MOLECULAR DETECTION AND FACTORS ASSOCIATED WITH SELECTED TICKBORNE ZOONOSES IN DOGS IN CHILANGA DISTRICT IN ZAMBIA

By

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DECLARATION

I, Pipina Anna Vlahakis, hereby declare that the work presented in this dissertation is my own original work. It is being submitted for the Degree of Master of Science in Medical Parasitology at the University of Zambia, Lusaka. It has not been submitted for any degree at this or any other University.

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SUPERVISOR:

I have read this dissertation and approved it for final submission.

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CERTIFICATE OF APPROVAL

This dissertation of **Pipina Anna Vlahakis** has been approved in partial fulfilment of the requirements for the degree of Master of Science in Medical Parasitology at the University of Zambia.

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Examiner 3	Signature	Date

ABSTRACT

Vector borne diseases, also referred to as zoonoses, have been responsible for some of the worst plagues of mankind. The close relationship between humans and animals increases chances of transmission of vector-borne zoonoses. Among some of these infections are pathogens transmitted by ticks which are currently considered to be second only to mosquitoes as vectors of human infectious diseases in the world. Tick-borne zoonoses are not considered a priority due to resource constraints and focus on other more fatal vector-borne diseases. The aim of this study was to determine the prevalence of specific canine associated tick-borne zoonotic pathogens including *Rickettsia* species*, Ehrlichia canis* and *Anaplasma phagocytophilum* in dogs.

A cross sectional study was conducted from February 2016 to September 2016. A total of 301 canine blood samples were collected from three localities in Chilanga district and analyzed at University of Zambia School of Veterinary Medicine. The Polymerase Chain Reaction (PCR) was performed using species specific primers targeting a portion of the 16S rRNA gene for the *Anaplasma* spp and *Ehrlichia canis* and *gltA* gene for *Rickettsia species*. Approximately nine positive samples of *Anaplasma* spp and 15 positive samples of *E. canis* were sequenced using the Genetic Analyzer 3130 Applied Biosystems (AB, USA). Epidemiological data was analyzed using the Chi-square test and logistic regression in SPSS version 20.

Out of the total 301 dogs sampled, 79.7% had ticks on them. The prevalence of *Rickettssia* spp was 0.3%, *Ehrlichia canis* was 34.9% and that of *Anaplasma spp* was 9%. The risk factors found to be associated with increased prevalence of infection with tick-borne pathogens of zoonotic importance included breed of dog, use of the dog, its level of confinement as well as the method of tick control used by the owner. For *E. canis* infections, the factors that were found to increase the risk of infection by zoonotic tick-borne pathogens were use of non-conventional tick control methods and functions dogs were occupied with specifically hunting as well as roaming with odds ratios of 2.0, 10.0 and 3.0, respectively. For *Anaplasma* spp infection, age and breed were found to be factors influencing infection. Dogs younger than one year old were more likely to be infected (OR = 2.4) compared to the older dogs aged one year and above. Mongrels were nine times more likely to be infected by *Anaplasma* spp compared to the cross breeds. After sequencing, *Ehrlichia* spp. in our study showed 100% similarity to *Ehrlichia canis*. Two types of *Anaplasma* spp. were detected, the first type was similar to *Anaplasma platys* and the second type was similar to *Anaplasma* spp *closely related to A. phagocytophilum* detected in South African Dogs. The results of this study indicated that tick-borne zoonotic pathogens are prevalent in canines in Chilanga District and this portrays a potential risk of human exposure because of their companionship with dogs. Therefore, there is need to raise awareness of the dangers to the communities about the threat of these pathogens as well as the remedial measures they can easily use to reduce the risk to themselves through quality care of their animals.

Key words: Vector-borne diseases, *Rickettsia* species, *Anaplasma* species, *Ehrlichia* species.

DEDICATION

I dedicate this dissertation to my husband, for his love and spiritual support and for encouraging me to soldier on when it was rough.

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ABBREVIATIONS

A	*Anaplasma*
ATBF	African Tick Bite Fever
CNS	Central Nervous System
CVBD	Canine Vector-borne Diseases
DNA	Deoxyribonucleic Acid
DBS	Dried Blood Spots
E	*Ehrlichia*
EDTA	Ethylene Diamine Tetraacetic Acid
HGA	Human Granulocytic Anaplasmosis
HE	Human Granulocytic Ehrlichiosis
HME	Human Monocytic Ehrlichiosis
HUCZC	Hokkaido University Centre for Zoonosis Control
IFA	Indirect Immunofluorescence Assay
MEGA	Molecular Evolutionary Genetic Analysis
MIF	Micro Immunofluorescence
PCR	Polymerase Chain Reaction
R	*Rickettsia*
RMSF	Rocky Mountain spotted fever
SATREPS	Science and Technology Research Partnership for Sustainable Development
SFG	Spotted Fever Group
SOM	School of Medicine
SP	Specie (singular)
SPP	Species (plural)
TE	Tris-EDTA
UNZA	University of Zambia
UTH	University Teaching Hospital
WB	Western Blotting

CHAPTER 1

INTRODUCTION

1.1 Background

Tick-borne diseases are currently an emerging and re-emerging problem affecting tropical and semi-tropical regions especially within the last 30 years (Parola *et al*., 2005; de la Fuente and Kocan, 2006; Piesman and Eisen, 2008; Nicholson *et al*., 2010; Vesco *et al*., 2011; Estrada-Pena *et al.*, 2012). Viral (Colorado tick fever), bacterial (anaplasmosis and protozoal (babesiosis) pathogens are known to be transmitted by ticks (Kim *et al*., 2006) resulting in medical and veterinary concerns (Dantas-Torres *et al.,* 2012). These ticks and the wildlife they feed on are reportedly the main reservoirs of some of the important tick-borne diseases reported the World over (Dantas-Torres *et al*., 2012), passing on the infections to domestic animals and pets when these venture into areas shared with wildlife (Dantas-Torres *et a*l., 2012). Tick-borne diseases cause life-threatening clinical conditions in dogs, and these have potential to result in human infection (Shaw *et al.,* 2001), rendering the name zoonotic infections or diseases. The close bonding and relationship between man and dogs put man at the risk of contracting these infections (Harrus and Baneth, 2005; Gubler 2009). There are approximately 800 tick species (spp) worldwide (Berrada and Telford, 2009) and currently, more than 40 of these are widely distributed across Africa, indicating the potential distribution of tick-borne zoonoses across the continent (Walker *et al*., 2003).

Anaplasmosis, ehrlichiosis, and babesiosis are among the most common tick-borne zoonoses whose transmission to humans is closely associated with dogs (Baneth *et al*., 2012). Ehrlichiosis caused by *Ehrlichia ruminantium* (*E. ruminantium*), *Ehrlichia canis* (*E. canis*) and *Anaplasma phagocytophilum* (*A. phagocytophilum*); babesiosis caused by *Babesia microti*; relapsing fever caused by *Borrelia duttonii* (*B. duttonii*) and rickettsioses caused by *Rickettsia africae (R. africae), Rickettsia aeschlimannii (R. aeschlimannii)* and *Rickettsia conorii* (*R. conorii*) have all been reported in Europe, Asia, Africa and Southern Africa in particular (Beugnet and Marie, 2009; Chitanga *et al.,* 2014). Of these tick-borne zoonoses reported within the region, *A. phagocytophilum, E. canis, Ehrlichia chaffeensis* (*E. chaffeensis*) and *R. conorii* have been reported to be associated with dogs and their owners hence making them of public health significance (Otranto *et al.,* 2009 part two ; Otranto *et al.*, 2010)

1.2 Statement of the problem

Human beings and dogs have both been reported to be exposed and susceptible to infections caused by many of the same tick-borne pathogens (Nicholson *et al.,* 2010). Among these recognised zoonotic tick-borne pathogens are rickettsial pathogens such as *A. phagocytophilum, Anaplasma platys* (*A. platys*)*, E. canis* and *R. conorii* (Nicholson *et al.,* 2010; Dantas-Torres *et al.,* 2012). Companion animals such as dogs act as reservoirs for human tick-transmitted infectious agents (Shaw *et al*., 2001), which are reportedly on the increase worldwide (Piesman and Eisen 2008).

Zoonotic tick-borne diseases have been reported to be prevalent in different parts of Zambia in wildlife carnivores and domestic dogs, livestock as well as human beings (Okabayashi *et al.,* 1999; Makala *et al.,* 2003; Nakayima *et al.,* 2014; Williams *et al.,* 2014). Although these diseases have been reported in many parts of Africa including Angola, South Africa, Botswana, Kenya, Ivory coast and many others, there is still little information on these infections in dogs in other parts of Africa (Inokuma *et al.,* 2005), which includes Zambia. The occurrence of the tick-borne pathogens under investigation in this study has not been surveyed in dogs in Zambia and those that have been investigated have been found to be absent.

Tick-borne diseases are not considered a priority in Zambia due to resource constraints and focus on other more fatal vector-borne diseases such as malaria (Makala *et al*., 2003). There is a possibility of misdiagnosis of some fever associated infections managed as malaria or other infections due to similar clinical presentation (Makala *et al*., 2003). Many health practitioners fail to consider the possibility that they may be dealing with zoonotic diseases or ignore the public health implications this type of infection may cause (Cripps, 2000). The impact of zoonotic diseases is rarely investigated especially in animals such as dogs and this makes it difficult to estimate their contribution to human infections (Cripps, 2000). There is need therefore to determine the prevalence of tick-borne zoonotic diseases in low economic status countries such as Zambia.

1.3 Justification of the problem

Dogs have been shown to be competent reservoir hosts of a number of zoonotic agents and due to their close interaction with humans, they can readily serve as a source of infection to humans (Otranto *et al.,* 2009). Generally, the frequency and range of zoonotic tick-borne diseases (TBDs) is increasing worldwide affecting both dogs and human beings (Nicholson *et*

al., 2010). Important zoonotic TBDs including anaplasmosis, ehrlichiosis, and rickettsioses are attracting more attention from physicians and veterinarians in Europe (Dantas-Torres *et al.,* 2012). The knowledge of occurrence and the prevalence of these diseases are necessary for the determination of the local risk and familiarising medical practitioners and veterinarians with not only the prevalence of these pathogens, but also the emergence of new infections and the prediction of possible vector-borne outbreaks (Kamani *et al.,* 2013).

World Organisation for Animal Health (OIE) recognises zoonotic diseases as being both a global and regional issue, driven by human activities and behaviour and recommends the need for coordination between public health and Veterinary sectors (WHO, 2004). Vector-borne diseases affecting human beings such as malaria, dengue fever, among others compose high priority in public health and veterinary services in developing countries. However, in low-income countries such as Zambia, zoonotic canine vector-borne diseases (CVBDs) are of low priority in terms of control despite the high risk of transmission to humans (Otranto *et al.,* 2009). Both developing and developed countries have scanty, unreliable and out-dated information on the distribution of arthropods and CVBDs probably because of inadequate veterinary diagnostic services and lack of surveillance (Dantas-Torres, 2008). Documentation of infection in dogs which are arguably more often in contact with ticks than humans should encourage veterinary professionals to warn owners of an increased risk of TBDs (Nicholson *et al.,* 2010).

1.5 Objectives

1.5.1 General objectives

To determine the prevalence of selected zoonotic tick-borne pathogens and the factors associated with occurrence of these pathogens in dogs in Chilanga district using molecular methods.

1.5.2 Specific objectives

1.5.2.1 To detect the prevalence of *E. canis, Anaplasma* spp *and Rickettsia* spp from blood collected from dogs in Chilanga district.
1.5.2.2 To determine the phylogenetic relationships of the pathogens detected from dogs in Chilanga district.

1.5.2.3 To determine the risk factors associated with the occurrence of *E. canis, Anaplasma* spp *and Rickettsia* spp in dogs.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview of Tick-borne Diseases

Vector-borne diseases have an effect on human and animal health and the global economy, contributing 17% to the burden of all infectious diseases (Fabruary, 2015). Canine vector-borne diseases (CVBDs) such as tick-borne diseases constitute a group of globally distributed and rapidly spreading illnesses that are caused by a range of pathogens transmitted by arthropods such as ticks and mosquitoes among others (Otranto *et al.,* 2009). Tick-borne diseases constitute serious economic and human health problems, with tick-borne zoonoses emerging worldwide (Nicholson *et al.,* 2010; Piesman and Eisen 2008; Vesco *et al.,* 2011). Dogs are commonly known as man's best friend based on their close relationship with humans and they are also known to be loyal, hunters, guards and great companions (Derr and Mark, 2013). The increasingly close relationship with humans poses a concern for human public health because dogs are competent reservoir hosts of several zoonotic agents and can serve as a readily available source of nutrition for many blood-feeding arthropods (Otranto *et al.,* 2009). Both dogs and humans are susceptible to tick-borne pathogens such as *A. phagocytophilum,* which causes anaplasmosis*, E. canis* which causes ehrlichiosis*, R. conorii*, *R. africae* and *R. aeschlimanii* which cause rickettsioses among other spotted fever group pathogens (Pretorius and Birtles, 2004; Rutherford *et al.,* 2004; Nicholson *et al*. 2010). These pathogens and the diseases they cause have been reported in ticks, humans and animals in different parts of Africa especially in Northern, Eastern and Southern Africa (Parola *et al*., 2013).

2.2 Description and Aetiology Tick-borne Diseases

2.2.1 Rickettsioses

Rickettsiosis is a febrile illness caused by obligate intracellular pathogens of genus *Rickettsia.* Rickettsiosis is mostly transmitted by ticks, but can also be transmitted by fleas, lice and mites (Parola *et al*., 2005). The species and associated human clinical diseases vary depending on the geographical locations, despite the worldwide distribution (Sahni *et al*., 2009).

