Molecular Characterization of *Anaplasma* spp. in Cattle and Sable Antelope from Lusaka and North-Western Provinces of Zambia

By

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A Dissertation Submitted to the University of Zambia in Partial Fulfillment of the requirements for the degree of Master of Science in Tropical Infectious Diseases and Zoonoses

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I, Rhodasi Mwale, student number 2019103699, do hereby declare to the senate of the University of Zambia that this dissertation is my original work and has not been submitted for a degree award in any other University

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CERTIFICATE OF APPROVAL

This dissertation by Rhodasi Mwale has been approved as fulfilling the requirements for the award of the degree Master of Science in Tropical Infectious Diseases and Zoonoses by the University of Zambia

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ABSTRACT

Rickettsiales of the genus *Anaplasma* are globally distributed tick-borne pathogens of animals and humans with complex epidemiological cycles involving ticks and vertebrate hosts. This study aimed to detect and characterize *Anaplasma* species present in Zambia with a focus on the infection risk posed by the translocation of sable antelope (*Hippotragus niger)* from North Western Province to Lusaka Province. The study utilized archived DNA samples from cattle (Chongwe District) and sable antelope (Kasonso Busanga). Additionally, a knowledge, attitudes and practices (KAP) survey was conducted in Chongwe District to contextualize the possibility of spill-over of *Anaplasma* from introducing wild ruminants to a low prevalence area. From the one-hundred samples examined, *Anaplasma* species were detected by DNA sequencing in 7% (4/53) of cattle samples and 23.25% (10/47) of sable samples. *A.marginale* (4), was detected in both cattle and sable while *A.ovis* (7) and *A.platys* (2) were detected from sable antelope only. The knowledge, attitudes and practices survey determined that the awareness of anaplasmosis among farmers was moderately low (43%). This may be a consequence of low prevalence of *Anaplasma* in cattle in Chongwe. Phylogenetic analysis of the 16S rRNA partial gene sequence revealed that *A.ovis* and *A.marginale* samples were likely genetically related regardless of the host i.e 100% and 96.9% bootstrap support, respectively. This suggests a likelihood of transmissibility between sable and cattle.This study postulates that the introduction of sable antelope infected with multiple *Anaplasma* species poses a risk to animals and humans in Chongwe District.

Keywords: anaplasmosis, tick-borne diseases, *Anaplasma* species in cattle, *Anaplasma* species in wildlife, molecular epidemiology

DEDICATION

This study is dedicated to my father, who didn’t get to live long enough to see me complete it but was a never ending oasis of encouragement and to my family and friends, who have had to bear the burden with me and provide support.

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LIST OF ABBREVIATIONS

TBD’s	Tick-borne diseases
GMA	Game management area
HGA	Human granulocytic anaplasmosis
EGA	Equine granulocytic anaplasmosis
TBF	Tick-borne fever
DNA	Deoxyribonucleic acid
rRNA	Ribosomal ribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
CAT	Card agglutination test
cELISA	Competitive Enzyme-linked immunosorbent assay
Msp5	Major surface protein 5
CFT	Complement fixation test
PCR	Polymerase chain reaction
nPCR	Nested PCR
qPCR	Quantitative real-time PCR
RLB	Reverse line blot hybridization (RLB) assay
MLST	Multi-locus sequence typing
WGS	Whole genome sequencing
NWP	North Western Province
CD	Chongwe District
KAP	Knowledge, attitudes and practices
OIE	World Organization for Animal Health
bTB	Bovine tuberculosis

ECF	East Coast fever

CHAPTER ONE

1. INTRODUCTION

1.1. **Background**

For decades, after anaplasmosis was described in cattle in 1910, it was considered a disease of cattle (*Anaplasma marginale*) and small ruminants (*Anaplasma ovis*). In 2001, the species was reorganized using phylogenetic analysis of the *Anaplasmatacae* family. This reorganization led to the realization of extent of *Anaplasma* host diversity and zoonotic potential (Dumler et al, 2001; Rar et al 2020). Since then, researchers have been keen on describing the genus at molecular level and determining vectors and wildlife reservoirs. These studies of *Anaplasma* have unearthed a worrying pattern of increased incidence, expanding geographic distribution, and the discovery of new pathogenic agents and variants that are a pressing threat to human health (Parola et al., 2005; Chitanga et al., 2014; Lorusso et al., 2016; Banović et al., 2021; Yang et al., 2021).

Currently, the six recognized species of *Anaplasma* are; *A.marginale*, *Anaplasma centrale* and *A.ovis*, *Anaplasma phagocytophilum*, *Anaplasma platys* and *Anapalasma bovis* (Rar and Golovljova, 2011; Atif, 2016; Rar et al., 2021). Several candidate species are also under consideration. Two newly described species, *Anaplasma capra* and *Anapalasma odocoelei*, are yet to be included in the list of Prokaryotic names with Standing in Nomenclature (Rar et al 2021). Of all these, *A.marginale*, *A.bovis* and *A.phagocytophilum* are the most aggressive agents of disease and most relevant (Atif, 2015). The most widespread of the *Anaplasma* species is *A. marginale* and though it can infect diverse animal species it is known to cause disease only in cattle and giraffes (Hove *et al.*, 2018). Other species, most notably *A. phagocytophilum*, are agents of disease in a diverse range of animal species including humans (Fuente et al., 2005; Parola et al., 2005; Kocan et al., 2015).

Anaplasma species infection presents with varying clinical manifestations and outcomes in different hosts ranging from asymptomatic to severe illness and even death (Atif, 2016; Rar et al., 2021). In domestic ruminants it can have devastating economic consequences and its geographic foci has extended over the years to previously non-endemic areas (Atif et al., 2015). Introduction of *Anaplasma* to naive hosts due to movement of persistently infected livestock, expansion of the wildlife-livestock interface and subsequent establishment of competent vectors are all implicated in the extension of geographic foci of *Anaplasma* species (Atif et al., 2015). The discovery and increasing incidence of zoonotic *Anaplasma* species is also of great concern (Stuen, Granquist and Silaghi, 2013; Matei *et al.*, 2019)

Anaplasma species have complex epidemiologic cycles in endemic areas involving several tick-vectors, wildlife and domesticated animals (Kocan et al., 2015; Battilani et al., 2017). The complexity of these epidemiological cycles magnify the challenge of anaplasmosis control and the search for wildlife reservoirs is in a bid to understand and break these cycles. *Anaplasma* species have been detected worldwide in wild ungulates including several antelope species, suggesting they may have an important role in maintenance and spread of *Anaplasma* (Worthington and Bigalke, 2001). While studies have demonstrated the infectivity of *Anaplasma* species obtained from wildlife to domestic animals through inoculation, the role of wildlife as reservoirs and their risk to domestic animals is still widely unexplored (Rar and Golovljova, 2011; Koh et al., 2018). As wild animals may have a role in the genetic mutation of *Anaplasma* species and subsequent spillover of new and more pathogenic strains to humans and domestic animals, molecular studies in wild animals, especially wild ungulates have gained traction in the past decade (Dahmani *et al.*, 2017; André, 2018).

In Zambia, anaplasmosis is considered endemic and several epidemiological studies have documented its prevalence in livestock (Makala *et al.*, 2003; Simuunza *et al.*, 2011).

Although *Anaplasma* species have also been detected in dogs and other wild hosts in Zambia, molecular data is scanty (Simuunza *et al.*, 2011; Chitanga, Gaff and Mukaratirwa, 2014; Vlahakis *et al.*, 2018). With different species and genetic variants differing in their pathogenicity and host and cell tropism, there is a need in Zambia to describe the molecular epidemiology of *Anaplasma* with a focus on genetic diversity and potential wildlife reservoirs.

1.2. **Statement of the problem**

Anaplasmosis is an economically important tick-borne disease and its prevalence is established in Zambia i.e 36% to 83% across the country (Simuunza et al., 2011). However, data on the species and strains of circulating *Anaplasma* is scanty. Furthermore, neither the animal reservoirs of *Anaplasma*, nor their potential to carry zoonotic *Anaplasma* species has been explored. This is particularly disconcerting as wildlife conservation practices in Zambia include the translocation of wild animals to new areas and their re-introduction to others thus widening the human-livestock-wildlife interface and putting the human and livestock population at risk of new pathogens. Additionally, the influence of farming practices on the spread and maintenance of anaplasmosis in cattle is poorly understood.

1.3. **Justification of the study**

Possible detection of *Anaplasma* in sable will provide evidence of its circulation in wild ruminants and the phylogenetic relationships between species detected in cattle and sable will allow us to make inferences on transmission potential. Additionally, the possible detection of *A. phagocytophilum* and *A. platys* will provide information on the risk of emergence of zoonotic anaplasmosis in Zambia.

1.4. Study Objectives

1.4.1. General Objective

To describe the epidemiology and genetic diversity of *Anaplasma* species in cattle and sable antelope.

1.4.2. Specific Objective

1. To detect and identify *Anaplasma* in cattle and sable antelope.

2. To determine genetic diversity and relatedness of *Anaplasma* from cattle and sable antelope.

3. To assess the knowledge, attitudes and practices of livestock farmers in relation to anaplasmosis.

1.5. **Research questions**

1. What are the species of Anaplasma found in cattle and sable antelope in Zambia?
2. Are these species of *Anaplasma* genetically related?
3. What are the practices, attitudes and knowledge levels of livestock farmers regarding anaplasmosis *Anaplasma*?

1.5.1. Are knowledge levels of livestock farmers associated with anaplasmosis prevention practices or activities?

CHAPTER TWO

2. LITERATURE REVIEW

2.1. General Overview of *Anaplasma*

Anaplasma species are coccoid to ellipsoidal, non-motile, pleomorphic, obligate intracellular alpha-proteobacteria that infect diverse cell types, worldwide (Atif et al., 2016: Battilani et al., 2017). They reside and replicate in membrane-bound vacuoles (morula) within the cytoplasm of eukaryotic host cells of the hematopoietic cell line (Battilani et al., 2017; Rar et al., 2021). While described as gram-negative, *Anaplasma* stains better with Romanowsky stains and are detected microscopically in Geimsa stained blood smears (Battilani et al., 2017).

Anaplasma belong to the family Anaplasmatacae which contains the genera *Neorickettsia, Wolbachia, Ehrlichia* and *Anaplasma* (Figure 2.1). Originally, the genus *Anaplasma* contained *A.marginale* and *A.centrale* but has expanded based on phylogenetics to include four other species, three of which (*A.bovis, A.phagocytophilum* and *A.platys*) were reclassified from *Ehrlichia* (Kocan et al., 2015; Dumler *et al.*, 2001).

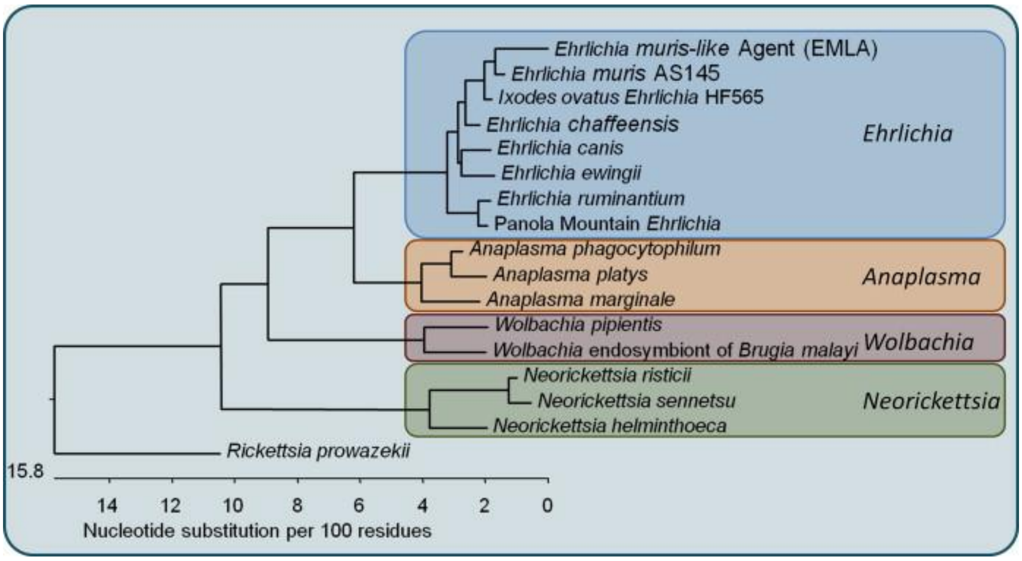

Figure 2.1: Phylogenetic relationships between the members of the genera, Ehrlichia, Anaplasma, Wolbachia and Neorickettsia within the family Anaplasmataceae based on 16 S ribosomal RNA gene sequences (Thirumalapura and Walker., 2015).

A.ovis, *A.marginale* and *A.centrale* are erythrocytic parasites, *A.bovis* infects monocytes, *A.platys* infects platelets and *A.phagocytophilum* infects granulocytes (Atif et al., 2016; Battilani et al., 2017; Rar et al., 2021).

The *Anaplasma* life cycle (Figure 2.2) involves tick vectors and vertebrate hosts with reproduction occurring in both (Kocan et al., 2003; Battilani et al., 2017). Vertebrate hosts can become persistently infected and act as reservoirs for the reinfection of tick-vectors (André, 2018; Matei *et al.*, 2019).

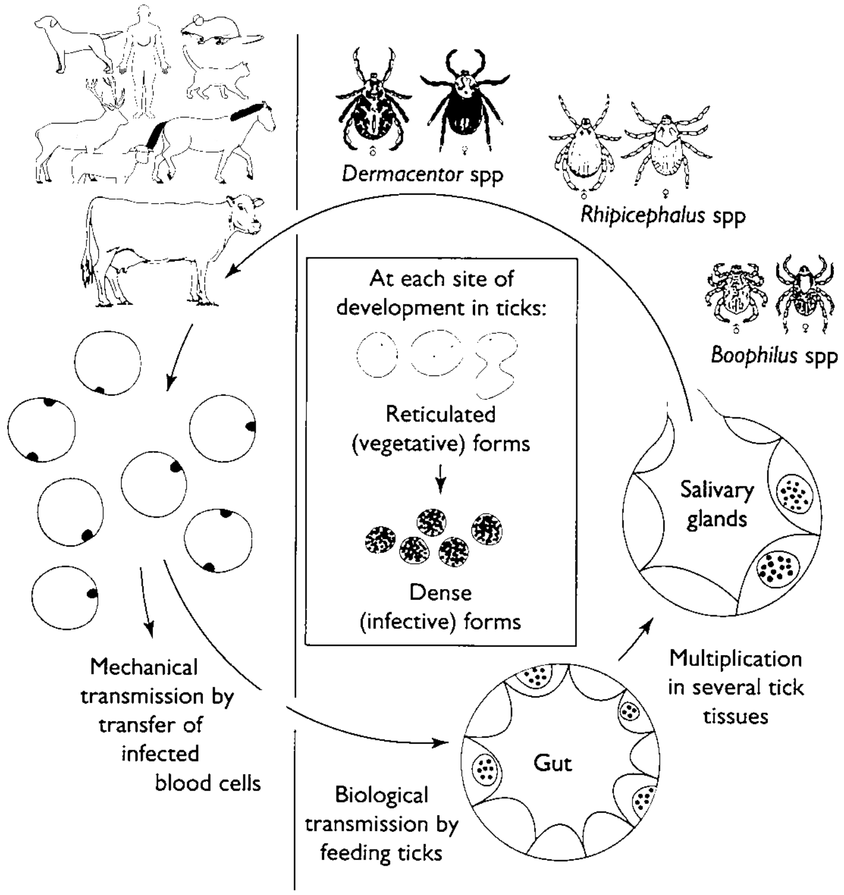

Figure 2.2: Life cycle of Anaplasma species. Modified from Kocan et al., (2003).

The main vectors, Ixodid ticks, transmit *Anaplasma* transstadially and the lack of transovarial transmission limit their role as reservoirs of infection (Stuen et al., 2013). Once infected, *Anaplasma* multiplies in the midgut and salivary glands of the ticks and a new host is infected when the tick takes its next blood meal. (Atif, 2016; Battilani et al., 2017). Different tick vectors are implicated in the life cycle of *Anaplasma* species. Important tick vectors include ticks of the genera *Rhipicephalus, Ixodes, Dermacentor* and *Amblyomma* (Rar and Golovljova, 2011; Atif, 2016; Battilani et al., 2017). However, *Anaplasma* has been detected in several other tick species although their vector competence is still under debate (Rar et al., 2021).

