTGGCTCAG	27F
16S_For_bc1007	F2	GCATC**TCTGTATCTCTATGTG**AGRGTTYGATYMTGGCTCAG	27F
16S_For_bc1008	F3	GCATC**ACAGTCGAGCGCTGCG**AGRGTTYGATYMTGGCTCAG	27F
16S_For_bc1012	F4	GCATC**ACACTAGATCGCGTGT**AGRGTTYGATYMTGGCTCAG	27F
16S_For_bc1015	F5	GCATC**CGCATGACACGTGTGT**AGRGTTYGATYMTGGCTCAG	27F
16S_For_bc1020	F6	GCATC**CACGACACGACGATGT**AGRGTTYGATYMTGGCTCAG	27F
16S_For_bc1022	F7	GCATC**CACTCACGTGTGATAT**AGRGTTYGATYMTGGCTCAG	27F
16S_For_bc1024	F8	GCATC**CATGTAGAGCAGAGAG**AGRGTTYGATYMTGGCTCAG	27F
16S_Rev_bc1033	R1	GCATC**AGAGACTGCGACGAGA**RGYTACCTTGTTACGACTT	1492R
16S_Rev_bc1035	R2	GCATC**CAGAGAGTGCGCGCGC**RGYTACCTTGTTACGACTT	1492R
16S_Rev_bc1044	R3	GCATC**CGCGCGTCGTCTCAGC**RGYTACCTTGTTACGACTT	1492R
16S_Rev_bc1045	R4	GCATC**AGAGAGTACGATATGT**RGYTACCTTGTTACGACTT	1492R
16S_Rev_bc1054	R5	GCATC**TCTGTAGTGCGTGCGC**RGYTACCTTGTTACGACTT	1492R
16S_Rev_bc1056	R6	GCATC**ATGTGCGTGTGTGTCT**RGYTACCTTGTTACGACTT	1492R
16S_Rev_bc1057	R7	GCATC**CTCTCAGACGCTCGTC**RGYTACCTTGTTACGACTT	1492R
16S_Rev_bc1059	R8	GCATC**TATCTCAGTGCGTGTG**RGYTACCTTGTTACGACTT	1492R

[a] red font= buffer sequence, bold font= MID sequence (barcodes), blue font= key sequence. Degenerate base identities: R = A or G; Y = C or T; M = A or C

[b] 27F = 5'-AGR GTT YGA TYM TGG CTC AG-3'; 1492R= 5'-RGY TAC CTT GTT ACG ACT T-3'

Appendix 3

Table A3: *Anaplasma*/*Ehrlichia* group-specific qPCR assay results.

Sample ID	Wildlife host	*Anaplasma*/*Ehrlichia* qPCR assay		16S rRNA gene amplification for microbiome analysis[b]
		qPCR result	Ct value (Mean±SD)[a]	
B53	African buffalo	Negative	-[c]	ND[d]
B54	African buffalo	Negative	-	ND
B55	African buffalo	Positive	33.78 ± 0.10	Positive
B56	African buffalo	Positive	31.33 ± 0.19	Positive
B57	African buffalo	Positive	32.10 ± 0.16	Positive
B58	African buffalo	Positive	34.91 ± 0.22	Positive
B59	African buffalo	Positive	31.88 ± 0.16	Positive
B60	African buffalo	Positive	37.01 ± 0.19	Negative
B61	African buffalo	Positive	33.17 ± 0.17	Positive
B66	African buffalo	Positive	38.73 ± 0.26	Negative
B67	African buffalo	Negative	-	ND
B68	African buffalo	Positive	37.19 ± 0.27	Negative
B69	African buffalo	Positive	38.29 ± 0.30	Negative
B70	African buffalo	Positive	32.79 ± 0.19	Positive
B71	African buffalo	Positive	36.85 ± 0.20	Negative
B72	African buffalo	Negative	-	ND
B73	African buffalo	Negative	-	ND
B74	African buffalo	Positive	33.66 ± 0.23	Positive
B75	African buffalo	Negative	-	ND
B76	African buffalo	Negative	-	ND
B77	African buffalo	Negative	-	ND
B78	African buffalo	Negative	-	ND
B79	African buffalo	Positive	32.06 ± 0.21	Positive
B80	African buffalo	Positive	34.64 ± 0.31	Positive
B81	African buffalo	Positive	39.24 ± 0.33	Negative
B82	African buffalo	Positive	39.22 ± 0.31	Negative
B83	African buffalo	Positive	34.13 ± 0.17	Positive
C4	African buffalo	Positive	33.77 ± 0.18	Positive
C5	African buffalo	Positive	32.13 ± 0.24	Positive
C6	African buffalo	Positive	38.94 ± 0.26	Negative
Mean Ct value ± SD (African buffalo):			35.04 ± 2.74	
B25	Impala	Negative	-	ND
B31	Impala	Positive	37.57 ± 0.19	Negative
C33	Impala	Negative	-	ND
C34	Impala	Positive	37.89 ± 0.24	Negative
C35	Impala	Positive	27.01 ± 0.12	Positive
C36	Impala	Positive	29.49 ± 0.16	Positive
C37	Impala	Positive	32.39 ± 0.19	Positive
C38	Impala	Positive	28.86 ± 0.18	Positive

C40	Impala	Negative	-	ND
C41	Impala	Positive	30.64 ± 0.16	Positive
C42	Impala	Positive	26.97 ± 0.11	Negative
C43	Impala	Positive	38.86 ± 0.34	Negative
C44	Impala	Positive	34.00 ± 0.18	Positive
C48	Impala	Positive	37.28 ± 0.31	Negative
C49	Impala	Positive	30.56 ± 0.14	Positive
C50	Impala	Positive	32.61 ± 0.16	Positive
C51	Impala	Positive	30.46 ± 0.18	Positive
C54	Impala	Positive	32.77 ± 0.11	Positive
C55	Impala	Positive	31.43 ± 0.17	Positive
C56	Impala	Positive	33.08 ± 0.10	Positive
C57	Impala	Positive	33.12 ± 0.17	Positive
C58	Impala	Positive	30.54 ± 0.12	Positive
C59	Impala	Positive	28.55 ± 0.10	Positive
C60	Impala	Positive	31.96 ± 0.14	Positive
C61	Impala	Positive	28.85 ± 0.10	Positive
C62	Impala	Positive	33.89 ± 0.12	Positive
C63	Impala	Positive	33.10 ± 0.11	Positive
C64	Impala	Negative	-	ND
C65	Impala	Positive	29.86 ± 0.12	Positive
C66	Impala	Positive	28.39 ± 0.12	Positive
Mean Ct value ± SD (Impala):			31.93 ± 3.27	
A57	Kudu	Negative	-	ND
A72	Kudu	Negative	-	ND
A73	Kudu	Positive	34.35 ± 0.20	Positive
A74	Kudu	Negative	-	ND
A75	Kudu	Negative	-	ND
A76	Kudu	Negative	-	ND
A77	Kudu	Positive	32.60 ± 0.14	Positive
A78	Kudu	Negative	-	ND
A79	Kudu	Positive	33.84 ± 0.23	Positive
A80	Kudu	Positive	35.46 ± 0.24	Negative
A81	Kudu	Negative	-	ND
A82	Kudu	Positive	36.45 ± 0.24	Negative
A83	Kudu	Negative	-	ND
A84	Kudu	Negative	-	ND
A85	Kudu	Negative	-	ND
A86	Kudu	Negative	-	ND
A87	Kudu	Negative	-	ND
B3	Kudu	Positive	34.77 ± 0.21	Positive
B4	Kudu	Positive	35.94 ± 0.17	Positive
B5	Kudu	Negative	-	ND
B6	Kudu	Positive	33.16 ± 0.14	Positive
B7	Kudu	Negative	-	ND
B8	Kudu	Negative	-	ND
B24	Kudu	Negative	-	ND
B85	Kudu	Positive	37.59 ± 0.32	Negative

B86	Kudu	Positive	36.50 ± 0.20	Negative
B87	Kudu	Negative	-	ND
B88	Kudu	Positive	34.53 ± 0.19	Negative
B95	Kudu	Negative	-	ND
C31	Kudu	Negative	-	ND
Mean Ct value ± SD (Kudu):			35.02 ± 1.53	
A29	Wild dog	Negative	-	ND
A30	Wild dog	Negative	-	ND
A31	Wild dog	Negative	-	ND
A32	Wild dog	Negative	-	ND
A33	Wild dog	Negative	-	ND
A35	Wild dog	Negative	-	ND
A36	Wild dog	Negative	-	ND
A37	Wild dog	Negative	-	ND
A40	Wild dog	Negative	-	ND
A41	Wild dog	Negative	-	ND
A43	Wild dog	Negative	-	ND
A49	Wild dog	Negative	-	ND
A50	Wild dog	Negative	-	ND
A52	Wild dog	Negative	-	ND
A54	Wild dog	Negative	-	ND
A55	Wild dog	Negative	-	ND
A88	Wild dog	Negative	-	ND
A90	Wild dog	Negative	-	ND
A92	Wild dog	Negative	-	ND
A96	Wild dog	Negative	-	ND
A100	Wild dog	Negative	-	ND
B30	Wild dog	Positive	35.11 ± 0.14	Positive
B43	Wild dog	Negative	-	ND
C7	Wild dog	Negative	-	ND
C73	Wild dog	Negative	-	ND
C82	Wild dog	Negative	-	ND
D19	Wild dog	Negative	-	ND
D20	Wild dog	Negative	-	ND
D25	Wild dog	Negative	-	ND
D26	Wild dog	Negative	-	ND
HH4	Wild dog	Negative	-	ND
Mean Ct value ± SD (Wild dog):			35.11 ± 0.00	
A58	Zebra	Negative	-	ND
A59	Zebra	Positive	35.36 ± 0.14	Negative
A60	Zebra	Negative	-	ND
A61	Zebra	Positive	35.80 ± 0.16	Negative
A62	Zebra	Negative	-	ND
A63	Zebra	Positive	36.54 ± 0.16	Negative
A64	Zebra	Negative	-	ND
A65	Zebra	Negative	-	ND
A66	Zebra	Negative	-	ND
A67	Zebra	Positive	35.84 ± 0.15	Negative

A68	Zebra	Negative	-	ND
A69	Zebra	Positive	33.52 ± 0.12	Positive
A70	Zebra	Positive	31.54 ± 0.11	Positive
A71	Zebra	Positive	33.95 ± 0.12	Positive
A95	Zebra	Negative	-	ND
B9	Zebra	Negative	-	ND
B10	Zebra	Positive	33.55 ± 0.15	Positive
B11	Zebra	Negative	-	ND
B12	Zebra	Negative	-	ND
B13	Zebra	Positive	36.32 ± 0.18	Negative
B14	Zebra	Negative	-	ND
B15	Zebra	Negative	-	ND
B16	Zebra	Negative	-	ND
B17	Zebra	Negative	-	ND
B18	Zebra	Negative	-	ND
B19	Zebra	Positive	35.26 ± 0.17	Negative
B20	Zebra	Negative	-	ND
B21	Zebra	Negative	-	ND
B22	Zebra	Positive	36.57 ± 0.19	Negative
B23	Zebra	Negative	-	ND
HH1	Zebra	Positive	32.92 ± 0.12	Positive
HH2	Zebra	Positive	35.71 ± 0.15	Positive
Mean Ct value ± SD (Zebra):			34.84 ± 1.58	
A17	Warthog	Negative	-	ND
A18	Warthog	Negative	-	ND
A19	Warthog	Negative	-	ND
A20	Warthog	Negative	-	ND
A21	Warthog	Negative	-	ND
A22	Warthog	Negative	-	ND
A23	Warthog	Negative	-	ND
A24	Warthog	Negative	-	ND
A25	Warthog	Negative	-	ND
A26	Warthog	Positive	36.83 ± 0.19	Negative
A42	Warthog	Negative	-	ND
A47	Warthog	Positive	33.59 ± 0.14	Positive
A89	Warthog	Negative	-	ND
A97	Warthog	Negative	-	ND
A99	Warthog	Negative	-	ND
B90	Warthog	Positive	35.71 ± 0.16	Positive
B97	Warthog	Negative	-	ND
C20	Warthog	Positive	33.07 ± 0.13	Positive
C21	Warthog	Positive	32.92 ± 0.11	Positive
C22	Warthog	Positive	35.66 ± 0.14	Negative
C23	Warthog	Negative	-	ND
C24	Warthog	Positive	38.79 ± 0.32	Negative
C25	Warthog	Positive	35.34 ± 0.17	Positive
C26	Warthog	Positive	33.51 ± 0.15	Positive
C27	Warthog	Positive	36.74 ± 0.20	Positive

C28	Warthog	Positive	37.50 ± 0.22	Negative
C29	Warthog	Positive	34.60 ± 0.17	Positive
C30	Warthog	Positive	36.76 ± 0.16	Negative
C52	Warthog	Negative	-	ND
C53	Warthog	Negative	-	ND
Mean Ct value ± SD (Warthog):			35.46 ± 1.84	
B1	Hyena	Negative	-	ND
B2	Hyena	Negative	-	ND
B52	Hyena	Negative	-	ND
B64	Hyena	Negative	-	ND
B65	Hyena	Negative	-	ND
B84	Hyena	Negative	-	ND
B89	Hyena	Negative	-	ND
B91	Hyena	Negative	-	ND
B94	Hyena	Negative	-	ND
B98	Hyena	Negative	-	ND
B100	Hyena	Negative	-	ND
C1	Hyena	Negative	-	ND
C2	Hyena	Negative	-	ND
C10	Hyena	Negative	-	ND
C11	Hyena	Negative	-	ND
C12	Hyena	Negative	-	ND
C13	Hyena	Negative	-	ND
C14	Hyena	Positive	37.15 ± 0.19	Negative
C16	Hyena	Positive	32.40 ± 0.11	Positive
C67	Hyena	Positive	36.14 ± 0.17	Negative
C70	Hyena	Positive	30.96 ± 0.15	Positive
C71	Hyena	Negative	-	ND
C74	Hyena	Negative	-	ND
C79	Hyena	Negative	-	ND
C81	Hyena	Positive	24.61 ± 0.12	Positive
C84	Hyena	Negative	-	ND
C85	Hyena	Positive	36.61 ± 0.14	Negative
C98	Hyena	Negative	-	ND
D15	Hyena	Negative	-	ND
D16	Hyena	Positive	35.34 ± 0.19	Negative
HH3	Hyena	Negative	-	ND
Mean Ct value ± SD (Hyena):			33.32 ± 4.47	
A1	Leopard	Positive	31.73 ± 0.11	Positive
A2	Leopard	Negative	-	ND
A3	Leopard	Negative	-	ND
A4	Leopard	Negative	-	ND
A5	Leopard	Negative	-	ND
A6	Leopard	Negative	-	ND
A7	Leopard	Negative	-	ND
A8	Leopard	Negative	-	ND
A9	Leopard	Negative	-	ND
A10	Leopard	Negative	-	ND

A11	Leopard	Negative	-	ND
A12	Leopard	Positive	35.69 ± 0.17	Positive
A13	Leopard	Negative	-	ND
A14	Leopard	Positive	34.10 ± 0.13	Positive
A15	Leopard	Negative	-	ND
A16	Leopard	Positive	38.89 ± 0.22	Negative
A27	Leopard	Positive	36.94 ± 0.18	Negative
A28	Leopard	Negative	-	ND
A34	Leopard	Positive	35.03 ± 0.17	Positive
A39	Leopard	Negative	-	ND
A48	Leopard	Positive	36.17 ± 0.16	Negative
A53	Leopard	Negative	-	ND
A56	Leopard	Positive	32.56 ± 0.11	Positive
A93	Leopard	Negative	-	ND
A94	Leopard	Negative	-	ND
B32	Leopard	Positive	31.98 ± 0.10	Positive
B33	Leopard	Positive	33.06 ± 0.16	Positive
B42	Leopard	Positive	34.68 ± 0.14	Positive
B45	Leopard	Positive	33.64 ± 0.17	Positive
C32	Leopard	Negative	-	ND
Mean Ct value ± SD (Leopard):			34.54 ± 2.14	
A38	Lion	Negative	-	ND
A44	Lion	Negative	-	ND
A45	Lion	Negative	-	ND
A46	Lion	Negative	-	ND
A51	Lion	Negative	-	ND
A91	Lion	Negative	-	ND
A98	Lion	Negative	-	ND
B46	Lion	Negative	-	ND
B50	Lion	Negative	-	ND
B63	Lion	Negative	-	ND
B93	Lion	Negative	-	ND
B99	Lion	Positive	33.99 ± 0.16	Positive
C3	Lion	Negative	-	ND
C8	Lion	Positive	34.94 ± 0.15	Positive
C9	Lion	Positive	35.69 ± 0.19	Positive
C15	Lion	Negative	-	ND
C19	Lion	Positive	35.37 ± 016	Positive
C39	Lion	Positive	33.45 ± 0.13	Positive
C47	Lion	Negative	-	ND
C72	Lion	Negative	-	ND
C73	Lion	Negative	-	ND
C76	Lion	Positive	28.54 ± 0.11	Positive
C78	Lion	Negative	-	ND
C94	Lion	Negative	-	ND
C95	Lion	Negative	-	ND
C96	Lion	Negative	-	ND
C97	Lion	Negative	-	ND

C99	Lion	Negative	-	ND
C100	Lion	Negative	-	ND
HH5	Lion	Negative	-	ND
HH6	Lion	Negative	-	ND
HH7	Lion	Negative	-	ND
HH8	Lion	Negative	-	ND
HH9	Lion	Negative	-	ND
Mean Ct value ± SD (Lion):			33.66 ± 2.65	
B26	Elephant	Negative	-	ND
B27	Elephant	Negative	-	ND
B28	Elephant	Negative	-	ND
B29	Elephant	Negative	-	ND
B34	Elephant	Negative	-	ND
B35	Elephant	Positive	36.50 ± 0.14	Negative
B36	Elephant	Negative	-	ND
B37	Elephant	Negative	-	ND
B38	Elephant	Positive	34.39 ± 0.18	Positive
B39	Elephant	Negative	-	ND
B40	Elephant	Negative	-	ND
B41	Elephant	Negative	-	ND
B43	Elephant	Negative	-	ND
B44	Elephant	Negative	-	ND
B47	Elephant	Negative	-	ND
B49	Elephant	Negative	-	ND
B51	Elephant	Negative	-	ND
B62	Elephant	Negative	-	ND
B92	Elephant	Negative	-	ND
B96	Elephant	Negative	-	ND
C17	Elephant	Negative	-	ND
C18	Elephant	Negative	-	ND
C45	Elephant	Negative	-	ND
C46	Elephant	Negative	-	ND
C68	Elephant	Positive	34.11 ± 0.19	Positive
C69	Elephant	Positive	34.80 ± 0.18	Negative
C77	Elephant	Positive	37.24 ± 0.26	Negative
C80	Elephant	Negative	-	ND
D5	Elephant	Negative	-	ND
D14	Elephant	Negative	-	ND
Mean Ct value ± SD (Elephant):			35.41 ± 1.38	
C83	Rhinoceros	Negative	-	ND
C86	Rhinoceros	Negative	-	ND
C87	Rhinoceros	Negative	-	ND
C88	Rhinoceros	Negative	-	ND
C89	Rhinoceros	Negative	-	ND
C90	Rhinoceros	Negative	-	ND
C91	Rhinoceros	Negative	-	ND
C92	Rhinoceros	Negative	-	ND
C93	Rhinoceros	Negative	-	ND

D1	Rhinoceros	Negative	-	ND
D2	Rhinoceros	Negative	-	ND
D3	Rhinoceros	Negative	-	ND
D4	Rhinoceros	Negative	-	ND
D6	Rhinoceros	Negative	-	ND
D8	Rhinoceros	Negative	-	ND
D9	Rhinoceros	Negative	-	ND
D10	Rhinoceros	Negative	-	ND
D11	Rhinoceros	Negative	-	ND
D12	Rhinoceros	Negative	-	ND
D13	Rhinoceros	Negative	-	ND
D17	Rhinoceros	Negative	-	ND
D18	Rhinoceros	Negative	-	ND
D21	Rhinoceros	Negative	-	ND
D22	Rhinoceros	Negative	-	ND
D23	Rhinoceros	Negative	-	ND
D24	Rhinoceros	Positive	36.55 ± 0.16	Negative
D27	Rhinoceros	Positive	37.89 ± 0.18	Negative
D28	Rhinoceros	Negative	-	ND
D29	Rhinoceros	Positive	35.63 ± 0.11	Negative
HH10	Rhinoceros	Negative	-	ND
HH11	Rhinoceros	Negative	-	ND
HH12	Rhinoceros	Negative	-	ND
HH13	Rhinoceros	Negative	-	ND
HH14	Rhinoceros	Negative	-	ND
HH15	Rhinoceros	Negative	-	ND
Mean Ct value ± SD (Rhinoceros):			36.69 ± 1.14	

[a] The qPCR assay was performed in triplicate and the mean Ct value and the standard deviation (SD) for each sample are presented. The mean Ct value and standard deviation values for each species are also presented.
[b] Conventional 16S rRNA amplification for microbiome analysis was performed using barcoded universal 16S rRNA primers (27F and 1492R).
[c] No Ct value was obtained.
[d] Not done.