Rickettsial diseases are among the oldest known vector-borne diseases distributed globally. Rickettsioses have led to morbidity and mortality that has had a major effect on the military activities and the public health in general for more than 2000 years (Kelly *et al*., 2002; Parola and Rauolt, 2006). These diseases are incapacitating, sometimes deadly but often unrecognized diseases cause fevers in a susceptible population (Kelly *et al.,* 2002). Different types of ticks play a role in the transmission of these diseases in dogs and human beings. These bacteria can be broadly divided into two groups which include spotted fever group transmitted by ticks or mites and the typhus group mainly transmitted by lice or fleas, with the spotted fever group being of importance in sub-Saharan Africa (Cazorla *et al.,* 2008). The range and importance of the recognized tick-associated rickettsial pathogens have increased dramatically in the past 25 years, reporting 26 *Rickettsia* spp with validated and published names (Parola *et al.,* 2005). *Rickettsia africae*, *R. aeschlimannii* and *R. conorii* are spotted fevers of medical importance and have been recorded in both Eastern and Southern Africa in ticks, domestic animals, livestock and humans (Cazorla *et al.,* 2008). The typhus group includes the Epidemic typhus and murine typhus which are caused by *R. prowazekii* and *R. typhi* respectively. Murine typhus and epidemic typhus are difficult to differentiate due to similar clinical symptoms, but epidemic typhus is more severe than murine typhus (Yu and Walker, 2012). Epidemic typhus, also known as louse-borne typhus is distributed worldwide and was one of the man's major scourges and regularly played a decisive role in wars in Europe from the 15th through 20th centuries (Conlon, 2007).

2.2.2 Ehrlichiosis

Ehrlichia is Gram-negative obligatory intracellular coccobacilli of the Anaplasmataceae family, causing ehrlichiosis in domestic animals and humans. The pathogen has long been considered a veterinary pathogen, but recent reports have shown the pathogens to be zoonotic (Dumler *et al.,* 1995; Dantas-Torres *et al.,* 2008). The genus *Ehrlichia* is classified into five species and these include *E. canis, E. muris, E. chaffeensis, E. ewingii* and *E. ruminantum* (Nicholson *et al.,* 2010). The pathogen *E. canis* causes monocytic ehrlichiosis in dogs while *E. ruminantum* is an agent of heartwater in ruminants. The species in the *E. canis* genogroup, *E. ewingii* and *E. chaffeensis* have also been implicated in human diseases (Nicholson *et al*., 2010). These bacteria are considered the agents responsible for the emergence of Human Monocytic Ehrlichiosis (HGE). Ehrlichiosis is a world-wide distributed zoonosis (Perez *et al.,* 2005), but mainly in tropical and subtropical regions due to the geographical distribution of its vector tick Ixodidae *R. sanguineus* (Andereg and Passos, 1999). *Ehrlichia canis* and *E.*

chaffeensis are globally the two most common causes of ehrlichiosis. Canine monocytic ehrlichiosis (CME) was first recognized as a disease in dogs in 1935 and has since been reported worldwide ever since (Donatien and Lestoquard, 1935). In 1996, *E. canis was* first isolated and molecularly characterized from asymptomatic humans in Venezuela although its role as a pathogen was not immediately recognized until 2006 (Perez *et al.,* 2006). *Ehrlichia canis* is now considered incompatible human illness in endemic areas when 78% prevalence has been detected in dogs from those areas (Diniz *et al.,* 2007).

2.2.3 Anaplasmosis

Anaplasma phagocytophilum is an obligate intracellular bacterium transmitted by Ixodes ticks. It belongs to the family *Anaplasmataceae*. It resides within the granulocytes, particularly neutrophils (Lillinie *et al.,* 2006). *Anaplasma phagocytophilum* was initially referred to as the HGE agent when identified in 1990, but was later described as Human Granulocytic Anaplasmosis (HGA) (Chen *et al*., 1994), based on its high genetic homology, presence of shared antigens and common biological characteristics with HGE (Dumler *et al.,* 2001). Human granulocytic anaplasmosis was first recognised in a Wisconsin patient who died with a severe febrile illness 2 weeks after a tick bite (Chen *et al*., 1994). By 1999 over 600 cases in the United States of America were reported and these have since increased and are now recognised in Europe and other continents (McQuiston *et al*., 1999). Another organism in the same genus that causes anaplasmosis is *Anaplasma platys,* which was first detected in the United States of America (USA) in Florida in 1979 by Harvey *et al*. It is an obligate intracellular rickettsial organism, transmitted by the brown dog tick (*Rhipicephalus sanguineus*) and it infects platelets causing cyclic thrombocytopenia in dogs (Rymaszewska and Grenda, 2008; Matei *et al*., 2016). *Anaplasma platys* is known as a pathogen of low virulence, often in association with other infections or diseases (Gaunt *et al*., 2010).

2.3 Susceptible Species of Tick-borne Pathogens

Cattle and goats are the most important domestic hosts, although sheep, horses, donkeys, giraffes, buffaloes, antelopes and warthogs are also frequently infected by *R. africae* (Althaus *et al.,* 2010). Dogs and human beings have been reported to be susceptible species of *R. conorii* (Solano-Gallego *et al.,* 2006; Pennisi *et al.,* 2012). Dogs, human beings, cattle and camels have also been reported to be susceptible for *R. aeschlimanii* (Raoult *et al*., 2002; Kernif *et al.,* 2012). Dogs, other wild canids such as wolves and foxes, jackals, cats and human beings are susceptible to infection with *E. canis* (Harvey *et al.,* 1979; Perez *et al.,*

1996; Breitschwerdt *et al.,* 2002; Perez *et al.,* 2006). A number of mammals are susceptible to *A. phagocytophilum*. Anaplasmosis has been described in dogs, horses, cattle, sheep, goats, llamas, cats and humans (Mazepa *et al.*, 2010; Eberts *et al.,* 2011; Gorna *et al.,* 2013). Human beings and domestic animals are considered incidental hosts (Poitout *et al.*, 2005).

2.4 Distribution of Ticks and Tick-borne Diseases

2.4.1 Rickettsioses

Tick-borne spotted fever group rickettsial pathogens are globally distributed in ticks, animals and humans. They are distributed in America, Europe, Asia and Africa. *Rickettsia africae* which cause African tick-borne fever (ATBF) has been reported in North and Central America, Pacific island Asia, North, East and sub-Saharan Africa (Parola *et al.,* 2013). Tick surveys in the North and Central America revealed infection rates ranging from 7% to 62% (Robinson *et al.,* 2009; Kelly *et al.,* 2010). Most of the human cases were common in travelers, tourists and deployed military members who had a history of having visited *R. africae* endemic regions (Jensenius *et al.,* 2003; Owen *et al.,* 2006). In sub-Saharan Africa, *R. africae* has been reported in 22 countries in ticks and/or humans. Among travelers returning from sub-Saharan Africa, ATBF was the second most frequent cause of infection after malaria. It was detected in ticks in Djibouti, Guinea, Senegal, Nigeria, Liberia and Democratic Republic of Congo (DRC) (Portilla *et al.,* 2007; Socolovschi *et al.,* 2007; Mediannikov *et al.,* 2010; Mediannikov *et al.,* 2012; Ogo *et al.,* 2012).

Rickettsia conorii has been detected in Europe, North Africa and sub-Saharan Africa (Parola *et al.*, 2013). *Rickettsia conorii* causes Mediterranean Spotted Fever (MSF) which has been reported to be endemic in southern Europe, but sporadic in northern and central Europe (Parola *et al*., 2005). A seroprevalence rate of 15-72% was detected in dogs in areas where MSF is endemic (Pennisi *et al.,* 2012). The closeness of seroactive dogs and humans was reported as a risk for humans to be infected with MSF (Solano-Gallego *et al.,* 2006). From 2005, *R. conorii* has been reported in 14 countries including Serbia, Romania, Italy, Spain, Slovakia and Malta. Fatal cases have been reported in Portugal, France, Greece, Bulgaria and Turkey (Botelho-Nevers *et al.,* 2005; Kuloglu *et al.,* 2006; Sousa *et al.*, 2008; Papa *et al*., 2010; Baltadzhiev and Popivanova, 2012). In North Africa, the pathogen was reported in Algeria, Tunisia and Morocco among others. In Algeria, 49% of patients were diagnosed with severe disease some of which resulted in neurological disturbances and death. Approximately

76.5% to 95.2% of the cases were found to have direct contact with dogs and 30.8% to 50.3% cases had a history of tick bites (Kernif *et al*., 2012). Finally in sub-Saharan Africa *R. conorii* has been reported to be prevalent in nine countries of this region including; Zimbabwe, Senegal, Uganda, Swaziland, Kenya and South Africa in ticks from livestock, dogs and humans (Yoshikawa *et al*., 2005; Buchau *et al.,* 2006; Socolovschi *et al.,* 2007; Mediannikov *et al*., 2010; de Almeida *et al*., 2010). Okabayashi *et al*. (1999) reported 3%, 23.1% and 16.7% of *R. conorii* infections in humans from Kasama, Chipata and Senanga districts in Zambia in 1999. In 2014, Nakayima *et al*. (2014) detected 39.8% of *Rickettsia spp* in baboons and vervet monkeys in Zambia.

Rickettsia aeschlimannii was reported in ticks in Asia and humans in Europe, North Africa and sub-Saharan Africa. In Europe this pathogen was reported in ticks in Croatia, Spain, southern France, Portugal, Italy, Russia, Cyprus, Germany, Turkey and the Greek island of Cephalonia (Fernandez *et al*., 2003; Mura *et al*., 2008; Shpynov, 2009; Rumer *et al.,* 2011; Gargili *et al.,* 2012; Chochlakis *et al*., 2012). This pathogen has also been collected from ticks from camels and/or cattle in Tunisia, Sudan, Egypt and Algeria and from humans in Algeria and Morocco where the first human cases of *R. aeschlimannii* was detected in North Africa (Raoult *et al.,* 2002; Mokrani *et al*., 2008; Sarih *et al*., 2008; Kernif *et al*., 2012). *Rickettsia aeschlimannii* was also found prevalent in South Africa, Senegal and Nigeria in ticks from different species that include cattle, goats, sheep and horses (Parola *et al*., 2005; Mediannikov *et al*., 2010; Reye *et al.,* 2012).

2.4.2 Ehrlichiosis

Dumler *et al.* (2005) reported that ehrlichioses causes significant morbidity and mortality in dogs and people globally. *Ehrlichia canis* has been reported in outbreaks in the previous years in Singapore, Malaysia and Vietnam (Shaw and Day, 2005). Other countries that have reported *E. canis* include India, United States of America (USA), Portugal, Turkey, Spain and Israel. Dogs infected with this pathogen manifests a wide range of clinical signs varying from mild weight loss to a severe and often fatal haemorrhagic syndrome and damage to the system of the host if treatment is not administered on time (Lakshmanan *et al*., 2007).This pathogen was detected in 22% of ticks in Nigeria as reported by Kamani *et al.* (2013) and 29.5% also in ticks in Uganda (Proboste *et al*., 2015). Canine Monocytic Ehrlichiosis caused by *E. canis* is commonly reported in veterinary practice and has been found in humans in South Africa and Mozambique (Brouqui *et al.,* 1994, Inokuma *et al.,* 2005). In animals, *E.*

canis was detected around Eastern and Southern Africa. In Malawi a 22% seroprevalence was reported in pre-owned dogs, 36.4 % in Kenya, 22.5% South Africa, 22% Malawi, 5.8% Angola, Botswana, Zimbabwe and 7.3% in Zambia in dogs from the veterinary clinic (Matjila *et al*., 2008; Sibanda *et al.,* 2012; Nalubamba *et al*., 2014; Kitaa *et al*., 2014; Cardoso *et al.,* 2016; Alvåsen, 2016; Matei *et al.,* 2016).

2.4.3 Anaplasmosis

Anaplasma phagocytophilum infection has a global geographic distribution in both humans and domestic animals (Diniz and Breitschwerdt, 2012). Among the countries in which *A. phagocytophilum* is endemic include the upper Midwest, New England, western coast especially northern California, eastern and northeastern regions of the USA, British Colombia, Poland, Germany, Finland, Scandinavia, Bangladesh, South America and the Baltic countries (Swanson *et al*., 2006; Kohn *et al*., 2011; Talukder *et al.,* 2013; Berzina *et al.,* 2013; Keesing *et al.,* 2014; Sainz *et al.,* 2015). This pathogen has also been reported in North Africa, in Tunisia, Algeria and Egypt in ticks, humans and horses (M'ghirbi *et al*., 2008; Ghafar and Amer, 2012; M'ghirbi *et al.,* 2012; Azzag *et al*., 2015). Eastern and southern Africa reported *A. phagocytophilum* in South Africa, Angola and Zambia in livestock, monkeys, vervet monkeys and dogs (Inokuma *et al.,* 2005; Mtshali *et al.,* 2012; Nakayima *et al* 2014; Cardoso *et al.,* 2016).

Anaplasma platys, another agent of Anaplasmosis have been reported in North Portugal, Grenada and Japan in both dogs and ticks (Inokuma *et al.,* 2003; Yabsley *et al.,* 2008; Cardoso *et al*., 2010). This pathogen has also been detected in different parts of Africa that include Kenya and Ivory Coast (Matei *et al.,* 20016).