Transmission of *Anaplasma* is also possible through mechanical and iatrogenic means. Biting insects, e.g. horseflies are an important source of infection in cattle herds especially in regions where the only competent vectors are one host ticks. Blood contaminated veterinary fomites play an important role in transmission in the absence of sufficient numbers of biological vectors. (Kocan et al., 2000; Aubry and Geale, 2011; Kocan *et al.*, 2012)

2.2. **Anaplasmosis: Clinical Signs and Pathogenesis**

Anaplasmosis is a general term for disease caused by infection with *Anaplasma* species. The clinical presentation and pathogenesis of *Anaplasma* infection depend on the *Anaplasma* species and infected cells (Atif 2016; Rar et al., 2021).

The intraethoricytic *Anaplasma* species, *A.marginale* and *A.ovis* present with mild to severe haemolytic anaemia, fever, pale mucous membranes, jaundice, brownish urine, decreased milk production, abortion, hyper excitability, weight loss and mortality during acute phase of the infection (Aubrey and Geal , 2011; Atif et al., 2015; Rar et al., 2021). The clinical signs result from the massive phagocytosis of infected erythrocytes by the reticuloendothelial system (Aubrey and Geal., 2011). *A.marginale* infection in young animals and calves infection is often subclinical but is associated with severe illness and poor outcomes in older cattle (Kocan et al., 2003; Aubrey and Geal , 2011; Rar et al., 2021). The control methods for anaplasmosis include; control of movement of infected animals, elimination of carrier cattle by treatment, vector control, prevention of iatrogenic transmission, vaccination and antibiotic prophylaxis in endemic areas (Kocan et al., 2012; Aubrey and Geal , 2011). Tetracylines are used for treatment of anaplasmosis in cattle (Kocan et al., 2012; Aubrey and Geale , 2011).

Anaplasma platys infects platelets and causes a clinical syndrome in dogs originally named canine infectious cyclic thrombocytopenia (CICT, (Atif, 2016; Ben Said et al., 2019). Infection in dogs can be subclinical or severe with symptoms including lethargy, fever, anorexia, and bleeding disorders (Atif, 2016; Ben Said et al., 2018; Rar et al., 2021). Highly pathogenic strains of *A. platys* cause serious uveitis and pancytopenia after direct or autoimmune damage to platelets (Rar et al., 2021). In humans, clinical signs of *A.platys* infection are non-specific from headaches and muscle pains (Arraga-Alvarado et al., 2014) to one reported case of migraines and progressively severe seizures (Rar et al., 2021). Doxycycline is the drug of choice for treatment (Atif, 2016)

Anaplasma bovis infects monocytes of diverse hosts and has been detected in both domestic and wild ruminants (Atif, 2016; Rar et al., 2021). In stressed or naive cattle, infection is characterized by fever, drop in milk production, weakness, obvious weight loss, pale mucous membranes, enlargement of prescapular lymph nodes and death (Atif, 2016). There is currently no prescribed treatment for *A.bovis* anaplasmosis (Atif, 2016; Rar et al., 2021).

Anaplasma phagocytophilum infects the granulocytes and is the agent of human granulocytic anaplasmosis (HGA), equine granulocytic anaplasmosis (EGA) and tick-borne fever (TBF, (Ben Said et al., 2018; Rar et al., 2021). Specific strains and variants are responsible for illness in specific hosts (Battilani et al., 2017; Rar et al., 2021). The cells most often infected are the eosinophils and neutrophils (Rar et al., 2021).

TBF is a disease of domestic ruminants characterized by fever, weakness, anorexia, abortion, and a drop in milk production. It is significant problem in Europe and has been reported in Africa, China and South Korea (Ben Said et al., 2018; Fernandes et al., 2019; Rar et al., 2021). It infects cattle, sheep and goats with varying degrees of severity (Rar et al., 2021). TBF is a particular nuisance due to its immmunosuppressive effect and infected animals are prone to *Staphylococcus aureus* induced tick pyemia which can result in high rates of mortality (Atif, 2016). Sheep harbour the pathogen for years and are excellent reservoirs for *A.phagocytophilum* (Rar et al., 2021).

EGA is more widely distributed with cases recorded in the USA, Canada, South America, Europe, Asia, and Africa (Ben Said et al., 2018; Rar et al., 2021. It is an acute febrile illness with clinical symptoms that include high fever, depression, anorexia, ataxia, icterus, and lower limb edema (Atif, 2016; Rar et al., 2021. HGA is an emerging zoonotic disease with increasing incidence in USA and Europe with clinical manifestations ranging from mild febrile illness to severe fever (Atif, 2016; Ben Said et al., 2018; Rar et al., 2021). HGA often presents with non-specific fever, chills, headache, and myalgia (Rar et al., 2021). Oxytetracyline is effective in horses and other ruminants and HGA responds to oxytetracycline, rifampin, doxycycline, hyclate and levofloxacin (Atif, 2016)

2.3. ***Anaplasma* in Livestock**

The species of *Anaplasma* that cause disease differs between cattle and small ruminants although either can be accidental hosts of multiple *Anaplasma* species e.g. *A. ovis* has been detected in cattle (Rar et al., 2021). The implication of these findings on the epidemiology of anaplasmosis is still under study. (Fernandes et al., 2019; Rjeibi et al., 2018) Cattle can be infected by several *Anaplasma* species, namely; *A. marginale, A. centrale, A.bovis, A.phagocytophilum* and to a lesser extent, *A.ovis* and *A.platys.* Infection with *A.ovis* and *A.platys* are asymptomatic and rare (Atif, 2016; Rar and Golovljova, 2011).

A.marginale is one of the most prevalent tick-borne diseases of cattle in the world and results in massive economic losses in naive cattle (Aubry and Geale, 2011). It is of great economic importance in countries that rely on livestock production and although its impact is difficult to ascertain due to co-infections with other tick-borne diseases it is estimated to cause millions of dollars in losses per year. (Aubry and Geale, 2011; Fernandes et al., 2019)

Animals that recover from the acute phase of anaplasmosis become persistently infected and act as reservoirs of the disease (Atif., 2015; Rat et al., 2021). There are several strains of *A. marginale* differing in morphology, antigenic characteristics, protein sequence and biological transmission (AL-Hosary et al., 2020; de la Fuente et al., 2005;de la Fuente et al., 2003).

The presence of multiple strains of *A.marginale* in a herd indicates unrestricted movement of cattle and tick vectors and has implications for control of the disease (AL-Hosary et al., 2020; Fernandes et al., 2019).

Of the other *Anaplasma* species, only *A.bovis* and *A.phagocytophilum* cause disease in cattle (Rar et al., 2021). *A. phagocytophilum* causes tick-borne fever in domestic ruminants which is a problem in Europe and although the agent has been isolated in cattle in Africa, there are no reports of clinical disease in domestic ruminants (Atif et al., 2015; Atif et al., 2016; Rar et al., 2021). Teshale et al., 2018, sampled cattle from 6 African countries and recorded a prevalence of 2.73%. Although *A.bovis*, like *A.phagocytophilum* can infect a wide range of animals, it causes clinical disease only in cattle (Rar et al., 2021).

A.centrale does not cause symptomatic infection and its presence in cattle prevents the development of severe *A.marginale* anaplasmosis (Mtshali et al., 2007). For this reason, it is used as a live vaccine in select countries to prevent severe anaplasmosis e.g. South Africa.

An intraerythrocytic pathogen of small ruminants, *A.ovis* is often considered a disease of importance in poor countries (Belkahia et al., 2015; Renneker et al., 2013).

2.4. *Anaplasma* in Wildlife

Although *Anaplasma* can infect a wide host of wild vertebrates, the role of wild animals in the epidemiology of anaplasmosis is still under study (Berggoetz et al., 2014; Beyer and Carlyon, 2015; Dugat et al., 2015; Mtshali et al., 2015).

A.marginale has been detected in several wild animals in Africa including wildebeest, sable antelope, buffaloes, impalas and giraffes (Worthington and Bigalke, 2001). Interestingly, only giraffes have been shown to have clinical disease. Water buffalo and African buffalo have been explored as reservoirs of *A. marginale* (Kocan et al., 2000; Rar and Golovljova, 2011).

In a study in Zambia, *A.marginale* was detected in one free-ranging impala and one puku on a game ranch. However this study relied on microscopic examination of blood smears which is not as sensitive as molecular methods. (Munang'andu et al., 2012) Additionally, *Anaplasma* species have been detected in non-human primates in the country (Nakayima et al., 2014). Although they are few documented studies in wildlife, the evidence points to the sylvatic circulation of *Anaplasma* species in the country.

The distinction between veterinary and zoonotic *A.phagocytophilum* variants based on phylogenetic analysis of the *groEL* and *ankA* genes makes it possible to determine animal reservoirs of *A.phagocytophilum* (Atif, 2015; Atif, 2016; Di Domenico et al., 2016). In the USA and Europe, the red deer, roe deer and white-tailed deer have been determined to be important reservoirs for both zoonotic *A.phagocytophilum-ha* and veterinary *A.phagocytophilum*-v1 (Atif, 2016; Olafson et al., 2018; Rar et al., 2021). Studies of *A.phagocytophilum* in wildlife in Africa are limited but it has been detected in non-human primates in Zambia (Nakayima et al., 2014).

A.bovis has been detected in roe deer, red deer, buffalo, marsh deer, sika deer, Korean water deer, Mongolian gazelle and some non-ungulates (Amer et al., 2019; Atif, 2016). In South

Africa, the insectivores - Eastern rock sengis, have been proposed as a reservoir of *A.bovis* (Harrison et al., 2013). There isn't much data on this species in wildlife on the rest of the continent and so far it has been detected only in cattle outside of South Africa (Ben Said et al., 2018; Fernandes et al., 2019; Rjeibi et al., 2018).

Studies of *A.ovis* in wildlife are rare all over the world but it has been detected in wild ruminants e.g. roe deer, red deer, bighorn sheep, sika deer and mule deer (Rar et al., 2021).

2.5. ***Anaplasma* in Humans**

Being genetically diverse, pathogenicity of *Anaplasma* in humans is thought to depend on the infecting strain and not species (Stuen et al, 2013; Dugat *et al.*, 2015; Matei *et al.*, 2019). So far, this has been extensively studied only for *A.phagocytophilum* which is an important emerging zoonosis in Europe and the USA (Stuen et al., 2013; Matei et al., 2019; Rar et al., 2021). The variant or ecotype of *A.phagocytophilum* that infects humans, *A.phagocytophilum-ha,* is distinct from the veterinary variant *Ap-v1* (Atif, 2015; Dugat et al., 2015; Rar et al., 2021). Currently, no human illness has not been reported in Africa but there is evidence of zoonotic *Anaplasma* in animals (Fernandes et al., 2019; Teshale et al., 2018; Vlahakis et al., 2018).

While only *A.platys* and *A.phagocytophilum* are recognized as zoonotic *Anaplasma* species, symptomatic infection with other species has been demonstrated in humans (Arraga-Alvarado et al., 2014). A single human case of non-fatal 11 day-fever with *A.ovis* was reported in Cyprus (Chochlakis et al., 2010) and human infection with a candidate species, *A.capra* has been reported in China (Beyer et al., 2015; Yang et al., 2018).

2.6. **Interplay between wild and domestic animals**

In 1935, Nietz successfully infected black wildebeest with *A.marginale* from cattle demonstrating that infection can occur between domestic and wild animals. Thomas et al., (1982) inoculated sheep and cattle with *Anaplasma* species from sable antelope which were morphologically similar to *A.marginale*. The experiment resulted in no infection in splenectomized cattle and asymptomatic infection in one sheep. Studies of *Anaplasma* in modern times are conducted at molecular level to determine phylogenetic relationships and infer transmission patterns and transmissibility between animal hosts (Atif, 2015; Berggoetz et al., 2014; Matei et al., 2019; Neitz, 1935; Thomas et al., 1982).

In a study done in Botswana there was a significant difference between prevalence of *A.marginale* and other tick-borne diseases between buffalo from the Okavango Delta (OD) and Chobe National Park (CNP). Two major differences were noted; the OD has water all year round and there is no mixing of buffalo herds while buffalo herds from Zambia, Zimbabwe and Botswana mix freely in CNP at the Chobe river in the dry season. Additionally, the OD is cordoned off from cattle by a game fence while in the CNP cattle and wildlife mix freely. This exemplifies the risk of interspecies transmission of pathogens at a wildlife-livestock interface (Eygelaar et al., 2015)

Tonetti et al., 2009 detected *A.marginale* in a captive gemsbok in a wildlife reserve in an area of South Africa with *A.marginale* endemic in cattle. The gemsbok were originally from an area where *A.marginale* is not present. This susceptibility of gemsbok to infection and their infestation with multiple species of competent *A.marginale* vectors give rise to the risk of spreading *A.marginale* from cattle to gemsbok and into non-endemic areas. (Tonetti et al., 2009).

Khumalo et al, (2016) notably found no difference in infection rates and genetics between cattle from cordoned and non-cordoned areas suggesting that direct contact is not necessary for transmission. Smaller mammals especially rodents and birds have been suggested elsewhere to be involved in spreading *Anaplasma* across large areas. (Harrison et al., 2013; Khumalo et al., 2016; Matei et al., 2019).

Wild ungulates are competent hosts for many tick-borne diseases and since tick-control is impractical for wildlife, their presence in an area poses a risk for tick-borne diseases (Dugan et al., 2006; Eygelaar et al., 2015; L'Hostis and Seegers, 2002; Tonetti et al., 2009). With the expansion of conservation practices and game farming on the African continent, there is an increasing wildlife interface forcing contact between livestock and potential disease reservoirs (Munang'andu et al., 2012).

2.7. **Detection and Characterization of *Anaplasma* spp.**

2.7.1. **Microscopy**

The oldest and routinely used detection method is examination of Geimsa stained thin blood smears. Like all microscopic methods, this method is more sensitive during acute disease and with high parasite load (Atif, 2016). In Geimsa stained thin film blood smears, *A. marginale,* and *A. centrale* appear as dense, deep purple, vacuole-bound, near-circular inclusion bodies, with a diameter ranging from 0.3 to 1 µm (Hove *et al.*, 2018, (Figure 2.3).

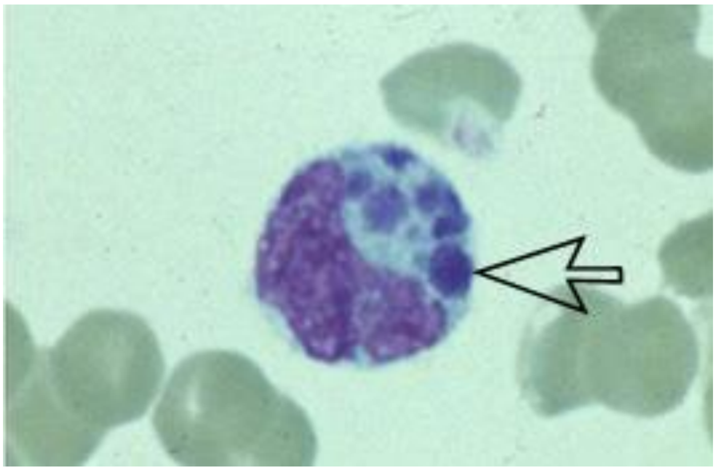

Figure 2.1: Anaplasma phagocytophilum morula in an immature granulocyte. Source: http://www.cdc.gov/anaplasmosis/symptoms/index.html.

Microscopy has a low sensitivity but is the most widely available diagnostic test and is still in use (Aubry and Geale, 2011; Ben Said et al., 2018; Hove *et al.*, 2018; AL-Hosary *et al.*, 2020).

2.7.2. **Serological tests**

Major surface protein 5 (Msp5) based enzyme-linked immunosorbent assay (ELISA), complement fixation and the card agglutination test (CAT) are genus specific serological tests used for *Anaplasma* detection (Aubry and Geale, 2011; Kocan et al., 2015). Msp5 competitive ELISA (cELISA) has medium sensitivity, cannot distinguish between *Anaplasma* spp and has a moderate chance of false negatives (Khumalo *et al.*, 2016). CAT is sensitive for *A. marginale* and gives results within minutes, and is therefore used in field conditions to screen for anaplasmosis in cattle herds (OIE Manual, 2018). CAT however has the problem of non-specific reactions, interpretation of results and reproducibility since preparation of the antigen which is the suspension of *A. marginale* varies from batch to batch and laboratory to laboratory (Battilani *et al.*, 2017). Since members of the family *Anaplasmataecae* share antigenic properties, genus wide *Anaplasma* serological tests also react with other members of the family e.g. *Erhlichia* species. Therefore, cross reactivity, serological tests that target specific *Anaplasma* species e.g. *A.marginale* are preferred, as genus wide tests have lower specificity (Ybañez and Inokuma, 2016; Battilani *et al.*, 2017; Hove et al., 2018).