Appendix 4

Table A4: List of samples collected from cattle at the IA@UP Experimental Farm and test results for the *A. marginale* whole genome sequencing study.

Animal number	*Anaplasma* duplex qPCR		Number of *A. marginale* genotypes	*A. marginale msp1α* genotype	RLB result
	A. marginale qPCR (Ct)	*A. centrale* qPCR (Ct)			
1917	38.0	Negative	Did not test		Did not test
1935	31.2	Negative	Did not test		Did not test
1911	30.7	Negative	1	A	Did not test
1937	Negative	Negative	Did not test		Did not test
1835	Negative	Negative	Did not test		Did not test
1806	Negative	Negative	Did not test		Did not test
1724	28.9	Negative	1	A	E/A catch-all, *A. marginale*
1620	28.8	Negative	1	A	E/A catch-all, *A. marginale*
1734	Negative	Negative	Did not test		Did not test
1824	31.5	Negative	1	A	Did not test
1924	31.5	Negative	Did not test		Did not test
1929	33.2	Negative	Did not test		Did not test
1928	29.2	Negative	1	A	E/A catch-all, *A. marginale*
1921	29.8	Negative	1	A	E/A catch-all, *A. marginale*
1829	32.2	Negative	Did not test		Did not test
1807	29.0	Negative	1	A	E/A catch-all, *A. marginale*
1813	Negative	Negative	Did not test		Did not test
1817	Negative	Negative	Did not test		Did not test
15116	Negative	Negative	Did not test		Did not test
1519	Negative	Negative	Did not test		Did not test
1916	Negative	Negative	Did not test		Did not test
1925	31.3	Negative	Did not test		Did not test
1940	29.7	Negative	1	A	E/A catch-all, *A. marginale*
1906	Negative	Negative	Did not test		Did not test
1826	Negative	Negative	Did not test		Did not test
1914	26.2	Negative	2		Did not test
1812	Negative	Negative	Did not test		Did not test
1627	26.9	Negative	1	A	E/A catch-all, *A. marginale*
1621	Negative	Negative	Did not test		Did not test
1704	36.9	Negative	Did not test		Did not test
1913	Negative	Negative	Did not test		Did not test
1915	29.8	Negative	1	A	E/A catch-all, *A. marginale*
1909	26.9	Negative	1	A	E/A catch-all, *A. marginale*
1939	29.9	Negative	1	A	E/A catch-all, *A. marginale*
1923	38.0	Negative	Did not test		Did not test
1814	Negative	Negative	Did not test		Did not test
1904	Negative	Negative	Did not test		Did not test
1622	34.1	Negative	Did not test		Did not test

1708	26.9	Negative	1	A	E/A catch-all, *A. marginale*
1402	Negative	Negative	Did not test		Did not test
1941	31.4	Negative	1	A	Did not test
1933	Negative	Negative	Did not test		Did not test
1912	33.5	Negative	Did not test		Did not test
1910	33.4	Negative	Did not test		Did not test
1818	Negative	Negative	Did not test		Did not test
1810	33.0	Negative	Did not test		Did not test
1633	31.0	Negative	Did not test		Did not test
1713	Negative	Negative	Did not test		Did not test
1741	28.1	Negative	1	A	E/A catch-all, *A. marginale*
1703	27.0	Negative	1	A	E/A catch-all, *A. marginale*
1516	Negative	Negative	Did not test		Did not test
20006	Negative	Negative	Did not test		Did not test
20005	33.2	Negative	Did not test		Did not test
P472	Negative	Negative	Did not test		Did not test
1901	Negative	Negative	Did not test		Did not test
2007	Negative	Negative	Did not test		Did not test
1908	28.9	Negative	1	A	Did not test
20018	Negative	Negative	Did not test		Did not test
1832	Negative	Negative	Did not test		Did not test
1732	29.7	Negative	1	A	Did not test
2003	29.7	Negative	1	B	E/A catch-all, *A. marginale*, T/B catch all, T. catch all
P443	Negative	Negative	Did not test		Did not test
20015	32.2	Negative	Did not test		Did not test
1417	28.8	Negative	1	A	Did not test
1834	38.0	Negative	Did not test		Did not test
1841	34.6	Negative	Did not test		Did not test
20012	29.7	Negative	Did not test		Did not test
1804	28.8	Negative	1	A	Did not test
1827	Negative	Negative	Did not test		Did not test
20017	Negative	Negative	Did not test		Did not test
P445	Negative	29.7	Did not test		Did not test
1720	Negative	Negative	Did not test		Did not test
1925	32.6	Negative	Did not test		Did not test
1744	Negative	Negative	Did not test		Did not test
P480	Negative	Negative	Did not test		Did not test
1728	31.0	Negative	1	A	Did not test
1918	31.0	Negative	Did not test		Did not test
1630	29.1	Negative	1	A	Did not test
1821	31.7	Negative	Did not test		Did not test
P508	Negative	Negative	Did not test		Did not test
1945	32.9	Negative	Did not test		Did not test
20002	31.7	Negative	1	B	E/A catch-all, *A. marginale*
1604	32.5	Negative	Did not test		Did not test
1938	30.2	Negative	Did not test		Did not test

1836	Negative	Negative	Did not test	Did not test
1828	30.7	Negative	Did not test	Did not test
1936	Negative	Negative	Did not test	Did not test
1826	Negative	Negative	Did not test	Did not test
1725	32.0	Negative	Did not test	Did not test
1919	Negative	Negative	Did not test	Did not test
1840	32.3	Negative	Did not test	Did not test
1737	33.0	Negative	Did not test	Did not test

Appendix 5

Research Outputs

Scientific Publications

The following scientific publications were generated during the course of this research study:

Makgabo, S.M., Brayton, K.A., Biggs, L., Oosthuizen, M.C. and Collins, N.E. 2023. Temporal Dynamics of *Anaplasma marginale* Infections and the Composition of *Anaplasma* spp. in Calves in the Mnisi Communal Area, Mpumalanga, South Africa. *Microorganisms*, 11(2), p.465.

Makgabo, S.M., Brayton, K.A., Oosthuizen, M.C., Collins, N.E. 2023. Unravelling the diversity of *Anaplasma* species circulating in selected African wildlife hosts by targeted 16S microbiome analysis. *Current Research in Microbial Sciences*. p.100198..

Scientific Conferences

The following talks and posters based on the work done during the course of this research study were presented at conferences:

Makgabo, S.M., Brayton, K.A., Oosthuizen, M.C., Collins, N.E. 2022. Identification of *Anaplasma* species in wild animal species in the Kruger National Park and surrounding game reserves using a bacterial microbiome approach. Faculty Day 2022, Faculty of Veterinary Science, University of Pretoria, South Africa. 20 October 2022 (ORAL PRESENTATION).

Makgabo, S.M., Brayton, K.A., Oosthuizen, M.C., Collins, N.E. 2022. Temporal dynamics of *Anaplasma marginale* infection in calves at the wildlife-livestock interface in the Mnisi communal area, Mpumalanga, South Africa. Faculty Day 2022, Faculty of Veterinary Science, University of Pretoria, South Africa. 20 October 2022 (POSTER PRESENTATION).

Makgabo, S.M., Brayton, K.A., **Oosthuizen, M.C.**, Collins, N.E. (2022). Identification of *Anaplasma* species in wild animal species in the Kruger National Park and surrounding game reserves using a bacterial microbiome approach. Paper presented at the 24th International Congress on Parasites of Wildlife (ICPOW)- 50th Annual Conference of the Parasitological Society of Southern Africa (PARSA). Kruger National Park, South Africa. 11-15 September 2022 (POSTER PRESENTATION).

Makgabo, S.M., Brayton, K.A., Oosthuizen, M.C., Collins, N.E. 2022. Identification of *Anaplasma* species in wild animal species in the Kruger National Park and surrounding game reserves using a bacterial microbiome approach. TTP.10 - Tick and Tick-borne Pathogen Conference. Murighiol, Danube Delta, Romania. 27-31 August 2022 (ORAL PRESENTATION).

Makgabo, S.M., Brayton, K.A., Oosthuizen, M.C., **Collins, N.E**. 2022. Temporal dynamics of *Anaplasma marginale* infection in calves at the wildlife-livestock interface in the Mnisi communal area, Mpumalanga, South Africa. TTP.10 - Tick and Tick-borne Pathogen Conference. Murighiol, Danube Delta, Romania. 27-31 August 2022 (POSTER PRESENTATION).

Makgabo, S.M., Brayton, K.A., Oosthuizen, M.C., Collins, N.E. 2022. Identification of *Anaplasma* species in wild animal species in the Kruger National Park and surrounding game reserves using a bacterial microbiome approach. The International Intracellular Bacteria Meeting (ESCCAR) 2022. Lausanne, Switzerland. 23-26 August 2022 (ORAL PRESENTATION). **Best Oral Presentation**.

Makgabo, S.M., Brayton, K.A., Oosthuizen, M.C., **Collins, N.E**. 2022. Temporal dynamics of *Anaplasma marginale* infection in calves at the wildlife-livestock interface in the Mnisi communal area, Mpumalanga, South Africa. The International Intracellular Bacteria Meeting (ESCCAR) 2022. Lausanne, Switzerland. 23-26 August 2022 (POSTER PRESENTATION).

Makgabo, S.M., Brayton, K.A., Oosthuizen, M.C., Collins, N.E. 2022. Identification of *Anaplasma* species in wild animal species in the Kruger National Park and surrounding game reserves using a bacterial microbiome approach. International conference for Association of Institutions for Tropical Veterinary Medicine (AITVM) and the Society for Tropical Veterinary

Medicine (STVM), Virtual. 17 May 2022 (ORAL PRESENTATION). **Best Oral Presentation**.

Appendix 6

Research and Animal Ethics Approvals

Research and Animal ethics approvals for the PhD study '**Genetic diversity of *Anaplasma marginale* in cattle and in putative novel *Anaplasma* species from wildlife in Mpumalanga, South Africa**' issued by the Research and Animal Ethics Committee, University of Pretoria.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Tick-borne disease dynamics in calves at the wildlife-livestock interface in the Mnisi Community Programme (MCP) area, Mpumalanga Province, South Africa (PILOT)**
PROJECT NUMBER	**V041-16**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Mr. SM Makgabo**

STUDENT NUMBER (where applicable)	**UP_11150752**
DISSERTATION/THESIS SUBMITTED FOR	**MSc**

The animals will go back to the owners after the pilot study is completed

ANIMAL SPECIES	**Bovine**	
NUMBER OF ANIMALS	**14**	
Approval period to use animals for research/testing purposes		**May 2016 –May 2017**
SUPERVISOR	**Dr. N Collins**	

KINDLY NOTE:

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

APPROVED	Date 30 May 2016
CHAIRMAN: UP Animal Ethics Committee	Signature

S4285-15

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science

Research Ethics Committee 16 April 2020

CONDITIONALLY APPROVAL

Ethics Reference No	**REC252-19**
Protocol Title	**Isolation and genome sequence analysis of Anaplasma species**
Principal Investigator	**Mr SM Makgabo**
Supervisors	**Dr NE Collins**

Dear Mr SM Makgabo,

We are pleased to inform you that your submission has been conditionally approved by the Faculty of Veterinary Sciences Research Ethics committee, subject to other relevant approvals.

Please note the following about your ethics approval:

1. Please use your reference number (REC252-19) on any documents or correspondence with the Research Ethics Committee regarding your research.
2. Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
3. Please note that ethical approval is granted for the duration of the research as stipulated in the original application for post graduate studies (e.g. Honours studies: 1 year, Masters studies: two years, and PhD studies: three years) and should be extended when the approval period lapses.
4. The digital archiving of data is a requirement of the University of Pretoria. The data should be accessible in the event of an enquiry or further analysis of the data.

Ethics approval is subject to the following:

1. The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.
2. **Applications using Animals:** FVS ethics recommendation does not imply that AEC approval is granted. The application has been pre-screened and recommended for review by the AEC. Research may not proceed until AEC approval is granted.

Note: Conditionally approved pending obtaining all other relevant approvals (and to ensure that rerouting to AEC is not delayed). Please note that the MoU (project agreement) between UP and ARC-OVR, DAFF Section 20 and the various permissions required from the organizations from which wildlife blood samples will be obtained (i.e. SANParks, Ezemvelo KZN Wildlife, MPTA, Dinokeng Game Reserve, etc. as well as the Onderstepoort Wildlife Centre, UP), will have to be provided before final approval.

We wish you the best with your research.

Yours sincerely

PROF M. OOSTHUIZEN
Chairperson: Research Ethics Committee

100 YEARS OF VETERINARY EDUCATION

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UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science

Research Ethics Committee 03 May 2021

LETTER OF APPROVAL

Ethics Reference No	**REC252-19**
Protocol Title	**Isolation and genome sequence analysis of Anaplasma species**
Principal Investigator	**Mr SM Makgabo**
Supervisors	**Dr NE Collins**

Dear Mr SM Makgabo,

We are pleased to inform you that your submission conforms to the requirements of the Faculty of Veterinary Sciences Research Ethics committee.

Please note the following about your ethics approval:

1. Please use your reference number (REC252-19) on any documents or correspondence with the Research Ethics Committee regarding your research.
2. Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
3. Please note that ethical approval is granted for the duration of the research as stipulated in the original application (for Post graduate studies e.g. Honours studies: 1 year, Masters studies: two years, and PhD studies: three years) and should be extended when the approval period lapses.
4. The digital archiving of data is a requirement of the University of Pretoria. The data should be accessible in the event of an enquiry or further analysis of the data.

Ethics approval is subject to the following:

1. The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.
2. **Applications using Animals:** FVS ethics recommendation does not imply that AEC approval is granted. The application has been pre-screened and recommended for review by the AEC. Research may not proceed until AEC approval is granted.

We wish you the best with your research.

Yours sincerely

PROF M. OOSTHUIZEN
Chairperson: Research Ethics Committee

100 YEARS

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UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
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Faculty of Veterinary Science
Animal Ethics Committee

11 May 2020

Approval Certificate
New Application

AEC Reference No.:	REC252-19
Title:	Isolation and genome sequence analysis of Anaplasma species
Researcher:	Mr SM Makgabo
Student's Supervisor:	Dr NE Collins

Dear Mr SM Makgabo,

The **New Application** as supported by documents received between 2020-01-31 and 2020-05-04 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2020-05-04.

Please note the following about your ethics approval:

1. The use of species is approved:

Species and Samples	**Number**
Cattle (OVI)	3
Wild ruminants (various reserves)	1000 (to be reported on)
Samples Blood (Wild ruminants)	 1000
Blood collection and smears -every day (Cattle)	Max blood to collected not to exceed 800 ml per animal per 100kg

2. Ethics Approval is valid for 1 year and needs to be renewed annually by 2021-05-11.
3. Please remember to use your protocol number (REC252-19) on any documents or correspondence with the AEC regarding your research.
4. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.
Yours sincerely

Prof V Naidoo
CHAIRMAN: UP-Animal Ethics Committee

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Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Animal Ethics Committee

7 June 2021

AEC Reference No.: REC252-19
Title: Isolation and genome sequence analysis of Anaplasma species

Researcher: Mr SM Makgabo

Dear Mr SM Makgabo,

Please note the following about your **Amendment** Animal Ethics application:

The UP AEC has reviewed this project/application and had no ethical concerns with project. For regulatory reasons you are not allowed to start your study until the final Section 20 approval/permit has been made available to the AEC office.

THE FINAL CERTIFICATE OF APPROVAL WILL BE ONLY PROVIDED AFTER RECEIVING THE SECTION 20 PERMITS.

1. Please remember to use your protocol number **(REC252-19**) on any documents or correspondence with the AEC regarding your research.
2. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.
Yours sincerely

Prof V Naidoo
CHAIRMAN: UP-Animal Ethics Committee

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Lefapha la Diseanse tša Bongakadiruiwa

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Animal Ethics Committee

18 July 2022

Approval Certificate
Annual Renewal
(EXT2)

AEC Reference No.: REC252-19 Line 3
Title: Isolation and genome sequence analysis of Anaplasma species
Researcher: Mr SM Makgabo
Student's Supervisor: Dr NE Collins

Dear Mr SM Makgabo,

The **Annual Renewal** as supported by documents received between 2022-03-11 and 2022-06-27 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2022-06-27.

Please note the following about your ethics approval:

1. The use of species is approved:

Species	Approved
Cattle (OVI)	3
Cattle - Holstein Friesian cows (@Innovation Africa)	20
Mammalia – Wild ruminants (Screening for all anaplasmosis causing pathogens)	1000 (to be reported on)
Samples	**Approved**
Holstein Friesian - EDTA blood samples (samples from live animals)	100

2. Ethics Approval is valid for 1 year and needs to be renewed annually by 2023-07-18.
3. Please remember to use your protocol number (REC252-19) on any documents or correspondence with the AEC regarding your research.
4. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
5. All incidents must be reported by the PI by email to Ms Marleze Rheeder (AEC Coordinator) within 3 days, and must be subsequently submitted electronically on the application system within 14 days.
6. The committee also requests that you record major procedures undertaken during your study for own-archiving, using any available digital recording system that captures in adequate quality, as it may be required if the committee needs to evaluate a complaint. However, if the committee has monitored the procedure previously or if it is generally can be considered routine, such recording will not be required.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

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Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa

We wish you the best with your research.

Yours sincerely

Prof V Naidoo
CHAIRPERSON: UP-Animal Ethics Committee

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Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa

UNIVERSITEIT VAN PRETORIA
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Faculty of Veterinary Science
Animal Ethics Committee

03 July 2023

Approval Certificate
Amendment 2

AEC Reference No.:	REC252-19 Line 4
Title:	Isolation and genome sequence analysis of Anaplasma species
Researcher:	Mr SM Makgabo
Student's Supervisor:	Dr NE Collins

Dear Mr SM Makgabo,

The **Amendment** as supported by documents received between 2023-05-26 and 2023-06-26 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2023-06-26.

Please note the following about your ethics approval:

1. The use of Animals and samples is approved:

   We previously identified four A. marginale-positive cattle, **1627, 1909, 20002 and 1708**, at the Innovation Africa @ UP (IA@UP) Experimental Farm that were infected with a single Anaplasma marginale genotype and were negative for other tick-borne hemoparasites.