2.5 Risk Factors of Tick-borne Diseases

The risk of tick-borne infection is greatly associated with being bitten by a tick and host population dynamics (Dantas-Torres, Chomel and Otranto, 2012). Traveling or residing within endemic areas increases the chance of infection for both companion animals and human beings (Baba *et al.,* 2012). Humans, domestic animals and companion animals are at high risk of infection when they enter tick infested areas like wooded areas with wild animals infested with ticks and areas with long grass (Dantas-Torres *et al.,* 2012). Outdoor activities of pets and pet owners also increase the chance of infection with tick-borne pathogens. Tick activity is generally heightened during the spring and summer months because the

temperatures are favorable for survival (Delgado and Cármenes, 1995; Gubler, 2009). Cases of blood transfusion and organ transplantation have been recorded as methods of transmission although they are very rare (Linden and Bianco, 2001). Different studies have reported age, outdoor activities, tick infestation history, age and exposure to farms as some of the risk factors (Jones *et al.,* 2002; Kamani *et al.,* 2013)

2.6 Vectors and Transmission of Tick-borne Diseases

2.6.1 Rickettsioses

Rickettsial diseases are mainly transmitted by arthropod vectors, including ticks, mites, fleas, and lice (Glaser *et al.,* 2010). Typhus group rickettsiae are transmitted to humans through the faeces of lice and fleas while the spotted fever group rickettsiae are predominantly transmitted by ticks (Socolovschi *et al.,* 2009; Azad and Beard, 1998). Ixodides are the main vectors of SFG rickettsiae. Ixodides also known as hard ticks are bloodsucking arthropods throughout all of their developmental stages, except from some of the adult male ticks in some species (Brouqui *et al.,* 2007). During a blood meal, tick-borne rickettsia is inoculated into the skin of the host from the arthropod's saliva. Interestingly some cases of tick-borne rickettsioses have been reported to be also transmitted by transfer of rickettsiae to the conjunctiva by fingers contaminated with infectious tick hemolymph or organs after crushing a tick that has been removed from a dog or human (Valbuena and Walker, 2009).

Ambylomma hebraeum and Ambylomma variegatum are the main vectors that are involved in the transmission of *R. africae* causing African tick-bite fever (ATBF) in humans (Jensenius *et al.,* 2003). *Rhicephalus sanguineus* is a tick vector and potential reservoir commonly known for transmitting *R. conorii* and causing MSF. Other recognized vectors include *Rhipicephalus evertsi evertsi, R. mushamae, Rhipicephalus simus* and *Haemaphysalis leachi* resulting in infection in horses, cattle and dogs (Parola, Paddock and Raoult, 2005; Mediannikov *et al.,* 2010). *Rhipicephalus aeschlimanni's* main vector is *R. appendiculatus*, although the pathogen has also been detected in *H. marginatum marginatum*, *H. marginatum rufipes, Rhipicephalus sanguineus, Rhipicephalus turanicus, Rhipicephalus bursa* (Parola *et al.,* 2001; Bitam *et al.,* 2006; Parola *et al.,* 2013).

The transovarial and transstadial transmission of Spotted fever group (SFG) rickettsiae within tick vectors in nature ensures rickettsial survival without requiring the complexity inherent in an obligate multihost reservoir system (Gonzalez *et al.,* 1992; Nabeth *et al.*, 2004).

Rickettsiae in the ticks' salivary glands transmit the infection to vertebrate hosts during feeding. Therefore, as larvae, nymphs and adults may all be infective for susceptible vertebrate hosts resulting in infection (Brouqui *et al.,* 2007).

2.6.2 Ehrlichiosis

Ticks have the primary role in the transmission process of *Ehrlichia* pathogens and act as vectors, carrying the pathogenic bacteria to and from their multiple hosts (Ganguly *et al.,* 2008). *Ehrlichia canis* is mainly transmitted by *R. sanguineus*, the brown dog tick which has a worldwide disease distribution in dogs, wild canids and humans (Shipov, 2008). *Dermacentor variabilis* has also been reported to be a vector transmitting *E. canis* (Johnson *et al.,* 1998). Other *Ehrlichia* species such as *E. chaffeensis* are prominently transmitted by *Ambylomma americanum* ticks, which are vectors to humans and dogs. *Ehrlichia ewingii* is most importantly transmitted by *A. americanum* and is also known to be transmitted by *D. variabilis* and *R. sanguineus* in dogs and deer (Murphy *et al.,* 1998; Dumler *et al*., 2007). *Ehrlichia canis* is transmitted transstadially and not transovarially in both *R. sanguineus* and *D. variabilis* (Johnson *et al.,* 1998; Bremer *et al.,* 2005). The lifecycle of *Ehrlichiae* is complex and involves a tick, vector and mammalian host. Tick nymphs or larvae are infected with *E. canis* after constantly feeding on an infected dog. Transstadial transmission occurs to subsequent stages of the tick vector. Mammalian hosts such as dogs and humans are infected through salivary gland secretions during blood feeding resulting in Canine Monocytic Ehrlichiosis or HME. Transmission of disease through blood transfusion has also been reported (Groves *et al.,* 1975; Ettinger, Feldman and Edward, 1995).

2.6.3 Anaplasmosis

Tick vectors of different species transmit anaplasmosis. The bacterium is maintained in a transmission cycle by ticks of *Ixodes persulcatus* complex (Stuen *et al.,* 2013; Swanson *et al.,* 2006). These ticks have a worldwide distribution but are mainly encountered in the northern hemisphere (Swanson *et al.,* 2006; Woldehiwet, 2010). Ixodes species involved in the transmission of the Anaplasmosis varies according to the geographic area and these includes *I. scapularis* in the eastern United States, *I. pacificus* in the western United States, *I. ricinus* in Europe, and probably *I. persulcatus* in parts of Asia. This tick species has a geographic area that extends into Japan and is considered the primary vector in Asia (Sainz *et al*., 2015; Swanson *et al.,* 2006). The brown dog tick (*Rhipicephalus sanguineus*) and *Dermacentor* species transmit *A. platys*. The organism is transmitted to humans and other domestic animals

such as dogs (Annen *et al.,* 2012). *A. phagocytophilum* is transmitted to the host during the bite of a nymphal or adult tick infected during previous larval or nymphal stages (Doudier *et al.,* 2010; Nicholson *et al.,* 2010; Diniz and Breitschwerdt, 2012). Unlike the adult stage, nymphs are very small size (approximately 1 mm) and often feed much longer, therefore, increasing the risk to transmit *A. phagocytophilum*. Nymphs and adult ticks contaminated in a previous stage are able to contaminate susceptible hosts during the following blood meals because of transstadial transmission (Swanson *et al.,* 2006; Heyman *et al*., 2010).

2.7 Pathogenesis and Clinical Presentation of Tick-borne Diseases

2.7.1 Rickettsioses

There are several steps that result from the interaction between rickettsiae and the host cell of mammals such as recognition, entry, phagosome escape, growth, actin-based motility, cell-to-cell spread and cell lysis. The transmitted rickettsiae are hematogenously disseminated and the bacteria preferentially infect endothelial cells lining the small blood vessels after the rickettsial entry into the dermis (Walker *et al.,* 2003; Balraj *et al.,* 2009). Pathogenesis is fundamentally due to irreversible destruction of the cells by the replicating bacteria, due to the fact that rickettsiae do not produce soluble toxins (Teysseire *et al.,* 1995). Rickettsiae have the ability to infect any type of nucleated cells and their main target cells are endothelial cells whose destruction results in leakage of fluid from the bloodstream due to increased vascular permeability and this is more critical in the lungs and brain (Walker *et al.,* 2003). The fluid is collected in the surrounding tissue, resulting in edema that leads to organ and tissue damage (Walker *et al*., 2003).

Disease manifestation is similar in dogs and humans. Clinical signs vary with the causative agent and patient though common symptoms that typically develop within 1–2 weeks of infection include fever which occurs 2-3 days after tick attachment, headache, malaise, myalgia, rash, nausea, abdominal pains and vomiting. Many rickettsioses are accompanied by a maculopapular, vesicular, or petechial rash or sometimes an eschar at the site of the tick bite (this is common in spotted fever group rickettsioses). African tick-bite fever is typically milder than some other rickettsioses while Mediterranean spotted fever is a potentially life-threatening rickettsial infection (Eremeeva and Dasch, 2012).

2.7.2 Ehrlichiosis

The process of invasion of *E. canis* into their host cells includes cell adhesion, internalisation and intracellular proliferation, followed by exocytosis and intercellular spreading (McBride and Walker, 2011). *Ehrlichia canis* target and infect the mononuclear phagocytic cells once the pathogen is transmitted to a host. Monocytes or blood cells are the most infected cells within the human or canine host. Other cells that have been reported to be infected with *E. canis* include; lymphocytes, promyelocytes and metamyelocytes (Ismail *et al.,* 2010). *Ehrlichia* maintain and ensure their survival because they possess the capability to reprogram the systems and mechanisms of defense employed by the host cell (Ismail *et al.,* 2010). Documented mechanisms of immune invasion by *E. canis* include inhibition of bacterial trafficking to lysosomes and formation of phagolysosomes through lysosomal fusion, decreased reactive oxygen species (ROS), inhibition of host cell apoptosis, down-regulation of major histocompatibility (MHC) class II receptors on monocytes/macrophages, and recombination of outer membrane protein genes leading to antigenic (McBride and Walker, 2011; Liu *et al*., 2012). The involvement of immune mechanisms in the pathogenesis of ehrlichiosis is shown by the presence of haemolytic anaemia, platelet-bound anti-platelet antibodies and circulating immune complexes (Waner *et al*., 2000; Harrus *et al.,* 2001). Pathological findings in canines and humans have also described ehrlichial DNA present within the liver, lymph, spleen, heart, brain, Lungs and kidneys (Ganguly and Mukhopadhayay, 2008; Waner and Harrus, 2013). The highest density of ehrlichia is found within the bone marrow in the most severe chronic stages of the disease (Waner and Harrus, 2013).

There are mainly three different stages of clinical signs for CME which include acute, subclinical and chronic stages. Acute diseases last between 3 to 5 weeks. Thrombocytopenia and anemia are the most commonly observed acute clinical manifestations (Harrus and Waner, 2011). Other signs of the acute phase include fever, anorexia, depression, lymphadenopathy and splenomegaly. Other acute signs such as ocular discharge, pale mucous membranes, hemorrhagic tendencies, or neurological signs rarely occur (Harrus, Waner and Bark, 1997). In the subclinical phase the animal doesn't have prominent signs but may show slight anemia that lasts several years (Waner *et al*., 1997). The dog can become an asymptomatic carrier of the infection, eliminate *Ehrlichia* from the body or the infection may progress to the chronic phase. The chronic stage is characterized by recurrent clinical and hematological signs including thrombocytopenia, anemia, and pancytopenia (Skotarczak,

2003). Other clinical manifestations in dogs may include weight loss, depression, petechiae, pale mucous membranes, edema, and lymphadenopathy among other signs. Severe cases result in death from massive hemorrhage, severe debilitation, or secondary infections especially when the dogs fail to respond to antibiotic therapy (Hess *et al.,* 2006).

The clinical manifestations of HME and HGE are similar to that of dogs. The acute disease is characterized by non-specific signs such as a high fever, headache, myalgia, anorexia, digestive and respiratory disturbances, depression, dyspnea, lethargy, anorexia, lymphadenomegaly, splenomegaly and hemorrhagic tendencies (Bakken and Dumler, 2000; Komnenou *et al.,* 2007). The second phase is the subclinical phase which does not have prominent symptoms (Fishbein 1994; Bakken and Dumler, 2000). The chronic phase is characterized by signs and symptoms including; depression, weight loss, pale mucous membranes, abdominal pain, hemorrhage, lymphadenopathy, splenomegaly, increased lung sounds, hepatomegaly, arrhythmias, pulse deficits, polyuria, stiff, swollen and painful joints, ocular abnormalities and neurological disturbances among others (Bakken and Dumler, 2000). Ehrlichioses may be severe and even fatal, particularly in patients with underlying immunosuppression. Leukopenia, thrombocytopenia and elevated liver enzymes are frequent laboratory abnormalities (Fishbein 1994; Bakken and Dumler, 2000).

2.7.3 Anaplasmosis

Anaplasma phagocytophilum, being an intracellular microorganism uses different strategies to avoid intracellular killing by the host. Neutrophil and special protein-ligand P-selectin are present on the host cell and this mediates phagocytosis (Carlyon and Fikrig, 2003). In order to ensure its intracellular survival and replication, *A. phagocytophilum* colonizes intracellular environment by evading or subverting phagolysosome formation, it actively dysregulates key neutrophil bactericidal functions and delays neutrophil apoptosis allowing the pathogen to form morulae (Carlyon and Fikrig, 2003; Garyu *et al*., 2005). The pathogen also decreases endothelial adherence and transmigration of neutrophils, which normally occurs through selectin-mediated rolling, cellular activation, and binding via surface integrin molecules (Carrade *et al*., 2009). The damaged neutrophil function predisposes to the development of secondary opportunistic infections in livestock (sheep and cattle), dogs and humans (Carrade *et al*., 2009). Splenic lymphoid depletion, macrophage aggregates and apoptosis within the liver, paracortical lymphoid hyperplasia and hemophagocytic cells within tissues of the

reticuloendothelial system have all been described in humans regardless of the limited information available in the pathogenesis of humans (Lepidi, Bunnell and Martin, 2000).

The pathogenic impact of *A. platys* is not clearly defined in dogs. *Anaplasma platys* infect platelets leading to abnormalities of primary hemostasis. The geographic origin of the *A. platys* strains results in differences in the severity of anaplasmosis caused by this pathogen (Shaw *et al.,* 2001).