2.7.3. **Molecular Methods**

A number of molecular methods have been developed over the last two decades for the detection of *Anaplasma* species. These include conventional polymerase chain reaction (PCR), nested PCR (nPCR), quantitative real-time PCR (qPCR) and a reverse line blot hybridization (RLB) assay. The sensitivities of these assays depend on the molecular target used (Rar et al., 2021). The RLB hybridization assay has been used extensively for the routine screening of cattle and wildlife samples in South Africa and has the advantage of detecting *Anaplasma, Ehrlichia, Babesia* and *Theileria* parasites in a single reaction (Atif, 2016; Battilani *et al.*, 2017; Hove et al., 2018).

Due to the high variability of genes from species to species, no single PCR assay is specific for all *Anaplasma* (Battilani et al., 2017). Nested PCR assays are widely used to combat this problem with a non-specific 16S rRNA in the first reaction and several species specific internal primers in the nested reaction. However, this approach sometimes results in false negatives due to sequence differences in the forward and reverse priming regions e.g. in certain *A. marginale* strains (Hove et al, 2017). In South Africa, Hove at al., (2017) determined qPCR to be the most sensitive PCR assay for detecting *A. marginale* and *A. centrale.*

2.8. **Molecular epidemiology**

2.8.1. **Genomic features of *Anaplasma* species**

The complete genome of *Anaplasma* has been characterized for all species except *Anaplasma bovis* (Rar et al., 2021). It is a single circular chromosome between 1.2 to 1.5kb which encodes between 949 (*A.marignale*) to 1411 (*A.phagocytophilum*) genes. (Battilani *et al.*, 2017; Rar et al., 2021). This small genome size compared to other bacteria is attributed to the fact that the host cell fulfills some of the bacterial functions (Battilani *et al.*, 2017). Several targets are used for multi locus and single locus genetic studies (Atif., 2015: Rar at al., 2021).

2.8.2. **Gene Targets for single locus genetic studies**

2.8.2.1. 16S rRNA

Gene sequence analysis of the 16S rRNA gene is widely used for bacterial taxonomy and to determine novel bacterial species (Janda and Abbott, 2007). In Rickettsiales, this gene is highly conserved and PCR based amplification is non-specific (Battilani *et al.*, 2017). Although studies of the 16S rRNA gene provide limited information on species strain diversity particularly for *A. marginale and A. centrale,* it is adequate for interspecies differentiation (Belkahia et al., 2015; Hove et al., 2018; Inokuma et al., 2001). Interestingly the differentiation between the zoonotic and animal variants of *A. phagocytophilum*, (*Ap-ha* and *Ap-v1*) are based on a two nucleotide difference in the 16S rRNA and msp4 gene (Dugat et al., 2015).

2.8.2.2. Genes encoding major surface proteins

Much of what is known about the major surface protein coding genes is derived from studies of *A. marginale* and *A. phagocytophilum* (Battilani et al., 2017). There is limited information on *msps* from *A. centrale, A. ovis* and *A. platys* and none have been published for *A. bovis*. Of importance to genetic diversity studies of *A. marginale* are the *msp1* complex, *msp2* and *msp4* (Atif, 2015; Battilani et al., 2017; Hove et al., 2018; Khumalo et al., 2016).

msp1α is extensively used for strain typing of *A. marginale* and its ortholog *msp1αS* in *A. centrale* (Hove et al., 2018; Khumalo et al., 2016). The pattern of tandem repeats at the 5' end of the gene differs from strain to strain and makes it useful in diversity studies and to elucidate host pathogen interactions and co evolution (Hove et al., 2018). However, it has limited use in phylogeography and the msp1β is preferred for phylogenetic analysis of strains (Rar et al., 2021). Because *A. marginale* shows genotypic exclusion of closely related strains in cattle and ticks, the co-existence of multiple *A. marginale* strains in a herd suggests importation of new genotypes through the unrestricted movement of infected cattle or ticks (Batillani et al., 2017).

Unlike *msp1, msp2* has orthologs in all studied *Anaplasma* species. Antigenic variation of *msp2* in *A. marginale* occurs when cattle and ticks become persistently infected allowing it to elude the immune system and contributing to maintenance of infection (Atif, 2016; Battilani et al., 2017; Junsiri et al., 2020). For this reason, genetic variation in *msp2* provides information on the endemicity of *A. marginale.* Phylogenetic analysis of *A. phagocytophilum msp2* is important to differentiate between strains from ruminant and non-ruminant hosts (Rar et al., 2021).

The *msp4* is a highly conserved single gene with orthologs in all *Anaplasma* species which provides limited phylogeographic information in certain regions (Battilani et al., 2017). Phylogenetic studies of *msp4* in *A. marginale* and *A. phagocytophilum* in the USA have provided geographic information but studies across multiple countries could not provide the same geographic discrimination between strains (Atif, 2016). They could, however, segregate geographically at species level. *Msp4* therefore has limited phylogeographic use within certain world regions (Atif, 2016; Fernandes et al., 2019; Hove et al., 2018; Ruybal et al., 2009).

2.8.2.3. groESL operon

Spanning a region encoding heat shock proteins *groEL* and *groES*, the *groESL* operon called the bacterial common antigen, has the same basic structure in all bacteria and is a useful molecular clock (Battilani et al., 2017) The reorganization of the *Anaplasma* species was partly based on the *groEL* and it was used in the first molecular phylogenetic study of *A. bovis* (Battilani et al., 2017; Rar et al.,2021). It is a popular single locus target for genetic diversity studies of *A. marginale* and *A. phagocytophilum.* Using the *groEL,* four *A. phagocytophilum* ecotypes have been identified with ecotype I exclusively zoonotic and ecotype II containing variants from domestic and wild ruminants among other animals (Atif, 2015, 2015; Battilani et al., 2017; Ben Said et al., 2019; Rar and Golovljova, 2011).

2.8.2.4. AnkA gene

Ankyrin (*Ank*) genes are broadly conserved in *Anaplasma* and *Ehrlichia* species and have been described in *A. marginale, A. ovis* and *A. phagocytophilum* (Atif, 2015, 2016; Battilani et al., 2017). Ankyrins are a family of scaffolding proteins that link the membrane-bound proteins to the underlying cytoskeleton and play a role in pathogenesis of bacteria (Al-Khodor et al., 2010). The strain variable *AnkA* gene of *A. phagocytophilum* is believed to play a role in pathogenesis in animals and humans and in host specific adaptations (Battilani et al., 2017; Rar et al., 2021; Stuen et al., 2013)). In 2017, five *ankA* clusters of *A. phagocytophilum* had been identified with the first cluster including variants from domestic and wild ruminants, humans, dogs and cats. Interestingly clusters 4 and 5 include variants from single host species i.e. roe deer and rodents respectively (Battilani et al., 2017). By 2021, 12 distinct clusters had been described and this host specific clustering of the *ankA* gene provides valuable information for *A. phagocytophilum* studies (Rar et al., 2021).

2.8.3. Multi-locus Sequence Typing (MLST)

Multi-locus sequencing is based on PCR amplification and sequencing of several loci that encode housekeeping genes (Battilani et al., 2017). Generally these loci consist of seven genes with a slow molecular clock (Battilani et al., 2017; Rar et al 2021). Two MLST schemes are available for *A.phaocytophilum* (Rar et al 2021). One scheme contains the nine loci ie. a*nkA, groESL, gyrA, msp4, pled, polA recG, typA,*and an intergenic regions (Chastagner et al., 2014: Rar et al., 2021). It identified 3 clusters of *A.phagocytophilum* with 3 epidemiologic cycles Another spans seven loci with the housekeeping genes, *atpA, dnaN, glyA, fumC, mdh , pheS, and sucA* and delineates *A.phagocytophilum* into 311 sequence types in 8 clusters (Huhn et al., 2014; Rar et al., 2021).

2.8.4. Whole Genome Sequencing of *Anaplasma* spp

In the study of *Anaplasma,* whole genome sequencing (WGS) is most often used for analysis of *A.phagocytophilum* strains (Battilani et al., 2017). In order to understand the genetic differences that determine *A.phagocytophilum* wide host range and pathogenicity, WGS is applied (Battilani et al., 2017; Rar et al., 2021). Using WGS it has been determined that zoonotic strains have conserved genome structures and the *drhm* gene locus has been identified as a potential genetic marker for human-infective strains (Battilani et al., 2017).

2.9. Role of Knowledge, Attitudes and Practices Survey in *Anaplasma* control

Knowledge, Attitude and Practices (KAP) surveys are questionnaire based quantitative method that are often used to measure the extent of a known situation and establish baselines for future assessments. KAP can be used as a stand-alone epidemiological tool or to complement information obtained from other types of studies e.g laboratory based studies or interventions. Questionnaires provide quantifiable insights from a target group and are particularly useful in assessing risks and determining risk behaviors. Regarding tick-borne diseases, control is hinged on the preventive practices to maintain a disease free herd and prevent spread of infection (Kocan et al., 2000; Aubrey and Geale , 2010). In developing countries, due to economic constraints, control of livestock diseases has fallen on the livestock owner (Sungirai et al., 2016). Since farmers play a pivotal role in the control and spread of livestock diseases it is necessary to understand the baseline knowledge and practices. This information, added to the detection of *Anaplasma* spp in sable antelope, would ascertain the possibility of spillover of disease from wildlife by determining the farmer's readiness to respond to, and prevent and anaplasmosis outbreak.

2.10. **Knowledge gaps**

The genus *Anaplasma* is broad and with the differences in pathogenicity, virulence, wide range of hosts and vectors, there are a lot of gaps in our understanding of their epidemiology.

Because of their ability to asymptomatically infect several animal species, the search for animal reservoirs of *Anaplasma* has had limited success. While some wildlife, e,g. buffalo for *A.marginale,* white footed mice for *A.phagocytophilum* have been conclusively named as reservoirs, they are not the only players in these ecosystems (Dugan et al., 2006; Miranda et al., 2021). Because of the involvement of different tick and vertebrate hosts it is difficult to establish complete and detailed transmission cycles in any ecosystem.

Concerning tick-vectors, although *Anaplasma* has been demonstrated in ticks of several genera, the vector competence of some is still in doubt (Rar et al., 2021). Additionally, in some ecosystems vector transmission appears to be secondary to mechanical transmission (Dahmani et al., 2017; Mtshali et al., 2015; Rar and Golovljova, 2011).

When it comes to pathogenicity and virulence, very little is understood about *Anaplasma* (Battilani et al., 2017). It is however important to understand the difference in pathogenicity of domestic versus wildlife derived strains particularly in areas of widening wildlife-livestock interface. As much as infectivity across domestic and wildlife has been demonstrated, the transmission cycles that would be involved in spillover and spillback events in nature are incomplete (Aubry and Geale, 2011; Battilani et al., 2017; Kocan et al., 2015).

While the movement of animals, domestic or wild, between tick-free and tick-infested areas are a significant risk factor for *Anaplasma* infection, there is hardly any attention paid to the risk posed by movement of wildlife in Africa (Eygelaar et al., 2015; Munang'andu et al., 2012; Olafson et al., 2018). For example, a risk analysis report for the importation of sable antelope from Zambia to South Africa did not include analysis of risk of *Anaplasma* spp (Sergant E., 2014). Overall, *Anaplasma,* though endemic in Zambia is not studied extensively and data on the circulating species, wildlife reservoirs, risk factors, economic impact and molecular epidemiology of this important tick-borne disease is scanty (Makala et al., 2003; Nakayima et al., 2014; Simuunza et al., 2011; Vlahakis et al., 2018).

This study thus aimed to identify and characterize *Anaplasma* species and explore the role of sable antelope as possible reservoirs of the disease. With the increase in ex-situ wildlife-livestock interface due to conservation practices and game ranching, knowledge of pathogens carried by wildlife will add to the concerted effort to prevent the spillover of pathogens from wildlife to humans and livestock. Furthermore, this information will provide a basis for further study into the molecular epidemiology of *Anaplasma* species and its impact on livestock production.

CHAPTER THREE

3. MATERIALS AND METHODS

3.1. Study Area

This study was carried out with samples from Kasonso Busanga game management area (GMA, (North Western Province) and Chongwe District (Lusaka Province).

Kasonso Busanga GMA (E 25°00' to E 26°15' and S13°45' to S14°30') located in the northwestern part of the Kafue National Park (Figure 3.1) is part of the greater Kafue ecosystem. This area is characterized by open grasslands and plains, dense thickets, miombo woodland and swampy areas. The GMA is also endowed with a diverse and large number of wildlife species that include elephants, Hippopotamus, Warthog, Buffalo, Puku, Impala, Oribi, Reedbuck, Lechwe, Sable, Roan, Hartebeest, Waterbuck, Zebra, Eland, Wildebeest, Lion, Leopard and Duikers. The greater Kafue ecosystem is the primary source of wildlife for stocking game ranches and reserves and is also a hot spot for arthropod-borne diseases (Lindsey et al., 2016). Lusaka National Park (E28°20' to E28°26' and S15°31' to S15°33') on the other hand, is a small wildlife reserve in Lusaka District bordering Kafue and Chongwe districts. Chongwe District (Figure 3.1), in particular, is a peri-urban district with the majority of residents living in homesteads and relying on subsistence and livestock farming (Pinchoff et al., 2015: Squarre et al., 2020). Chongwe has a mix of traditional, smallholder and commercial cattle farmers rearing goats, sheep, pigs and game animals, with a majority of traditional farmers rearing goats alongside cattle.

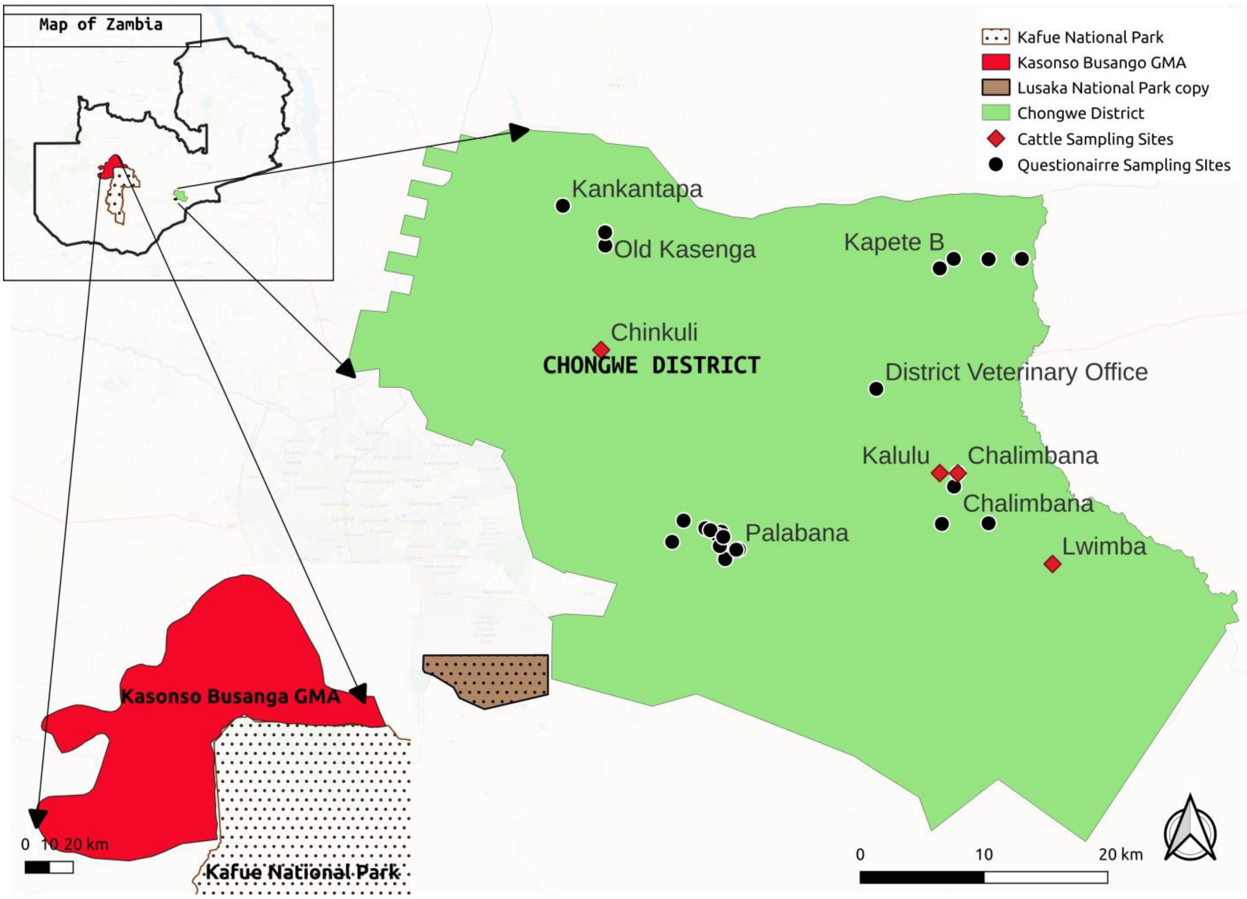

Figure 3.1: Map of Zambia showing its provinces and areas sampled. On the map of Chongwe district (green), red diamonds represent cattle samples, black circles are areas where farmers were sampled. Sable antelope were sampled from Kansoso Busanga game management area (red) * Drawn with QGIS software 2022

3.2. Sampling Methods

3.2.1. Cattle and Sable Antelope

This molecular analysis was carried out on archived DNA samples, stored at -80C, of cattle blood previously collected from Chongwe district in 2017 and sable antelope blood from antelopes that were translocated from Kasonso Busanga Game Management area, North Western Province to Lusaka National Park in 2016. Antelope blood was collected prior to their relocation to Lusaka National Park and are thus being considered in this study as a sample of North Western Province.