   These four (4) animals to be bled once off 100 ml from each animal

Samples	No Approved	Additional Required	Total Approved
Cattle - EDTA blood samples - Innovation – Live animals (100 ml each)	0	4	4

2. Please note that the approved date(s) from the original application certificate / annual renewal certificate will be applicable to this amendment.
3. Please remember to use your protocol number (REC252-19) on any documents or correspondence with the AEC regarding your research.
4. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
5. **All incidents** must be reported by the PI by email to Ms Marleze Rheeder (AEC Coordinator) within 3 days, and must be subsequently submitted electronically on the application system within 14 days.
6. The committee also requests that you record major procedures undertaken during your study for own-archiving, using any available digital recording system that captures in adequate quality, as it may be required if the committee needs to evaluate a complaint. However, if the committee has monitored the procedure previously or if it is generally can be considered routine, such recording will not be required.

Room 6-13, Arnold Theiler Building, Onderstepoort
Private Bag X04, Onderstepoort 0110, South Africa
Tel +27 12 529 8434
Fax +27 12 529 8321
Email: marleze.rheeder@up.ac.za

Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Animal Ethics Committee

03 July 2023

Approval Certificate
Annual Renewal
(EXT3)

AEC Reference No.:	REC252-19 Line 5
Title:	Isolation and genome sequence analysis of Anaplasma species
Researcher:	Mr SM Makgabo
Student's Supervisor:	Dr NE Collins

Dear Mr SM Makgabo,

The **Annual Renewal** as supported by documents received between 2023-06-12 and 2023-06-26 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2023-06-26.

Please note the following about your ethics approval:

1. The use of species is approved:

Species	Approved
Cattle - OVI	3
Cattle - Holstein Friesian cows (@ Innovation Africa farm)	20
Mammalia various - Screening for all anaplasmosis causing pathogens (to be reported)	1000
Samples	Approved
Holstein Friesian - EDTA blood samples (@ Innovation Africa farm)	100

2. Ethics Approval is valid for 1 year and needs to be renewed annually by 2024-07-03.
3. Please remember to use your protocol number (REC252-19) on any documents or correspondence with the AEC regarding your research.
4. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
5. All incidents must be reported by the PI by email to Ms Marleze Rheeder (AEC Coordinator) within 3 days, and must be subsequently submitted electronically on the application system within 14 days.
6. The committee also requests that you record major procedures undertaken during your study for own-archiving, using any available digital recording system that captures in adequate quality, as it may be required if the committee needs to evaluate a complaint. However, if the committee has monitored the procedure previously or if it is generally can be considered routine, such recording will not be required.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

Room 6-13, Arnold Theiler Building, Onderstepoort
Private Bag X04, Onderstepoort 0110, South Africa
Tel +27 12 529 8434
Fax +27 12 529 8321
Email: marleze.rheeder@up.ac.za

Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa

Appendix 7

DALRRD Section 20 Permit

Permission to conduct research under Section 20 of the Animal Diseases Act, 1984 (Act number 35 of 1984) for the research project '**Genetic diversity of *Anaplasma marginale* in cattle and in putative novel *Anaplasma* species from wildlife in Mpumalanga, South Africa**' issued by the Department of Agriculture, Land Reform and Rural Development (DALRRD), Pretoria, South Africa.

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

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

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
Reference: 12/11/1/1/6

Sekgota Marcus Makgabo
Department of Veterinary Tropical Diseases
Faculty of Veterinary Science
University of Pretoria

Dear Mr/Ms Makgabo,

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

Your fax / memo / letter/ Email dated 24 August 2016, requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers.

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

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. All potentially infectious material utilised or collected during the study is to be destroyed at the completion of the study. Records must be kept for five years for audit purposes. A dispensation application may be made to the Director Animal Health in the event that any of the above is to be stored or distributed;
3. The study must be conducted in compliance with the Veterinary and Para-Veterinary Professions Act 1982 (Act No. 19 of 1982);
4. Bovine blood and tick samples may only be transported to Hans Hoheisen Wildlife Research Station (HHWRS) under a veterinary movement permit;
5. Genomic DNA extracted from the collected bovine blood and tick samples may be transported to the Department of Veterinary Tropical Diseases laboratory under a veterinary movement permit;
6. All samples must be packaged and transported in accordance with the National Road Traffic Act, 1996 (*Act* No. 93 of 1996).

Title of research/study: Tick-borne disease dynamics in calves at the wildlife-livestock interface in the Mnisi Community Programme (MCP) area,

Mpumalanga Province, South Africa.

Researcher (s): Sekgota Marcus Makgabo
Institution: Department of Veterinary Tropical Diseases
Your Ref./ Project Number: VO41-16
Our ref Number: 12/11/1/1/6

Kind regards,

Maja.

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date: 2016 -09- 23

SUBJECT: Tick-borne disease dynamics in calves at the wildlife-livestock interface in the Mnisi Community Programme (MCP) area, Mpumalanga Province, South Africa.

agriculture, land reform & rural development
Department:
Agriculture, Land Reform and Rural Development
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Land Reform & Rural Development
Private Bag X138, Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@dalrrd.gov.za
Reference: 12/11/1/1/6 (1734 LH)

Sekgota Marcus Makgabo
University of Pretoria, Faculty of Veterinary Science
Department of Veterinary Tropical Diseases
M35 Road
Onderstepoort, Pretoria
Tel: 078 324 7789
E-mail: marcusmakgabo.mm@gmail.com

Dear Sekgota Marcus Makgabo,

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

Your application dated 23 October 2020, received by us on 9 November 2020, requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers. I am pleased to inform you that permission is hereby granted to perform the following study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. This permission is given upon finding the biosecurity of the research project as described to be acceptable to DALRRD.
3. The research project is approved as per the application form dated 23 October 2020 and the correspondence thereafter. Written permission from the Director: Animal Health must be obtained prior to any deviation from the conditions approved for this research project under this Section 20 permit. Please apply in writing to HerryG@dalrrd.gov.za;
4. If required, an application for an extension must be made by the responsible researcher at least one month prior to the expiry of this Section 20 permit. Please apply in writing to HerryG@dalrrd.gov.za;
5. No part of this research project may begin until the valid ethical approval has been obtained in writing from the relevant South African authority;

SUBJECT: S20 PERMISSION FOR: Identification of *Anaplasma* species in wild animal species in South Africa using bacterial microbiome analysis

6. Only biobanked samples from Hans Hoheisen Research Station and the Veterinary Wildlife Services Biobank, Skukuza may be used for this research study, and no field samples may be collected;
7. DNA may only be extracted at the Skukuza State Veterinary Services laboratory or Hans Hoheisen Wildlife Research station, and only extracted DNA may be removed to the Department of Veterinary Tropical Diseases (DVTD) laboratory;
8. All potentially infectious material utilised or generated during or by the research project is to be destroyed at completion of the study;
9. Only a waste disposal company registered for the disposal of biohazardous waste may be used for the removal of all potentially infectious waste from the research project; Records must be kept for five years for auditing purposes;
10. Extracted DNA may be stored under access control in the DVTD freezer facilities of the Research and Training Laboratories;
1. DNA libraries of 16S PCR products generated from the DNA may be sent to the Washington State University's Genomic Sequencing Core, in the United States of America, and may only be exported in full compliance with the requirements of the importing country.
11. Stored samples may not be outsourced for research without prior written approval from the Director: Animal Health.
12. Should samples be used for further research, written approval from the Director: Animal Health must be obtained prior to start of project

Title of research/study: Identification of *Anaplasma* species in wild animal species in South Africa using bacterial microbiome analysis
Researcher: Sekgota Marcus Makgabo
Institutions: University of Pretoria, Faculty of Veterinary Science, Department of Veterinary Tropical Diseases; Hans Hoheisen Research Station; Skukuza State Veterinary Services laboratory; Veterinary Wildlife Services biobank
Permit Expiry date: 31 December 2023
Our ref Number: 12/11/1/1/6 (1734 LH)
Your ref: REC252-19

Kind regards,

Maja.

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date: 2020 -12- 10

SUBJECT: S20 PERMISSION FOR: Identification of *Anaplasma* species in wild animal species in South Africa using bacterial microbiome analysis

agriculture, land reform & rural development
Department:
Agriculture, Land Reform and Rural Development
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Land Reform and Rural Development
Private Bag X138, Pretoria 0001
Enquiries: Ms Marna Laing • Tel: +27 12 319 7442 • Fax: +27 12 319 7470 • E-mail: MarnaL@dalrrd.gov.za
Reference: 12/11/1/1/6 (1461 AC) (1)

Mr Sekgota Marcus Makgabo
Department of Veterinary Tropical Diseases
Faculty of Veterinary Science
University of Pretoria
Onderstepoort
E-mail: marcusmakgabo.mm@gmail.com ; nicola.collins@up.ac.za

Dear Mr Makgabo,

RE: AMENDMENT OF SECTION 20 APPROVAL IN TERMS OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984) FOR: "ISOLATION AND GENOME SEQUENCE ANALYSIS OF ANAPLASMA SPECIES IN SOUTH AFRICA: TOWARDS THE DEVELOPMENT OF MORE SPECIFIC SERODIAGNOSTIC TESTS"

The Section 20 permit that was issued for the above mention study on 1 June 2020 is hereby revoked and replaced by this amended Section 20 permit. Permission is hereby granted to perform the following study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. The study is approved as per the application form received on 19 February 2020 and the correspondence thereafter. Written permission from the Director: Animal Health must be obtained prior to any deviation from the conditions approved for this study under this Section 20 permit. Please apply in writing to MarnaL@dalrrd.gov.za;
3. The study must be conducted in compliance with the Veterinary and Para-Veterinary Professions Act 1982 (Act No. 19 of 82);
4. Blood samples may be collected from cattle at the University of Pretoria Innovation Africa @UP (previously known as Hatfield Experimental Farm), for which a state veterinary letter has been received;
5. It is the researcher's responsibility to remain in contact with the relevant state veterinarian to ensure that the disease status of the area has not changed when collecting samples. Records must be kept for five years for auditing purposes;

6. Samples must be packaged and transported in accordance with the National Road Traffic Act, 1996 (Act No. 93 of 1996);
7. Blood samples may be sent to the Research and Training Laboratories of the DVTD (Labs 2-58, 2-46, 2-44, 2-51) for PCR screening for *Anaplasma marginale* and *A. Centrale,* as well as other haemoparasites, including *Anaplasma bovis, A. phagocytophilum, Anaplasma sp. Omatjenne, Ehrlichia ruminantium, Babesia bovis, B. bigemina, Theileria parva, T. taurotragi, T. velifera, T. Mutans,* using a Reverse Line Blot (RLB) hybridisation assay. DNA extraction and amplification of relevant *A. marginale* and *A. centrale* positive blood samples may be conducted;
8. The detection of any controlled or notifiable animals diseases must be reported to the responsible state veterinarian;
9. All potentially infectious material utilised, collected or generated during the study is to be destroyed at the completion of the study using the specified registered waste contractor;
10. Records must be kept for five years for auditing purposes;
11. Extracted *A. marginale* and *A. centrale* DNA may be sent to Washington State University's Genomic Sequencing Core for whole genome sequencing. All requirements as specified by the importing country must be duly met;
12. Extracted *A. marginale* and *A. centrale* DNA may be stored under access control in the freezers of the Research and Training Laboratory of the DVTD. Stored samples may not be used for further research or be outsourced without prior written approval from the Director: Animal Health;
13. If required, an application for an extension must be made by the responsible researcher at least one month prior to the expiry of this Section 20 approval.

Title of research/study: "*Isolation and genome sequence analysis of Anaplasma species in South Africa: towards the development of more specific serodiagnostic tests".*
Researcher: Mr Sekgota Marcus Makgabo
Institution: Department of Veterinary Tropical Diseases, University of Pretoria
Our ref Number: 12/11/1/1/6 (1461 AC) (1)
Your ref: REC252-19
Expiry date: December 2023

Kind regards,

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date: 24/2/2022.

– 2 –

SUBJECT: *RE: AMENDMENT OF SECTION 20 APPROVAL IN TERMS OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984) FOR: "ISOLATION AND GENOME SEQUENCE ANALYSIS OF ANAPLASMA SPECIES IN SOUTH AFRICA: TOWARDS THE DEVELOPMENT OF MORE SPECIFIC SERODIAGNOSTIC TESTS"*

Appendix 8

Scientific Manuscripts

microorganisms

MDPI

Article

Temporal Dynamics of *Anaplasma marginale* Infections and the Composition of *Anaplasma* spp. in Calves in the Mnisi Communal Area, Mpumalanga, South Africa

S. Marcus Makgabo [1,*], Kelly A. Brayton [1,2], Louise Biggs [3], Marinda C. Oosthuizen [1] and Nicola E. Collins [1,*]

[1] Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort 0110, South Africa
[2] Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, WA 99164, USA
[3] Department of Production Animal Studies, University of Pretoria, Onderstepoort 0110, South Africa
* Correspondence: marcusmakgabo.mm@gmail.com (S.M.M.); nicola.collins@up.ac.za (N.E.C.)

Abstract: Bovine anaplasmosis, caused by *Anaplasma marginale*, is one of the most important tick-borne diseases of cattle. *Anaplasma marginale* is known to be present in the Mnisi community, Mpumalanga Province, with frequent cases of anaplasmosis reported. This study investigated the infection dynamics in calves (n = 10) in two habitats in the study area over 12 months. A duplex real-time PCR assay targeting the *msp1β* gene of *A. marginale* and the *groEL* gene of *A. centrale* confirmed the presence of *A. marginale* in five calves in a peri-urban area from the first month, but in only two calves at the wildlife–livestock interface and only after six months. These results were confirmed by 16S rRNA microbiome analysis. Over 50 *A. marginale msp1α* genotypes were detected in the calves along with five novel Msp1a repeats. Calves in the peri-urban area were more likely to be infected with *A. marginale* than calves in the wildlife–livestock interface. Cattle management, acaricide treatment, and cattle density could explain differences in infection prevalence in the two areas. Our results revealed that most calves were superinfected by distinct *A. marginale* strains within the study period, indicating continuous challenge with multiple strains that should lead to robust immunity in the calves and endemic stability in the area.

Keywords: *Anaplasma marginale*; *msp1α* gene; wildlife–livestock interface; genotyping; tick-borne diseases; detection; diagnosis

Citation: Makgabo, S.M.; Brayton, K.A.; Biggs, L.; Oosthuizen, M.C.; Collins, N.E. Temporal Dynamics of *Anaplasma marginale* Infections and the Composition of *Anaplasma* spp. in Calves in the Mnisi Communal Area, Mpumalanga, South Africa. *Microorganisms* **2023**, *11*, 465. https://doi.org/10.3390/microorganisms11020465

Academic Editor: Gereon R. M. Schares

Received: 30 December 2022
Revised: 26 January 2023
Accepted: 27 January 2023
Published: 13 February 2023

1. Introduction

Bovine anaplasmosis is a tick-borne disease (TBD) caused by the obligate intracellular pathogen, *Anaplasma marginale* [1]. Bovine anaplasmosis occurs predominantly in cattle; however, infection can also occur in ruminants such as sheep, goats, African antelopes, Cape buffalo, and some species of deer [1].

Bovine anaplasmosis caused by *A. marginale* is prevalent throughout the world occurring in Africa, the Americas, Asia, Australia, the Caribbean, and Europe [2]. The disease is amongst the three most economically important TBDs of cattle resulting in mortality and morbidity, decreased milk and meat production, and expensive control measures [3–6]. The economic impact of bovine anaplasmosis in South Africa has been estimated at approximately R115 million ($US9.6 million) per year due to mortalities [7], but this does not take into account costs associated with treatment and control. In other parts of the world, costs arising from anaplasmosis have been estimated from USD 300 to USD 800 million [2]. Clinical signs caused by infection with *A. marginale* are characterized by fever, progressive anaemia, weight loss and abortion, as well as icterus that may result in mortality. The

closely related organism, *A. centrale*, usually causes asymptomatic infections and is currently used as a vaccine for cattle in South Africa [8–10]. Animals under one year of age are usually asymptomatic to infection with *A. marginale* [11,12]. However, older animals are more likely to react severely and fatally upon challenge [1].

Biological transmission of *A. marginale* to naïve cattle occurs by feeding ticks, while mechanical transmission occurs by biting flies or blood-contaminated instruments [13,14]. Transplacental transmission of *A. marginale* has also been reported [1,15]. Although the transmission of *A. marginale* in South Africa has not been extensively studied, five tick species, *Rhipicephalus decoloratus*, *R. microplus*, *R. evertsi evertsi*, *R. simus*, and *Hyalomma marginatum rufipes*, have been shown experimentally to transmit anaplasmosis and could therefore account for the widespread distribution of the disease [4,10]. *Rhipicephalus decoloratus* has long been regarded as the main vector [10], with *R. microplus* increasing in importance as a vector due to its recent spread into most South African provinces [16,17].

The genetic diversity of *A. marginale* from many countries around the world has been characterized using a genotyping method based on sequence analysis of the single-copy *msp1α* gene that codes for the major surface protein 1a (Msp1a) [18–20]. Msp1a is regarded as a determining marker for *A. marginale* transmission between cattle and ticks as it has been shown to be involved in the adhesion of the pathogen to tick cells and bovine erythrocytes [1]. The genotyping method uses differences in the number and sequence of tandem repeats located at the N-terminus of the Msp1a protein to differentiate between strains. The *msp1α* genotyping method was first described in 1990, and since then >300 genotypes have been reported worldwide [21,22]. In South Africa, a diversity of *A. marginale* genotypes has also been identified [19,20,23–25].

The presence of single *msp1α* genotypes in infected cattle is a well-documented phenomenon [13,26], but infection with multiple *A. marginale* strains (superinfection) has been less well studied. More recently, both co-infection and superinfection of cattle with multiple genetically distinct strains of *A. marginale* have been shown to be important drivers of *A. marginale* infection [27–30]. Co-infection and superinfection were recently shown to drive the development of complex infection with *A. marginale* under natural transmission conditions in Ghana [31].

In the South African context, bovine anaplasmosis is currently widespread and endemic throughout the cattle-farming areas in all South African provinces, except for the Northern Cape, where the vector is mostly absent [4,6,24,25]. Data collected through the Health and Demographic Surveillance System in Livestock (HDSS) established in the study area of the Mnisi community, indicate the presence of *A. marginale* in cattle, with frequent bovine anaplasmosis cases reported at villages that abut provincial and private game reserves (the wildlife–livestock interface) [32]. The Mnisi community is a sprawling area that provides an opportunity to study natural *A. marginale* infection dynamics at both more densely populated peri-urban villages and at villages at the wildlife–livestock interface [33].

To understand *A. marginale* strain diversity, infection dynamics, and the frequent nature of clinical cases of anaplasmosis in the Mnisi community, ten calves were examined from birth for a period of 12-months in a peri-urban area and at a wildlife–livestock interface.

2. Materials and Methods

2.1. Ethical Consideration

The study was carried out in strict accordance with the conditions and guidelines of the Animal Ethics Committee of the Faculty of Veterinary Science (reference number: V041-16). Permission to perform the study under Section 20 of the Animal Disease Act of 1984 was granted by the South African Department of Agriculture, Forestry and Fisheries (currently Department of Agriculture, Land Reform and Rural Development) (reference number: 12/11/1/1/6).