In dogs, the most common clinical signs and symptoms in the first to the second week of infection caused by *A.phagocytophilum* include lethargy and fever (Poitout *et al.,* 2005; Kohn *et al.,* 2008). Other symptoms include anorexia, reluctance to move and lameness. Less common clinical manifestations are charaterized by polydipsia, pale mucous membranes, gastrointestinal signs including vomiting and diarrhea, and hemorrhage manifested as mucosal petechiae, melena, or epistaxis (Poitout *et al.,* 2005; Kohn *et al.,* 2008). Chronic disease manifestations in both humans and dogs are still a subject of ongoing investigations (Carrade *et al*., 2009).

Human Granulocytic Anaplasmosis (HGA) clinical signs begin 10-14 days after the incubation period of the pathogen. Initial symptoms usually include; fever, headache and myalgia which are the most common signs (Dumler *et al.,* 2005). Less frequently reported symptoms include nausea, diarrhea, muscle pain, malaise, chills, abdominal pains, cough and confusion (Dumler *et al.,* 2005). Central nervous system involvement is rare although few cases of meningoencephalitis have been reported. Severity varies from person to person and people with compromised immunities such as HIV patients or those undergoing immunosuppressive therapies may suffer from more severe cases (Kohn *et al.,* 2008; Carrade *et al.,* 2009).

Infection with *A. platys* in dogs commonly occurs in blood platelets resulting in failure for blood to clot properly leading to bleeding disorders, bruising on the gums and belly and spontaneous nosebleeds (Egenvall *et al.,* 1997). A study in Venezuela reported by Arraga-Alvarado *et al.* (2014) reported signs and symptoms in humans that included fever, headaches, fatigue, anorexia, lethargy, muscle pain, primary hemostatic disorders as the pathogen destroys platelet cells of the host, mild anemia, and lymphadenomegaly among others.

2.8 Diagnosis of Tick-borne Diseases

2.8.1 Rickettsioses

The symptoms used in the clinical diagnosis of tick-borne spotted fever group rickettsioses typically include fever, headache, muscle pain, rash, local lymphadenopathy and the presence of an inoculated eschar at the site of the tick bite (Parola, Paddock and Raoult, 2005). Different laboratory diagnostic methods are used to detect tick-borne rickettsia. Culture is the gold standard method for microbiological diagnosis though agents that cause tick-borne rickettsial diseases are obligate intracellular pathogens and must be isolated from patient samples using cell culture techniques that are not readily available. Isolation of rickettsiae is also used for diagnosis using buffy coat preparations of heparinized or EDTA-anticoagulated whole blood, skin biopsy specimens, or arthropods (Parola, Paddock and Raoult, 2005). Serological tests are the most frequently used and available methods of diagnosis. The serological tests used include microimmunofluorescence (MIF) assay, indirect Immunofluorescence antibody (IFA), the Weil-Felix test, Cross-absorption (CA) and Western blotting (WB). Indirect immunofluorescence antibody assays are the reference standard for serologic confirmation of rickettsial infection (Raoult *et al.,* 2002). Cross-reactivity often exists among antigens of pathogens within the same genus and occasionally in different genera making this one of the major limitations of serology (Parola and Raoult, 2001). The sensitivity of detection by culture can be lower than molecular or serologic techniques depending on expertise (Sanogo *et al.,* 2003; Sanguioni *et al.,* 2005).

Rickettsiae can be detected occasionally in tissue specimens of numerous organs such as the liver, spleen, kidney, heart, meningeal membranes, or skin of humans and animals by various histochemical stains, including Giemsa or Gimenez stains (Green, Walker and Cain, 1978; La Scola *et al.,* 1997). Molecular methods such as PCR and sequencing methods are now used as sensitive and rapid tools to detect and identify rickettsiae in blood and skin biopsy specimens throughout the world. Primers amplifying sequences of several genes, including *ompA*, *ompB*, *gltA*, and *gene D*, have been used (Fournier *et al.,* 1998, Mathai *et al*., 2001, Sekeyova *et al.,* 2000; Fournier *et al.,* 2003; Brouqui *et al.,* 2004).

2.8.2 Ehrlichiosis

The different stages of CME and HME and the multiple clinical manifestations make clinical diagnosis of ehrlichiosis challenging (Harrus and Waner, 2011). Regardless of this, ehrlichiosis is usually suspected when a compatible history (living in or travelling from an

endemic region, previous tick exposure) is associated with typical clinical signs such as high fever, depression, lethargy, anorexia, lymphadenomegaly, splenomegaly among others and characteristic haematological and biochemical abnormalities are present (Harrus and Waner, 2011). Haematological analysis is an essential part of the diagnostic process, with thrombocytopaenia being a characteristic feature in both dogs and humans (Harrus and Waner, 2011). On blood smear evaluation, the demonstration of the typical cytoplasmic *E. canis* morulae in monocytes is considered diagnostic (Harrus and Waner, 2011). However, microscopy is difficult and time consuming, with a reported success rate of only about 4% (Woody and Hoskins, 1991). Different serological tests are used for the diagnosis of this disease and these include indirect immunofluorescence antibody (IFA) test for anti-*E. canis* IgG antibodies. The test is considered as the serological gold standard, indicating exposure to *E. canis* (McBride *et al.,* 2003). Enzyme-linked immunosorbent assays (ELISA) have also been found to be useful in the diagnosis of the disease (Harrus *et al.,* 2002). Western immunoblot is a more specific serological technique which can assist in characterizing the infecting agent and has proved useful in distinguishing between infections with *E. canis* and *E. ewingii* (Harrus and Waner, 2011). Isolation is also another method used, but the growth of *ehrlichia* species is time and labour consuming and also requires specific laboratory equipment and trained staff. It is used more in research laboratories and less as a diagnostic tool (Keysary *et al*., 2001). Molecular diagnostic tests such as PCR and sequencing are sensitive methods for detecting and characterizing *E. canis* DNA (Harrus and Waner, 2011). The 16S rRNA and the *p30* are most commonly targeted genes for PCR assays (Stich *et al.,* 2002).

2.8.3 Anaplasmosis

The diagnosis of HGA follows a certain diagnostic criteria for confirmation of the disease (Carrade *et al.,* 2009). This criterion includes clinical signs, laboratory findings indicating the presence of granulocytic anaplasmosis and detection of morulae within neutrophils, isolation of *A. phagocytophilum* from blood, a 4-fold increase or decrease in the antibody titer within 4 weeks and a positive PCR test result using specific *A. phagocytophilum* primers (Bakken and Dumler, 2008). The above criteria are also used in dogs (Carrade *et al.,* 2009). The finding of morulae within neutrophils in peripheral blood from a dog in an endemic area highly indicates the presence of *A. phagocytophilum* although the morulae cannot be distinguished from those of other *Ehrlichia* spp. such as *E. ewingii* unless other tests such as PCR are used for confirmation (Carrade *et al.,* 2009). Serologic assays for *A. phagocytophilum*, including

the widely used recombinant *Msp2* assay may also test positive for *A.platys* infection in dogs (Bowman *et al.,* 2009). Culture may be the most sensitive diagnostic modality for detection of acute infection in human patients but is rarely used in dogs (Aguero-Rosenfeld, 2002). Diagnosis can also be achieved using different serological tests such as immunofluorescent antibody (IFA) techniques and ELISA in both dogs and humans (Magnarelli *et al.,* 2001). Molecular techniques such as PCR and DNA sequencing are considered as the gold standard test on whole blood. These tests are usually used for confirmation. Sequencing of the PCR product helps determine whether *A. phagocytophilum* is the infecting species. The target genes used for PCR include 16S rRNA gene or the outer surface protein genes including *msp2 (p44)* (Egenvall *et al.,* 2000). *Anaplasma platys* is mainly detected using serology, including ELISA and IFAT (Arraga-Alvarado *et al.,* 2014).

2.9 Treatment of Tick-borne Diseases

Before confirmation of diagnosis, empirical antibiotic therapy should be prescribed in any suspected tick-transmitted rickettsioses, ehrlichiosis and anaplasmosis. Doxycycline remains the treatment of choice for spotted fever rickettsioses, anaplasmosis and ehrlichiosis in both dogs and people in adults and children too (Neer *et al.,* 2002; Wormser *et al.,* 2006; Dantas-Torres, 2007). Physicians and veterinarians should not wait for diagnostic test results because tests performed early in the course of the infection may be negative and delayed treatment may result in serious disease and long-term sequelae or death (CDC, 2017). The dose of doxycycline recommended for the treatment of tickborne rickettsial diseases is 100 mg twice daily for adults and 2.2 mg/kg body weight twice daily both orally or intravenously for children weighing <100 lbs (45 kg) (Biggs *et al.,* 2016). Tetracycline is the only other recommended drug used to treat rickettsial pathogens. Doxycycline has advantages over tetracycline and these include; its twice-daily oral dosing, better patient tolerance, highly efficacious and the relatively lower risk of adverse drug effects for children less than 8 years of age and no reports of post therapy relapse (Biggs *et al.,* 2016). The only alternative drug used to treat rickettsioses, ehrlichiosis and anaplasmosis is chloramphenicol though it is rarely used because of its hematologic effects (Feder, Osier and Maderazo, 1981; Frensenius-kabi, 2008). Studies have shown that the use of chloramphenicol is not effective in the treatment of ehrlichiosis and anaplasmosis (Brouqui and Raoult, 1992; Klein, Nelson and Goodman, 1997). Doxycycline has been used successfully to treat tick-borne rickettsial diseases in several pregnant and lactating women, but in cases of life-threatening allergies to doxycycline and in some pregnant patients, alternate antibiotics such chloramphenicol is

advised (Biggs *et al*., 2016). Rifampin has been used successfully in several pregnant women and children with anaplasmosis, and studies suggest that this drug appears effective against all *Anaplasma* species (Poitout *et al.,* 2005). Rifampin is not considered in the treatment of spotted fever (Biggs *et al.,* 2016).

2.10 Impact of Tick-borne Diseases

The impact of tick-borne diseases on human beings, livestock and canines has continued to grow worldwide (Jongejan and Uilenberg, 2004; Nicholson *et al.,* 2010). Tick-borne diseases have caused socio-economic and health loss. Several countries have reported great economic loss due to the infection of livestock with tick-borne diseases resulting in low milk and meat production, as well as death (Jongejan and Uilenberg, 2004; Kivaria, 2006; Ndhlovu, Makaya and Penzhorn, 2009). Tick-borne pathogens such as *E. canis* have a worldwide distribution and infection is usually fatal. The impact of these infections have increased and spread over the years especially in dogs belonging to tourists travelling to warm regions. Human international travel is among the largest and most rapidly evolving industries worldwide and travel associated with tick-borne diseases are increasingly emerging as common subjects of international travel medicine (Jongejan and Uilenberg, 2004; Sutherst, 2004). The range of tick-borne zoonotic diseases emerging in the temperate parts of the world poses an ever increasing public health risk. Increase in outdoor activities in rural tick infested areas has led to increased infection in human beings (Jongejan and Uilenberg, 2004). Human tick-borne infections are usually underestimated with regards to morbidity and their impact on public health resulting to increased chronic infections and death (Jensenius *et al.,* 2003).

2.11 Prevention and Control of Tick-borne Diseases

Raising public awareness and enhancing early disease detection, building upon the current information on local epidemiology, possible risk factors, and the clinical features and symptomatology of the diseases are all important in the prevention and control of tick-borne diseases. Prevention of tick bites is the most important step to avoiding infection. Personal protection is important such as long-sleeve clothing of light colour should be worn to improve protection against vector attachment, as ticks are easily seen (Piesman *et al.,* 2008). Clothing can also be impregnated with acaricides such as permethrin and decontaminated immediately after leaving a tick-infested environment. Application of insect repellents (Meta-N, N-diethyltoluamide, DEET) to skin and clothing at regular intervals is important even

though repellents such as DEET are effective but only for a short period (Shultz, 2006; Elgart *et al,* 2004; Elston *et al.,* 2004).

Reducing exposure to ticks by cutting of vegetation and applying an insecticide with residual action to sites of small mammal infestation, followed by the removal and avoiding of potential breeding sites of reservoir hosts can be done to control vector populations. This sequence of actions prevents the vector from switching from small mammal hosts to humans (Stafford, 2007). People that have been to highly infested tick areas should check for ticks regularly when they are there and remove those using fine-tipped tweezers, forceps, commercial tick removal devices or gloved hands. Rapid tick removal is likely to reduce the infection risk regardless of the uncertainty in the minimum attachment time for transmission of the infection (Needham, 1985; Felz *et al.,* 1999). Bare hands should not be used to remove ticks due to the risk of exposure to the tick's fluids or feces. Various tick-transmitted disease organisms can enter the body through cuts in the skin or mucous membranes when bare hands are used to remove ticks. Other than gloves other barriers can be used to shield the hands such as tissue or paper towel (CDC, 2015). Tick bite area should be cleaned with soap and water, alcohol or an iodine swab. People should not squeeze, crush or puncture ticks (CDC, 2015). Use of hot matches or petroleum jelly may stimulate it to release additional saliva and increase the risk of infection hence they should not be used (Needman, 1985). After removal of ticks from pets or humans, one should disinfect the tick bite site and wash their hands with soap and water (Needman, 1985).

Acaricides, anti-tick vaccines, biological controls such as entomopathogenic fungi and modification of tick habitats also play a role in the reduction of tick populations (Samish , 2008; Willadsen, 2008). Preventing engorgement of the ticks on dogs is important in prevention and control of the tick and the well-being of the dog. Treatments with fipronil (in sprays and spot-on), amitraz (often in flea and tick collars), permethrin (sprays and shampoos) and deltamethrin (shampoos) are known to be effective (Otranto and Wall, 2008). Medical attention should be sought as soon as possible, should one develop a fever after visiting risk areas. Physicians and veterinarians must be up to date about new discoveries and developments to improve their clinical practice (Dantas-Torres *et al.,* 2012).