3.2.2. Farms/ Households

Among the Veterinary camps in Chongwe District, those that were reported to have sporadic to epidemic cases of anaplasmosis were selected. These included, were possible, the areas from which the cattle blood was collected in 2017 (Chalimbana, Chinkuli, Kalulu and Lwimba, Figure 3.1).

In these identified areas, farms and households where cattle are reared were selected at random. If a household/farm was unable or unwilling to participate the next household/farm available within the same area or village was selected. Some farmers were interviewed from local dip tanks.

The questionnaire had 46 questions that collected information on knowledge of farmers on tick-borne diseases and anaplasmosis, history of *Anaplasma* outbreaks in the area, contact patterns of livestock and humans with wildlife in the area and farm management practices that may be associated with occurrence of *Anaplasm*a in cattle (Appendix F). Respondents cames from Palabana, Chalimbana, Kanakantapa, Kepete B and Kasenga. Clarifying statements and respondents recommendations were noted in writing were applicable e.g reasons given for the quality of vet services. Knowledge was assessed on the basis of questions about treatment, prevention and health effects. Awareness was defined as the respondent being aware of the existence of the disease e.g answering yes to the question, 'Have you ever heard of anaplasmosis or gall-sickness in cattle?'

3.3. Sample Size

This study utilized purposive sampling such that no statistical calculation was used to determine sample size. For the molecular study, all 100 available DNA samples, 57 cattle and 43 sable antelope, from the areas of interest that were in storage at UNZA, School of Veterinary Medicine were included. Additionally, farmers and livestock herders were purposively selected from (1) areas with reports of suspected anaplasmosis according to the District Veterinary office and (2) areas from which the cattle samples originated.

Sample Processing

3.3.1. DNA extraction from cattle and sable antelope whole blood.

DNA was extracted from archived whole blood in EDTA tubes collected in September and October 2016 from wild sable antelopes (n=47) to facilitate their translocation from the Kafue National Park to Lusaka National Park, 474 km away (Figure 3.1) and whole blood from cattle (n=53), collected from Chongwe district in February 2017 as part of routine surveillance of tick-borne diseases (Figure 3.1). Whole genomic DNA (n=100) was then extracted from whole blood using a commercial DNA extraction kit (Thermo Scientific, CA, USA) according to the manufacturer's instructions and stored at −20°C.

3.3.2. PCR Detection of Anaplasmateacae

For detection of Anaplasmateacae, primers ANA- F 5' - 3' and ANA-R 5' - 3' targeting the 16S rRNA variable region 1 as described by Giudice et al., (2012) were used (Table 3.1) This primer set is non-specific for members of the family Anaplasmataceae including *Ehrlichia, Wolbachia* and *Neoricketssia* (Stuen et al., 2013). Therefore, samples detected by PCR that were not be confirmed by sequencing were classified in this study as unidentified Anaplasmataceae.

Table 3.1: Primer Details

Primer name	Sequence	Ta	Amplicon size	Reference
ANA -F	5'CAGAGTTTGATCCTGGCTCAGAACG'3	48°C	421bp	Stuen et al.,
ANA-R	5'GAGTTTGCCGGGACTTCTTCTGTA'3			

Conventional PCR was performed on ABI Biosystems' Veriti™ 96-Well Fast Thermal Cycler following the protocol for OneTaq® 2X Master Mix with Standard Buffer (New England Biolabs) (Appendix I). All PCR reactions were made up to a final volume of 20 μL which contained 1x PCR buffer, 2.5 mM MgCl2, 0.16 mM dNTP's premixed, 0.04 μM of each primer, and 0.75 units of Taq Polymerase. Amplification was performed with an initial denaturation at 95˚C for 1 minute, followed by 35 cycles of 95˚C for 30 seconds and annealing for 30 seconds at 48˚C. Extension was done at 72˚C for one minute with a final extension at 72˚C for 10 minutes.

3.3.3. Separation of *Anaplasma* 16S rRNA amplicons

PCR products were separated on 1.5% agarose gel stained with ethidium bromide. Four microliters (4 uL) of a 100bp DNA ladder was loaded into the first well of the gel. Molecular grade water was used as a no-template control (NTC) and loaded into the last well. Finally, four (4 uL) of PCR product was loaded in the rest of the wells. Although it is good practice to include a positive control or quantitative standard for the PCR reaction it is not absolutely required (Lorenz et al., 2012). As no positive controls were available no PCR positive control was added. However, the DNA ladder would act as a positive guide for the success of gel electrophoresis. The agarose gels were run for 30 minutes and bands visualized under a UV light using the benchtop BioDoc-It^{TM} Imaging system (Analytik Jena, US). The specimen band sizes were compared to the 100 bp DNA ladder in well M (Figure 4.1 and 4.2). The expected band size was 467bp.

3.3.4. PCR Product Purification

PCR products were purified with the Zymo Research DNA Clean and Concentrator kit (Zymo Research, USA) according to the manufacturer's instructions. Briefly, DNA binding buffer was added in a ratio of 5:1 of the PCR product followed by two wash steps with 200ul of washing buffer. Afterwards, 30ul of DNA elution buffer was added to the spin columns which were then incubated at room temperature for one minute. The purified DNA was collected in sterile, labeled 1.5ml eppendorf tubes. To ensure DNA concentration and purity were in range of the Big Cycle Terminator v3.1 cycle sequencing kit recommended optimum (3-10 ng0 the purified amplicons were tested using the NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, USA)

3.3.5. Cycle Sequencing of 16S rRNA gene

Purified DNA was subjected to cycle sequencing with the Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA) according to the manufacturer's instructions. Briefly, for a final reaction volume of 20 uL, 5 uL of DNA template was added to Big Dye mix, 5X Sequencing buffer, primer and deionised water. Forward or reverse primer was added to the relevant tubes. The thermocyler conditions were set to a 45 second incubation at 96˚C, and 28 cycles of denaturing at 96˚C for 10 seconds, annealing at 50˚C and extension for two minutes at 60˚C. After successful cycle sequencing, 2 uL of 0.125 mM EDTA and 3M sodium acetate were added to each tube and after mixing by tapping, 90 uL of 100% ethanol were added. Following incubation to 10 minutes in the dark, at room temperature, the tubes were centrifuged for 20 minutes at 15000 RPM. The supernatant was carefully pipetted out and 200 uL of 70% ethanol was added, followed by a five minute centrifugation step and pipetting step. This was repeated. The tubes were wrapped in aluminium foil and dried in a vacuum dryer for 10 minutes.

3.3.6. Capillary Electrophoresis

Ten microliters of highly ionized formaldehyde (HiDi) was added to each tube. The tubes were vortexed and denatured for two minutes at 95˚C and then finally resolved on the ABI 3500 genetic analyzer (Applied Biosystems™, USA).

3.4. Molecular Data Analysis

<u>Specific Objective 1: To detect and identify *Anaplasma* from cattle and translocated sable antelope</u>

Sequence trace files were viewed in BioEdit and good chromatograms (minimum background interference and distinct peaks) were chosen for editing. Sequences were edited by trimming both ends. Sequences with unresolved base calls and/or overlapping peaks were mapped onto reference 16SrRNA sequences and manually edited. These were then assembled using BioEdit (pairwise alignment - allow ends to slide) and consensus sequences were subjected to BLAST analysis on the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm the *Anaplasma* sequence identity. Identities of above 90% and low E-values were selected as identities.

Specific Objective 2: To determine genetic diversity and relatedness of *Anaplasma* from cattle and sable antelope.

Homologous sequences were downloaded from GenBank to use as reference sequences for phylogenetic analysis. These references were aligned with study sequences using the ClustalW1.6 multiple sequence alignment algorithm in MEGA X (Kumar et al., 2018). The alignments were exported to PHYLIP format and phylogenetic analysis was done using PhyML online (Guindon et al., 2010). A maximum likelihood tree was constructed with a Generalized time reversible (GTR) model, gamma distributed rates among sites and 1000 transfer bootstrap replicates (Lemoine et al., 2018). The resulting tree was rooted with a *Wolbachia pipentis* outgroup sequence who’s 16S rRNA gene was determined to be sufficiently divergent from *Anaplasma* species but not too distant on the evolutionary tree (Thirumalapura and Walker., 2015). The tree was annotated using the Interactive Tree of Life tool, iTOL (Letunic and Bork., 2019: https://itol.embl.de).

3.5. Knowledge, Attitude and Practices Data Collection

Specific Objective 3: To assess the knowledge, attitudes and practices of cattle farmers towards *Anaplasma* in cattle and determine cattle contact with wildlife

A coded, semi-structured questionnaire was administered to selected household/farms. After deployment on the first six informants, the questionnaire was adjusted to include extra options for questions with multiple answers e.g. for a question asking what kind of cattle are prone to tick infestation, additional options of ‘any’ and ‘don’t know’ were added. The changes are highlighted in Appendix F.

3.6. Statistical Analysis

3.6.1. **Study Variables**

In the molecular study the categorical variables, *Anaplasma* detection (PCR result) and *Anaplasma* species were the dependent variables. The location or origin of the samples and the animal host or species were independent variables. In the KAP survey, variables and covariates of interest included scores for knowledge of tick-borne diseases, knowledge of anaplasmosis, knowledge of zoonotic diseases, attitude, area or veterinary camp,education level and age category. Scoring criteria is detailed in Appendix H.

3.6.2. Data Entry and Pre-processing

Data was entered into MS Excel and coded using the codebook (Appendix G). The continuous variables, age, number of cattle and attitude score were transformed into categorical variables as categories were more informative than absolute values (Appendix H). Age was split into three age categories; 16-35 years, 36 -50 years, and greater than 50 years old. Cattle holding was split into herd sizes of; 1-10, 11-20 and greater than 20 animals. A likert scale was used to record attitude and this was transformed into a score ranging from zero to six (Appendix G & H). This score was then split into three categories; favorable (4-6), neutral (4) and unfavorable (<3) attitude.

3.6.3. **Statistical Analysis**

R-Studio was used to draw contingency tables and for graphical analysis. To explore association or correlation between categorical variables, a two-sided fisher’s exact test at a 95% confidence level was applied. Associations were tested between education level and. knowledge of anaplasmosis, knowledge of anaplasmosis and area, attitude scores and area, attitude scores and knowledge of anaplasmosis and, awareness of anaplasmosis and knowledge of anaplasmosis.

3.6.4. Ethical Considerations

Ethical clearance was obtained from Excellence in Research Ethics and Science

(ERES) Converge hub (Appendix A) and the National Health Research Authority (Appendix C).

CHAPTER FOUR

4. RESULTS

4.1. Detection of *Anaplasmatacae* and *Anaplasma*

All of the one hundred archived DNA samples were tested for *Anaplasma.* Fifty-seven of them came from cattle and 43 from sable antelopes. Thirty nine were PCR positive (Table 4.1) for Anaplasmatacaea but only 14 (36%) were confirmed to be positive for *Anaplasma* species with sequencing while 25 (63%) had no or bad signal during sequencing. Four cattle samples and 10 sable antelope samples were confirmed positive (Table 4.1). Cattle positive samples (Figure 4.1) showed brighter bands on gel electrophoresis compared to sable antelope samples (Figure 4.2).

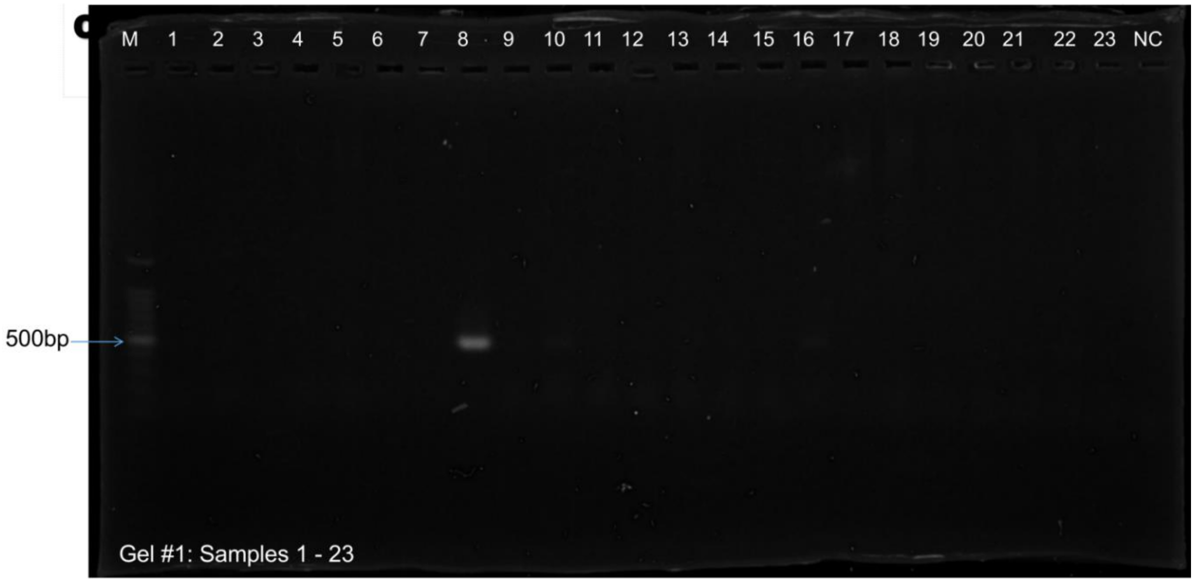

Figure 4.1:1.5% agarose gel electrophoresis of cattle antelope samples PCR products. M corresponds to the 100bp DNA ladder, NC is the negative control and lanes 1-23 are test samples. The expected band size was 467bp and lane nine shows a positive result. Lanes 1-8 and 10 - 23 were negative. The gels were stained with ethidium bromide for visualization under UV light

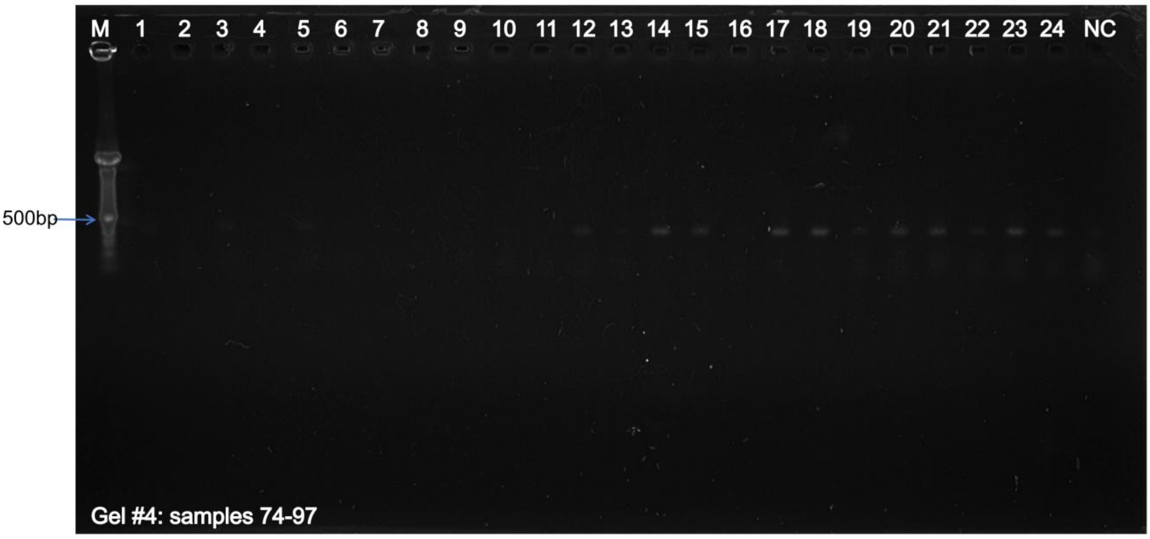

Figure 4.2: 1.5% agarose gel electrophoresis of sable antelope samples PCR products. M corresponds to the 100bp DNA ladder, NC is the negative control and lanes 1-24 are test samples. The expected band size was 467bp and lanes 12, 14-15, and 17-24 show positive results. Lanes 1-11, 13 and 16 were negative. The gels were stained with ethidium bromide for visualization under UV light.