2.2. Study Area

The Mnisi community (24.8205° S, 31.1710° E) is situated in the north-eastern corner of the Bushbuckridge Municipality, Mpumalanga Province, South Africa (Figure 1). The community shares 75% of its boundary with adjacent wildlife areas, including the Andover and Manyeleti provincial game reserves and the Timbavati and Sabi Sand private game reserves. There are no fences between these reserves, including the Kruger National Park (KNP), such that game can freely roam between them. Livestock farming is the main agricultural activity in the area with more cattle than any other livestock species. The project was conducted in three villages, Eglington, Utha A, and Dixie. Eglington village is in a peri-urban area, while Utha A and Dixie are located at the wildlife–livestock interface close to the border with Manyeleti provincial game reserve.

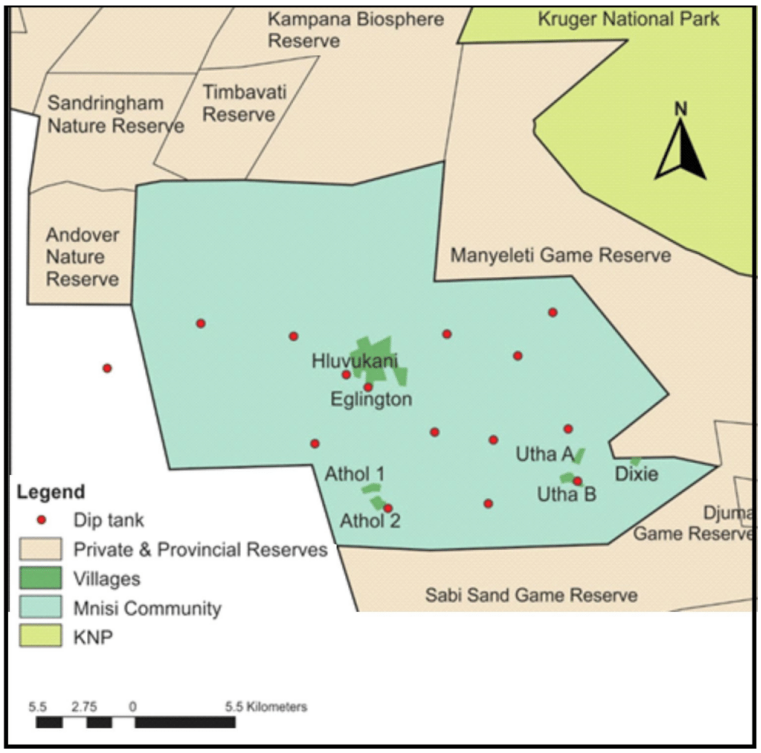

Figure 1. Map of the Mnisi communal area, showing the location of the three villages where the study was conducted, Eglington, Utha A, and Dixie, relative to various wildlife reserves and the Kruger National Park (KNP).

Over 40,000 people live in the Mnisi community. Eglington village is a peri-urban area, situated approximately 11.5 km away from the Manyeleti Game Reserve, 12.1 km from the Andover Game Reserve, and 15.1 km from the Timbavati Game Reserve. Each day, cattle herders collect the cattle from the owners' homes where they are kept in kraals overnight, and they are taken to a fully protected and fenced cattle grazing camp, where the chosen calves grazed during the study period. The Eglington cattle grazing camp is located approximately 16 km away from Manyeleti Game Reserve, 13 km from Andover Game Reserve, and 15.1 km from Timbavati Game Reserve. Utha A and Dixie villages are only 2 km apart and are located close to the wildlife–livestock interface being, respectively,

approximately 2.4 km and 0.5 km away from the Manyeleti Game Reserve. In this area, cattle grazing camps are located adjacent to the Manyeleti Game Reserve and cattle are often seen grazing alongside wildlife separated only by the game fence, which is the only barrier between livestock and the abundant wildlife populations in the game reserves. Due to the study area being in the foot and mouth disease (FMD) protection zone, animals can move between villages in the zone only with permission from the state vet, however, trading of livestock out of the zone is not permitted. There is therefore little animal cross-traffic between villages. The community is characterized by a human health centre, animal health clinic, and shopping complex in Hluvukani, where people from the different villages gather. There is human cross-traffic in the study area, with villagers, commuters, researchers, and veterinarians travelling freely between villages.

Due to the study area being in the FMD protection zone, and the proximity of wildlife species, which harbour and facilitate the spread of ticks and tick-borne diseases between wildlife, livestock and humans, comprehensive disease surveillance measures are implemented in the area by local veterinary services, mainly in the form of cattle dip tanks built throughout the region, which every cattle herd must visit for dipping and FMD inspection once a week. The dip consists of the Delete® X5 acaricide which is used on cattle, sheep, and goats, for the prevention and treatment of ectoparasite infestation. The farmers in the Mnisi community do not vaccinate their cattle against bovine anaplasmosis.

2.3. Animals

Ten local mixed breed *Bos taurus* calves (0–1 months of age, 6 males and 4 females) were monitored for a period of one year. Three of the ten calves were situated in Utha A (with a total of 715 cattle and a cattle density of 128 cattle/km^2) and two were in Dixie (with a total of 137 cattle and cattle density of 27 cattle/km^2); these two villages are located approximately 2.4 km and 0.5 km away, respectively to the wildlife–livestock interface. The remaining five cattle were based in Eglington village (with a total of 1009 cattle and a cattle density of 194 cattle/km^2); this is a peri-urban area, located 11.5 km away from the border with Manyeleti Game Reserve. The local veterinary services used the following different methods of acaricide treatment in the two areas: the plunge method of dipping cattle was used at Eglington (the peri-urban area), as well as Utha A (wildlife–livestock interface), while the hand spraying method was used at Dixie village (wildlife–livestock interface) due to water-shortages. The study required farmers with a relatively small herd of cattle who do not dip their cattle privately.

2.4. Study Design and Sample Collection

This longitudinal study was conducted between November 2016 (when the calves were 0–1-month-old) and October 2017. Whole blood samples were collected in 10 mL Vacutainer® ethylenediaminetetraacetic acid (EDTA) tubes from the ten calves once a month for 12 months according to the 12 time-point collection timeline (Figure 2).

2.5. Genomic DNA Extraction and Quantitative Real-Time PCR (qPCR) Assay

Genomic DNA was extracted from the samples collected from all time-points using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA was eluted in 100 µL elution buffer and stored at −20 °C. Genomic DNA samples were screened for the presence of *A. marginale* and *A. centrale* using a duplex qPCR assay targeting the *msp1β* gene of *A. marginale* and the *groEL* gene of *A. centrale* [34]. Primers, AM-For (5′-TTG GCA AGG CAG CAG CTT-3′), AM- Rev (5′-TTC CGC GAG CAT GTG CAT-3′) and a probe, AM-Pb (6-FAM-TCG GTC TAA CAT CTC CAG GCT TTC AT-BHQ1) were used to amplify and detect a 95 bp fragment of the *msp1β* gene of *A. marginale* while primers, AC-For (5′-CTA TAC ACG CTT GCA TCT C-3′), AC-Rev (5′-CGC TTT ATG ATG TTG ATG C-3′) and probe AC-Pb (LC610-ATC ATC ATT CTT CCC CTT CCC CTT TAC CTC GT-BHQ2) were used to amplify a 77 bp fragment of the *groEL* gene of *A. centrale*. Reactions were performed in a 20 µL final reaction volume comprising

4 µL FreshStart Taqman mix (Roche Diagnostics, Midrand, South Africa), 0.5 µL UDG, 0.6 µM of the *A. marginale*-specific primers, 0.9 µM of the *A. centrale*-specific primers, 0.2 µM of each probe, and 2.5 µL of template DNA (approximately 200 ng). The duplex assay was performed on a LightCycler v2 (Roche Diagnostics, Mannheim, Germany), using the thermal cycling conditions described previously [34]. Positive control for the *A. centrale* assay was DNA extracted from the *A. centrale* vaccine strain obtained from Onderstepoort Biological Products (OBP), Pretoria, South Africa.

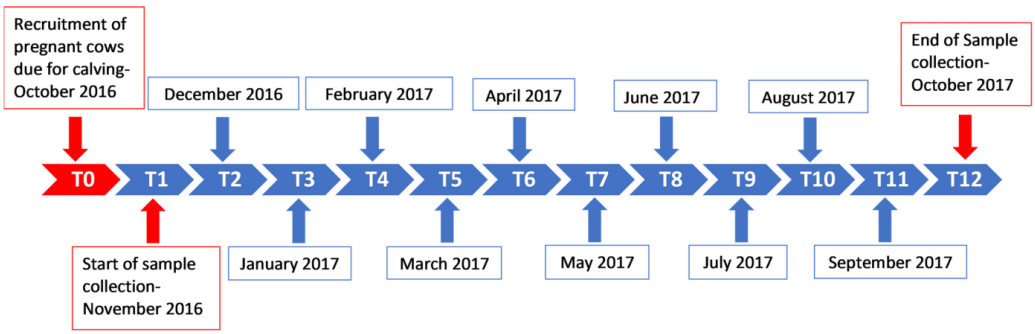

Figure 2. Sample collection timeline for the study. Samples were collected monthly from the ten calves for a period of one year (T1—November 2016, T2—December 2016 and T12—October 2017), from the ages of 0–1 months old (0–1 M) to 11–12 months old (11–12 M). T(x) = time point (month number).

Field sample C14 (obtained from cattle in the Mnisi Community area, Mpumalanga) was used as the positive control for the *A. marginale* assay. Nuclease-free water was used as a negative control for the assay. Results were analyzed using the LightCycler Software version 4.0 (Roche Diagnostics, Mannheim, Germany). Samples were run in triplicate per time-point to ensure reproducibility and repeatability of the results. A published linear range of detection and assay efficiency [25,34] were used to quantify the level of *A. marginale* rickettsaemia which was expressed as infected red blood cells (iRBC) per mL of blood.

2.6. Amplification, Cloning and Sequencing of the A. marginale Msp1α Gene

The repeat-containing variable region of the *A. marginale msp1α* gene was amplified using primers 1733F (5′-TGT GCT TAT GGC AGA CAT TTC C-3′) and 2957R (5′-AAA CCT TGT AGC CCC AAC TTA TCC-3′) [35]. Amplifications were performed in a 25 µL final reaction volume and consisted of 1x Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific, Johannesburg, South Africa), 0.5 µM of each primer, and 2.5 µL of template DNA (approximately 200 ng). The thermal cycling parameters used were modified from those previously reported [35] and comprised a pre-PCR denaturation at 94 °C for 3 min and *Taq* activation at 98 °C for 10 s, followed by 30 cycles of 98 °C for 1 s, 69.1 °C for 5 s, and 72 °C for 18 s, and a final extension at 72 °C for 1 min. PCR products were analysed by electrophoresis on a 1.5% agarose gel (1 × TAE buffer, pH 8.0), stained with ethidium bromide and viewed under UV light. All positive PCR products were purified using the Omega Bio-tek® DNA purification kit (Whitehead Scientific, Modderfontein, South Africa) according to the manufacturer's instructions. Purified PCR products were cloned into pJET 1.2 (Thermo Fisher Scientific, Johannesburg, South Africa). Recombinant clones were screened by colony PCR using vector specific primers, pJET1.2F and pJET1.2R; clones which yielded a product of 610 bp or greater were selected for sequencing. Fifteen recombinant clones per calf per time-point were sequenced bidirectionally on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) at Inqaba Biotechnical Industries, Pretoria or at the Central Analytical Facility, Stellenbosch University. *Anaplasma marginale msp1α* nucleotide sequences generated in this study were named

according to a naming to a proposed naming scheme [22] and deposited in GenBank under accession numbers OQ384772–OQ384912 and are also available under BioProject accession number PRJNA929355.

2.7. Characterization of A. marginale Msp1a Repeats and Msp1α Genotypes

Msp1a sequences were trimmed, assembled, edited, and aligned using CLC Genomics Workbench 20.0.4 (Qiagen, https://digitalinsights.qiagen.com/, accessed on 17 January 2023). The RepeatAnalyzer command line software tool [22] was used to identify, store, curate, and analyse Msp1a repeats and *A. marginale msp1α* genotypes. Novel repeats that were not recognized by RepeatAnalyzer were designated UP37 to UP42. The Msp1a repeat structure determines the *msp1α* genotype of a strain.

2.8. 16S rRNA Gene Amplification and PacBio Sequencing

In order to determine the composition and diversity of *Anaplasma* species present in the ten calves by T12, the full-length 16S rRNA gene (V1–V9 variable regions) was amplified in triplicate from the ten DNA samples collected at T12 using modified barcoded 16S rRNA gene specific primers, 27F: (5′-AGR GTT YGA TYM TGG CTC AG-3′) and 1492R: (5′-RGY TAC CTT GTT ACG ACT T-3′) recommended for the PacBio Sequel II sequencing instrument (Pacific Biosciences, Menlo Park, CA, USA) [36,37]. Reactions were performed in triplicate in a final volume of 25 µL containing 1 X Phusion Flash® High Fidelity Master Mix (Thermo Fisher Scientific, South Africa), 0.15 µM of each forward and reverse primer, and 5 µL of DNA (approximately 400 ng). DNA extracted from the *A. centrale* vaccine strain (Onderstepoort Biological Products, South Africa) was used as a positive control and molecular grade water as a negative control. Cycling conditions included 98 °C for 30 s, followed by 35 cycles of 98 °C for 10 s, 60 °C for 30 s, and 72 °C for 30 s and a final extension at 72 °C for 10 min. Amplicons were visualized under UV light after electrophoresis on a 1.5% agarose gel stained with ethidium bromide. Amplicons were purified using the QIAquick® PCR purification kit (Qiagen) according to the manufacturer's instructions and submitted to the Genomics Sequencing Core at Washington State University, Pullman, WA, USA for circular consensus sequencing (CCS) on the PacBio (Pacific Biosciences, Menlo Park, CA, USA) platform.

2.9. Analysis of Anaplasma 16S rRNA Sequences Identified by Microbiome Sequencing

The 16S rRNA amplicon sequence data was curated and filtered using SMRT Link software 8.0 according to a minimum barcode score of 70 and 99% precision. Final Fasta and Fastaq data sets were analyzed using the Ribosomal Database Project (RDP) 16S classifier [38,39] for *Anaplasma* genus level classification of the sequences with a 95% confidence interval. Sequences were further classified to the *Anaplasma* species level using a customized NCBI BLASTn database of all known and published *Anaplasma* spp. sequences downloaded from GenBank using the command line application. Sequences were further filtered and excluded based on sequence length (minimum of 1275 bp), quality, and sequence identity in Microsoft Excel [39,40]. Since some distinct *Anaplasma* species are known to have more than 98.7% shared sequence identity, and *A. platys*, *Anaplasma* sp. Mymensingh, "*Candidatus* Anaplasma camelii" and *Anaplasma* sp. Omatjenne share more than 99.5% 16S rRNA gene sequence identity, it is not possible to resolve these organisms to species level [40]. Thus, only 16S rRNA sequences that were identical to previously published sequences were classified to species level; the remainder were reported as putative novel *Anaplasma* species and/or genotypes. The raw microbiome data from the ten calves is available at the Sequence Read Archive (SRA) with BioProject accession number PRJNA929355.

2.10. Sequence and Phylogenetic Analysis

The *Anaplasma* 16S rRNA gene sequences identified by microbiome sequencing were aligned with reference sequences from GenBank. *Anaplasma* 16S rRNA sequences from representative genome sequences as well as the most closely related sequences from cattle

and other ruminants in South Africa and worldwide, as identified by BLAST analysis, were selected to construct the phylogenetic tree. *Anaplasma* 16S rRNA sequences from wildlife were included [41]. The extent of sequence variation was analysed using CLC Genomics Workbench (Qiagen). Alignments were further trimmed using Bioedit 7 [42]. Jmodel test 1.3 [43] predicted the HKY85 (Hasegawa–Kishino–Yano, 85) evolutionary model [44–46] as the best fit model for the 16S rRNA gene sequences. Phylogenetic trees for the 16S rRNA gene were constructed using the neighbor-joining and maximum likelihood (ML) method in MEGA 7 with bootstrap analysis using 1000 replicates to estimate the confidence levels of the tree branches [47], as well as Bayesian inference in Mr Bayes 3.2 [48]. The *Anaplasma* 16S rRNA nucleotide identified in this study were deposited in GenBank under accession numbers OQ348128-OQ348132, with BioProject accession number PRJNA929355.

3. Results

The *A. marginale* and *A. centrale* duplex qPCR was used to determine the presence of *Anaplasma* species in the calves. *Anaplasma marginale* was detected in seven of the 10 (70%) calves recruited to the study. Of the seven calves that tested positive for *A. marginale*, five were in the peri-urban area (Eglington village), while only two were located at the wildlife–livestock interface, both in Utha A village.

Four of the calves in the peri-urban area were already infected with *A. marginale* at the first time point (T1), and by the second time-point (T2), all five calves in this area tested positive (Figure 3A). The two calves that tested positive for *A. marginale* at the wildlife–livestock interface became infected only at T7 and T8, while the remaining three calves at the wildlife–livestock interface were either infected at levels below the detection limit of the assay (250 copies per reaction) or were not infected with *A. marginale* at (Figure 3B). *Anaplasma centrale* was not detected in any of the calves.

Table 1. *Anaplasma marginale msp1α* genotypes identified from infected calves over the 12-month study period.

Calf No.[a]	No. of Genotypes	Size (bp)	No. of Msp1a Repeats	Genotype	Number Allocated to Genotype	Genotype #, If Previously Detected in Study
1 (EG1)	13	949	5	171-2;UP3 [b] 172-2;UP4 61 172-2;UP4 172-2;UP4	1	
		700	2	τ 10	2	
		697	2	171-2;UP3 172-2;UP4	3	
		866	3	171-2;UP3 172-2;UP4 61	4	
		836	6	UP37 [c] UP31 UP31 UP31 UP31 UP31	5	
		781	2	τ UP31	6	
		787	3	τ 10 22-2	7	
		610	1	UP38 [c]	8	
		893	5	61 172-2;UP4 61 172-2;UP4 172-2;UP4	9	
		781	3	61 172-2;UP4 169-2	10	
		781	3	61 172-2;UP4 172-2;UP4	11	
		781	3	171-2;UP3 172-2;UP4 172-2;UP4	12	
		697	2	172-2;UP4 172-2;UP4	13	
2 (EG2)	4	784	3	UP39 [c] 10 UP31	14	5, 7
		781	3	179-2 169-2 172-2;UP4	15	
3 (EG3)	4	787	3	84 172-2;UP4 172-2;UP4	16 [d]	
		959	5	34 3 36 36 38	17 [d]	
		958	5	13 27 36 3 38	18	
		700	2	13 27	19	

Table 1. *Cont.*

Calf No.[a]	No. of Genotypes	Size (bp)	No. of Msp1a Repeats	Genotype	Number Allocated to Genotype	Genotype #, If Previously Detected in Study
4 (EG4)	12	1040	6	34 36 36 3 36 38	20	17 [d]
		1037	6	UP40 [c] β β β β F	21	
		959	5	34 36 36 27 18	22	
		954	5	MZ2 3 UP41 [c] 36 38	23	
		880	4	3 β 36 3	24	
		962	5	42 43 43 25 31	25	
		1131	7	34 3 UP1 43 43 25 31	26	
		1026	6	UP40 [c] β β β Is9;78 31	27	
		965	5	84 172-2;UP4 172-2;UP4 172-2;UP4 172-2;UP4	28	
		870	4	τ 22-2 13 18	34 [b]	
		705	2	34 3	41	
5 (EG5)	9	689	2	UP40 [c] β	29	17 [d], 21, 41
		1001	5	UP40 [c] β β β F	30	
		875	4	42 43 25 31	31	
		790	3	42 UP42 [c] 27	32	
		791	3	H M 27	33 [c]	
		602	1	UP40 [c]	42	
6 (UT1)	4	919	5	UP5 UP6 25 31 31	35	17 [d], 33 [d], 34 [d]
7 (UT2)	9	1075	7	UP5 UP6 25 31 UP6 27 18	36	16 [d], 33 [d], 34 [d], 35
		787	3	84 61 31	37	
		863	4	UP5 UP6 25 31	38	
		1202	8	UP5 UP6 25 31 UP6 25 31 31	39	
		955	4	84 Is9;78 31 31	40	

[a] Calves 1–5 were in the peri-urban area, Eglington; Calves 6–7 were in the wildlife–livestock interface, Utha A and Dixie. [b] Msp1a repeats denoted with a semicolon (e.g., 171-2;UP3) have been given two names in the literature. [c] indicates a novel Msp1a repeat (red). [d] *msp1α* genotypes that occur in both areas.