CHAPTER 3

MATERIALS AND METHODS

3.1 Study Sites

The study was conducted in Chilanga District in Lusaka province, Zambia. The District is located 20 km south of Lusaka, the capital city of Zambia. It also shares boarders with Kafue District to the south. The district is divided into four Veterinary Camps and these include Mapepe, Chilogolo, Mwembeshi and Kasupe although samples were only collected from Mapepe, Chilogolo and Mwembeshi (Figure 3.1).

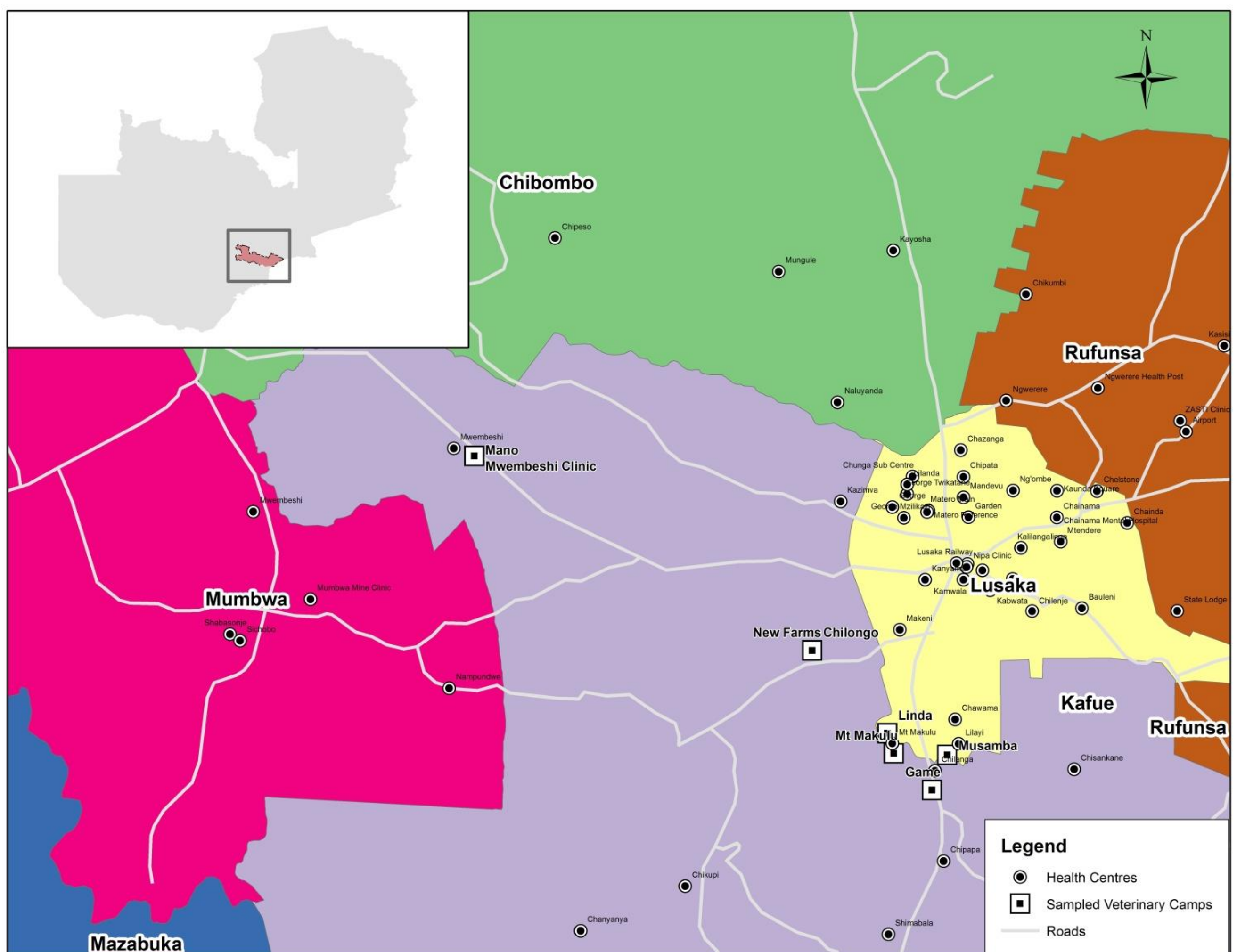

Figure 3.1 Map of Sampling sites

3.2 Study Design and Sampling of Dogs

The study was a cross-sectional in design that was conducted between February and September 2016.

3.2.1 Inclusion Criteria

All dogs regardless of age and gender

3.2.2 Exclusion Criteria

Dogs without consent from their owners were not included in the study.

3.3 Sample size

Sample size formula:

The sample size was determined using the formula below as described in Eng (2003):

$N=Z^2xP(1-P)/d^2$ at CI=95%

Where:

N=Sample required

Z=Statistic (1.96)

P=Expected prevalence in population based on previous prevalence (22.5%) which represents the total prevalence of dogs found with *E. canis, Anaplasma spp* and *Rickettsia spp* in South Africa (Mtshali *et al*., 2012)

d=Absolute error (0.05)

This resulted in a sample size of 268 dogs.

3.3.1 Sampling

Systematic random sampling was used to collect blood samples from the dogs. Three hundred and one (301) dog samples were collected from Mapepe (136), Chilogolo (17) and Mwembeshi (148) veterinary camps in Chilanga district. The sample size was initially two hundred and eight (268) based on calculations using a previous prevalence from a South African study as illustrated above (Mtshali *et al*., 2012). South Africa was one of the nearest countries in a similar region as Zambia that had conducted a similar study and hence the use of a previous prevalence from this study. Two to three days were allocated to each study area depending on the size of the veterinary camp. Some of the dogs that were sampled came from

the same household. Three hundred and one samples were collected based on the dogs that were brought for vaccination against rabies. In Mapepe Veterinary Camp samples were collected from Mt Makulu (15°32'46.3"S 28°14' 23.6"E), Linda (15°31'57.1"S 28°14'06.7"E), Game (15°34'15.6"S 28°16'01.5"E) and Musamba (15°32'49.1"S 28°16'40.4"E) areas. In Chilogolo Veterinary Camp, samples were collected from New farms (15° 28'34.7"S 28° 10'53.1"E) and Chilogolo (15° 28'35.0"S 28° 10'53.0"E).

3.4 Questionnaire administration

A semi-structured questionnaire was administered to dog owners at the time of blood sampling so as to obtain information on possible risk factors of the diseases under study. The questionnaire was administered by the researcher. Three hundred and one questionnaires were given to dog owners. Information collected included the use of the dogs, management and care including tick control, sex and age of the dogs.

3.5 Blood Collection

Consent to collect blood from the dogs was sought from the dog owners after explaining the purpose of our study. Dogs were manually restrained and blood was collected from the cephalic vein. Blood collection was done by veterinary assistants who were professionally trained and certified to do so. About two mls of blood was collected into labeled EDTA tubes. The collected blood was carried on ice in cooler boxes to the laboratories at the University of Zambia, School of Veterinary Medicine where they were stored at -30 ^{o}C until DNA extraction.

3.6 DNA Extraction

DNA extraction was done using QIAmp DNA Extraction mini kit (QIAGEN Sample and Assay Technologies, Germany) according to the manufacturer's instructions. Briefly, about 20 µl of QIAGEN Protease (or proteinase K) was pipetted into a 1.5 ml microcentrifuge tube. Whole blood was left on a rack to thaw. Two hundred micro-litres of whole blood was then added to the microcentrifuge tubes with protease. The sample was then lysed using 200 µl of AL (chaotropic salts) buffer, vortexed and incubated at 56°C for 10 minutes. Two hundred micro-litres of 100% ethanol was added to the mixture and then transferred to the QIAmp mini spin column after vortexing. The sample was washed with 750 µl AW1 buffer (low concentration of chaotropic salts) and then 750 µl AW2 buffer (Tris-based 70% ethanol solution and sodium azide used as a perservative). Finally buffer AE (10 mM Tris·HCl; 0.5

mM EDTA; pH 9.0) was added to mini-column for elution. Fifty micro-litres of the extracted DNA were then stored in 1.5 ml eppendorf tubes at -30°C until needed for analysis. DNA concentration after extraction was determined using a Nanodrop Q 5000 micro-volume Spectrophotometer (Thermo Scientific, Wilmington, USA). Samples that had low DNA concentration were re-extracted from the whole blood.

3.7 Polymerase Chain Reaction (PCR)

PCR was done using Veriti®Thermocycler (Applied Biosystems, U.S.A.). For all the PCR, the reaction mixture included 12.5 µl of Dream Taq (1.25 u) (Dream Taq buffer, dNTPs of 0.4 mM each and 4 mM $MgCl_2$), 1.25µl of the forward and reverse primers (0.5 µM each), 8 µl nuclease free water and 2 µl DNA template with a concentration ranging between 10 pg-1 ug which were pipetted into 0.2 ml PCR tubes. The mixture was well mixed by moving the tube gently at an angel of 180°. The tubes were then put into the Veriti®Thermocycler (Applied Biosystems, U.S.A.). The general cycling conditions were as follows: initial denaturation was at 95°C for 1 min, denaturation at 95°C for 30s, specific annealing temperature as indicated in Table 3.1, initial extension at 72°C for 1 min and the final extension was at 72°C for 5 minutes. The number of cycles from the final denaturation to the initial extension for the specific pathogens is included in Table 3.1. The PCR reaction mix was kept at 4°C.

3.7.1 *Rickettsia* species

Detection of *Rickettsia* pathogens by nested PCR targeted *gltA* gene as described by Gaowa *et al*. (2013). PCR amplification was done using the primer sequence in Table 3.1. The expected fragment size for *Rickettsia* pathogens was 540 bp.

3.7.2 *Ehrlichia canis*

Extracted DNA from each blood sample was tested in individual nested PCR amplifications with primers that amplify a portion of the 16S rRNA gene (Murphy *et al*., 1998). The primers used for both the primary and secondary reactions are indicated in Table 3.1. The expected fragments for this pathogen were 344, 396 and 506 bp. This variability was due to the fact that DNA samples sometimes exist in different shapes and hence migrate at different speed (Tirabassi *et al*., 2012).

3.7.3 *Anaplasma* species

PCR targeting a portion of the 16S rRNA gene was performed according to Welc-Faleciak *et al*. (2009) with primers indicated in Table 3.1. The expected fragment was 250 bp.

Table 3.1. **Primer Sequences used to amplify the Target Pathogens**

Pathogen	**Primer sequences (5' - 3')**	**Annealing temperature**	**Number of cycles**	**References**
Ehrlichia canis	**ECC**-AGAACGAACGCTGGCGGCAAGC **ECB**-ATTACCGCG GCTGCTGGCA **ECAN5-**CAATTATTTATAGCCTCTGG CTATAGG **HE3**-TATAGGTACCGTCAT TATCTTCCCTAT	65°C 55°C	35	Murphy *et al*. (1998)
***Anaplasma* species**	**EHR-521** TGTAGGCGGTTCGGTAAGTTAAAG **EHR-747**GCACTCATCGTT TACAGGGTG	60°C	40	Welc-Faleciak *et al*. (2009)
Rickettssia spp	***gltA*–Fc** CGAACTTACCGCTATTAGAATG ***gltA*–Rc** CTTTAAGAGCGATAGCTTCAAG	55°C	40	Gaowa *et al.* (2013)

3.8 Agarose Gel Electrophoresis

All PCR products were separated by gel electrophoresis in TAE buffer (Tris-base 2M, Acetic acid 2M, 0.005M EDTA and distilled water) on an ethidium bromide pre-stained 1% agarose gel for visualization. A molecular weight ladder was loaded into the first lane of the gel. One micro-litre of loading dye was added to 3 µl of PCR product which was then loaded into the additional wells on the gel. The samples were left to run for 25 minutes at 100V. The products were viewed using a Benchtop 3UV transilluminator (BioDoc-it Imaging system UVP, CA, U.S.A). The sizes of the PCR amplification products were determined relative to a 100 bp molecular weight standard (Takara DNA ladder). Known positive samples were used as positive controls for each pathogen tested. Negative controls were also included and no

DNA template was added to the negative control mixture instead, nuclease free water was added. A clear band of expected fragment size was considered a positive result.

3.8.1 DNA Purification

The purification of the amplified DNA fragments was done by centrifugation at 16,000 x *g* using Promega Wizard®Plus SV (Promega Corporation, Madison, U.S.A) mini prep DNA purification system Kit. Fifteen micro-litres of membrane binding solution was added to 15 µl of the PCR product. The product was then transferred to the mini-column assembly and incubated for one minute and then centrifuged at 16,000 x *g* for one minute. After discarding the flow-through and replacing the collection tube, 700µl membrane wash solution (10mM potassium acetate pH 5.0, 80% ethanol and 16.7µM EDTA pH 8.0) was added to the mini-column and centrifuge at 16,000 x *g* for a minute. The flow through was discarded and the mini column was reinserted into the collection tube. This stage was repeated with 500 µl membrane wash solution and centrifuged at 16,000 x *g* for five minutes. The column assembly was re-centrifuged for a minute without the lid to allow for evaporation of any ethanol residual. The mini column was transferred to a clean 1.5 ml microcentrifuge tube. This was followed by adding 50 µl of nuclease-free water to the mini-column and incubated at room temperature for a minute. The purified DNA was then centrifuged at 16,000 x *g* for one minute and then stored at -20°C.