Table 4.1: Distribution of positive molecular results by host type & Anaplasma species

	PCR Positive	Confirmed	Proportion
Cattle n=57	9 (16%)	4	7
A.marginale		4	7
Sable n=43	30 (70%)	10	23.25
A.marginale		1	2.33
A.ovis		7	16.28
A.platys		2	4.65
Total N=100	39	14	14

4.2. Phylogenetic Analysis

Phylogenetic analysis revealed that the study sequences formed four clusters with reference sequences (Figure 4.3). The majority of the sable and cattle derived sequences were delineated by clusters, except for sequence NWP72S (*A.ovis*) from sable and sequence CD65B (*A. marginale*) from cattle that formed a minor cluster with 82.2% bootstrap support. In addition, sequence NWP48S *A.platys* sequence from sable clustered with cattle derived *A.platys* sequence from India. Overall the rest of the study sequences were separated into clusters by species type regardless of the host of origin except for the above-mentioned sequences.

Figure 4.3: Phylogenetic tree of *Anaplasma* sequences based on a partial 16S rRNA gene. Phylogenetic analysis was based on the Maximum Likelihood method and General Time Reversible model (Kumar and Nei. 2000) with 1000 bootstrap replicates (shown as percentages on nodes). The scale bar indicates an evolutionary distance of 0.1 nucleotides per position in the sequence. Sequences shaded green are sable antelope derived and those in yellow are cattle derived.

4.3. .Knowledge, Attitudes and Practices Survey Results

The average distance of sampling sites from Lusaka National Park was 29.5 kilometers (95% CI = 23.21 -35.69). Fifty-seven farmers from Chongwe District responded, of whom only four were female (7%). Many of the respondents (46%) were between 16 and 35 years and had at least a basic education (Figure 4.4). The two most common purposes for cattle rearing were for draft (40%) and dairy (48%).

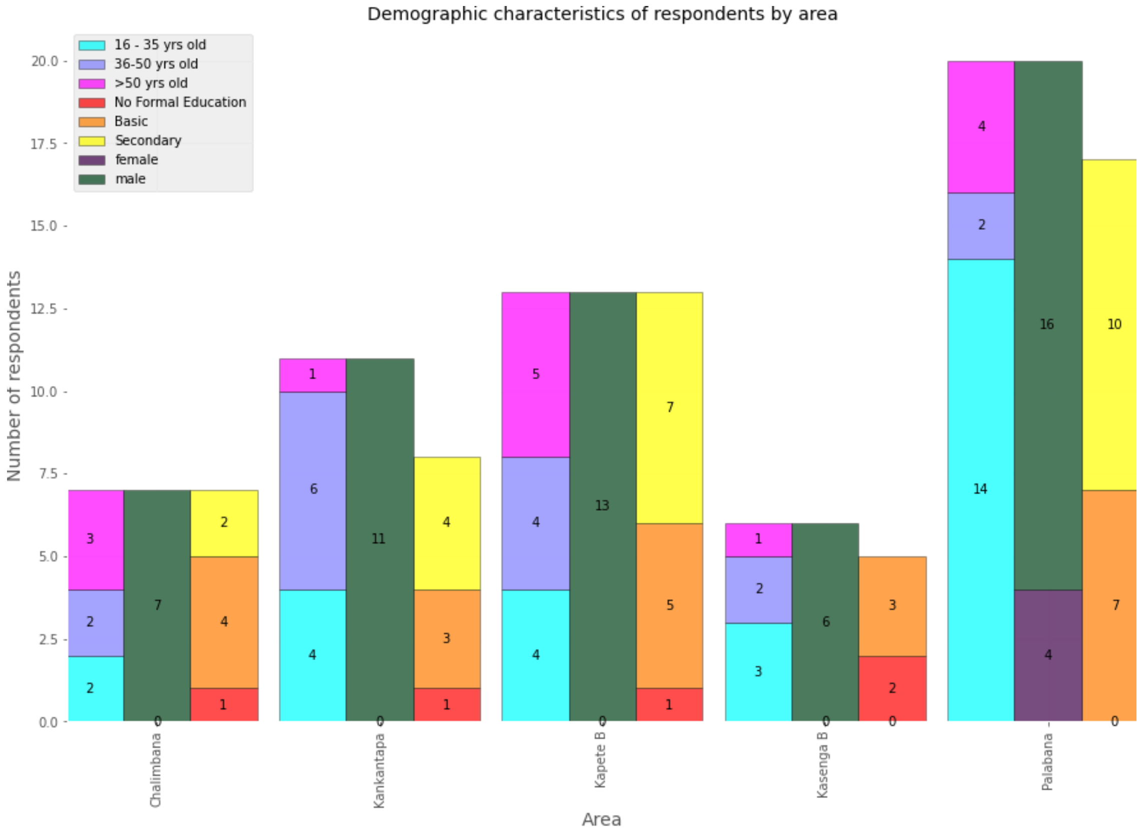

Figure 4.4: Demographic characteristics of KAP respondents. Numbers shown on bars are absolute values

4.4. **Knowledge and Awareness of Tick-Borne Diseases.**

As shown in Table 4.2, 80% of respondents were aware of tick-borne diseases but only one had adequate knowledge about TBD’s. There were no associations between awareness of TBD’s. with age (p-value=0.4995, fisher’s exact test), education level (p-value=0.5378), fisher’s exact test, cattle holding (p-value = 0.5668, fisher’s exact test) , and provision of veterinary services and knowledge (p-value= 0.0539, fisher’s exact test). As only one farmer had adequate knowledge of TBD’s no test of association could be measured i.e zero values in contingency table.

Table 4.2:KAP Respondents Awareness and Knowledge of TBDs and Anaplasmosis

Variable	**Response**	
Awareness of TBDs N= 55	No	Yes
n	11	44
Proportion	20.00%	80.00%
TBD knowledge N= 44	Inadequate	Adequate
n	43	1
Proportion	97.73%	2.27%
Aware of anaplasmosis N= 57	No	Yes
n	27	30
Proportion	47.37%	52.63%
Anaplasmosis knowledge N= 30	Inadequate	Adequate
n	17	13
Proportion	56.67%	43.33%
Knowledge of TBD’s from wildlife 57	Inadequate	Adequate
n	27	30
Proportion	47.37%	52.63%

4.4.1. Knowledge and Awareness of Anaplasmosis

A modest 43.33% of the respondents aware of anaplasmosis had adequate knowledge (Table 4.2). Farmers in all areas reported suspected incidences of anaplasmosis (Figure 4.5). There was an association between awareness of anaplasmosis with knowledge of anaplasmosis (p-value 0.0032, fisher’s exact test) and with area (p-value = 0.0089, fisher’s exact test). Awareness of anaplasmosis was not associated to education level (p-value = 0.1486, fisher’s exact test) but was associated to age (p-value = 0.0052, fisher’s exact test) and awareness of TBDs (p-value = 0.0104, fisher’s exact test). No association was found between knowledge of anaplasmosis with area (p-value = 0.4392, fisher’s exact test) and age (p-value = 0.0267, fisher’s exact test).

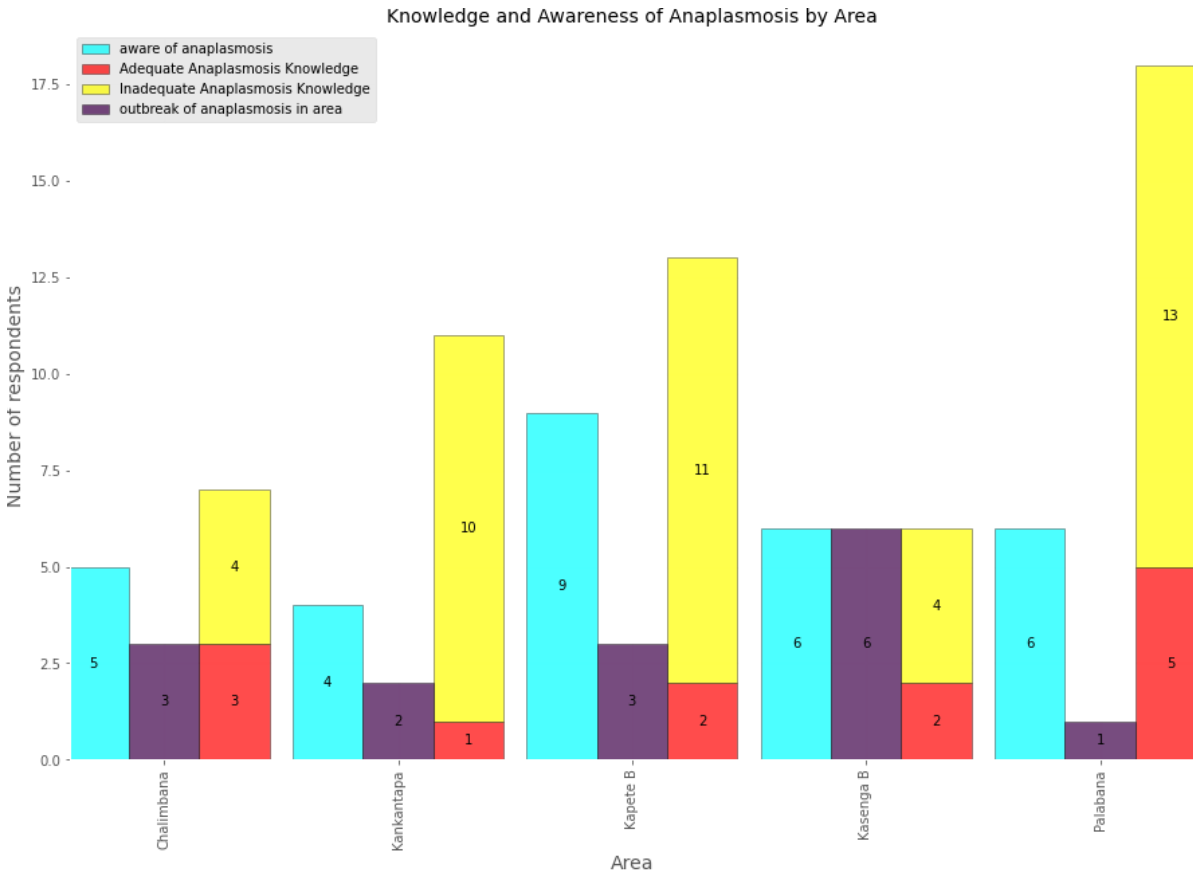

Figure 4.5: Knowledge, Awareness and Reports of Anaplasmosis by Area.

4.4.2. Knowledge of Wildlife risk of TBDs

Sixteen, (28.1%) of the respondents reported the presence of wildlife in the area in which they graze their cattle. The most sighted wild ruminants were impala (9) and puku (7) with rabbits and rodents sighted in 100% of the areas. There were no associations between age knowledge of wildlife risk of TBDs with age (p-value = 0.2906, fisher's exact test). Associations between knowledge of TBDs from wildlife with knowledge of TBDs, education level, awareness of TBDs, and area could not be tested due to null values in the contingency table.

4.4.3. Self -Reported Farming Practices

All respondents reported that they apply acaricides to their animals at least weekly, using either hand spraying (41), spray race (13) or dip tanks (3). Animals were open air tethered and free grazing is practiced by all. Communal grazing grounds were used and cattle freely mingle with other animals except for 15 farms in Palabana which had paddocks.

4.4.4. **Attitude of Farmers Towards TBDs and TBD Control**

Fisher's exact test found no associations between attitude scores and age (p-value = 0.9344, fisher's exact test), education level (p-value = 0.1389, fisher's exact test), ,awareness of TBDs (p-value = 0.8237, fisher's exact test), availability of veterinary services (p-value = 0.1448, fisher's exact test). A mild association was found between attitude and area (p-value = 0.02012, fisher's exact test).

CHAPTER FIVE

5. DISCUSSION

In this study, three species of *Anaplasma*, *A.marginale*, *A.platys* and *A.ovis* were detected. All three were detected in sable antelope and only *A.marginale* in cattle. In Zambia, *A.platys* and *A.marginale* have been documented in cattle (*A.marginale,* (Simuunza et al., 2011) and dogs (*A.platys*, (Vlahakis et al., 2018), however no records of *A.ovis* detection exist.

The proportion of cattle samples positive for Anaplasmatacaecae (PCR) and *A.marginale* (sequencing) in cattle in this study was 16% and 7% respectively (Table 4.1). Simuunza et al., (2011), documented a prevalence in cattle of 41.1% (wet season) to 74.3% (dry season) for both single and mixed infections in Lusaka Province. Single infections were as low as 9.2% (dry season) and 2% (wet season). Prevalence of mixed infection were not provided but 42 different combinations of mixed infections of tick-borne diseases were observed with low prevalence for each combination. All the archived cattle samples used in this study were also positive for *Theileria parva* and this could explain the prevalence observed. It should be noted, however, that results from the KAP survey also indicate a low prevalence of anaplasmosis in Chongwe area as evidenced by the farmers awareness of the disease (43.33%, Table 4.2). Of course, since the clinical presentation of bovine anaplasmosis resembles other livestock diseases and definitive or molecular diagnosis is rarely employed, KAP results are a biased measure of awareness of disease.

Anaplasmataceae was detected in 70% and *Anaplasma* species in 24 % of sable antelope samples(Table 4.1). This finding of high proportion of positives and diversity of species infecting sable is consistent with other studies in wild ruminants (Atif, 2016; Rar et al., 2021). In southern Spain, de La Fuente et al., (2003) found 30% of roe deer infected with *A.ovis*, and 18% with *A.phagocytophilum* but no *A.marginale* infection despite the cattle in the area having a 30% prevalence. In tsetsebe antelope in South Africa, no evidence of *Anaplasma* species infection was found (Brothers et al., 2010). Other studies in Asia and Europe have had varying results in roe deer, red deer, bighorn sheep, sika deer, mule deer and white tailed deer (Rar et al., 2021). What is consistent is the detection of two or more species of *Anaplasma* in the sampled wild ruminants, suggesting wild ruminants are susceptible to infection by many *Anaplasma species* (Atif, 2016; Olafson et al., 2018; Rar et al., 2021).

While *A.marginale* has been detected in other wild ruminants on the continent, including sable antelope, there are no documented findings of *A.platys* and *A.ovis* (Berggoetz et al., 2014; Khumalo et al., 2018). This may be due to the fact that studies of *A.ovis* are generally scarce and most molecular studies are designed to target *A.marginale* and *A.phagocytophilum* specific genes. (Rar et al., 2021). In Mozambique, a molecular study in cattle revealed infection with *A.platys* and *A.ovis* in addition to *A.marginale, A.bovis*, *A.centrale* and *A. phagocytophilum* adding to the evidence that cattle can be atypically infected by non-bovine *Anaplasma* species and serve as reservoirs in endemic areas (Fernandes et al., 2019).

It has been recently postulated that a number of *A.platys* isolated or amplified from atypical hosts are genetically distinct from canine *A.platys* species and have been coined *A.platys*-like species (Rar et al., 2021). However, *A-platys* like species are closely related with *A.platys* and cannot be distinguished on the basis of 16S rRNA. What’s more, these *A.platys*-like species in atypical hosts are considered the likely culprit in zoonotic infection (Ben Said et al., 2017: Rar et al., 2021). Further study would be required to determine if the species detected in sable antelope in this study are *A.platys* or *A.platys*-like and rule out their zoonotic potential.

The detection of *Anaplasma* species in sable antelope in this study raises the question of their being a possible wildlife reservoir and a subsequent danger to livestock (*A.marginale, A.ovis)* and humans (*A.platys)* in surrounding areas. With the inadequate knowledge of both TBDs and anaplasmosis in the sampled areas of Chongwe District, *Anaplasma* may readily spread if introduced. And although farmers self-reported 100% acaricide treatment of their animals, TBDs, particularly East Coast Fever are prevalent in the area, suggesting inadequate tick control methods (Simuunza et al., 2011: Muleya et al., 2022).