The levels of *A. marginale* infection fluctuated over the course of the 12-month study period, exhibiting the cyclic rickettsaemia known to occur in persistently infected animals [2,49]. In calves in the peri-urban area, the rickettsaemia ranged from 4×10^6 to 3×10^9 iRBC/mL from time of infection to a year. The levels of rickettsaemia in the two calves at the wildlife–livestock interface ranged from 2×10^6 to 2×10^7 iRBC/mL in the five and six months of infection.

3.1. *Anaplasma Marginale Msp1α Genotype Analysis in the Calves for a Period of a Year*

A total of 406 *msp1α* nucleotide sequences were generated from the seven *A. marginale*-positive calves and, in total, 42 unique *msp1α* genotypes were generated from the seven calves over the 12-month study period; however, several of the genotypes occurred in more than one animal (Table 1). Of the total number of *A. marginale* genotypes generated from the seven calves, 76.4% were identified in the five calves at the peri-urban area and 23.6% were identified in the two calves at the wildlife–livestock interface. Calves were infected with four to 13 genotypes (Table 1). Of the 42 *msp1α* genotypes identified, only four occurred in both areas (Table 1).

The *A. marginale msp1α* genotypes identified in the seven calves were made up of a total of 56 Msp1a repeats; 50 of these have been reported previously while six sequences are novel Msp1a repeats detected for the first time in this study (Figure 4). While only three of the *msp1α* genotypes occurred in both areas, 47.4% of the Msp1a repeats were common to both areas; a further 47.4% of the Msp1a repeats were identified only in calves at the peri-urban area while 5.2% were unique to calves at the wildlife–livestock interface. The six novel Msp1a repeats (named UP37-UP42) were all identified in calves from the peri-urban area.

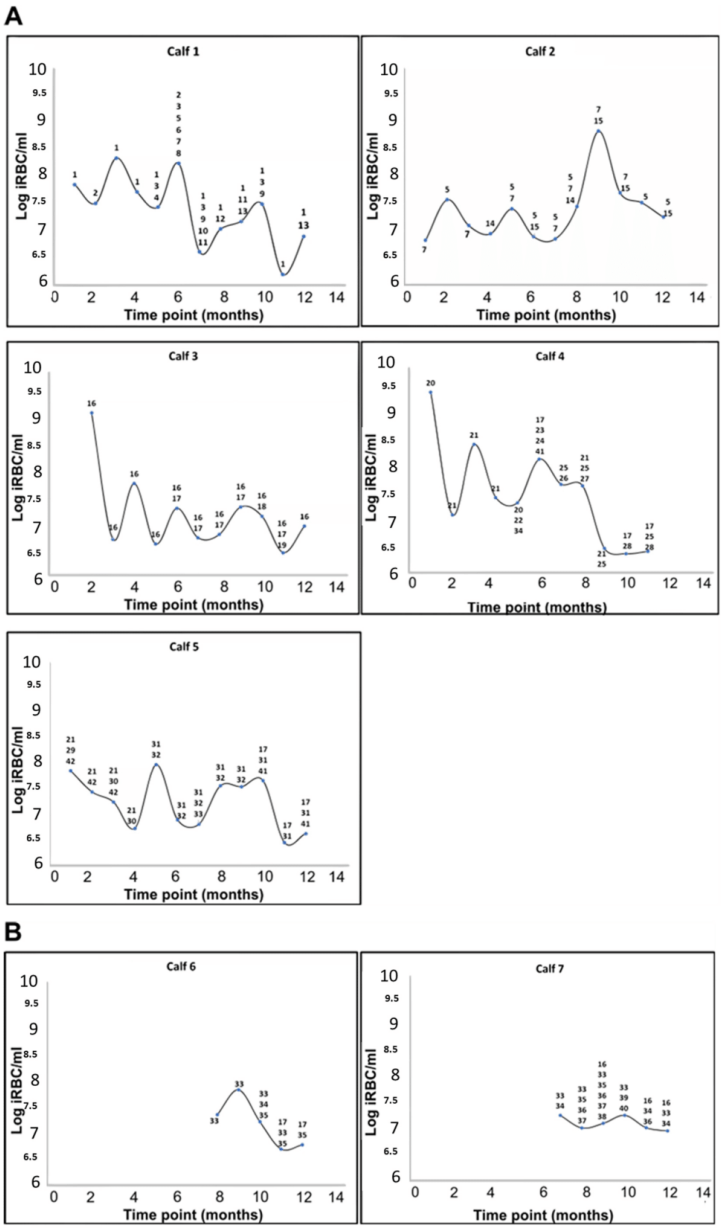

Figure 3. Cyclic *A. marginale* rickettsaemia in calves from the Mnisi community as determined by qPCR [25,34]. The level of infection is expressed as the log of infected red blood cells (RBC) per mL (iRBC/mL) of blood. (**A**) Calves infected with *A. marginale* at the peri-urban area. (**B**) Calves infected with *A. marginale* at the wildlife–livestock interface. The number and type of *A. marginale msp1α* genotypes detected at each time-point are indicated for each calf; genotypes were assigned numbers as shown in Table 1.

Figure 4. Novel Msp1a repeat sequences detected in this study. Six novel Msp1a repeats (UP37-UP42) were identified in the five calves located in the peri-urban area of the Mnisi community, Mpumalanga. Msp1a sequences were aligned using BioEdit. Conserved amino acid residues in the alignment are highlighted by white text on a black background, while variable residues are shown by black text on a white background.

3.2. Occurrence of A. marginale Multi-Strain Infections in the Calves

The complexity of *A. marginale* infection in the calves was determined by single/co-infection or superinfection events over the 12-month period (Figure 3). Detection of one or multiple genotypes at the initial time-point was defined as either single or co-infection, respectively. Detection of additional genetically distinct genotypes in the calves over time was defined as superinfection.

Animals in the peri-urban area acquired four to thirteen *msp1α* genotypes over the 12-month period. At the initial time-point, four of the five calves in the peri-urban area were infected with a single *A. marginale* genotype (or other genotypes were below the level of detection) and one was co-infected with more than two genotypes (Figure 3A). Superinfection with distinct *msp1α* genotypes occurred in all five calves during the study period. The same trend of infection was observed in the two calves that eventually became infected with *A. marginale* at the wildlife–livestock interface (Figure 3B). Although they only became positive for *A. marginale* from time-point T6 and T7, they were either singly infected or co-infected at the beginning but became superinfected with distinct *msp1α* genotypes over time (Figure 3B).

3.3. The Composition of Anaplasma spp. in the Ten Calves

PacBio CCS sequencing of 16S rRNA gene amplicons from the final sample taken from each of the ten calves generated a total of 57,683 raw nucleotide sequences that were classified in the genus *Anaplasma* using the RDP 16S classifier. Of these, 55,079 sequences were classified to *Anaplasma* species level using a customized 16S *Anaplasma* NCBI BLASTn database.

From the 55,079 16S rRNA sequences classified to *Anaplasma* species level, 87% of those were identified in calves at the peri-urban area and only 13% were identified in calves at the wildlife–livestock interface. The raw sequences were randomly sub-sampled to a total of 9950 sequences to equalize the sequencing depth, with 995 sequences analyzed per sample. A total of three *Anaplasma* species were identified in the 10 calves. They consisted mostly of *A. platys*-like 16S rRNA sequences (83.3%), followed by *A. marginale* (16.6%) and *Anaplasma boleense* (<0.1%) as highlighted in Table 2.

A. marginale and *A. platys*-like 16S rRNA gene sequences were the most abundant sequences identified in the *Anaplasma* infected calves and frequently occurred as a co-infection. The *A. platys*-like sequence was detected in four of the five calves at the peri-urban area and in three of the calves at the wildlife–livestock interface. Of the three calves that tested negative for *A. marginale* at the wildlife–livestock interface, two were also negative for other *Anaplasma* spp., whilst the third was infected with the *A. platys*-like organism. In terms of *Anaplasma* spp. infections, calf-4 at the peri-urban area that died at T11, was only infected with *A. marginale*.

Table 2. The percentage of 16S rRNA sequences of each *Anaplasma* spp. identified.

Calf No.	*A. platys*-like	*A. marginale*	*A. boleense*
1 (EG1)	67.3	32.3	0.4
2 (EG2)	98.7	1.3	0
3 (EG3)	56.0	43.9	0.1
4 (EG4)	0	100	0
5 (EG5)	68.5	31.3	0.2
6 (UT1)	7.0	93.0	0
7 (UT2)	95.3	4.7	0
8 (DI1)	0	0	0
9 (DI2)	0	0	0
10 (DI3)	100	0	0

3.4. 16S rRNA Phylogenetic Analyses

The phylogenetic relationships between *Anaplasma* spp. 16S rRNA gene sequences identified in this study and other published sequences are shown in Figure 5. The phylogenetic tree topologies obtained using three tree algorithms were very similar, and the maximum likelihood tree was chosen as a representative tree. The *A. marginale* sequences had 100% identity to *A. marginale* St Maries [50] and had 99.9% sequence identity to *A. marginale* sequences identified in the various wildlife hosts in the Kruger National Park (KNP) [41]. A minority of sequences had 99.8% identity with *A. boleense* [51], and 99.4% identity with *Anaplasma* sp. KNP9, a novel *Anaplasma* species recently identified in wildlife from KNP [41]. The *A. platys*-like sequences were closely related to *Anaplasma* sp. Omatjenne [52] with 99.7% identity, *A. mymensingh* [53] with 99.9% identity, and "*Candidatus* Anaplasma camelii" [54] with 99.6%. They had 99.7–99.9% identity to *Anaplasma* sp. KNP2, a novel *Anaplasma* species recently identified in wildlife from the Kruger National Park [41].

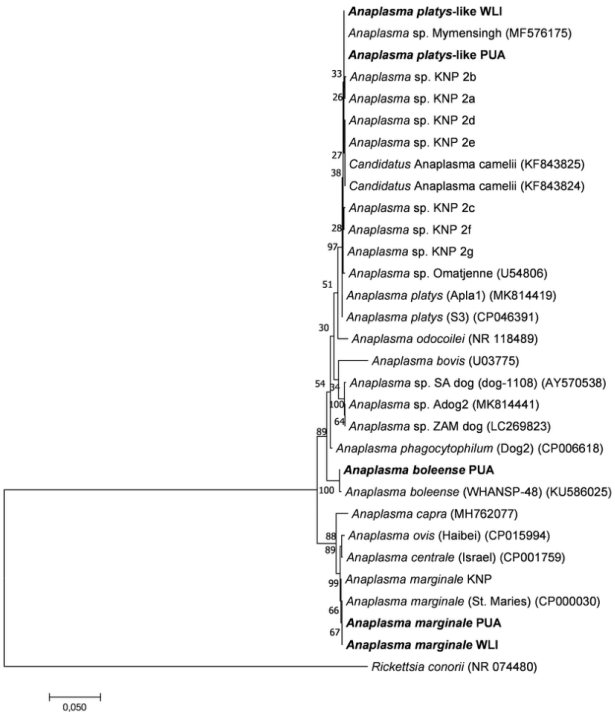

Figure 5. Maximum likelihood phylogenetic tree showing the phylogenetic relationships between *Anaplasma* 16S rRNA gene sequences obtained from the ten calves included in this study (in bold) and

previously published *Anaplasma* 16S rRNA gene sequences. Near full-length 16S rRNA gene sequences of approximately 1328 bp in length were used to construct the tree. The numbers associated with each node indicate the percentage of 1000 bootstrap replications supporting the node. Phylogenetic analyses were conducted in MEGA7 with an HKY85 evolutionary model. Sequences with abbreviations PUA, WLI, and KNP highlight that the sequences were retrieved from animals in the peri-urban area, wildlife–livestock interface and Kruger National Park, respectively. *Rickettsia conorii* was used as the outgroup. The scale bar highlights a 5% nucleotide sequence divergence.

4. Discussion

The presence of *A. marginale* in cattle in the Mnisi community was expected, since the pathogen is currently widespread and endemic in cattle in eight of the nine South African provinces [4,24,25] and is known to occur in most cattle farming areas in the country [10,55]. However, *A. marginale* was detected in only seven of the ten calves in the 12-month study period. Our results further revealed that *A. marginale* infects calves early in their lives or during intra-uterine development [1], since 50% of the calves were infected at T1 and T2, and they did not show clinical symptoms for the duration of the study. This agrees with previous findings [11,12], showing that calves up to 12 months of age are not clinically affected by anaplasmosis. The fact that three of the five calves at the wildlife–livestock interface were not infected was a surprising result. The bovine anaplasmosis cases observed at the wildlife–livestock interface in the Mnisi communal area might thus be attributed to a localised lack of endemic stability since calves at the wildlife–livestock interface are not continually infected with *A. marginale* in their first year when natural immunity is higher. The level of infection (number of infected red blood cells) in the calves that were infected in the two areas did not appear to be significantly different; however, our sample size is very limited and a larger study with more animals would be required to confirm these findings.

Although the Mnisi community is a non-anaplasmosis vaccinating area, absence of *A. centrale* infections was not expected, as *A. centrale* was previously detected in cattle in the study area [33], furthermore the natural circulation *A. centrale* infection was previously observed in buffalo (*Syncerus caffer*), zebra (*Equus quagga burchelli*), warthog (*Phacochoerus africanus*), and lion (*Panthera leo*) in the KNP [56–58].

The calves that were infected with *A. marginale* from both areas of the Mnisi community displayed complex *A. marginale* infections driven by co-infection and superinfection, with four to thirteen *msp1α* genotypes detected per animal over the 12-month period, indicating continuous challenge with multiple strains over time that should lead to robust immunity in these animals. Our results are similar to recent findings, where complex *A. marginale* infection with two to six strains per animal was detected in 97% of naïve calves that were introduced into an *A. marginale* infected herd in southern Ghana [31]. Another study from a high *A. marginale* prevalence region in Mexico, showed that up to six *A. marginale* genotypes could be detected per animal using *A. marginale msp1α* genotyping for strain characterization [30]. Although our small sample size might have skewed the results, our findings highlight differences in temporal *A. marginale* infection dynamics between the villages, with all five of the calves at Eglington village (a peri-urban area) being infected at T1 and T2, but only two of the three calves at Utha A (at the wildlife livestock–interface) infected at T5 and T6, and no infection detected in the remaining three calves (one at Utha A and two at Dixie at the wildlife–livestock interface). Factors such as cattle density and management, which differ at the three villages, may drive the dynamics of *A. marginale* infection, with a lack of early *A. marginale* infection at the wildlife–livestock interface resulting in the frequent clinical cases in the area. The rapid migration of *R. microplus* ticks (larvae and adult ticks) from infested to un-infested cattle has been implicated in the interstadial transmission of *A. marginale* [59]; furthermore, attachment of three infected *R. microplus* ticks is sufficient for transmission of *A. marginale* from infected to naïve cattle [60], while a single *Dermacentor andersoni* infected tick is sufficient for transmission [61]. Therefore, transmission of *A. marginale* is more likely to occur in areas where cattle density is higher, due to increased opportunities for migration of vector ticks from *A. marginale*-infected to

uninfected cattle, thus increasing the chances of transmission in the area. Additionally, different methods of acaricide treatments are used in the two study areas, and this might have had an effect on the disease transmission dynamics observed. The frequency of cattle dipping in the Mnisi communal area is greatly affected by water shortages. Cattle in the peri-urban site, Eglington village, as well as Utha A, at the wildlife–livestock interface are dipped using the plunge method of dipping cattle, while a hand spraying method is used at Dixie village (at the wildlife–livestock interface). Several factors, such as the inability to clean and empty the dip tank resulting in a heavily silted dip tank, and incorrect mixing ratios of water and the acaricide, have been shown to be the prime causes of tick control failure at communal plunge dip tanks [62,63] such as the ones used at Eglington and Utha A. Thus, the hand spraying method, as is used at Dixie village, may be more effective in controlling tick infestation and thus preventing disease transmission, than the plunge method of cattle dipping where the concentration of the acaricide in the dip tank might not be consistent.

Our findings further indicate the presence of other *Anaplasma* species circulating in the calves, which mainly comprised an *A. platys*-like organism that is closely related to a novel *Anaplasma* species recently identified in wildlife in the Kruger National Park [41]. Very low levels of *A. boleense* 16S rRNA sequences were also detected in the calves, which were also previously detected in cattle in the area [33]. The high levels the *A. platys*-like organism present in the calves suggest the presence of ticks responsible for the transmission of this organism in the area. It has been postulated that exposure to closely related non-pathogenic organisms might provide some cross-protection against the pathogenic species in cattle and thus decrease the pathogenicity of the infection [64]. It is thus possible that infection with the *A. platys*-like organism might confer heterologous protection against *A. marginale* in cattle in the area, thus contributing to endemic stability of *A. marginale*. An experimental study conducted in Kenya [65] showed that cattle are highly susceptible to infection by less pathogenic *Anaplasma* species from wildlife hosts. Cattle having recovered from anaplasmosis caused by *Anaplasma* species from wildlife showed slight protection against subsequent infection with *A. marginale*. Future studies should be aimed at confirming these observations and further determining the mechanisms underlying heterologous protection against bovine anaplasmosis by closely related non-pathogenic species.

5. Conclusions

Complex *A. marginale* infection in the Mnisi community is driven by co-infection and superinfection. Factors such as cattle density and management, which differ at the three villages, may drive the temporal dynamics of the infection. A localized lack of endemic stability at the wildlife–livestock interface could result in clinical cases caused by challenge with *A. marginale* at a later point in life. Our findings suggest that cattle in the Mnisi community are exposed to other *Anaplasma* spp. which might confer cross-protection against the pathogenic *A. marginale* infection and might suggest that other, previously unrecognized *Anaplasma* species could contribute to the control of bovine anaplasmosis in South Africa. While our results suggest that there are differences in the time-course of infection in calves in different areas of the Mnisi community, it should be noted that only five calves were examined from each area. A future in-depth longitudinal study in more villages of the Mnisi community with a larger sample size is recommended to confirm and further analyze the dynamics of *A. marginale* infections in the Mnisi communal area, especially at the wildlife–livestock interface.

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Institutional Review Board Statement: The study was carried out in strict accordance with the conditions and guidelines of the Animal Ethics Committee of the Faculty of Veterinary Science (reference number: V041-16)**.** Permission to perform the study under Section 20 of the Animal Disease Act of 1984 was granted by the South African Department of Agriculture, Forestry and Fisheries (currently Department of Agriculture, Land Reform and Rural Development) (reference number: 12/11/1/1/6).Ethical clearance for this study was approved on 30 May 2016.

Informed Consent Statement: Permission to conduct research in the chosen villages was granted by the village leaders. Informed consent was further granted by the cattle owners.

Data Availability Statement: All the sequences generated in this study are available under BioProject accession number PRJNA929355. *Anaplasma msp1α* and 16S rRNA nucleotide sequences generated in this study were deposited in GenBank under accession numbers OQ384772–OQ384912 and OQ348128-OQ348132 respectively. The raw microbiome data from the ten calves is available at the Sequence Read Archive (SRA) with BioProject accession number PRJNA929355.