3.8.2 Sequencing

Positive samples of *Anaplasma spp* and *E. canis* were then sequenced using the Genetic Analyzer 3130 Applied Biosystems (AB, USA). The sequencing mixture included 0.5 µl Big dye, 5X buffer, 0.33 µl primers, 12.67 µl Distilled water (DW) and 3 µl DNA template. The cycling conditions were as follows: initial denaturation at 96°C for 1 min, 25 cycles of denaturation at 96°C for 10 seconds, annealing at 50°C for 0.05 seconds and extension at 60°C and was held at 4°C.

3.9 Statistical and Phylogenetic Analysis

All PCR results were manually entered in Microsoft Excel with 0 or 1 indicating negative and positive results respectively and later transferred to SPSS version 20 (IBM, USA) for analysis. Descriptive statistics were generated for each of the variables under study. The Chi-square test or Fisher's Exact test was used to test for associations between categorical variables. Stepwise binary logistic regression was used to determine the predictors of dogs being positive to tick-borne infections on PCR. All variables with p-values ≤ 0.250 were

included in the model. A non-significant Hosmer and Lemeshow Test ($p > 0.05$) and a significant Omnibus Test of Model Coefficients ($p < 0.05$) were used to determine whether the model fitted the data.

The sequences were all aligned manually using ATGC software (GENETYX Corporation, Tokyo, Japan). The BLAST program was used for comparing and analysing the nucleotide sequences (http://www.ncbi.nlm.nih.gov/BLAST). Molecular evolutionary genetics analysis (MEGA) software version 6 (Tamura *et al*., 2013) was used for the construction of phylogenetic trees for *E. canis* and *Anaplasma spp* using the likelihood method. The bootstrap confidence level used for the phylogenetic trees was 95%.

3.10 Ethical Clearance

Ethical clearance was sought from University of Zambia Biomedical Research and Ethics committee (UNZABREC). The ethics clearance certificate reference number was 009-09-15 (appendix 3). Dogs were also vaccinated against rabies after blood collection as an incentive to the owners. Minimal pain was inflicted on the dogs when drawing blood as specialised personnel were used. The positive results were only shared with veterinary assistants in the veterinary camps and dog owners for the purpose of treatment of the dogs. The standard of treatment is often dewormers, antibiotics such as doxycycline which are administered by the veterinary assistants. The data was stored in boxes before being transferred to Microsoft excel and this was later used to determine the risk factors associated with the occurrence of the tickborne pathogens. Permission to use the School of Veterinary Medicine laboratories was sought through the Department of Disease Control.

CHAPTER 4

RESULTS

4.1 Prevalence of tick-borne pathogens in dogs

A total of 301 dogs were sampled in this study, of which 174 were male and 127 were female. Diagnosis of infection with any of the selected tick-borne zoonoses was based on the identification of a positive band on agarose gel upon electrophoresis of PCR product (Figures 4.1, 4.2 and 4.3). Among the dogs sampled, the overall prevalence with any of the tick-borne zoonotic pathogens was 44.2% (95% Cl= 38.6% to 49.8%). The prevalence of infection in male dogs was 47.1% (95%Cl= 39.7%54.5%) whereas for females the prevalence was 40.2% (95% Cl= 31.7% to 48.7%).

The most commonly isolated pathogen in this study was *Ehrlichia canis,* which was isolated at an overall prevalence of 34.9% (95% Cl= 29.5% to 40.3%), followed by *Anaplasma* spp which had overall infection prevalence of 9% (95% Cl= 5.8% to 12.3%).

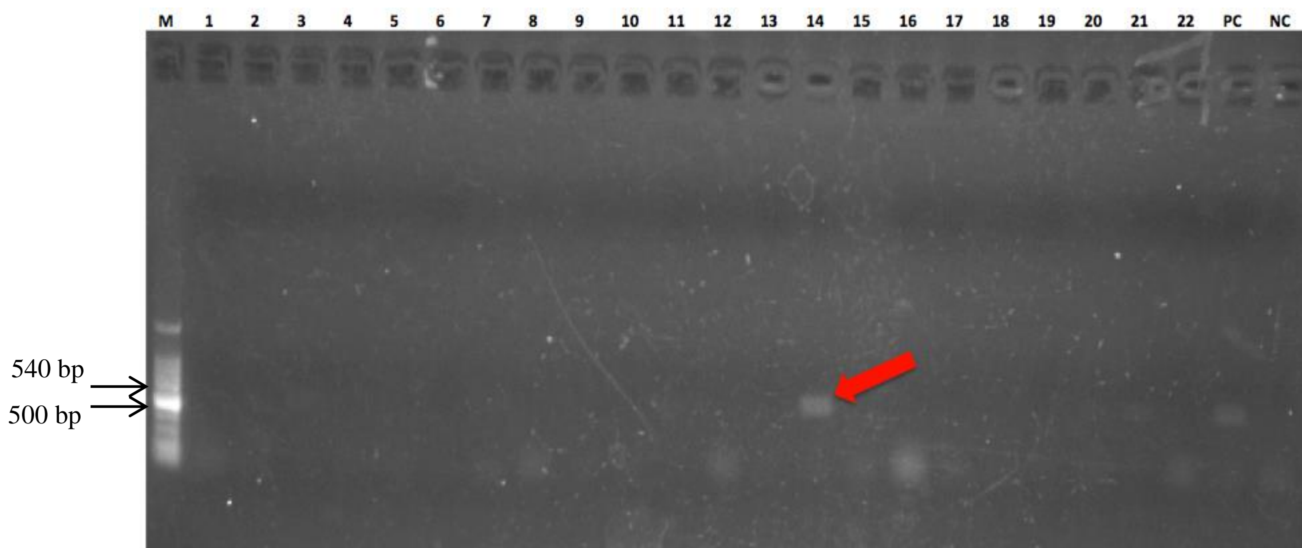

Figure 4.1. Agarose gel electrophoresis of PCR products of *Rickettsia* spp. M - 100 bp ladder; PC- positive control of *Rickettsia* spp*;* NC-negative control, lanes 1-22 tested samples. The expected fragment size for *Rickettssia* spp was 540 as shown in the agarose gel electrophoresis image.

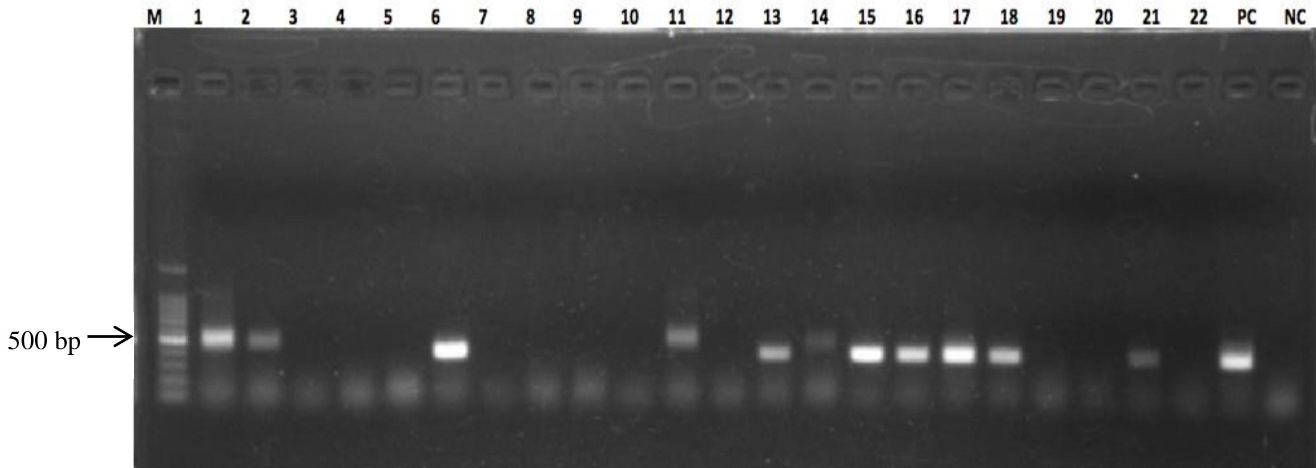

Figure 4.2. Agarose gel electrophoresis of PCR products. M - 100 bp ladder; PC- positive control of *Ehrlichia canis;* NC- negative control, lanes 1-22 samples tested samples. The expected fragment bands of this pathogen are 344, 396 and 506 based on the primers ECAN 5 and HE3.

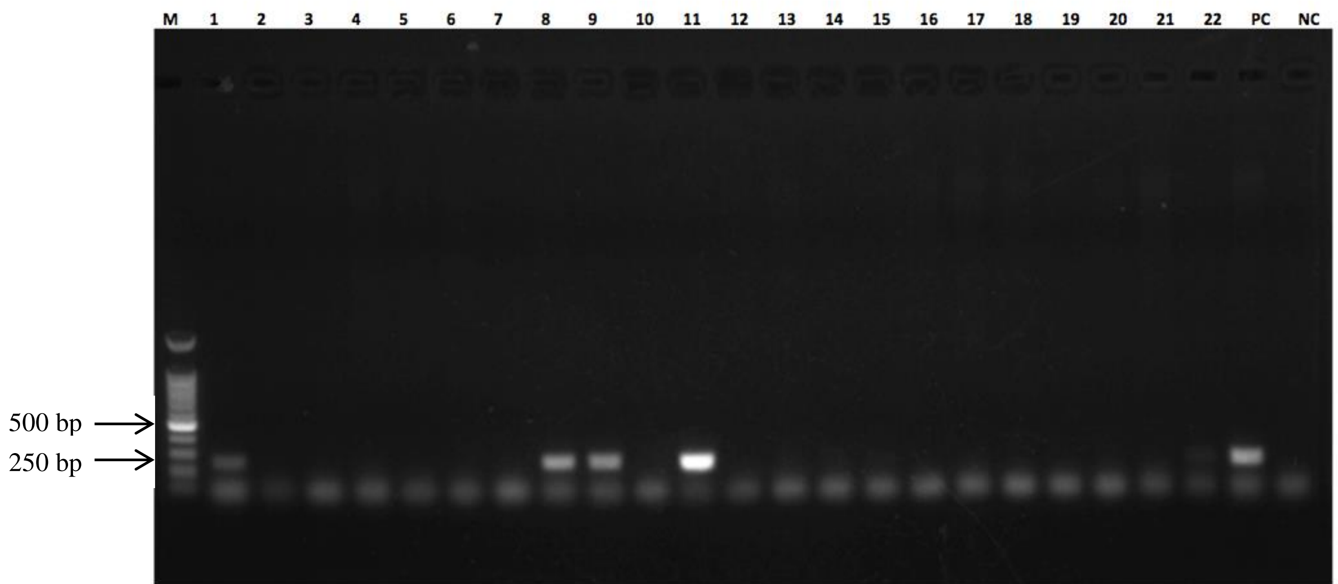

Figure 4.3. Agarose gel electrophoresis of PCR products M - 100 bp ladder; PC-positive control of *Anaplasma* spp*;* NC-negative control, lanes 1-22 tested samples. The expected band was 250 bp.

4.1.1 Results of *Rickettsia* species

Only one dog in the study was found to be infected with *Rickettsia* pathogens (1/301) giving an overall prevalence of 0.3%. The descriptive statistics of factors associated with infection with this pathogen are shown in Table 4.1. None of these factors was found to be significantly associated with the prevalence of *Rickettsia* pathogens in dogs in Chilanga district ($p > 0.05$).

Table 4.1. **Prevalence of *Rickettsia* species according to associated risk factors**

Term	*n*	Prevalence (%)	CI_{95}	p-value
Sex				
Female	127	0		
Male	174	0.6	-0.55- 1.75	1.000
Age				
Young	74	1.4	-1.28- 4.08	
Adult	227	0	0.00	0.246
Breed				
Cross	25	0		
Exotic	10	0	0.00	
Mongrel	266	0.4	-0.36- 1.16	1.000
Area				
Chilogolo	17	0		
Mapepe	136	0.7	-0.7- 2.1	
Mwembeshi	148	0	0.00	0.508
Body Condition				
Normal	227	0.4	-0.42-1.22	
Thin	74	0	0.00	1.000
Use of dogs				
Pet	37	0		
Guard	160	0.6	-0.6- 1.8	
Hunt	105	0	0	1.000
Roaming dogs				
No	149	0.7	-0.64-2.04	
Yes	152	0	0	0.495
Previous disease				
No	268	0.4	-0.36- 1.16	
Yes	33	0	0.00	1.000
Vet visits				
No	202	0		
Yes	99	1	-0.96- 2.96	0.329
Presence of ticks				
No	61	1.6	-1.55-4.75	
Yes	240	0	0.00	0.203
Other control measures				
No	232	0.4	-0.41-1.21	
Yes	69	0	0.00	1.000
Dipping				
No	170	0.6	-0.56-1.76	
Yes	131	0	0.00	1.000
Use of Acaricides				
No	27	0.4	-1.98- 2.78	
Yes	31	0	0.00	1.000
Nothing				
No	234	0.4	-0.41-1.21	
Yes	67	0	0.00	1.000
Vaccination				
No	183	0	0.00	
Yes	118	0.8	-0.81-2.41	0.392

CI_{95}: Confidence intervals (95%); p-value: probability

4.1.2 Results on *Ehrlichia canis*

The results of the prevalence of *E. canis* are shown in Table 4.2. Only the area of sampling was significantly associated with *E. canis* prevalence (p=0.001) in the univariate analysis. Of the *E. canis* infected dogs, 36.2% (95% Cl=29.06-43.34) were males and 33.1% (95% Cl=24.92-41.28) were females.