A fair number of respondents (52.63%, Table 4.2) were able to recognise the risk of disease from wildlife. There is documented evidence of transmission between wild and domestic ruminants. Tonetti et al., (2009) observed that the *A.marginale* positive gemsbok may have acquired the disease from the livestock in that area while Berggoetz et al., (2014) documents transmission between wild and domestic ruminants. Although twenty-eight percent of the farmers reported spotting wild ruminants (impala and puku) in their areas the general consensus was that the risk was low as they did not graze their animals near wildlife. However, it has been shown that immediate proximity is not a required factor in the transmission of tick-borne diseases as small animals e.g. rodents and insectivores have been implicated in the geographic spread of TBDs (Stuen et al., 2013; Berggoetz et al., 2014; Bargul et al., 2021). As all the farmers reported, there are regular sightings of rabbits and rodents in the study area which may have a role in spreading ticks across large areas. Eygelaar et al., (2015), however, documents a difference in infection rates between buffalo and cattle from non-cordoned and cordoned areas and postulated that free mingling is cardinal to an increased prevalence of anaplasmosis.

It is not possible to conclude on the genetic relatedness of the detected *Anaplasma* species from phyologenetic analysis of the 16S rRNA alone. However, the proximity of the sequences on the phylogenetic tree suggests a genetic link i.e. the same species from the same host are found in the same cluster. Except of course for the one *A.marginale* (NWP64S) sable sample which clustered with the *A.ovis* sable sequences instead of the *A.marginale* reference sequences (96.6% bootstrap support). Another anomaly was a cattle *A.marginale* (CD35) and a sable *A.ovis* (NWP65S) sequence that formed a minor cluster suggesting genetic similarity (82.2% bootstrap support). This clustering of *A.marginale* with *A.ovis* is similar to results obtained in the study in Mozambique on the basis of 16S rRNA (Fernandes et al., 2019). There are still a lot of knowledge gaps in *Anaplasma* genetics and whether or not different species exchange genes is undetermined (Battilani et al., 2017). If so, there is a possibility of pathogenicity gene exchange between *A.ovis* and *A.marginale* with undetermined consequences. It should be noted, however, that this apparent relatedness could simply be due to the difficulties associated with 16S rRNA to distinguish between certain *Anaplasma species* as exemplified by *A.marginale* and *A.centrale s*(Belkahia et al., 2015; Hove et al., 2018; Inokuma et al., 2001).

Knowledge levels of both TBDs and anaplasmosis were found to be inadequate in the area. It is interesting to note that of those aware of anaplasmosis, 20% had adequate knowledge (p-value = 0.003, fisher's exact test) but only 2% of those aware of TBDs had adequate knowledge (untested). This can be attributed to the scope of questions in the survey with a larger proportion of possible responses for TBD knowledge, particularly on the health and production effects of ticks compared to the number of questions testing for knowledge of anaplasmosis. In contrast, a study in Bhutan found that many farmers were able to describe or list the health effects of tick infestation (Namgyal, et al., 2021).

It was interesting to note that education level (p-value = 0.1389), and age (p-value = 0.9344),_did not have a significant association to attitude towards TBD control but area/veterinary camp did (p-value = 0.02012). However this did not seem linked to the availability of veterinary services (p-value = 0.1448, fisher's exact test). Munyeme et al., (2010) reported stark differences in level of farmers awareness to bovine tuberculosis (bTB) even within similar areas suggesting that more complex factors may be at play. The bTB study had an overall conclusion that farmers are more aware of bTB in high prevalence areas due to experiencing animal losses. It should be noted, however, that although significance of the observed pattern of low knowledge could not be tested it is consistent with the conclusion from Munyeme et al., (2010) that high prevalence is a prerequisite for better knowledge and awareness.

Certain constraints in this study's research design limit the confidence of interpreting statistically significant results. Firstly, the molecular study relied solely on archived samples which pose difficulties when it comes to PCR amplification and sequencing i.e samples failed. These blood samples also represent a different timeline from the KAP study (about 3 years apart) and the prevalence on the ground may have changed. Additionally, the study utilized non-probability sampling and obtained a low sample of fifty-seven. This limited the use of inferential statistical tests i.e null values in contingency tables and some associations were not tested. Farmers in some areas were unwilling to cooperate with the veterinary officers and this led to a low turn out. Additionally as many farmers rent out their animals for draft purposes they could not be reached as the survey was conducted during planting season and they were out in fields. These areas were unaccesible due to poor state of the roads and only a few farmers were reached in the fields.

KAP itself as a tool for epidemiological research has several weaknesses mainly due to bias in responses and interviewer bias. This study attempted to reduce interviewer bias by limiting the interviews to two. In addition, the questionnaire was adjusted after the first six responses to allow more responses. The researchers were also unable to administer the questionnaire in one of the locations from which the cattle samples originated (Chinkuli).

CHAPTER SIX

6. CONCLUSIONS AND RECOMMENDATIONS

6.1. Conclusions

This study demonstrated that *Anaplasma* species are present in both cattle and sable antelope in Zambia. There is a notable difference in proportion in cattle from Chongwe and sable antelope translocated to Lusaka National Park from Northwestern Province. The three species detected in sable antelope, *A.marginale, A.ovis* and *A.platys* are also three of the most important species with implications for cattle, small ruminants, dogs and humans. These findings suggest that the introduction of sable antelope to Lusaka National Park introduced a risk of *Anaplasma* to the surrounding areas. With Chongwe's reported low prevalence of *Anaplasma* in cattle (Simuunza et al., 2011: Muleya et al, 2022) in addition to free grazing practices, mixed livestock farming and an observed gap in knowledge, spillover of *Anaplasma* may have devastating effects on livelihood in the area.

6.2. Recommendations

1. Efforts must be made to educate the farmers and residents of Chongwe district on tick-borne diseases including their zoonotic potential.

2. A study of the prevalence and diversity of *Anaplasma* species in cattle in North Western is needed to ascertain the role of sable antelope in the epidemiology of *Anaplasma.*

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APPENDICES

Appendix A: Ethical Clearance Letter

ERES CONVERGE

Lusaka Zambia
Tel. +260 955 155 633
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Cell: +260 977 493 220
Email: eresconverge@yahoo.co.uk

I.R.B. No. 00005948
F.W.A. No. 00011697

14th February, 2022.

Ref. No. 2022-Feb -004

The Principal Investigator
Ms. Rhodasi Mwale
University of Zambia
School of Veterinary medicines
Department of Disease Control
Lusaka, Zambia

Dear Ms Mwale

RE: MOLECULAR CHARACTERIZATION OF ANAPLASMA SPP.IN CATTLE AND SABLE ANTELOPE IN LUSAKA AND NOTH-WESTERN PROVINCE OF ZAMBIA.

Reference is made to your protocol submission. The IRB resolved to approve this study and your participation as Principal Investigator for a period of one year.

Review Type	**Ordinary**	Approval No. **2022-Feb-004**
Approval and Expiry Date	Approval Date: 14th February 2022	Expiry Date: 13th February, 2023
Protocol Version and Date	Version - Nil.	13th February, 2023
Information Sheet, Consent Forms and Dates	• English.	13th February, 2023
Consent form ID and Date	Version - Nil	13th February, 2023
Recruitment Materials	Nil	13th February, 2023
Other Study Documents	Data Collection Sheet, Focus Group Discussion.	13th February, 2023
Number of participants approved for study	-	13th February, 2023

Specific conditions will apply to this approval. As Principal Investigator it is your responsibility to ensure that the contents of this letter are adhered to. If these are not adhered to, the approval may be suspended. Should the study be suspended, study sponsors and other regulatory authorities will be informed.

Conditions of Approval

- No participant may be involved in any study procedure prior to the study approval or after the expiration date.
- All unanticipated or Serious Adverse Events (SAEs) must be reported to the IRB within 5 days.
- All protocol modifications must be IRB approved prior to implementation unless they are intended to reduce risk (but must still be reported for approval). Modifications will include any change of investigator/s or site address.
- All protocol deviations must be reported to the IRB within 5 working days.
- All recruitment materials must be approved by the IRB prior to being used.
- Principal investigators are responsible for initiating Continuing Review proceedings. Documents must be received by the IRB at least 30 days before the expiry date. This is for the purpose of facilitating the review process. Any documents received less than 30 days before expiry will be labelled "late submissions" and will incur a penalty.
- Every 6 (six) months a progress report form supplied by ERES IRB must be filled in and submitted to us.
- A reprint of this letter shall be done at a fee.

Should you have any questions regarding anything indicated in this letter, please do not hesitate to get in touch with us at the above indicated address.

On behalf of ERES Converge IRB, we would like to wish you all the success as you carry out your study.

Yours faithfully,
ERES CONVERGE IRB

Dr. Jason Mwanza
Dip. Clin. Med. Sc., BA., M.Sc., PhD
CHAIRPERSON

Appendix B: Study Site Permission Letter

REPUBLIC OF ZAMBIA
MINISTRY OF FISHERIES AND LIVESTOCK
DEPARTMENT OF VETERINARY SERVICES

P.O BOX 31
Chongwe

15th December,2021

The Assistant Dean – Post Graduate,
School of Veterinary Medicine,
University of Zambia,
Lusaka,

Dear Sir,

RE: Permision To Conduct Research In Chongwe District

Refer to the above subject matter and the letter from your office dated 13th December, 2021.

I write to inform you that the Department of Veterinary Services has no objection to your request to have Ms. Rhodasi Mwale to conduct research work. The officers will be available to work with her and provide any further assistance.

REPUBLIC OF ZAMBIA
MINISTRY OF FISHERIES
AND LIVESTOCK
15 DEC 2021
DISTRICT VETERINARY OFFICER
P.O. BOX 89, CHONGWE.

Yours Sincerely,

Dr. Mwaka Namwila
District Veterinary Officer

Cc: District Fisheries and Livestock Coordinator

Appendix C: National Health Research Authority Clearance

NATIONAL HEALTH RESEARCH AUTHORITY

Paediatric Centre of Excellence, University Teaching Hospital, P.O. Box 30075, LUSAKA

Chalala Office Lot No. 18961/M, Off Kasama Road, P.O. Box 30075, LUSAKA

Tell: +260211 250309 | Email: znhrasec@nhra.org.zm | www.nhra.org.zm

REF:NHREB00008/01/04/2022 **Date: 1st April, 2022**

The Principal Investigator
Ms Rhodasi Mwale,
University of Zambia,
Lusaka, Zambia.

Dear Ms Mwale,

Re: Request for Ethical Clearance and Authority to Conduct Research

The National Health Research Ethics Board (NHREB) is in receipt of your request for ethical clearance and authority to conduct research titled **"Molecular characterization of Anaplasma spp. in cattle and sable antelope in Lusaka and North-western Province of Zambia."**

I wish to inform you that following submission of your request to the Board, its review of the same and in view of the ethical clearance, this study has been **approved** on condition that:

1. **A Material Transfer Agreement is obtained and cleared by the National Health Research Ethics Board should there be any need for samples to be sent outside the country for analysis.**
2. The relevant Provincial and District Medical Officers where the study is being conducted are fully appraised;
3. Progress updates are provided to NHRA quarterly from the date of commencement of the study;
4. The final study report is cleared by the NHRA before any publication or dissemination within or outside the country;
5. After clearance for publication or dissemination by the NHRA, the final study report is shared with all relevant Provincial and District Directors of Health where the study was being conducted, and all key respondents.

Yours sincerely,

Dr Fusya Goma
Vice Chairperson
National Health Research Ethics Board

Appendix D: Information Sheet

THE UNIVERSITY OF ZAMBIA

INFORMATION SHEET

Title of Study: Molecular characterization of *Anaplasma* spp. in cattle and sable antelope in Lusaka and North-western Province of Zambia

Investigators:

Rhodasi Mwale - MSc Tropical Infectious Diseases and Zoonoses.

The University of Zambia, School of Veterinary Medicine

PO Box 32379

LUSAKA.

Email:rhodasimwale@outlook.com

Cell: 097 3 23 38 38

Background and rationale for the study:

Anaplasmosis is a disease of cattle and small ruminants that is spread through ticks. It can affect both wildlife and domestic animals but almost exclusively causes disease in domestic animals. In cattle, anaplasmosis can cause outbreaks on farms or in areas resulting in economic losses from sickness, reduced production levels, abortions and veterinary expenses. In order to understand the epidemiology of anaplasmosis in cattle and wildlife in the area, cattle farmers knowledge, attitudes and practices that may influence anaplasmosis prevalence, transmission and maintenance must be explored.

Purpose:

The information gathered in this interview will help the researcher determine the respondents knowledge of anaplasmosis, and the farming practices that may affect prevalence of and exposure to anaplasmosis and other tick-borne diseases.

Procedures:

The interviewer will ask a series of questions to find out information about your animal management practices, knowledge on ticks, tick-borne diseases and access to veterinary services.

Who will participate in the study?

This study is interested in households and farms involved in cattle rearing in Chongwe District and the Area around Lusaka National Park.

Risks/Discomforts:

You are not required to provide your names and your personal information will remain private..

Benefits:

Information gathered on anaplasmosis and it's risk factors in your cattle will help provide background data which will inform stakeholders on the occurrence of anaplasmosis in your area, and the need for control, preventive and educational activities.

Cost:

You will not incur any costs by participating in this study.

Compensation for participation in the study:

At this time, there is no monetary compensation for participating in the study.

Reimbursement:

You will not incur expenses, travel or otherwise and therefore, no reimbursement will be provided.

Questions:

If you have any questions regarding the study, feel free to contact the researcher using the details included above.

Questions about participants rights:

If you have questions concerning your rights as a research participant, you can contact the ERES Converge IRB offices using the details provided.

Statement of voluntariness:

You reserve the right to refuse to disclose any information and refusal to participate in this study. If you however do consent to this study we require you to sign the consent provided.

Confidentiality:

The results of this study will be kept strictly confidential, and used only for research purposes. Your identity will be concealed in as far as the law allows. Your name will not appear anywhere on the coded forms with the information. Paper and computer records will be kept under lock and key and with password protection respectively.

For any further questions, you may contact the Chairperson, ERES Converge IRB on the following details; Tel. 0955 155633/4, Meanwood Ibex Hill, LUSAKA

Appendix E: Participant Consent Form

STATEMENT OF CONSENT/ASSENT

....…***(interviewer name)*** has described to me what is going to be done, the risks, the benefits involved and my rights regarding this study. I understand that my identity will be concealed. I am aware that I may withdraw at any time. I understand that by signing this form, I do not waive any of my legal rights but merely indicate that I have been informed about the research study in which I am voluntarily agreeing to participate.

Name:………………………………………………………………

Signature /Thumbprint of participant.. ……………………………………………..

Age..…………… Date (DD/MM/YY)…………………………………….

Name of Interviewer……………………………………………………….……..

Signature of Interviewer …………………… Date (DD/MM/YY)………………………...

If you have questions, complaints, or problems because of participating in this study, you may contact Rhodasi Mwale (+260 973233838) or email: rhodasimwale@yahoo.com. All research is reviewed by an Ethics committee that works to protect your rights and welfare. If you have questions or concerns about your rights as a research participant you may contact, anonymously if you wish, ERES Converge IRB office, Meanwood Ibex Hill, Lusaka, Tell: 0955 155633/4.

Appendix F: KAP Questionnaire

Survey on the knowledge, attitudes and practices of cattle farmers regarding bovine anaplasmosis.

PART 1. QUESTIONNAIRE IDENTIFICATION INFORMATION

Questionnaire serial Number ________________________________

Province ________________________________

District ________________________________

Veterinary Camp ________________________________

Village/Farm ________________________________

Chief/Chiefdom ________________________________

Suburban/Location ________________________________

Date of Interview ________________________________

Interviewer (Name) ________________________________

Name of Head of Household (HH) ________________________________

Cell phone of interviewee or head of HH___________________________________

Relationship of the interviewee to HH head________________________________

Response/Questionnaire filter details codes:

Completed Partially done Not done at all

P. S Coordinates: UTM: X:______________ Y:________________, Zone:________.
WGS(x.x^{o}):

Distance and campus bearing of Farm from Lusaka National Park:

Section A: Sociodemographic Information

(Tick the answer and specify as required)

1	Name:	
2	Age (Years):	
3	Gender:	1 ☐ Male 2 ☐ Female
4	Education level:	1 ☐ Not attended any school 2 ☐ Attending/Attended Non-Formal Education 3 ☐ Primary level 4 ☐ High school 5 ☐ Secondary level 6 ☐ Graduate

Section B: Animal Population and Management
(Tick the answers and specify as required)

5. Number of cattle by breed
(Use breed specifications as applicable)

Animal species

Local
Crossbred
Purebred

Cattle

6

Type of husbandry practice the farm follows:

1		Stall feeding
2		Mix of stall feeding & free grazing
3		Mix of stall feeding & tethered grazing
4		All-time free grazing
5		Mixture of the above practices

7

Does the farm also have any of the following animals?