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Unravelling the diversity of *Anaplasma* species circulating in selected African wildlife hosts by targeted 16S microbiome analysis

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ABSTRACT

Organisms in the genus *Anaplasma* are obligate intracellular alphaproteobacteria. Bovine anaplasmosis, predominantly caused by *Anaplasma marginale*, is the most prevalent tick-borne disease (TBD) of cattle worldwide. Other *Anaplasma* species are known to cause disease; these include *A. ovis, A. platys* in dogs, *A. capra* in goats and humans, and *A. phagocytophilum* in humans. The rapid advancement of next-generation sequencing technologies has led to the discovery of many novel sequences ascribed to the genus *Anaplasma*, with over 20 putative new species being proposed since the last formal organization of the genus. Most 16S rRNA gene surveys for *Anaplasma* were conducted on cattle and to a lesser extent on rodents, dogs, and ticks. Little is known about the occurrence, diversity, or impact of *Anaplasma* species circulating in wildlife species. Therefore, we conducted a 16S rRNA gene survey with the goal of identifying *Anaplasma* species in a variety of wildlife species in the Kruger National Park and neighbouring game reserves, using an unbiased 16S rRNA gene microbiome approach. An *Anaplasma/Ehrlichia*-group specific quantitative real-time PCR (qPCR) assay revealed the presence of *Anaplasma* and/or *Ehrlichia* species in 70.0% (21/30) of African buffalo, 86.7% (26/30) of impala, 36.7% (11/30) of greater kudu, 3.2% (1/31) of African wild dog, 40.6% (13/32) of Burchell's zebra, 43.3% (13/30) of warthog, 22.6% (7/31) of spotted hyena, 40.0% (12/30) of leopard, 17.6% (6/34) of lion, 16.7% (5/30) of African elephant and 8.6% (3/35) of white rhinoceros samples. Microbiome sequencing data from the qPCR positive samples revealed four 16S rRNA sequences identical to previously published *Anaplasma* sequences, as well as nine novel *Anaplasma* 16S genotypes. Our results reveal a greater diversity of putative *Anaplasma* species circulating in wildlife than currently classified within the genus. Our findings highlight a potential expansion of the *Anaplasma* host range and the need for more genetic information from other important genes or genome sequencing of putative novel species for correct classification and further assessment of their occurrence in wildlife, livestock and companion animals.

1. Introduction

Bovine anaplasmosis is among the three most important tick-borne diseases (TBDs) of ruminants and results in major economic losses in food animal production globally (Uilenberg, 1995). This disease is mainly caused by the obligate intracellular rickettsial pathogen, *Anaplasma marginale*, which is currently widespread in cattle in South Africa (Hove et al., 2018; Makgabo et al., 2023). Several other species of *Anaplasma* have been reported to infect cattle in South Africa: these include *A. centrale, A. bovis, A. platys* and *Anaplasma* sp. (Omatjenne) (De Kock et al., 1937; Zweygarth et al., 2006; Harrison et al., 2011; Harrison et al., 2013; Khumalo et al., 2016; Kolo et al., 2020). Of the seven species included in the most recent reorganization of the genus *Anaplasma* (Dumler et al., 2001), four species, *A. marginale, A. bovis, A. centrale* and *A. phagocytophilum*, are known to cause anaplasmosis in cattle (Aktas and Özübek, 2017; Hove et al., 2018; Jurković et al., 2020; M'Ghirbi et al., 2016). Of these, *A. marginale* is the most important pathogen in cattle (Kocan et al., 2010). Although *A. bovis, A. centrale*,

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and *A. phagocytophilum* are widely known to cause subclinical disease in cattle, a clinical case of bovine anaplasmosis caused by *A. centrale* was reported in Europe in 2008 (Carelli et al., 2008). *Anaplasma ovis* mainly causes a subclinical disease characterized by fever in sheep and goats (Kuttler, 1984). *Anaplasma phagocytophilum* causes human granulocytic anaplasmosis in humans (Bakken et al., 1994), while *A. capra* infects both goats and humans (Li et al., 2015). *Anaplasma platys* is a pathogen that mostly infects platelets in dogs causing infectious cyclic thrombocytopenia (Abarca et al., 2007).

The past few decades have seen the global occurrence of several new, emerging and re-emerging tick-borne rickettsial pathogens of major public and veterinary health concern (Walker and Dumler, 1996; Dumler et al., 2001; Paddock and Childs, 2003; Li et al., 2015). A significant increase in the wildlife industry in South Africa over the past two decades has resulted in an increase in land use dedicated to wildlife and thus an increase in wildlife species in both game reserves and farming areas, thus resulting in an increase in wildlife-livestock interfaces in many parts of the country (Parker and Bernard, 2005; Smith and Parker, 2010; Jori et al., 2011; Horak et al., 2015). This, in turn, increases potential TBD transmission opportunities between wildlife, livestock and the humans who maintain them, through increased opportunities for ticks to move between them (Yusufmia et al., 2010; Caron et al., 2013; Mbizeni et al., 2013). Very little is known about the role played by wildlife hosts in the distribution and epidemiology of anaplasmosis in domestic animals, livestock and possibly in humans. *Anaplasma marginale, A. centrale* and/or *A. ovis* have been identified in several wild ruminant species in Africa, including African buffalo (*Syncerus caffer*), black wildebeest (*Connochaetes gnou*), blue wildebeest (*Connochaetes taurinus*), blesbok (*Damaliscus pygargus phillipsi*), grey duiker (*Sylvicapra grimmi*), nyala (*Tragelaphus angasii*), eland (*Taurotragus oryx*) and giraffe (*Giraffa camelopardalis*) (Neitz, 1935; Peirce, 1972; Augustyn, 1974; Kuttler, 1984; Smith et al., 1982; Ngeranwa et al., 1998; Potgieter and Stoltsz, 2004; Eygelaar et al., 2015; Khumalo et al., 2016). *Anaplasma bovis* has been identified in rock sengis (*Elephantulus myurus*) (Harrison et al., 2011; Harrison et al., 2013) and nyala (Pfitzer et al., 2011), and a sequence with 99% identity to *A. bovis* was identified in a *Rhipicephalus evertsi* tick collected on a gemsbok from the Sandveld nature reserve (Tonetti et al., 2009). *Anaplasma* sp. (Omatjenne) was identified in 33% of nyalas examined from four game ranches in northern KwaZulu-Natal (Pfitzer et al., 2011). It is clear that African wildlife harbor several *Anaplasma* spp., but the full range of *Anaplasma* spp. present in wildlife hosts is not known, and the importance of wildlife as a disease reservoir is unclear.

The rapid advancement of high-throughput sequencing technologies has enabled a massive increase in molecular, metagenomic, microbiome and taxonomic analyses, which have resulted in the discovery of a plethora of sequences ascribed to the genus *Anaplasma* worldwide. Over 20 putative *Anaplasma* species with unique 16S rRNA sequences have been identified from various hosts since the last formal organization of the genus (Dumler et al., 2001; Caudill and Brayton, 2022). These putative *Anaplasma* spp. have been reported from a variety of hosts including human, livestock and wildlife and/or tick and mosquito vectors from across the world (a list of the putative *Anaplasma* spp. is shown in Table S1, modified from Caudill & Brayton (2022)). Several novel *Anaplasma* 16S rRNA gene sequences have been reported in cattle, including a putative novel *Anaplasma* species from Uganda (Ikwap et al., 2010; Muhanguzi et al., 2010), "*Candidatus* Anaplasma boleense" (Guo et al., 2016; Fernandes et al., 2019; Kolo et al., 2020), *Anaplasma* sp. Saso, *Anaplasma* sp. Hadesa and *Anaplasma* sp. Dedessa (Hailemariam et al., 2017; Kolo et al., 2020), *Anaplasma* sp. Mymensingh (Roy et al., 2018; Kolo et al., 2020), and "*Candidatus* Anaplasma africae" (Dahmani et al., 2019). The phylogenetic relationships of these newly detected agents to known pathogens and their ability to serve as a source of cross-reaction in detection assays have not been well assessed.

The present study was aimed at using next-generation sequencing and bioinformatics to profile *Anaplasma* populations in selected wildlife species, to better understand the range and genetic diversity of *Anaplasma* species with potential for transmission to humans, livestock and companion animals.

2. Materials and methods

2.1. Ethics approval

The study was performed in accordance with the conditions of the Animal Ethics Committee of the Faculty of Veterinary Science, University of Pretoria (REC 252–19). Research and Material Transfer Agreements were obtained from the Scientific Services Committee of the South African National Parks (SANParks), Kruger National Park (KNP) (BMTA 005/20) and the Hans Hoheisan Wildlife Research Station (HHWRS). Permission to conduct research under Section 20 of the *Animal Disease Act* 35 of 1984 was granted by the Department of Agriculture, Land Reform and Rural Development (12/11/1/1/6 (1734 LH)).

2.2. Field samples

A total of 343 frozen EDTA blood samples collected from 11 free roaming wildlife species in the Kruger National Park and surrounding game reserves including the Timbavati Game Reserve, Klaserie Private Nature Reserve and Manyeleti Game Reserve were made available by the Veterinary Wildlife Services, Kruger National Park (SANParks) and HHWRS biobanks (Table 1). These were collected from African elephant (*Loxodonta africana*), African lion (*Panthera leo*), African wild dog (*Lycaon pictus*), Burchell's zebra (*Equus quagga burchelli*), African buffalo (*Syncerus caffer*), common warthog (*Phacochoerus africanus*), greater kudu (*Tragelaphus strepsiceros*), impala (*Aepyceros melampus*), leopard (*Panthera pardus*), spotted hyena (*Crocuta crocuta*) and white rhinoceros (*Ceratotherium simum*) from 2012 to 2020.

2.3. DNA extractions

DNA was extracted from 1 ml of blood from the SANParks and HHWRS biobanked samples using the Gentra Puregene Kit® (Qiagen) according to the manufacturer's instructions.

2.4. Anaplasma/Ehrlichia group-specific quantitative real-time PCR (qPCR) assay

A multiple sequence alignment of 16S rRNA reference sequences of all known species of *Anaplasma* and closely related species in the genera *Ehrlichia* and *Rickettsia* was created using CLC Genomics Workbench 20 (Qiagen). Primers, Ma16SF: (5′-ACA GAA GTC CCG GCA AA-3′), Ma16SR: (5′-TTG CCC CCT CCG TAT TAC C-3′) (Inqaba Biotech, South Africa) and a TaqMan MGB™ probe, Ma16SP: (FAM-5′-CCG TGC CAG C-3′-MGB) (Thermo Fisher Scientific, South Africa) were designed to target a 64 bp fragment in the V3 hypervariable region that is conserved between *Anaplasma* and *Ehrlichia* species. Reactions, performed in a final volume of 20 µl, contained 2 X TaqMan® Universal PCR Master Mix (Thermo Fisher Scientific, South Africa), 0.5 µM of each forward and reverse primer, 0.25 µM of TaqMan MGB™ probe and 2 µl of target DNA. The quantitative real-time PCR (qPCR) assays were performed on the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Cycling conditions included UNG incubation at 50 °C for 2 min, followed by AmpliTaq Gold pre-activation at 95 °C for 10 min, 45 cycles of denaturation at 95 °C for 20 s and annealing at 60 °C for 1 min. DNA extracted from the *Anaplasma centrale* and *Ehrlichia ruminantium* vaccine strain (Onderstepoort Biological Products, Pretoria, South Africa), *A. marginale* and *A. platys* field samples (confirmed by sequence analysis) collected, respectively, from the Proefplaas dairy farm, University of Pretoria and the Mnisi community, Mpumalanga province, were used as positive controls and molecular grade water as a negative control. Data was analyzed using the StepOnePlus™ software version

Table 1
Origin and number of blood samples collected from wildlife hosts.

Wildlife host	Sample type	Biobank	Origin	Year	Number of samples
Buffalo	EDTA-blood	SANParks	Kruger National Park	2019	30
Impala	EDTA-blood	SANParks	Kruger National Park	2020	30
Kudu	EDTA-blood	SANParks	Kruger National Park	2018/19	30
Wild dog	EDTA-blood	SANParks	Kruger National Park	2017/18	30
	EDTA-blood	HHWRS	Timbavati Game Reserve	2020	1
Zebra	EDTA-blood	SANParks	Kruger National Park	2018/19	30
	EDTA-blood	HHWRS	Private Owner	2020	2
Warthog	EDTA-blood	SANParks	Kruger National Park	2017/18/19	30
Hyena	EDTA-blood	SANParks	Kruger National Park	2019/20	30
	EDTA-blood	HHWRS	Timbavati Game Reserve	2020	1
Leopard	EDTA-blood	SANParks	Kruger National Park	2012–2019	30
Lion	EDTA-blood	SANParks	Kruger National Park	2018/19/20	29
	EDTA-blood	HHWRS	Timbavati Game Reserve	2019	5
Elephant	EDTA-blood	SANParks	Kruger National Park	2019/20	30
Rhinoceros	EDTA-blood	SANParks	Kruger National Park	2020	29
	EDTA-blood	HHWRS	Klaserie Nature Reserve	2020	4
	EDTA-blood	HHWRS	Manyeleti Game Reserve	2020	2
Total					**343**

2.2. The analytical specificity of the qPCR assay was determined by testing DNA samples from tick-borne haemoparasites of ruminants including *Rickettsia africae, Babesia bigemina, Babesia bovis* and *Theileria parva*. All DNA samples extracted from the wildlife blood samples included in this study were screened for the presence of *Anaplasma* (and *Ehrlichia*) species using the *Anaplasma/Ehrlichia* group-specific qPCR assay. Since this assay was developed for screening purposes, no Ct value cut-off for true positives was used.

2.5. 16S rRNA gene amplification and PacBio sequencing

The full-length 16S rRNA gene (V1-V9 variable regions) was amplified in triplicate from all *Anaplasma* and/or *Ehrlichia*-positive wildlife DNA samples using modified barcoded 16S rRNA gene-specific primers, 27F: (5′-AGR GTT YGA TYM TGG CTC AG-3′) and 1492R: (5′-RGY TAC CTT GTT ACG ACT T-3′) as recommended for the PacBio Sequel II platform (Pacific Biosciences, Menlo Park, CA) (Lane, 1991; Turner et al., 1999) (a list of the barcoded primers is shown in Table S2). Reactions were performed in triplicate in a final volume of 25 μl containing 1 X Phusion Flash® High Fidelity Master Mix (Thermo Fisher Scientific, South Africa), 0.15 μM of each forward and reverse primer and 5 μl of target DNA. To prevent contamination, master mixes were prepared in a dedicated master mix preparation laboratory where no DNA or PCR products are allowed. PCRs for each wildlife species were prepared on separate days. DNA extracted from the *A. centrale* vaccine strain (Onderstepoort Biological Products, South Africa) was used as a positive control and molecular grade water as a negative control. Cycling conditions included 98 °C for 30 s, followed by 35 cycles of 98 °C for 10 s, 60 °C for 30 s and 72 °C for 30 s and a final extension at 72 °C for 10 min. Amplicons were visualized under UV light after electrophoresis on a 1.5% agarose gel stained with ethidium bromide. Amplicons were purified using the QIAquick® PCR purification kit (Qiagen) according to the manufacturer's instructions and submitted to the Genomic Sequencing Core of Washington State University, Pullman, USA for circular consensus sequencing (CCS) on the PacBio (Pacific Biosciences, Menlo Park, CA) platform. Two sequencing libraries were prepared using 500 ng of pooled amplicons in each library and the SMRT Bell library 2.0 express kit. Samples were sequenced following standard annealing and loading conditions detailed in SMRT Link software 8.0 (Pacific Biosciences, Menlo Park, CA).

2.6. 16S Microbiome sequence analysis

The 16S rRNA amplicon sequence data were demultiplexed using SMRT Link software 8.0 according to a minimum barcode score of 70. Sequences were then trimmed and filtered using SMRT Link software 8.0, with the following filtering parameters: QV minimum at 0.9999 (QV40), min read length 500 bp, max read length 3000 bp and 4 passes. Final Fasta and Fastq data sets were analyzed using the Ribosomal Database Project (RDP) 16S classifier (Cole et al., 2009; Gall et al., 2016) for *Anaplasma* genus level classification of the sequences with a 95% confidence interval. Sequences classified in the genus *Anaplasma* were further used in a BLAST search against a local NCBI BLASTn customized database created from all known and published *Anaplasma* spp. sequences downloaded from GenBank using the command line application to establish the correct identity of the sequences. Sequences were further filtered and excluded based on sequence length (minimum of 1275 bp), quality and sequence identity in Microsoft Excel (Gall et al., 2016; Caudill and Brayton, 2022). Since some distinct *Anaplasma* spp. are known to have more than 98.7% shared sequence identity, and *A. platys, Anaplasma* sp. Mymensingh, "*Candidatus* Anaplasma camelii" and *Anaplasma* sp. Omatjenne share more than 99.5% 16S rRNA gene sequence identity, it is clear that 16S rRNA gene sequences cannot be used to resolve these organisms to species level (Caudill and Brayton, 2022). Thus, only 16S rRNA sequences that were identical to previously published sequences were classified to species level. The *Anaplasma* species classification was further examined using a newly developed single-nucleotide polymorphism method of identifying and classifying *Anaplasma* spp. (Caudill and Brayton, 2022).

2.7. Terminology

It is difficult to formally name *Anaplasma* species due to their obligate intracellular nature and the requirement to deposit viable cultures in two type collections in different countries, and many of the newly identified putative species have been molecularly detected from samples but not isolated in culture. While the new sequences may well represent novel species, additional sequence data is required for confirmation; we will therefore refer to the newly detected novel 16S rRNA sequences as "sequence type" (ST). Where we refer to the organisms represented by the newly detected sequence types, we will refer to them as putative *Anaplasma* species.

2.8. Sequence and phylogenetic analysis

16S rRNA gene sequences classified as *Anaplasma* were aligned with reference sequences from GenBank and the extent of sequence variation was analysed using CLC Genomics Workbench (Qiagen). Alignments were trimmed using CLC Genomics Workbench. The HKY85 (Hasegawa et al., 1985) evolutionary model (Guindon and Gascuel, 2003; Anisimova and Gascuel, 2006; Chevenet et al., 2006) was the best-fit model for the 16S rRNA gene sequences as determined by Jmodel test 1.3 (Darriba et al., 2012). Phylogenetic trees for the 16S rRNA gene sequences were constructed using the maximum likelihood (ML) method in MEGA 7 with a HKY85 substitution model, an estimated proportion of invariant sites and four gamma-distributed rate categories (Kumar et al., 2016).

All of the sequence data generated from this study have been registered in GenBank under the BioProject accession number: PRJNA965916. The raw microbiome sequence reads from *Anaplasma*-positive wildlife hosts are available at the Sequence Read Archive (SRA) under accession numbers SRX20180660 to SRX20180741. The near-full length *Anaplasma* 16S rRNA nucleotide sequences were deposited under GenBank accession numbers OQ909436 to OQ909508.

3. Results

3.1. The presence of Anaplasma/Ehrlichia species in African wildlife hosts

The *Anaplasma/Ehrlichia* group-specific qPCR assay based on the 16S rRNA gene revealed the presence of *Anaplasma/Ehrlichia* spp. in all eleven wildlife species examined (Fig. 1) (qPCR results for individual samples are shown in Table S3).