Table 4.2. **Prevalence of *Ehrlichia canis* according to associate risk factors**

Term	*n*	Prevalence (%)	CI_{95}	p-value
Sex				
Female	127	33.1	24.92-41.28	
Male	174	36.2	29.06-43.34	0.625
Age				
Adult	227	34.4	28.22- 40.58	
Young	74	36.5	25.53- 47.47	0.779
Breed				
Cross	25	16.0	1.63-30.37	
Exotic	10	50.0	19.01- 80.99	
Mongrel	266	36.1	30.33- 41.87	0.087
Area				
Chilogolo	17	0		
Mapepe	136	40.4	32.15- 48.65	
Mwembeshi	148	33.8	26.18- 41.42	0.001
Body Condition				
Normal	227	34.4	28.22-40.58	
Thin	74	36.5	25.53-47.47	0.421
Use of dogs				
Pet	37	24.3	10.48-38.12	
Guard	160	32.5	25.24- 39.76	
Hunt	105	42.3	32.85- 51.75	0.103
Roaming dogs				
No	149	34.9	27.25- 42.55	
Yes	152	34.9	27.32- 42.48	1.00
Previous disease				
No	268	35.1	29.39-40.81	
Yes	33	33.3	17.22- 49.38	1.00
Vet visits				
No	202	36.6	29.96-43.24	
Yes	99	31.3	22.17-40.43	0.440
Presence of ticks				
No	61	29.5	18.06-40.94	
Yes	240	36.2	30.12- 42.28	0.369
Other control	**measures**			
No	232	32.3	26.28- 38.32	
Yes	69	43.5	31.8- 55.2	0.113
Dipping				
No	170	33.5	26.4-40.6	
Yes	131	36.6	28.35- 44.85	0.626
Acaricides				
No	27	35.6	17.54-53.66	
Yes	31	29.0	13.03- 44.97	0.554
Nothing				
No	234	37.2	31.01-43.39	
Yes	67	26.9	16.28- 37.52	0.146
Vaccination				
No	183	37.2	30.2-44.2	
Yes	118	31.4	23.03-39.77	0.324

CI_{95}: Confidence intervals (95%) ; p-value: probability

4.1.3 Results of *Anaplasma* species

The results of prevalence of *Anaplasma* spp are shown in Table 5.3. The prevalence of this pathogen among the male dogs was 10.3% (95%Cl=5.78-14.82) while among the females dogs it was 7.1% (95% Cl=2.63-11.57). Only the mongrel breed of dogs was significantly associated with the prevalence of *Anaplasma* spp ($p = 0.007$) in the univariate analysis.

Table 4.3. **Prevalence of *Anaplasma* species according to associate risk factors**

Term	*n*	Prevalence (%)	CI_{95}	p-value
Sex				
Female	127	7.1	2.63-11.57	
Male	174	10.3	5.78- 14.82	0.415
Age				
Adult	227	14.9	10.27- 19.53	
Young	74	7.0	1.19- 12.81	0.058
Breed				
Cross	25	12.0	-0.74- 24.74	
Exotic	10	40.0	9.64- 70.36	
Mongrel	266	7.5	4.33- 10.67	0.007
Area				
Chilogolo	17	0		
Mapepe	136	11	5.74- 16.26	
Mwembeshi	148	8.1	3.7- 12.5	0.403
Body Condition				
Normal	227	7.5	4.07- 10.93	
Thin	74	13.5	5.71- 21.29	0.157
Use of dogs				
Guard	160	8.8	4.41- 13.19	
Hunt	105	6.7	1.92- 11.48	
Pet	37	16.2	4.33-28.07	0.219
Roaming dogs				
No	149	9.4	4.71-14.09	
Yes	152	8.6	4.14- 13.06	0.842
Previous disease				
No	268	9.3	5.82- 12.78	
Yes	33	6.1	-2.07-14.27	0.751
Vet visits				
No	202	8.9	4.97- 12.83	
Yes	99	9.1	3.43- 14.77	1.000
Presence of ticks				
No	61	11.5	3.49- 19.51	
Yes	240	8.3	4.81- 11.79	0.454
Other control	**measures**			
No	232	10.0	6.14- 13.86	
Yes	69	7.6	1.35- 13.85	0.471
Dipping				
No	170	9.3	4.93- 13.67	
Yes	131	6.5	2.28- 10.72	0.545
Acaricides				
No	27	9.3	-1.65-20.25	
Yes	31	6.5	-2.18-15.18	1.000
Nothing				
No	234	9.0	5.33- 12.67	
Yes	67	9.0	2.15- 15.85	1.000
Vaccination				
No	183	9.3	5.09- 13.51	
Yes	118	8.5	3.47-13.53	0.840

CI_{95}: Confidence intervals (95%); p-value: probability

4.2 Risk factors associated with tick-borne pathogens

Stepwise binary logistic regression was used to determine predictors of dogs being positive for the zoonotic pathogens under study. The risk factors found to be associated with a dog being positive for *E. canis* were dogs that are used for hunting and dogs that freely roam around the community (Table 4.4). For *Anaplasma* spp infection, age and breed were found to be factors associated with a dog being positive (Table 4.5). One hundred and twenty (40%) dogs had single infection. Thirteen dogs (4.3%) were found to have co-infection with *Anaplasma* spp and *E. canis* pathogens.

Table 4.4. **Logistic regression analysis results used to determine factors associated with *Ehrlichia canis* infection.**

Term	*n*	OR	CI_{95}	p-value
Area				
Chilogolo*	17			
Mapepe	136	0	0	0.998
Mwembeshi	148	1.46	0.71 – 3.02	0.305
Use of dogs				
Pet*	37			
Guard	160	2.24	0.89-5.62	0.086
Hunt	105	10.15	2.89-35.66	0.000
Roaming dogs				
No*	149			
Yes	152	2.92	1.21-7.02	0.017
Other control measures				
No*	232			
Yes	69	2.42	1.22– 4.81	0.012

*Reference Category

OR: Odds Ratio; CI_{95}: Confidence intervals (95%); p-value: probability

Dogs in Mwembeshi were 1.5 times more likely to be infected with *E. canis* compared to dogs in Chilogolo. The dogs that were used for hunting or for security were 10 times and 2.4 times more likely to be infected with *E. canis* respectively, compared to dogs that were used as pets. Roaming dogs were also reported to be associated with an increase in infection with *E. canis* by three times.

Table 4.5. **Logistic regression analysis results used to determine factors associated with *Anaplasma* species**

Term	*n*	OR	CI_{95}	p-value
Sex				
Female*	127			
Male	174	0.49	0.19 -1.27	0.14
Age				
Adult*	227			
Young	74	2.44	1.05-5.68	0.038
Breed				
Cross*	25			
Exotic	10	1.56	0.42-5.75	0.503
Mongrel	266	9.03	2.29-35.69	0.002

*Reference Category

CI_{95} : Confidence intervals (95%); OR :Odds ratios; p-value: probability

Young dogs approximated to be less than 1 year are 2.4 times more likely to be infected with *Anaplasma spp* compared to adult dogs. Mongrel dogs were reported to be 9 times more associated with the transmission of *Anaplasma spp* compared to the cross breeds.

4.3 Phylogenetic Analysis Results

A representative of the positive samples in this study were further characterized for speciation using sequencing and using samples already deposited in the NCBI GenBank database to construct phylogenetic tress.

4.3.1 *Ehrlichia canis* – Phylogenetic Tree

For the *Ehrlichia* species, a representative number of fifteen isolates from this study were found to have 100% similarity with *Ehrlichia canis* (accession number KR183819) based on partial nucleotide sequences of 16S rRNA (Figure 4.4). This indicated that the isolates in this study were all *E. canis*. The positive samples were randomly picked from Mapepe and Mwembeshi for sequencing. Chilogolo had no positive samples to sequence.

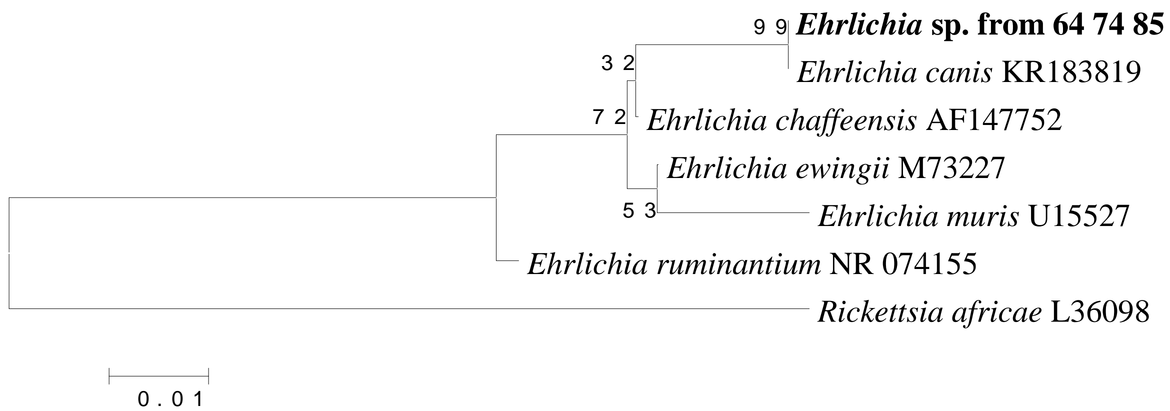

Figure 4.4. Phylogenetic relationships between the new *Ehrlichia* sp 64 74 85 (these numbers were used to identify the samples sequenced) detected in this study, *Ehrlichia* and *Rickettsia* spp based on partial nucleotide sequence of 16S rRNA. The numbers at nodes are the proportions of 1000 bootstrap resampling that support topology shown. The Scale bar represents 1% divergence.

4.3.2 *Anaplasma* species – Phylogenetic Tree

For *Anaplasma* spp nine isolates were sequenced. The sequence results showed that two (2) *Anaplasma* species were detected in this study and designated *Anaplasma* type 1 and *Anaplasma* type 2. *Anaplasma* species type 1 in this study had a 100% similarity with *Anaplasma platys* from GenBank (AF287153 and AY530806). The *Anaplasma* species type 2 in this study was found to be similar to an *Anaplasma* species isolated in South African dogs (Accession numbers AY570539, AY570538, and AY570540), which are closely related to *Anaplasma phagocytophilum* (Inokuma *et al*., 2005) (Figure 4.5).

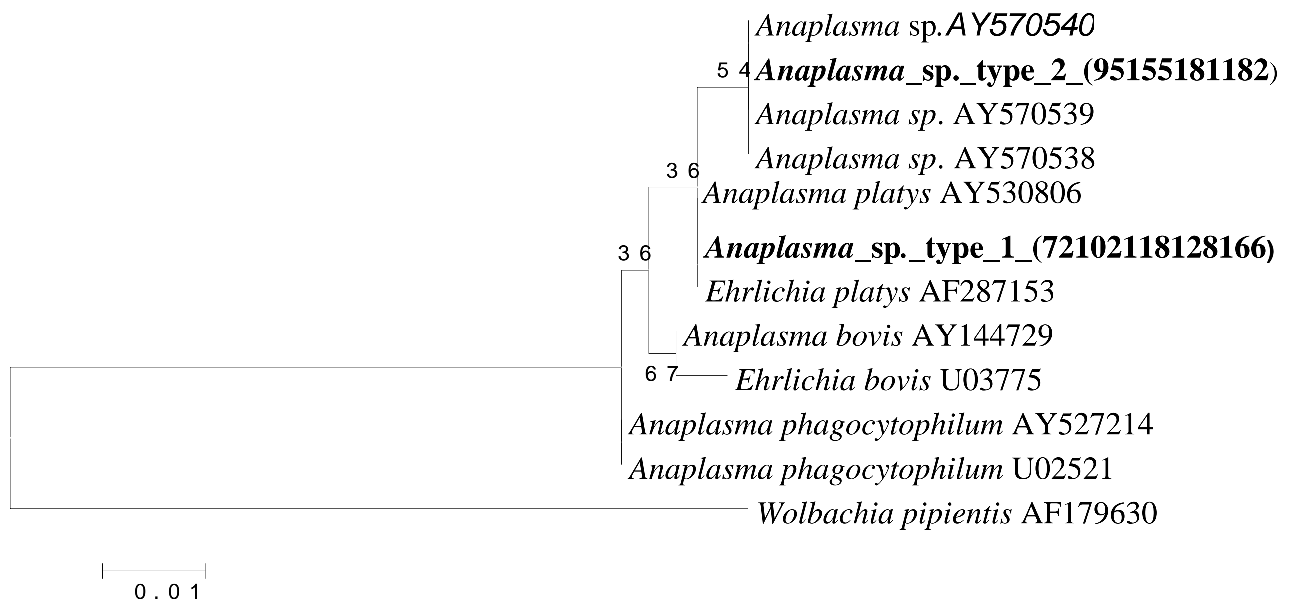

Figure 4.5. Phylogenetic relationships between *Anaplasma* type 1 (72102118128166) and *Anaplasma* type 2 (95155181182) detected in this study and *Anaplasma, Ehrlichia* and *Wolbachia* based on partial nucleotide sequence of the 16S rRNA gene using a maximum likelihood method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The scale bar represents 1% divergence.

CHAPTER 5

DISCUSSION

5.1 Discussion

In this study, PCR and phylogenetic analysis were used to determine the prevalence and genetic relationships of zoonotic tick-borne pathogens in Chilanga district of Lusaka Province in Zambia. This is the first study that has determined the presence of tick-borne zoonotic pathogens in dogs in Chilanga district in Zambia. An overall prevalence of 14.7% of zoonotic tick-borne pathogens was found in this study.