1		Goats
2		Sheep
3		Dogs
4		Game animals
5		None

8

Do the cattle mingle with the other animals on the farm?

1		Yes
2		No

Section C: Knowledge and awareness of Ticks and Tick-Borne Diseases in cattle

(Tick the answers and specify as required)

9

Have you ever seen a tick?

1		Yes
2		No

10

If yes, where do you commonly find these ticks on your farm?
Respondents can have more than one answer.

1		On the animals
2		In the forests
3		In the agriculture land
4		In the pastureland
5		All of the above

11

Have you heard of any tick-borne diseases in cattle?

1		Yes
2		No

If yes, move to the following question.

12

If yes, from where did you hear about the tick-borne diseases in cattle?
Respondents can have more than one answer.

1		Farmers' training program

2		Livestock officials visiting farms
3		Neighbors
4		Family members
5		Friends
6		Media (including social media)

Any other, please specify?

13

Have you also seen ticks on the body of other animals?

1		Yes
2		No

If no, go to question 15

14

If yes, in which species of animal did you see?
Respondents can have more than one answer.

1		Small ruminants (sheep and goats)
2		Dogs
3		Cats
4		Horses
5		Pigs
6		Poultry
7		Wild animals (including birds)

Knowledge of TBDs

15

Which places do you think you find the ticks most commonly?

1		Warm places
2		Cold places
3		Both types of places

16

Where do you think the cattle get ticks from?
Respondents can have more than one answer.

1		From the forest
2		From the grazing land
3		From the bedding materials
4		From fodder grasses
5		Don't know
6		All of the above

17

Which type of cattle do you think gets mostly infested by the ticks?

1		Native breeds
2		Exotic breeds
3		Don't know

18

Which of the cattle in the following do you think is most affected by tick infestation?

1		Adult
2		Heifers
3		Young calves
4		Old cattle
5		Don’t know
6		Any

19

Do you think ticks always stay on the body of a cattle unless removed?

1		Yes
2		No
3		Don’t know

20

What do you think is the health and production impacts of tick infestation in cattle?
Respondents can have more than one answer.

1		Bloodsucking
2		Bite wound
3		Anorexia
4		Loss of weight
5		Fever
6		Red or brown color urine
7		Hide damage
8		Loss of production
9		Don’t know
10		No effect
11		All the above

21

Do you think cattle can get diseases from the ticks?

1		Yes
2		No
3		Don’t know

TBD Knowledge Score

Section D: Knowledge and awareness of Anaplasmosis/Gallsickness
(Tick the answers as required)

22

Have you ever heard of anaplasmosis/gallsickness in cattle?

1		Yes
2		No

If No, move to Section E

23

Has there ever been an outbreak on anaplasmosis / gallsickness in the area?

1		Yes
2		No
3		Don't know

If No or Don't know, move to question 26

24

How long ago was this outbreak?

1		Within the past 6 months
2		Between 6 months and 1 year ago
3		Over a year ago
4		Don't know

25

Was there any veterinary / government response to the outbreak?

1		Yes
2		No
3		Don't know

Knowledge of Anaplasmosis/Gallsickness

26

Is there treatment for anaplasmosis/gallsickness

1		Yes
2		No
3		Don't know

27
Can a farmer prevent anaplasmosis/gallsickness in animals?

1		Yes
2		No
3		Don't know

Anaplasmosis Knowledge Score

Section E: Cattle Interaction With Wildlife
(Tick the answers as required)

28	Are there any wild animals in the area?	1 Yes 2 No 3 Don't know	
29	Have you encountered any of these wild animals in the area?	1 Impala 2 Buffalo 3 Puku 4 Kudu 5 Water buck	
30	Do you ever graze or water your animals in areas with wild animals?	1 Yes 2 No 3 Don't know	
31	Do you think cattle can get diseases from wild animals?	1 Yes3 2 No 3 Don't know	
32	Do you think cattle can get ticks from wild animals?	1 Yes 2 No 3 Don't know	

Section F: Farmers' Practices
(Tick the answers and specify as required)

33	What is the main purpose of rearing cattle in your household?	1 Family consumption of products 2 Income through the sale of products 3 Income through the sale of animals 4 As a source of manure 5 Draft purpose	

		6 ☐ Breeding purpose Any other, please specify:	
34	What type of cattle shed do you have?	1 ☐ Improved shed with CGI Sheet and concrete flooring 2 ☐ Improved shed with CGI Sheet and wooden flooring 3 ☐ Conventional Shed (built with local materials) 4 ☐ Open-air tethering *If Open-air tethering skip to question 39*	
35	If your cattle shed's floor is concrete, how often do you wash the flooring of your cattle shed?	1 ☐ Daily 2 ☐ Weekly 3 ☐ Every 2 weeks 4 ☐ Monthly 5 ☐ Never	
36	Do you use bedding materials in your cattle shed?	1 ☐ Yes 2 ☐ No *If No, proceed to question 39*	
37	If yes, how often do you change the bedding materials?	1 ☐ Every 3 days 2 ☐ Weekly 3 ☐ Monthly 4 ☐ Over a month/infrequently	
38	When do you mostly use bedding materials?	1 ☐ Dry Season 2 ☐ Cold Season 3 ☐ Rainy Season	
39	Which animal health problems are most important to you? *Select 3 of the problems*	1 ☐ Mastitis 2 ☐ Endoparasitism 3 ☐ Milk fever 4 ☐ Bacterial diseases (e.g., HS, BQ) 5 ☐ Tick infestation 6 ☐ Foot and mouth disease 7 ☐ Plant poisoning	
40	What are the main purposes that make you visit the veterinary centers? *Select 3 of the purposes*	1 ☐ To receive livestock production inputs 2 ☐ To receive medicines for sick animals 3 ☐ To receive deworming drugs 4 ☐ To receive acaricides 5 ☐ To seek advice on farming practices. 6 ☐ Do not visit any veterinary centers	

41	What control measures do you follow in your farm to address the problem of tick infestation in cattle? *Respondents can have more than one answer.*	1 ☐ Use acaricides 2 ☐ Don't let the animals out for grazing 3 ☐ Follow rotational grazing 4 ☐ Use homemade remedies 5 ☐ Manually remove the ticks. 6 ☐ Adopt good farm practices	
42	What is the frequency of applying acaricides on your farm?	1 ☐ Weekly 2 ☐ Every 2 weeks 3 ☐ Monthly 4 ☐ Occasionally	
43	When you use acaricides, which method of application do you follow?	1 ☐ Hand spraying 2 ☐ Hand dressing 3 ☐ Pour on 4 ☐ Spray race 5 ☐ Dipping 6 ☐ Mixed of above practices	
44	Do you check your body for ticks after handling the tick-infested cattle?	1 ☐ Always 2 ☐ Sometimes 3 ☐ Never	
45	Do you check your body for ticks after visiting the forests?	1 ☐ Always 2 ☐ Sometimes 3 ☐ Never	

Section G: Attitudes of the farmers
(Tick an appropriate column)

Start asking each question by "Do you agree?

Attitude Questions on Likert Scale	**1**	**2**	**3**	**4**	**5**
Proper use of synthetic acaricides can reduce the incidences of tick infestation in cattle.	Strongly agree	Disagree	No opinion	Agree	Strongly agree
Proper use of synthetic acaricides can reduce the incidences of anaplasmosis/tick-borne diseases in cattle.	Strongly agree	Disagree	No opinion	Agree	Strongly agree
Risk of the tick infestation can be	Strongly	Disagree	No opinion	Agree	Strongly

reduced by keeping the cattle always in the shed.	Disagree				Agree
Risk of tick diseases can be reduced by keeping the cattle always in the shed.	Strongly Disagree	Disagree	No opinion	Agree	Strongly Agree
Adopting good farm practices can reduce the risk of tick infestation (e.g., regular washing of floor, regular checking of animals, avoiding the use of bedding materials, etc.)	Strongly Disagree	Disagree	No opinion	Agree	Strongly Agree
Sterilising equipment for dehorning, castration and not re-using needles can reduce the incidences of anaplasmosis / gall sickness in cattle.	Strongly agree	Disagree	No opinion	Agree	Strongly agree
Attitude Score					

Section H:Access to Veterinary Services

(Tick the answers as required)

47	Do you receive any Veterinary Services (animal health extension services)?	1 ☐ Yes 2 ☐ No *If No, skip this section.*	
48	Who are the provider(s) of animal health extension (Veterinary) services in your area?	1 ☐ Veterinary Department 2 ☐ Community Livestock Auxiliary 3 ☐ NGO (Provide name) 4 ☐ Other (specify)	
49	What is your comment on the quality of service you receive?	1 ☐ Excellent 2 ☐ Very Good 3 ☐ Poor 4 ☐ Extremely Poor	

Appendix G: Codebook for KAP attitude, knowledge and awareness scores

q#	Variable/Question	Code	Description		
q2	Age	N/A	Ratio scale		
	Age Category	1 2 3	16-35 36-50 >50		
q3	Gender	1 2	Male Female		
q4	Education Level 1= No education 2=Basic education 3=Secondary Education 4=Tertiary Education	1 1 2 3 3 4	Not attended any school Attending/Attended Non-Formal Education Primary level High school Secondary level Graduate		
q5a	Cattle breed	 1 2 3	 local cross breed exotic	Yes 1 1 1	No 0 0 0
q5b	Number of cattle	1	Numerical, discrete		
q5b	Cattle Holding	1	1-10		
		2	11-20		
		3	>20		
q6	Type of husbandry practice	1 2 3 4 5	Stall feeding Mix of stall feeding & free grazing Mix of stall feeding & tethered grazing All-time free grazing Mixture of the above practices		
q7	Other animals on farm	1 2 3 4 5	Goats Sheep Dogs Game animals None		
q8	Cattle mingling with other animals	1 0	Yes No		
q9	Seen a tick	1 0	Yes No		
q10	Areas where ticks are found	 a b c d	 On the animals In the forests In the agriculture land In the pastureland	No 0 0 0 0	Yes 1 1 1 1

		tick all	All of the above	0	1
q11	Ever heard of TBDs	1 0	Yes No		
				No	Yes
q12	Source of TBD knowledge	a	Farmers' training program	0	1
		b	Livestock officials visiting farms	0	1
		c	Neighbors	0	1
		d	Family members	0	1
		e	Friends	0	1
		f	Media (including social media)	0	1
		g	Other	0	1
q13	Seen ticks on other animals	1 0	Yes No		
				No	Yes
q14	Other animals prone to ticks	a	Small ruminants (sheep and goats)	0	1
		b	Dogs	0	1
		c	Cats	0	1
		d	Horses	0	1
		e	Pigs	0	1
		f	Poultry	0	1
		g	Wild animals (including birds)	0	1
				No	Yes
q15	Temperature ticks prefer	a	warm	0	1
		b	cold	0	1
		c	don't know	0	1
				No	Yes
q16	Source of tick infestation on cattle	a	forests	0	1
		b	grazing land	0	1
		c	bedding materials	0	1
		d	fodder grasses	0	1
		e	don't know	0	1
				No	Yes
q17	Breeds affected by ticks	a	native	0	1
		b	exotic	0	1
		c	don't know	0	1
		d	any breed	0	1
				No	Yes
q18	Cattle most infested by ticks	a	adult	0	1
		b	heifers	0	1
		c	calves	0	1
		d	cold	0	1

		e f	don't know any	0 0	1 1
q19	Ticks stay on cattle unless removed	0 1 2	No Yes don't know		
q20		 a b c d e f g h i j k l	 bloodsucking bite wound anorexia loss of weight fever-illness red/brown urine hide damage loss of production don't know None No effect All of the above	No 0 0 0 0 0 0 0 0 0 0 0 0	Yes 1 1 1 1 1 1 1 1 1 1 1 1
q21	ticks cause disease in cattle	0 1 2	No Yes don't know		
q22	Heard of anaplasmosis	1 0	Yes No		
q23	Outbreak of anaplasmosis	0 1 2	No Yes don't know		
q24	How long ago was outbreak?	1 2 3 4	past 6 months 6months to 1 year over a year ago don't know		
q25	Vet response to the outbreak	0 1 2	No Yes don't know		
q26	Is there treatment for anaplasmosis?	0 1 2	No Yes don't know		
q27	Anaplasomis preventions	0 1 2	No Yes don't know		
q28	WIld animals in farming area	0 1 2	No Yes don't know		

				No	Yes
q29	Wild animals enountered by respondent	a	impala	0	1
		b	bufallo	0	1
		c	puku	0	1
		d	kudu	0	1
		e	water buck	0	1
q30	cattle grazing near wildlife	0 1 2	No Yes don't know		
q31	cattle can get diseases from wildlife	0 1 2	No Yes don't know		
q32	cattle can get ticks from wildlife	0 1 2	No Yes don't know		
q33	main purpose of cattle rearing in household	1 2 3 4 5 6 7	family consumption of products icome through sale of products income through sale of animals as source of manure draft purpose breeding purpose Other, specifiy		
q34	Type of cattle shed	1 2 3 4	Improved shed with CGI and concrete flooring Improved shed with CGI and wooden flooring conventional shed/ local materials open air tethering		
q35	washing frquency of concrete shed floors	1 2 3 4 5	daily weekly every 2 weeks monthly never		
q36	use of bedding material	1 0	yes no		
q37	frequency of changing bedding material	1 2 3 4	every 3 days weekly monthly once a month/infrequently		
q38	what season bedding	1	dry season		

	materials are used	2 3	cold season rainy season		
q39	animal health problems of concern to farmer			No	Yes
		a	mastitis	0	1
		b	endoparasitism	0	1
		c	milk fever	0	1
		d	bacterial diseases(HS, BQ, black leg)	0	1
		e	tick infestation	0	1
		f	foot and mouth disease	0	1
		g	plant poisoning	0	1
q40	purpose for visits to vet centre			No	Yes
		a	receive livestock production inputs	0	1
		b	receive medicines for sick animals	0	1
		c	receive deworming drugs	0	1
		d	receive acaricides	0	1
		e	seek advice on farming practices	0	1
		f	do not visit veterinary centres	0	1
q41	tick control measures used on farm			No	Yes
		a	acaricides	0	1
		b	don't let animals go out to graze	0	1
		c	follow rotational grazing	0	1
		d	homemade remedies	0	1
		e	manually remove ticks	0	1
		f	adopt good farming practices	0	1
q42	frequency of acaricide application	1	weekly		
		2	every 2 weeks		
		3	monthly		
		4	occasionally		
q43	mode of acaricide applicatiom	1	hand spraying		
		2	hand dressing		
		3	pour on		
		4	spray race		
		5	dipping		
		6	mix of above methods		
q44	checking self for ticks after handling animals	1	always		
		2	sometimes		
		3	never		

q45	checking self for ticks after visitng forests	1 2 3	always sometimes never		
q46	attitude score	N/A	Numerical, ratio		
q47	receives vet services	1 0	yes no		
q48	vet services provider	1 2 3 4	vet department community livestock auxillary NGO- provide name Other, specifiy		
q49	quality of vet services	1 2 3 4	excellent good poor extremely poor		

Appendix H: Scoring key for KAP attitude, knowledge and awareness scores

Variable	Questions	Response	Mark
Awareness of TBD	9, 11 total score possible	yes no	1 0 2
Knowledge of TBD	16,19-21 16 & 20 total score possible	yes no dont' know for each correct answer score	1 0 0 1 15
Knowledge of anaplasmosis	26,27 If any wrong, knowledge inadequate	yes no	1 0
Awareness of anaplasmosis	22	yes no	1 0
Knowledge of wildlife risk	31, 32	yes no	1 0
If any wrong, knowledge inadequate		Total	2
Attitude Score Calculation	Score a mark for agree or strongly agree Score 5-6; favorable Score 4: neutral Score <3:unfavourable	4, 5 1,2,3	1 0

Appendix I : OneTaq Polymerase Protocol

Protocol for One*Taq*® 2X Master Mix with Standard Buffer (M0482)

Overview

PCR

The Polymerase Chain Reaction (PCR) is a powerful and sensitive technique for DNA amplification (1). *Taq* DNA Polymerase is an enzyme widely used in PCR (2). The following guidelines are provided to ensure successful PCR using New England Biolabs' One*Taq* 2X Master Mix with Standard Buffer. These guidelines cover routine PCR. Amplification of templates with high GC content, high secondary structure or low template concentrations may require further optimization.

Protocol

Reaction setup:

We recommend assembling all reaction components on ice and quickly transferring the reactions to a thermocycler preheated to the denaturation temperature (94°C).