3.2. 16S rRNA gene amplification and PacBio CCS sequence analysis

Of the samples that tested positive using the *Anaplasma/Ehrlichia* group-specific qPCR assay, a visible 16S rRNA PCR product was obtained from 21 impala, 13 buffalo, 6 kudu, 6 zebra, 8 warthog, 3 spotted hyena, 9 leopard, 6 lion and 2 African elephant samples. No amplicon could be generated from the *Anaplasma/Ehrlichia*-positive wild dog or rhinoceros samples. PacBio CCS sequencing of the 16S rRNA gene amplicons revealed the presence of a total of 40,589 *Anaplasma* 16S nucleotide sequences. Further examination of the genus *Anaplasma* using the RDP 16S classifier and the customized 16S *Anaplasma* NCBI BLASTn databases resulted in the classification of 40,063 of these 16S rRNA nucleotide sequences to the *Anaplasma* species level. A total of 526 sequences were excluded based on sequence quality, length and sequence identity (Table 2).

3.3. Identification of Anaplasma species in wildlife hosts

From the 40,063 16S rRNA nucleotide sequences classified as *Anaplasma*, 13 distinct 16S rRNA sequences distinct were identified. Sequences with 100% identity to the 16S rRNA gene of known species were designated with the formal species name; novel 16S rRNA sequences were designated *Anaplasma* sequence type (ST) KNP-1 to KNP-9. *Anaplasma* spp. identified comprised 11,449 (28.6%) sequences of *Anaplasma* ST KNP-1, followed by 8107 (20.2%) of *Anaplasma* ST SA Dog, 6347 (15.8%) of *A. marginale*, 4361 (10.9%) of *Anaplasma* ST KNP-8, 4163 (10.4%) of *Anaplasma* ST KNP-6, 2597 (6.5%) of *Anaplasma* ST KNP-2, 2482 (6.2%) of *A. centrale*, 271 (0.7%) of *Anaplasma* ST KNP-4,

Table 2
PacBio CCS sequencing data of the 16S rRNA gene of *Anaplasma* spp. from wildlife hosts.

Wildlife species	Number of samples that yielded a visible 16S rRNA amplicon	Number of sequences classified as *Anaplasma*		Excluded sequences
		Genus (16S RDP)	Species (NCBI BLASTn)	
Impala	21	2786	2744	42
Buffalo	13	5458	5393	65
Kudu	6	1351	980	371
Zebra	6	2514	2513	1
Warthog	8	3498	3495	3
Hyena	3	31	31	0
Leopard	9	20,729	20,693	36
Lion	6	3715	3707	8
Elephant	2	507	507	0
Total	**74**	**40,589**	**40,063**	**526**

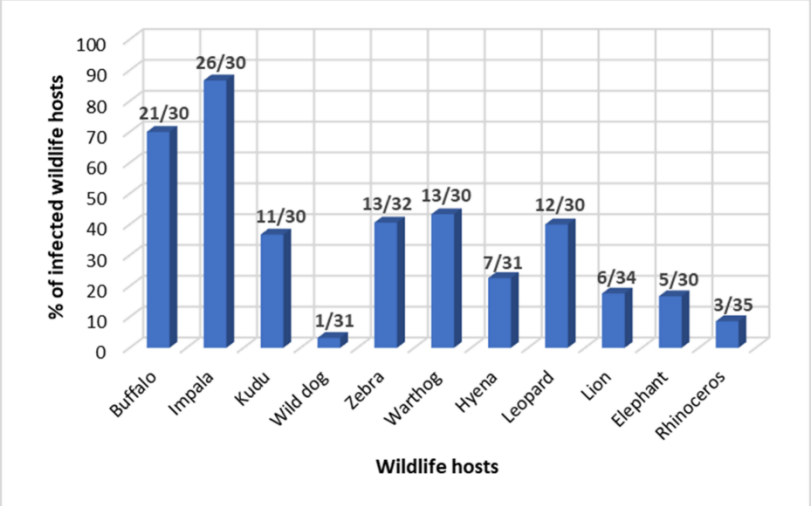

Fig. 1. Percentage of *Anaplasma/Ehrlichia*-positive samples from 11 wildlife species detected using the *Anaplasma/Ehrlichia* group-specific qPCR assay. The numbers above each bar indicate the number of *Anaplasma/Ehrlichia*-positive samples out of the total for each wildlife species included in the study.

206 (0.5%) of *Anaplasma* ST KNP-5, 55 (0.1%) of *Anaplasma* ST KNP-7, and less than 0.1% of *Anaplasma bovis* (14 sequences), *Anaplasma* ST KNP-9 (9 sequences), as well as *Anaplasma* ST KNP-3 (2 sequences).

Table 3 highlights the presence of the 13 *Anaplasma* 16S rRNA sequences in the different wildlife hosts. *Anaplasma* ST KNP-1, *Anaplasma* ST SA Dog and *Anaplasma* ST KNP-2 were detected in seven of the wildlife hosts, followed by *A. marginale* and *A. centrale* detected in four of the wildlife hosts. The remaining *Anaplasma* spp. and STs were detected in either three or two of the wildlife hosts.

3.4. Phylogenetic analyses of 16S rRNA gene sequences from wildlife hosts

The relationships between the near full-length *Anaplasma* 16S rRNA gene sequences identified in the wildlife hosts were revealed by phylogenetic analyses. Maximum likelihood phylogenetic trees of the 16S rRNA gene sequences resulted in two clades (Figs. 2 and 3), as highlighted in previous studies (Kolo et al., 2020; Caudill and Brayton, 2022). The first clade, which will be referred to as clade-1, includes *Anaplasma* spp. known to infect erythrocytes of livestock, such as *A. marginale, A. centrale* and *A. ovis*, while the second clade, which will be referred to as clade-2, includes *Anaplasma* spp. known to infect leukocytes and platelets, namely *A. platys, A. bovis* and *A. phagocytophilum* (Kolo et al., 2020; Caudill and Brayton, 2022).

Only four of the 13 sequences amplified belong to previously identified and described species; these were *A. bovis, A. centrale, A. marginale* and *Anaplasma* ST SA Dog (Fig. 2). *Anaplasma bovis* 16S rRNA gene sequences obtained from kudu and leopard samples were identical to the 16S rRNA gene sequence of *A. bovis* (U03775) from South Africa. *Anaplasma marginale* 16S rRNA gene sequences obtained from buffalo, zebra, leopard and lion samples were conserved and identical to *A. marginale* sequences previously reported from South Africa (AF414873) (Lew et al., 2003) and only varied by one nucleotide from the 16S rRNA gene of *A. marginale* St. Maries strain (CP000030) (Brayton et al., 2005). The *Anaplasma* sp. SA Dog sequences were identical to the *Anaplasma* sp. 16S rRNA sequences previously identified and described in dogs in Zambia and South Africa (LC269823 and MK814441, respectively) (Vlahakis et al., 2018; Kolo et al., 2020). The *A. centrale* sequences obtained from buffalo, zebra, warthog and lion samples were conserved and identical to the *A. centrale* vaccine strain from Israel (CP015994) (Herndon et al., 2010) and only varied by one nucleotide from the 16S rRNA sequence of the *A. centrale* vaccine strain from Australia (AF414868) (Lew et al., 2003).

Phylogenetic relationships between the 16S rRNA sequences of known *Anaplasma* species and the newly detected 16S rRNA sequences are shown in Fig. 3. Except for *Anaplasma* ST KNP-2, which is made up of several similar *A. platys*-like sequences, the unknown *Anaplasma* sequences identified formed monophyletic clades distinct from other validated reference *Anaplasma* spp. (Fig. 3). *Anaplasma* ST KNP-1 and *Anaplasma* ST KNP-3 grouped within clade-1. Two variants of *Anaplasma* ST KNP-1 were identified which formed a sister clade to the *A. ovis* group which includes *Anaplasma* sp. clone Mongolia. *Anaplasma* ST KNP-1a and *Anaplasma* ST KNP-1b had 99.9% sequence identity and had, respectively 99.5% and 99.6% shared sequence identity with *A. ovis* (GenBank accession no: CP015994) and 99.1% and 99.2% identity to *Anaplasma* sp. Mongolia (GenBank accession no: MK575506). *Anaplasma* ST KNP-1 was detected primarily in impala, but was also identified in buffalo, kudu, zebra, leopard, lion and African elephant samples. *Anaplasma* ST KNP-3 was obtained from buffalo and zebra samples and was closely related to *A. centrale*, with 99.6% identity. The two novel sequences, *Anaplasma* ST KNP-7 and *Anaplasma* ST KNP-8, were closely related, with 99.4% identity. The two sequences had, respectively, 99.4% and 99.6% shared sequence identity with *Anaplasma* sp. Om5 (GenBank accession no: LC558313) that was recently detected in *Ornithodoros moubata* ticks collected from African warthog burrows in Zambia (Qiu et al., 2021). *Anaplasma* ST KNP-7, *Anaplasma* ST KNP-8 and *Anaplasma* sp. Om5 and *Anaplasma* sp. Om5 formed a distinct sister group within clade-1 with less than 96.0% sequence identity to a multitude of sequences within the two prominent clades of *Anaplasma. Anaplasma* ST KNP-7 was detected in buffalo, kudu and leopard samples, while *Anaplasma* ST KNP-8 was obtained from warthog and lion samples and could be a variant of the putative novel *Anaplasma* sp. recently identified in *O. moubata* ticks (LC558313).

The remaining unknown *Anaplasma* sequences grouped in clade-2. *Anaplasma* ST KNP-6, identified in zebra, leopard and lion samples, grouped with *Anaplasma* sp. SA dog (GenBank accession no: AY570538) and *Anaplasma* sp. ZAM Dog (GenBank accession no: LC269823) 16S rRNA sequences with 99.6% and 99.8% identity, respectively, suggesting it might be a variant of these. *Anaplasma* ST KNP-9, found only in impala and leopard samples, grouped in a distinct clade with *Anaplasma* sp. boleense (GenBank accession no: KU586025) with 99.0% identity. *Anaplasma* ST KNP-4 and seven variants of *Anaplasma* ST KNP-2 all grouped with *A. platys* (GenBank accession no: CP046391) and have more than 99.5% shared 16S rRNA sequence identity. Similarly, Caudill and Brayton (2022) reported that *Anaplasma* sp. Mymensingh (GenBank accession no: MF576175), "*Candidatus* Anaplasma camelii" (GenBank accession no: 843,824) and *Anaplasma* sp. Omatjenne (GenBank accession no: U54806) all group with *A. platys* with more than 99.5% shared 16S rRNA sequence identity. Although *Anaplasma* ST KNP-5 also groups with *A. platys, Anaplasma* sp. Mymensingh, "*Candidatus* Anaplasma camelii" and *Anaplasma* sp. Omatjenne, it is less similar with 98.7%–99.1% identity between these sequences. *Anaplasma* ST KNP-4 sequences were obtained from kudu and leopard samples, while *Anaplasma* ST KNP-5 sequences were found in zebra and leopard. *Anaplasma* ST KNP-2 was identified from a variety of wildlife hosts, including impala, buffalo, kudu, zebra, leopard, lion and elephant.

Table 3
Percentage of each *Anaplasma* 16S rRNA sequence type identified in each wildlife host.

Anaplasma 16S rRNA sequence type	Percentage of each *Anaplasma* 16S rRNA sequence type								
	Impala ($n = 21$)	Buffalo ($n = 13$)	Kudu ($n = 6$)	Zebra ($n = 6$)	Warthog ($n = 8$)	Hyena ($n = 3$)	Leopard ($n = 9$)	Lion ($n = 6$)	Elephant ($n = 2$)
A. bovis	0	0	0.71	0	0	0	0.03	0	0
A. centrale	0	24.29	0	0.03	0.37	0	0	31.24	0
A. marginale	0	73.11	0	64.27	0	0	0.03	21.09	0
A. ST SA Dog	3.68	0.06	0	26.70	0.06	100	32.14	17.51	0
A. ST KNP-1	85.94	1.48	0.71	0.32	0	0	43.36	0.22	3.16
A. ST KNP-2	10.20	0.98	93.28	0.28	0	0	3.93	1.21	95.66
A. ST KNP-3	0	0.02	0	0.04	0	0	0	0	0
A. ST KNP-4	0	0	0.61	0	0	0	1.28	0	0
A. ST KNP-5	0	0	0	3.98	0	0	0.51	0	0
A. ST KNP-6	0	0	0	4.38	0	0	18.70	4.96	0
A. ST KNP-7	0	0.06	4.69	0	0	0	0	0	1.18
A. ST KNP-8	0	0	0	0	99.57	0	0	23.77	0
A. ST KNP-9	0.18	0	0	0	0	0	0.02	0	0

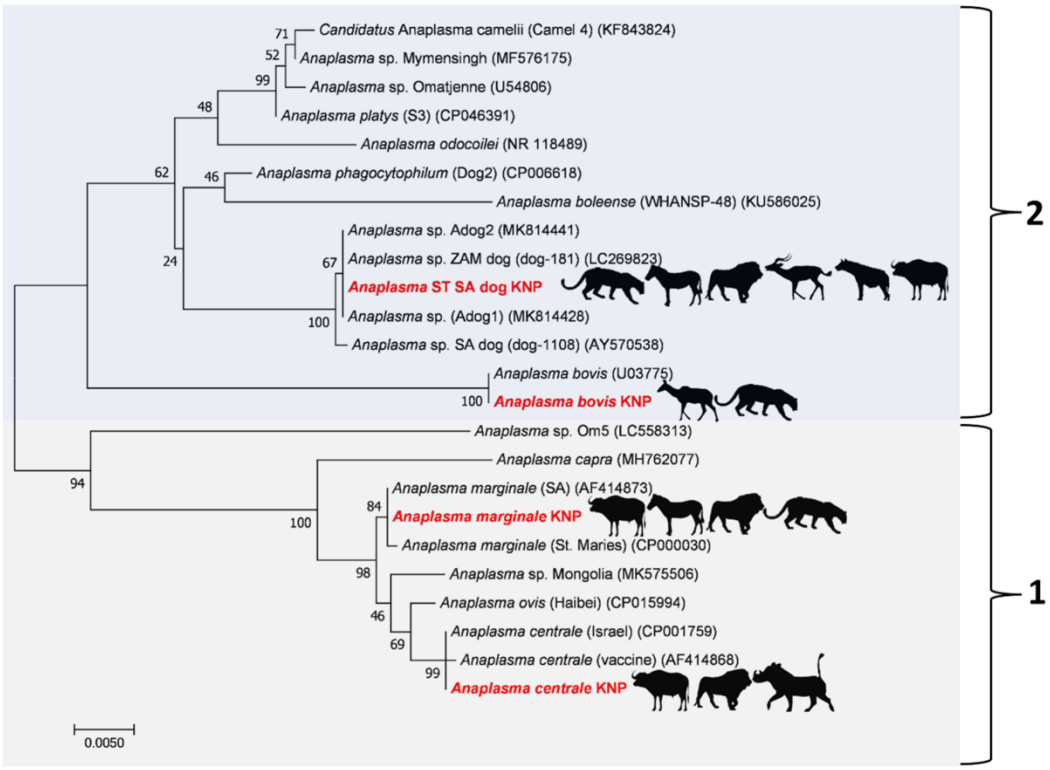

Fig. 2. Maximum likelihood phylogenetic tree based on 16S rRNA sequence data showing the relationships between *Anaplasma* species and highlighting sequences obtained in this study (shown in red) that were identical to known *Anaplasma* species. The tree is separated into the two prominent *Anaplasma* clades; clade-1, shaded in grey and clade 2, shaded in blue. Wildlife silhouettes indicate the hosts in which the sequences were identified. Near full-length 16S rRNA gene sequences of approximately 1328 bp in length were used to construct the tree. The numbers associated with each node indicate the percentage of 1000 bootstrap replications supporting the node. Phylogenetic analyses were conducted in MEGA7 with an HKY85 evolutionary model.

3.5. Analysis of Anaplasma 16S rRNA sequences detected in wildlife hosts using an Anaplasma species-discriminating single-nucleotide polymorphism method

The *Anaplasma* species-discriminating bases technique of identifying and classifying *Anaplasma* spp. as proposed by Caudill & Brayton et al. (2022) identified six species-specific bases that differentiate *Anaplasma* species within clade-1. The two 16S rRNA gene sequence variants of *Anaplasma* ST KNP-1 (*Anaplasma* ST KNP-1a and *Anaplasma* ST KNP-1b) varied by a single nucleotide which was not one of the six species-discriminating bases. The *Anaplasma* ST KNP-1 sequences varied from known species in two to four of the species-discriminating bases (Table 4). Similarly, *Anaplasma* ST KNP-7 and *Anaplasma* ST KNP-8 varied from the other *Anaplasma* sequences in clade-1 by two or three species-discriminating bases (Table 4). The six species-discriminating bases of *Anaplasma* ST KNP-3 differed from *A. centrale, A. ovis* and *Anaplasma* sp. Mongolia by one to five bases but were identical to those of *A. marginale*. However, *Anaplasma* ST KNP-3 grouped most closely with *A. centrale* sequences on phylogenetic analysis. Should further analysis of these putative novel species indicate that they are distinct from the known *Anaplasma* species, a new typing scheme will become necessary.

Caudill & Brayton (2022) proposed 14 bases to differentiate between species within clade-2. One of these 14 bases in the *Anaplasma* ST KNP-4 16S rRNA sequence differed from the *A. platys* sequence (Table 5), and there were other nucleotide differences elsewhere in the sequence. The *Anaplasma* ST KNP-5 sequence varied from *A. platys* and closely related species by two to eight *Anaplasma* species-discriminating bases, and *Anaplasma* ST KNP-6 varied from *A. platys* and closely related species by four to seven *Anaplasma* species-discriminating bases. *Anaplasma* ST KNP-9 varied from *A. platys* and closely related species by four to eight *Anaplasma* species-discriminating bases.

Seven variants of *Anaplasma* ST KNP-2 (a-g) 16S rRNA sequences were detected which group in clade-2. Although all of the *Anaplasma* ST KNP-2 variants, except for *Anaplasma* ST KNP-2 g, were identical to *A. platys* according to the single-nucleotide polymorphism method of classifying *Anaplasma* spp. (Table 6), the sequences differed from *A. platys* elsewhere in the full length 16S rRNA sequence. Similarly, three of the *Anaplasma* ST KNP-2 sequences (2b, 2d and 2e) were identical to "*Candidatus* Anaplasma camelii" according to the species-discriminating nucleotides, however, the full length 16S rRNA sequences differed elsewhere.

4. Discussion

This study provides insight into the diversity of *Anaplasma* species circulating in wildlife hosts in the Kruger National Park and surrounding game reserves. The most recent reorganization of the genus *Anaplasma* included seven species: *Anaplasma marginale, A. centrale, A. ovis, A. bovis, A. platys, A. phagocytophilum* and *A. caudatum* (Dumler et al., 2001). *Anaplasma capra* has been effectively published in the literature but not formally recognized (Li et al., 2015; Yang et al., 2017). More than 20

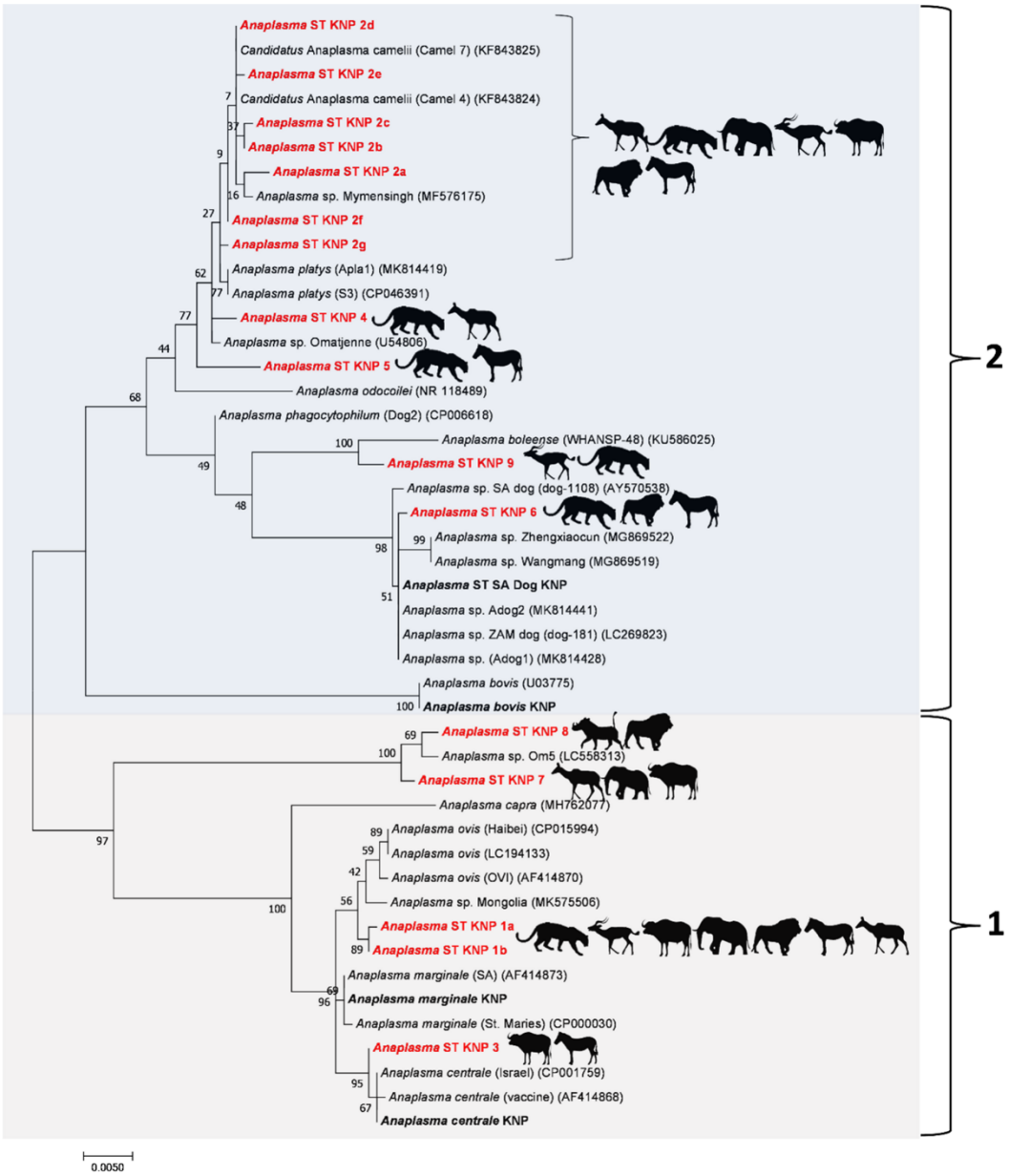

Fig. 3. Maximum likelihood phylogenetic tree based on 16S rRNA sequence data showing the phylogenetic relationships between the novel *Anaplasma* 16S rRNA sequence types identified (shown in red) and previously described *Anaplasma* species. The tree is separated into the two prominent *Anaplasma* clades; clade-1, shaded in grey and clade 2, shaded in blue. Wildlife silhouettes indicate the hosts in which the novel *Anaplasma* 16S rRNA sequences were identified. Near full-length 16S rRNA gene sequences of approximately 1328 bp in length were used to construct the tree. The numbers associated with each node indicate the percentage of 1000 bootstrap replications supporting the node. Phylogenetic analyses were conducted in MEGA7 with an HKY85 evolutionary model.

other putative *Anaplasma* species have been identified, mostly by 16S rRNA gene sequence analysis (Caudill and Brayton, 2022). We identified four previously known and nine novel *Anaplasma* genotypes in nine wildlife hosts, namely, African buffalo, impala, kudu, zebra, warthog, hyena, leopard, lion and elephant. The four known *Anaplasma* sequences identified were *A. marginale, A. centrale, A. bovis* and *Anaplasma* ST SA dog. The nine novel *Anaplasma* genotypes were genetically distinct from but closely related to known *Anaplasma* spp. based on the 16S rRNA gene sequence analysis. Although not much is known about the pathogenicity of *Anaplasma* species in wildlife hosts, subclinical infections of known

Table 4
Species-discriminating bases of clade-1 of the genus *Anaplasma*.

	Base Number*					
	144	156	220	265	274	1250
A. marginale	A	G	T	T	G	T
A. centrale	A	A	T	T	G	T
A. ovis	G	R†	Y†	C	T	T
Anaplasma sp. Mongolia	G	A	C	C	G	C
Anaplasma ST KNP-1a & b£	G	A	C	T	A	T
Anaplasma ST KNP-3	A	G	T	T	G	T
Anaplasma ST KNP-7	G	G	C	T	G	T
Anaplasma ST KNP-8	G	G	C	T	G	T

*Numbering and sequence alignment based on the *Anaplasma marginale* St. Maries 16S rRNA gene sequence. Differences between the six species-discriminating bases in *A. marginale* and the other *Anaplasma* spp. are highlighted by white text on a black background.
£Two variants with identical species-differentiating bases but differ elsewhere in the sequence.
†The degenerate position R denotes either A or G, while Y denotes either C or T.

Table 5
Species-differentiating bases of clade-2 of the genus *Anaplasma*.

	Base Number*													
	213	224	262	289	693	696	878	879	885	886	890	1052	1309	1358
A. platys	A	T	T	T	N†	T	R†	C	G	T	T	R†	Y†	C
A. sp. Mymensingh	A	T	T	T	C	T	A	C	G	T	T	A	C	C
A. sp. Omatjenne	A	C	T	T	C	T	R†	C	G	T	T	G	C	T
"*Candidatus* A. camelii"	A	T	T	T	C	T	A	C	G	T	T	A	T	C
A. odocoilei	G	A	G	C	A	A	G	T	A	C	C	G	C	C
A. ST KNP-4	A	T	T	T	C	T	G	T	G	T	T	G	T	C
A. ST KNP-5	A	T	T	T	A	A	G	C	G	C	T	A	C	C
A. ST KNP-6	A	T	T	C	C	T	A	T	A	T	C	G	C	C
A. ST KNP-9	A	T	T	T	A	A	A	T	A	T	T	G	C	T

*Numbering and sequence alignment based on the 16S rRNA gene from the *Anaplasma platys* strain S3 genome sequence. Differences between the 14 species-discriminating bases in *A. platys* and the other *Anaplasma* spp. are highlighted by white text on a black background.
†The degenerate position N denotes any possible nucleotide, while R denotes either A or G and Y denotes either C or T.

Table 6
Species differentiating bases of clade-2 of the genus *Anaplasma*.

	Base Number*													
	213	224	262	289	693	696	878	879	885	886	890	1052	1309	1358
A. platys	A	T	T	T	N†	T	R†	C	G	T	T	R†	Y†	C
A sp. Mymensingh	A	T	T	T	C	T	A	C	G	T	T	A	C	C
A sp. Omatjenne	A	C	T	T	C	T	R†	C	G	T	T	G	C	T
"*Candidatus* A. camelii"	A	T	T	T	C	T	A	C	G	T	T	A	T	C
A. odocoilei	G	A	G	C	A	A	G	T	A	C	C	G	C	C
A. ST KNP-2a	A	T	T	T	C	T	A	C	G	T	T	A	C	C
A. ST KNP-2b, d & e£	A	T	T	T	C	T	A	C	G	T	T	A	T	C
A. ST KNP-2c & f£	A	T	T	T	C	T	A	C	G	T	T	G	T	C
A. ST KNP-2g	A	C	T	T	C	T	A	C	G	T	T	G	C	C

*Numbering and sequence alignment based on the 16S rRNA gene from the *Anaplasma platys* strain S3 genome sequence. Differences between the 14 species-discriminating bases in *A. platys* and the other *Anaplasma* spp. are highlighted by white text on a black background.
£Two or three variants with identical species-differentiating bases which but differ elsewhere in the sequence. †The degenerate position N denotes any possible nucleotide, while R denotes either A or G and Y denotes either C or T.

Anaplasma species have been reported and wildlife are thus usually regarded as reservoir hosts (Neitz, 1935; Peirce, 1972; Augustyn, 1974; Kuttler, 1984; Smith et al., 1982; Ngeranwa et al., 1998; Potgieter and Stoltsz, 2004; Eygelaar et al., 2015; Khumalo et al., 2016; Sisson et al., 2023). *Anaplasma marginale* infections in cattle can cause disease with varying levels of severity, from icterus and anemia, to abortions and death, while *A. centrale, A. bovis* and *A. platys* are regarded as non-pathogenic in cattle and usually cause subclinical infection in these animals.

As expected, our results indicate that *A. marginale* and *A. centrale* are widespread in the African buffalo population in the Kruger National Park. These two tick-borne haemoparasites have previously been identified in African buffalo, as well as black wildebeest, blue wildebeest, eland and waterbuck (Henrichs et al., 2016; Khumalo et al., 2016; Sisson et al., 2017; 2023). Interestingly, we detected 16S rRNA sequences identical to *A. marginale* in zebra, leopard and lion, while 16S rRNA sequences identical to *A. centrale* were also found in zebra and warthog. These data may suggest an expansion of the potential host range for *A. marginale* and *A. centrale*, as they are regarded as ruminant-specific *Anaplasma* species. However, it should be noted that low numbers of *A. marginale* and *A. centrale* 16S rRNA sequences were detected in a minority of feline samples. Nevertheless, more work needs to be done in a larger population of felines to determine whether the detection of *A. marginale* and *A. centrale* was incidental or if felines are reservoir hosts.

Anaplasma sp. dog strain was initially detected and identified in three dogs at the Veterinary Teaching Hospital of the Medical University of South Africa (Inokuma et al., 2005). The 16S rRNA gene sequence, designated *Anaplasma* sp. SA dog (GenBank accession no: AY570539 and AY570540), has 99.8% sequence identity to the 16S rRNA sequence from an *Anaplasma* sp. identified in dogs in Zambia, designated *Anaplasma* sp. ZAM Dog (GenBank accession no: LC269823). The *Anaplasma* sp. ZAM Dog 16S rRNA sequence was detected in domestic dogs in Lusaka, Zambia (Vlahakis et al., 2018) and recently in dogs and *Rhipicephalus sanguineus* ticks in the Mnisi community, Mpumalanga, South Africa (Kolo et al., 2020). Kolo et al. (2022) suggested that the *Anaplasma* 16S rRNA sequences identified in dogs represent variants of a single novel organism and proposed that it be referred to as *Anaplasma* sp. SA dog (for *Anaplasma* sp. Southern Africa dog) until type material can be deposited. Little is known about this putative *Anaplasma* species. It groups closely with *A. phagocytophilum* and other *Anaplasma* 16S rRNA sequences identified in dogs in clade-2. In our study, a 16S rRNA gene sequence identical to the *Anaplasma* sp. ZAM Dog 16S rRNA sequence (GenBank accession no: LC269823) was widespread in the wildlife species examined; it was detected mainly in leopard, zebra and lion and to a lesser extent in impala, spotted hyena, buffalo and warthog. Although this *Anaplasma* species has thus far only been detected in dogs and associated ticks, our findings suggest additional wildlife hosts as possible reservoir hosts. Interestingly, *Anaplasma* ST KNP-6, detected in leopard, lion and zebra, also grouped in the *Anaplasma* sp. SA dog clade with 99.6% shared 16S rRNA sequence identity. Our data could therefore suggest that *Anaplasma* ST KNP-6, *Anaplasma* sp. ZAM Dog and *Anaplasma* sp. SA dog represent variants of the same species, however, additional genomic data is required to resolve this question, and, given their close relationship to *A. phagocytophilum* further work is required to determine their zoonotic potential.

We detected 16S rRNA sequences that are 100% identical to the *A. bovis* sequence (GenBank accession no: U03775) in kudu and leopard samples. Although *A. bovis* infection is mainly reported in cattle (Noaman and Shayan, 2010; Belkahia et al., 2015), little is known about the epidemiology of this agent. However, it is closely related to *A. phagocytophilum* and is regarded as a zoonotic agent that infects monocytes, it is usually associated with subclinical infection, and *Hyalomma* spp. are considered to be vectors of the organism (Donatien and Lestoquard, 1936). *Anaplasma bovis* was also detected in a population of eastern rock sengis (*Elephantulus myurus*) in South Africa, suggesting that sengis may be natural reservoir hosts of the organism (Harrison et al., 2013). It is thus possible that other rodent species are reservoir hosts of *A. bovis* and possibly other *Anaplasma* spp.

Of the nine novel *Anaplasma* 16S rRNA sequence types identified, *Anaplasma* ST KNP-1, *Anaplasma* ST KNP-3, *Anaplasma* ST KNP-7 and *Anaplasma* ST KNP-8 are found in clade-1, which is commonly referred to as the livestock clade, while *Anaplasma* ST KNP-2, *Anaplasma* ST KNP-4, *Anaplasma* ST KNP-5, *Anaplasma* ST KNP-6 and *Anaplasma* ST KNP-9 are found in clade-2, commonly referred to as the zoonotic clade. It should be noted that, since similarities above 98.70% occur between 16S rRNA sequences of known *Anaplasma* species (Caudill and Brayton, 2022), it is difficult to distinguish between *Anaplasma* species based on 16S rRNA sequences alone. Furthermore, it has previously been shown that the 16S rRNA gene sequences of *A. platys, Anaplasma* sp. Mymensingh, "*Candidatus* Anaplasma camelii" and *Anaplasma* sp. Omatjenne share more than 99.5% sequence identity and do not resolve these organisms to species level, although a high degree of intraspecies variance is evident based on the single-nucleotide polymorphisms used to distinguish the species within this clade (Caudill and Brayton, 2022). Many of the novel 16S rRNA sequences identified in our study are highly similar to previously reported *Anaplasma* sequences, and it is therefore not clear whether they represent novel species or variants of known or previously reported putative *Anaplasma* species. This will require further investigation, including the sequencing and phylogenetic analysis of other genes, or whole genome sequence analyses. It is evident that there is an urgent need to identify an alternative gene or genes for the discrimination of species within the genus *Anaplasma*.

Although *Anaplasma* species and strains infecting one host species might not necessarily infect and cause disease in other host species, we have noted the presence of some of the novel 16S rRNA sequences in a variety of wildlife hosts, suggesting that they may be able to infect multiple host species. Therefore, the ability of these newly identified agents to infect cattle, other domestic animals and possibly even humans should be assessed. Novel 16S rRNA sequences have already been identified in cattle in South Africa. These include "*Candidatus* Anaplasma boleense", *Anaplasma* sp. Mymensingh and *Anaplasma* sp. SA dog (Kolo et al., 2020) which were identified in cattle in the Mnisi community, which borders on the Manyeleti and Timbavati Game Reserves and the Kruger National Park. If the putative *Anaplasma* species identified in our study are found to infect livestock, they could affect the specificity of existing tests for detection of *A. marginale*. Furthermore, the presence of the novel 16S rRNA sequences in wildlife could impact on the use of existing tests for the detection of known *Anaplasma* species in wildlife.

Seroprevalence studies are often used to determine the prevalence of *A. marginale*. Current serological tests used to diagnose anaplasmosis include the competitive ELISA (cELISA), complement fixation test, card agglutination test (CAT) and IFA test, with only cELISA and CAT recommended for the diagnosis of anaplasmosis (Kocan et al., 1992; De la Fuente et al., 2005). The commercially available *Anaplasma* genus-specific cELISA kit (Knowles et al., 1996) uses recombinant major surface protein 5 (Msp5) as antigen. Since the Msp5 epitope is widely conserved between *Anaplasma* species (Munodzana et al., 1998; Hofmann-Lehmann et al., 2004; Dreher et al., 2005; Strik et al., 2007), the cELISA cannot be used for the specific detection of *A. marginale* if multiple species of *Anaplasma* are known to occur in cattle, frequently as co-infections (Zweygarth et al., 2006; Khumalo et al., 2016; Hove et al., 2018; Makgabo et al., 2023). Indeed, in many parts of the world assumptions are generally made by host species: if the test is positive in cattle, it is likely that it is detecting *A. marginale*, whilst a positive result from sheep or goats should indicate *A. ovis* infection. However, these assumptions can lead people astray (Da Silva et al., 2018), due to the broad cross-reactivity among known *Anaplasma* species from both clades (Munodzana et al., 1998; Hofmann-Lehmann et al., 2004; Dreher et al., 2005; Strik et al., 2007). We suspect the cELISA will also recognise the putative *Anaplasma* species identified in wildlife, although more work will be required to confirm this.

Based on our analysis, several primers and probes from nucleic acid-based assays targeting the 16S rRNA gene, such as the *A. platys* (Inokuma et al., 2001) and *A. phagocytophilum* (Kawahara et al., 2006) specific assays, as well the reverse line blot hybridization (RLB) assay (Georges et al., 2001; Bekker et al., 2002), would cross-react with some of the *Anaplasma* ST detected, ST KNP-2, ST KNP-4, ST KNP-5 as well as other previously described putative *Anaplasma* species, including *Anaplasma* sp. Omatjenne and *Anaplasma* sp. Mymensingh, while the *A. phagocytophilum* assay (Kawahara et al., 2006) would also cross-react with *Anaplasma* ST SA dog and *Anaplasma* ST KNP-6. The RLB *Anaplasma* sp. (Omatjenne) probe (Bekker et al., 2002) would cross-react with *Anaplasma* sp. Mymensingh, *Anaplasma* ST KNP-2 (all variants), *Anaplasma* ST KNP-4, as well as *Anaplasma* ST KNP-5. The *A. centrale*-specific probe (Georges et al., 2001) would cross-react with the two variants of *Anaplasma* ST KNP-1. The use of these assays to determine the presence of known *Anaplasma* species in wildlife should therefore be interpreted with caution. This highlights the necessity for more specific assays to be developed to assess the epidemiology of *Anaplasma* species more accurately.

The widely used duplex real-time assay to detect *A. marginale* and *A. centrale* infections in cattle (Decaro et al., 2008; Byaruhanga et al., 2016; Hove et al., 2018) is not based on the 16S rRNA gene but on the *A. marginale msp1β* gene and the *A. centrale groEL* gene (Decaro et al., 2008; Chaisi et al., 2017). While these assays have been used to detect *A. marginale* and *A. centrale* in wildlife (Khumalo et al., 2016), it remains to be seen whether these assays will cross-react with the putative novel *Anaplasma* species since nothing is known about their gene complement.

5. Conclusion

Our results revealed a greater genetic diversity of *Anaplasma* species circulating in wildlife hosts than currently classified within the genus *Anaplasma* and suggest potential for transmission to livestock or companion animals. Furthermore, these novel genotypes are phylogenetically similar to known *Anaplasma* spp. and may serve as a source of cross-reaction in the current detection assays. Although this data, including that of single-nucleotide polymorphisms used to distinguish between the different *Anaplasma* species within the two clades, may provide sufficient genetic divergence between these organisms to potentially suggest classification as separate species within the clade, there is a need for additional genetic data and genome sequencing of these putative species for correct *Anaplasma* species classification and to further determine their occurrence in livestock and companion animals.

CRediT authorship contribution statement

S. Marcus Makgabo: Methodology, Investigation, Formal analysis, Writing – original draft. **Kelly A. Brayton:** Conceptualization, Methodology, Supervision, Writing – review & editing. **Marinda C. Oosthuizen:** Supervision, Writing – review & editing. **Nicola E. Collins:** Conceptualization, Methodology, Supervision, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All of the sequence data generated from this study have been registered in GenBank under the BioProject accession number: PRJNA965916. *Anaplasma* 16S rRNA sequences were deposited under accession numbers OQ909436 to OQ909508. Additional data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.crmicr.2023.100198.

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