Add to a sterile thin-walled PCR tube:

Component	25 µl reaction	50 µl reaction	Final Concentration
10 µM Forward Primer	0.5 µl	1 µl	0.2 µM
10 µM Reverse Primer	0.5 µl	1 µl	0.2 µM
Template DNA	variable	variable	< 1,000 ng
One*Taq* 2X Master Mix with Standard Buffer	12.5 µl	25 µl	1X
Nuclease-free water	to 25 µl	to 50 µl	< 1,000 ng

Notes: Gently mix the reaction. Collect all liquid to the bottom of the tube by a quick spin if necessary. Overlay the sample with mineral oil if using a PCR machine without a heated lid.

Transfer PCR tubes to a PCR machine and begin thermocycling:

Thermocycling conditions for a routine PCR:

STEP	TEMP	TIME
Initial Denaturation	94°C	30 seconds

30 Cycles	94°C 45-68°C 68°C	15-30 seconds 15-60 seconds 1 minute/kb
Final Extension	68°C	5 minutes
Hold	4-10°C	

General Guidelines

1. Template:

   Use of high quality, purified DNA templates greatly enhances the success of PCR. Recommended amounts of DNA template for a 50 µl reaction are as follows:

DNA	Amount
genomic	1 ng–1 µg
plasmid or viral	1 pg–10 ng

2. Primers:

   Oligonucleotide primers are generally 20–40 nucleotides in length and ideally have a GC content of 40–60%. Computer programs such as Primer3 can be used to design or analyze primers. The final concentration of each primer in a PCR may be 0.05–1 µM, typically 0.2 µM

3. Mg^{++} and Additives:

   Mg^{++} concentration of 1.5–2.0 mM is optimal for most PCR products generated with One*Taq* DNA Polymerase. The final Mg^{++} concentration in 1X One*Taq* Master Mix with Standard Buffer is 1.8 mM. This supports satisfactory amplification of most amplicons. However, Mg^{++} can be further optimized in 0.2 mM increments using $MgCl_2$ (NEB# B9021).

   For amplification of difficult targets, like GC-rich sequences, we recommend One*Taq* 2X Master Mix with GC Buffer (NEB# M0483). Alternatively, DMSO or formamide may be used

4. Denaturation:

   An initial denaturation of 30 seconds at 94°C is sufficient to amplify most targets from pure DNA templates. For difficult templates such as GC-rich sequences, a longer denaturation of 2–4 minutes at 94°C is recommended prior to PCR cycling to fully denature the template. Alternatively, use One*Taq* Hot Start 2X Master Mix with GC Buffer. With colony PCR, an initial 2–5 minute denaturation at 94°C is recommended to lyse cells.

   During thermocycling a 15–30 second denaturation at 94°C is recommended

5. Annealing:

   The annealing step is typically 15–60 seconds. Annealing temperature is based on the T_m of the primer pair and is typically 45–68°C. Annealing temperatures can be optimized by doing a temperature gradient PCR starting 5°C below the calculated T_m. We recommend using NEB's Tm Calculator to determine appropriate annealing temperature for PCR.

6. Extension:

   The recommended extension temperature is 68°C. Extension times are generally 1 minute per kb. A final extension of 5 minutes at 68°C is recommended

7. Cycle Number:

   Generally, 25–35 cycles yield sufficient product. Up to 45 cycles may be required to detect low copy number targets.

8. PCR Product:

   The majority of the PCR products generated using One*Taq* DNA Polymerase contain dA overhangs at the 3´ end; therefore the PCR products can be ligated to dT/dU-overhang vectors.

References:

1. Saiki,R.K. et al (1985). *Science*. 230, 1350-1354.
2. Powell,L.M. et. al. (1987). *Cell*. 50, 831-840.

Links to this resource

Product Categories: One*Taq*® DNA Polymerases Products, *Taq* DNA Polymerase Products, Master Mixes Products

Applications: Fast PCR, Multiplex PCR, Specialty PCR, | More +

Related Products: One*Taq*® 2X Master Mix with Standard Buffer

Appendix J: Zymo Research DNA Clean and Concentrator

Protocol

Buffer Preparation

✓ *Before starting*: Add 24 ml 100% ethanol (26 ml 95% ethanol) to the 6 ml **DNA Wash Buffer** concentrate. Add 96 ml 100% ethanol (104 ml 95% ethanol) to the 24 ml **DNA Wash Buffer** concentrate.

✓ **DNA Wash Buffer** included with D4001S and D4001T is supplied ready-to-use and does not require the addition of ethanol prior to use.

Sample Processing

All centrifugation steps should be performed between 10,000 - 16,000 x g.

1. In a 1.5 ml microcentrifuge tube, add 2-7 volumes of **DNA Binding Buffer** to each volume of DNA sample (see table below). Mix briefly by vortexing.

Application	DNA Binding Buffer : Sample	Example
Plasmid, genomic DNA (>2 kb)	2 : 1	200 µl : 100 µl
PCR product, DNA fragment	5 : 1	500 µl : 100 µl
ssDNA[1] (*e.g.* cDNA, M13 phage)	7 : 1	700 µl : 100 µl

For efficient recovery of genomic or large DNA (> 20 kb to > 200 kb), use the **Genomic DNA Clean & Concentrator® (Cat. Nos. D4010, D4011)**.

2. Transfer mixture to a provided **Zymo-Spin™ Column**[2] in a **Collection Tube**.

3. Centrifuge for 30 seconds. Discard the flow-through.

4. Add 200 µl **DNA Wash Buffer** to the column. Centrifuge for 30 seconds. Repeat the wash step.

[1] For ssDNA purification, see **Appendix A** on page 10.
[2] The sample capacity of the column is 800 µl. Therefore, it may be necessary to load and spin a column multiple times if a sample has a volume larger than 800 µl.

8. F

5. Add ≥ 6 µl **DNA Elution Buffer**[3] or water[4] directly to the column matrix and incubate at room temperature for one minute. Transfer the column to a 1.5 ml microcentrifuge tube and centrifuge for 30 seconds to elute the DNA.

   Ultra-pure DNA is now ready for use.

---

[3] **DNA Elution Buffer**: 10 mM Tris-HCl, pH 8.5, 0.1 mM EDTA
[4] Elution of DNA from the column is dependent on pH and temperature. If water is used, make sure the pH is >6.0. Waiting 1 minute prior to elution may improve the yield of larger (> 6 kb) DNA. For even larger DNA (> 10 kb), the total yield may be improved by eluting the DNA with 60-70^{o}C **DNA Elution Buffer**.

Appendix K : Big Dye Terminator Cycle Sequencing Protocol

applied biosystems QUICK REFERENCE

BigDye™ Terminator v3.1 Cycle Sequencing Kit

Catalog Numbers 4337454, 4337455, 4337456, 4337457, 4337458

Pub. No. MAN0015666 **Rev.** A.0

Note: For safety and biohazard guidelines, see the "Safety" appendix in the *BigDye™ Terminator v3.1 Cycle Sequencing Kit User Guide* (Pub. no. 4337035). Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

This document is intended as a benchtop reference for experienced users of the BigDye™ Terminator v3.1 Sequencing Standard Kit (Cat. Nos. 4337454, 4337455, 4337456, 4337457, and 4337458). See the *BigDye™ Terminator v3.1 Cycle Sequencing Kit User Guide* (Pub. No. 4337035) for detailed instructions and troubleshooting.

Product description

The BigDye™ Terminator v3.1 Cycle Sequencing Kit provides pre-mixed reagents for Sanger sequencing reactions.

The kit includes BigDye™ Terminator v1.1 & v3.1 5X Sequencing Buffer , which is specifically optimized for use with the BigDye™ Ready Reaction mixes.

The kit has been formulated to deliver robust performance across a wide variety of DNA sequences while maximizing readlengths. When used in combination with Minor Variant Finder Software, the kit can also be used to detect variants as low as 5% in a sample (see *Minor Variant Finder Software User Guide* (Pub. No. MAN0014835).

Workflow

Prepare templates

Template quantity

Table 1 Recommended DNA quantities

DNA template	Quantity
PCR product:	
• 100–200 bp	1–3 ng
• 200–500 bp	3–10 ng
• 500–1000 bp	5–20 ng
• 1000–2000 bp	10–40 ng
• >2000 bp	20–50 ng
Single-stranded DNA	25–50 ng
Double-stranded DNA	150–300 ng
Cosmid, BAC	0.5–1.0 µg
Bacterial genomic DNA	2–3 µg

Sequencing templates should be purified before use in sequencing reactions. See **https://www.thermofisher.com/us/en/home/life-science/dna-rna-purification-analysis/dna-extraction.html** for a range of suitable kits.

Perform cycle sequencing

Set up the sequencing reactions

IMPORTANT! Protect dye terminators from light. Cover the reaction mix and sequencing plates with aluminum foil before use.

1. Completely thaw the contents of the BigDye™ Terminator v3.1 Cycle Sequencing Kit and your primers and store on ice.
2. Vortex the tubes for 2 to 3 seconds, then centrifuge briefly (2 to 3 seconds) with a benchtop microcentrifuge to collect contents at the bottom of the tubes.
3. Label microcentrifuge tubes "forward" and "reverse" and add components as indicated:

   IMPORTANT! Change pipette tips after each transfer.

   IMPORTANT! For control reactions use 4 µL of the control primers (0.8pmol/µL) in both 10 µL and 20 µL reactions.

Component	Standard reaction (20 µL)[1]		
	Quantity per reaction	Example Forward	Example Reverse
BigDye™ Terminator 3.1 Ready Reaction Mix	8 µL	8 µL	8 µL
Forward primer (3.2 µM)	3.2 pmol	1 µL	—
Reverse primer (3.2 µM)		—	1 µL
Deionized water (RNase/DNase-free)	Varies based on template and primer volume	9 µL	9 µL
Template	See "Template quantity" on page 1	2 µL[2],[3]	2 µL[2],[3]
Total volume	**20 µL**	**20 µL**	**20 µL**

[1] Reactions can be scaled to 10 µL for 384-well plates. Keep the primer concentration and volume the same as in 20 µL reactions.
[2] e.g., 150–300ng/µL of dsDNA
[3] Concentration of template may affect volume, if template volume differs please adjust the volume of water in the reaction mix.

   Note: Store on ice and protected from light.

4. Seal the plate with MicroAmp™ Clear Adhesive Film.
5. Vortex the plate for 2 to 3 seconds, then centrifuge briefly in a swinging bucket centrifuge to collect contents to the bottom of the wells (5 to 10 seconds) at 1,000 x g.

   Note: Bubbles may be present within the wells, but do not adversely affect the reaction.

Using BigDye™ Terminator v1.1 & v3.1 5X Sequencing Buffer to dilute sequencing reactions

Some cycle sequence reactions may be optimized using diluted BigDye™ Terminator Ready Reaction Mix. The BigDye™ Terminator Ready Reaction Mix is provided at a 2.5X concentration and can be diluted using BigDye™ Terminator v1.1 & v3.1 5X Sequencing Buffer to a final end reaction concentration of 1X.

Calculate the volume of BigDye™ Terminator v1.1 & v3.1 5X Sequencing Buffer to use:

0.5 * ((total reaction volume)/2.5) - volume of BigDye™ Terminator Ready Reaction Mix).

Note: If you use the BigDye™ Terminator v1.1 & v3.1 5X Sequencing Buffer without optimization, the quality of the sequence may deteriorate. We can not guarantee the performance of BigDye™ chemistry when it is diluted.

An example of a 0.5x diluted sequencing reaction is shown below:

Component	Diluted reaction (0.5X)		
	Quantity per reaction	Example Forward	Example Reverse
BigDye™ Terminator 3.1 Ready Reaction Mix	4 µL	4 µL	4 µL
BigDye™ Terminator v1.1 & v3.1 5X Sequencing Buffer	2 µL	2 µL	2 µL
Forward primer (3.2 µM)	3.2 pmol	1 µL	—
Reverse primer (3.2 µM)		—	1 µL
Deionized water (RNase/DNase-free)	Varies based on template and primer volume	11 µL	11 µL
Template	See "Template quantity" on page 1	2 µL[1],[2]	2 µL[1],[2]
Total volume	**20 µL**	**20 µL**	**20 µL**

[1] e.g., 150-300ng/µL of dsDNA

[2] Concentration of template may affect volume, if template volume differs please adjust the volume of water in the reaction mix.

Run the sequencing reactions

1. Place the tubes or plate(s) in a thermal cycler and set the volume.
2. Perform cycle sequencing:

Parameter	Stage/step				
	Incubate	Cycling (25 cycles)			Hold
		Denature	Anneal	Extend	
Ramp rate	—	1°C/second.			
Temperature	96°C	96°C	50°C	60°C	4°C
Time (mm:ss)	01:00	00:10	00:05	04:00[1]	Until ready to purify.

[1] Shorter extension times can be used for short templates.

Purify the sequencing reactions

Salts, unincorporated dye terminators, and dNTPs in sequencing reactions obscure data in the early part of the sequence and can interfere with basecalling.

Purify the sequencing reactions before capillary electrophoresis. See the *BigDye™ Terminator v3.1 Cycle Sequencing Kit User Guide* (Pub. No. 4337035) for recommended protocols.

Capillary electrophoresis

Capillary electrophoresis guidelines

- Resuspend sequencing reactions in 10-µL of Hi-Di™ Formamide.

  Do not heat samples to resuspend. Run samples as soon as possible after resuspension.

  Note: It is not necessary to resuspend samples purified with the BigDye XTerminator™ Purification Kit.
- Select the correct mobility file. Different dyes will have different mobility corrections required for adequate basecalling.

  If the wrong mobility file is used, this can be corrected with Sequencing Analysis Software.

Compatible sequencing instruments

- 310 Genetic Analyzer
- 3130/3130*xl* Genetic Analyzer
- 3500/3500xL Genetic Analyzer
- 3730/3730*xl* DNA Analyzer

Calibration

Matrix or sequencing standards provide a sample for multi-color spectral correction for the dye emission overlap of the BigDye™ Terminators.

Perform new spectral calibrations when an array is installed or capillaries are moved within the detection area to ensure and maintain the highest quality spectral calibration on your system.

See your specific instrument user guide for more information on calibration.

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For support visit **thermofisher.com/support** or email **techsupport@lifetech.com**

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Appendix L: Ethanol Precipitation Protocol

EDTA/NaOAc/Ethanol Precipitation of Cycle-Sequenced Products
Prepared by Amy Smith

This protocol continues from protocol **BDTv3.1 Cycle-Sequencing**

After Cycle Sequencing is complete, remove plate from thermocycler and place at 4˚C or –20˚C until ready to precipitate: Do not let plate sit in thermocycler overnight (low sample volume leads to easy evaporation).

EDTA helps to stabilize extension products during precipitation, and also helps to wash out unincorporated dyes from the completed reaction. NaOAc helps to prevent loss of small fragements due to EDTA, but does not effectively remove all unincorporated dyes. I only recommend using NaOAc if good signal from base 1 is absolutely necessary (very long fragments or no sequence overlap available). If NaOAc is not necessary, refer to the protocol **EDTA/Ethanol precipitation of Cycle-Sequenced Products**.

This protocol assumes a 10µl cycle-sequencing reaction. For larger (or smaller) volumes, alter volumes below proportionately.

1. To each well add 1µl 125mM EDTA to each well. Make sure EDTA dissolves into samples. Mix with pipette tips.
   *EDTA is a known XXX, handle with care. Prepare 125mM ahead of time: dissolve into 18MΩ water and filter sterilize. EDTA takes about 1hr to dissolve. EDTA can be dispensed into sterile boats or 0.2ml tubes for multi-channel pipetting, but do not save used aliquots.

2. Add 1µl 3M NaOAc to each well. Make sure NaOAc dissolves into samples. Mix with pipette tips.
   *NaOAc should be prepared regularly, but need not necessarily be made fresh. NaOAc is a known XXX, and is at pH 4.8 by glacial acetic acid. NaOAc solution is very vaporous; do not leave open on benchtop for prolonged periods.

3. Add 25µl 100% EtOH (from freezer) to each well. Mix with pipette tips.
   *you may use 95% EtOH, but will need to add more (32µl) to maintain final EtOH concentration (67-71%).

4. Replace sealing tape, seal very well and vortex lightly. Incubate at room temp for 15min.

5. Spin @ 2500g 30min.

6. Immediately flick plate contents into sink and lightly tap dry on paper towels.
   *if you do not immediately empty plate once spin has finished (5min or less), give samples a quick spin (2500g, 2min) to re-precipitate pellet

7. Invert plate onto paper towel and spin 185g for 1min to dry.