Whilst *Rickettsia* are considered the most widely distributed tick-borne zoonotic pathogens (Parola *et al.,* 2013), in this study a prevalence of 0.3% was detected. *Rickettsia* spp infection in humans, based on serological screening, has previously been reported in Zambia (Okabayashi *et al*., 1999). This indicates a real threat to human infection in the country. The very low prevalence of infection in dogs could be indicative that dogs may not be the main reservoirs of this pathogen. In a study conducted in Uganda (Proboste *et al.,* 2015) and Grenada (Yabsley *et al*., 2008), none of the dogs were found to have *Rickettsia* spp using molecular analysis. A study done in Nigeria (Kamani *et al.,* 2013) detected 8.8% of *Rickettsia* spp in dogs which were significantly higher than that detected in this study. This difference could be attributed to a higher rate of exposure of the dogs to ticks infected with *Rickettsia* spp compared to this study. Another difference between the results found in Nigeria and this study may have been due to the different types of PCR used. The study done by Kamani *et al.* (2013) used real time PCR to detect the *Rickettsia* spp while this study used nested PCR.

Ehrlichia canis was detected in 34.9% of the dogs in this study. It was the highest pathogen detected in dogs in Chilanga district. The prevalence in this study is much higher than the prevalence reported in other similar studies within the region. This is in contrast with the findings by Williams *et al.* (2014), in domestic and wild dogs in which *E. canis* was not detected. The locations of the study conducted by Williams were different from this study and hence the possible difference in vector pathogen distribution. The prevalence of *E. canis* molecularly detected in Portugal (Alexandre *et al.,* 2009) was 22% which was lower than that detected in this study but it was much lower in a study done in Malaysia (Nazari *et al.,* 2013) which only detected 2% *E.canis*. In contrast to this, 50% of this pathogen was detected in India, which was higher than in this study (Lakshmanan *et al*., 2007). A South African study

by Kolo *et al*. (2016) found a prevalence of *E. canis* in dogs to be 16%, in Angola it is reported to be 5.8% (Cardoso *et al*., 2016), whilst in Cape Verde the prevalence was reported to be 3.3% (Lauzi *et al*., 2016). The main vector species for *E. canis* (*Rhipicephalus sanguineus*) are widely distributed across all climatic regions of Africa because of its association with domestic dogs. The population of the vector is denser in warm and moist climates and is sparse in desert climates (Walker *et al*., 2003). The prevalence of *E. canis* in our study was higher probably due to a higher distribution of the vector in the areas studied. The observed differences would likely also point to a difference in the vector competency of the ticks in the different regions as a result of local microclimatic conditions, since many factors are known to influence vector competence (Harrus and Baneth, 2005). For example sampling was done at different times of the year in different countries and hence the difference in climate and distribution of vectors.

In this study the overall prevalence of *Anaplasma* spp of 9%, which were identified as *Anaplasma platys* and an *Anaplasma* spp South African dog strain closely related to *A. phagocytophilum* (Inokuma *et al*., 2005). This is the first study that reports both *A. platys* and the candidate *Anaplasma spp* South African dog strain within Zambia. *Anaplasma platys* have also been reported in Angola with a prevalence of 17.5% (Cardoso *et al*., 2016). Whilst the zoonotic potential *A. platys* pathogen has not been fully established, its close relation to the zoonotic *A. phagocytophilum* makes it a cause for concern. Vertical transmission of *A. platys* was recently reported (Latrofa *et al*., 2016) thus; the infection can easily be passed through generations, thus serving as a threat to the people over long periods of time as well.

The procedures used in the study done in Angola was similar (PCR) to those used in this study except from the study done in South Africa were Reverse line blot was utilised (Inokuma *et al*., 2005). Reverse line blot (RLB) assay makes it easier for the detection of multiple serovars that are difficult to identify by the more commonly used nested PCR and sequencing methods. This assay can analyze multiple numbers of parasitic samples relatively rapidly and inexpensively without specialized instruments compared to independent nested PCR reactions (Salih *et al.,* 2015).

Serological tests are the most widely used methods of diagnosis for tickborne pathogens (Nicholson *et al.,* 2010). Serological tests are limited by reduced ability to identify acute infection, difficulty in differentiating infection from prior exposure and species cross-reactivity. Polymerase Chain Reaction assays are very sensitive and specific making them

particularly appropriate for diagnosis of this group of diseases though they are limited by the fact that they cannot distinguish between acute infection and a carrier state. This molecular diagnostic test is also very expensive to run and not readily available in all laboratories compared to some serological tests. It also requires specialised personnel to run the tests (Shaw *et al.,* 2001).

There was 4.3% co-infection detected in *A. platys* and *E. canis* in this study. *Rickettsia sanguineus* is a competent vector for both *E. canis* and *A. platys* so they are likely to be found in the same geographic areas and also in similar hosts (Yabsley *et al.,* 2007). Dogs become co-infected when exposed to vectors infected with single pathogens at different points in time or to vectors concurrently infected with at least two pathogens, favouring the occurrence of co-infections (Otranto *et al.,* 2009). Co-infections have notable implications which include an increased and unexpected range of symptoms, more severe course of the disease or difficulties in the treatment and resistance to treatment. Usually multiple infections in the host may go undiagnosed mostly when conventional methods are used to detect these pathogens (Otranto *et al.,* 2009).

The potential role of dogs as reservoirs of tick-borne zoonotic pathogens has been reported (Otranto *et al*., 2009). High prevalence of zoonotic tick-borne pathogens in the dogs in this study area, gives an indication of the high threat level of these pathogens to humans, considering the close relationship between dogs and their owners. Various factors were tested in this study to ascertain their association with risk of dogs acquiring infection with tick-borne zoonotic pathogens. Amongst the factors that were shown to be risk factors were outdoor uses of dogs (hunting and free roaming) and the level of veterinary care given to these animals (type of tick control used). Being freely roaming and use of non-conventional and veterinary recommended tick control methods was associated with increased risk of acquiring tick-borne zoonotic pathogens. These risk factors increased the chance of dogs being positive for these pathogens because the dogs had high exposure to ticks. As such these results will help to raise awareness to the communities about the threat of these pathogens as well as the remedial measures they can easily use to reduce the risk to themselves through quality care of their animals.Ticks and tick-borne diseases are of major importance as they affect both animals and human beings causing loss of health and great economic loss worldwide (Jongejan and Uilenberg, 2004). Determining the local risk and epidemiology of tickborne pathogens present in Chilanga district will also allow for more studies to be conducted in different areas of Zambia so as to determine the national distribution and

abundance of tickborne pathogens and the risk it causes to human beings, domestic animals and also livestock. Tickborne infections can be fatal in human beings (especially in immunosuppressed patients) and companion animals e.g infection with *E.canis* causing death in dogs (Parola and Raoult, 2001; Jongejan and Uilenberg, 2004). These infections more often cause bleeding disorders, central nervous system dysfunction and kidney failure in dogs, and flu-like symptoms, including fatigue and headache, or even the loss of limbs in humans. Early diagnosis and treatment are essential to longer and higher-quality lives for dogs and human beings (Jongejan and Uilenberg, 2004).

Different drivers have greatly impacted the vulnerability of humans to tickborne diseases. These drivers include climate change, urbanization, deforestation, intensified agriculture, chemical pollution, travel and trade. Increased temperatures and high carbon dioxide concentrations result in increased pathogen development and vector longevity respectively (Sutherst, 2004). Urbanization leads to increased outer urban development, increasing contact between human beings and companion animals to bush areas. Chemical pollution affects the immunity of both humans and animals also increasing the chance of infection. Travel and trade have contributed to increased transfer of vectors and the pathogens they transmit hence increasing the risk of infection in the population. The combination of these drivers has the potential to cause multiple risks of infection (Sutherst, 2004). Rickettsial pathogen outbreaks have been reported worldwide in both human beings and dogs (Parola *et al.,* 2013). An Outbreak of canine monocytic ehrlichiosis was reported in Saudi Arabia in 2007 (Sacchini, Cessford and Robinson, 2007).

In many countries which include developing countries data on the epidemiology of arthropods such as ticks in dogs are often inadequate, unreliable and out-dated and this applies to Zambia. This can be attributed to poor developed veterinary diagnostic services and a lack of surveillance at local or regional levels (Dantas-Torres, 2008). In developing countries, the approach to canine and human vector-borne disease control is practically driven by the countries' economic and public health interest unlike developed countries that a more effective and sustainable approach (Nicholson *et al.,* 2010). The management of these tickborne diseases is important because it contributes to good health and the well-being of both dogs and human beings (Otranto *et al.,* 2009). Surveillance systems incorporating the human and veterinary public health structures are very important in the effective management of these diseases. The risk of pathogen transmission between human and dog population can be reduced by improving the diagnosis, treatment, prevention and control which focuses on

potential sources of infection, including the reduction of free-roaming dogs and the promotion of control programs against arthropod vectors and the pathogens and this can be applied to the Zambian setting (Otranto *et al.,* 2009). The best way to succeed in the management of tick-borne diseases is for public health authorities, parasitologists, physicians, veterinarians, epidemiologists, diagnosticians and molecular biologists to work together through multidisciplinary approaches (Otranto *et al.,* 2009).

The data from this research will help enrich the general body of knowledge with regards to tickborne diseases in Chilanga district and hopefully prompt a need to extend the research to others areas. The information in this study is necessary for veterinary and medical doctors to be aware of the existence of these pathogens and the clinical signs because morbidity and mortality as a result of tick-borne diseases increase substantially if there is any delay in the diagnosis and treatment of these diseases. This data can also be useful by clinicians in their differential diagnosis of diseases presenting with unknown fevers. This information can thus be used for the creation of policy for management of tickborne diseases in both humans and animals.

5.2 Conclusion

i) *Ehrlichia canis*, *Anaplasma spp* and *Rickettsia* species are all evidently prevalent in Chilanga district.
ii) Risk factors found to have been associated with Canine associated tick-borne zoonoses included dog age, breed, use of dogs for hunting, free roaming dogs and the use of conventional tick control measures.
iii) The sequence results detected two *Anaplasma* species which had 100% similarity with *Anaplasma platys* and species isolated in South African dogs. *Ehrlichia* species were found to have 100% similarity with *Ehrlichia canis.*
iv) Therefore there is an emerging need for further development of simple, robust, cheap, and appropriate approaches to detect and diagnose disease in different platforms.
v) Enhancing the knowledge base through additional studies is also important in the prevention and control of these diseases in this changing scenario of TBDs.

5.3 Recommendations

i) The close proximity of dogs and humans increase the possibility of transmission of these zoonotic infections to humans and hence extra caution should be taken by dog owners.

ii) Medical Doctors should always be alert and aware of the possibility of humans having tick-borne infections especially when malaria like symptoms are detected in patients thus tick-borne zoonoses should be considered as differential diagnosis in patients who present with unknown fever.

iii) There is need to raise awareness of the dangers posed by canine associated tick-borne zoonotic pathogens in Chilanga district to dog owners by talking to the people in the communities.

iv) More research in different areas of Zambia will help evaluate the prevalence of tick-borne zoonoses nationwide.

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Appendices

Appendix A

CONSENT

I ………………………………………………………………………………………… have been informed about the study entitled "Molecular detection of Tickborne zoonoses in dog in Chilanga district" by ……………………………………………………………….

I understand the purpose and procedures of the study.

I have been given an opportunity to answer questions about the study and have had answers to my satisfaction.

I declare that my participation in this study is entirely voluntary and that I may withdraw at any time.

If I have any further questions/concerns or queries related to the study I understand that I may contact the researcher on mobile number 0978961115 or email pipina.vlahakis@gmail.com.

If I have any questions or concerns about my rights as a study participant, or if I am concerned about an aspect of the study or the researchers then I may contact:

University of Zambia
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Biomedical Research Ethics Committee
Ridgeway Campus
P.O Box 50110
Lusaka, Zambia
Tel: 260-1-256067
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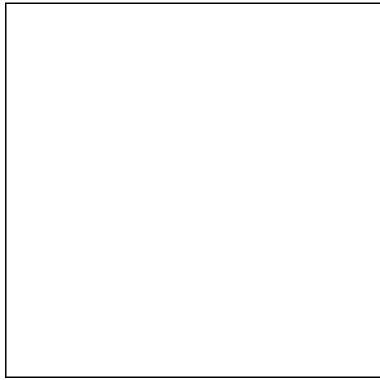

Thumb print of Participant ______________________ **Date**

______________________ ______________________
Signature of Participant **Date**

______________________ ______________________
Signature of Witness **Date**
(Where applicable)

______________________ ______________________

Signature of Translator **Date**
(Where applicable)

______________________ ______________________
Signature of Principle Investigator **Date**

Appendix B

QUESTIONNAIRE

Please tick to indicate your answer were options have been given. Clearly write the answers where options are not given.

OWNER'S DETAILS

First Name: ………………………………………………………………..

Middle Name: ……………………………………………………………

Last Name: ………………………………………………………………..

Gender: Male………… Female……….

Age: ……………………….

Residual Address: ………………………………………………………………………...

…………………………………………………………………………………………………..

Postal Address: ……………………………………………………………………………….

…………………………………………………………………………………………………….

Which Veterinary Camp are you Under ?

Mapepe…………………………

Chilogolo……………………….

Kasupe………………………….

Mwembeshi………………………

Phone Numbers: ……………………………………………………………………

…………………………………………………………………………………………………

Basic Questions

1. How many Dogs do you own?

………………………………….

2. What is the Dog for?

Pet………………………

Guard Dog………………

Other…………………….

3. Does the Dog go into the Bush?

Yes…………….. No………………

4. Do you take your Dogs to the Vet?

Yes…………….. No…………

5. How often do you take your Dogs to the Vet per year?

…………………………………….

PREVIOUS INFECTIONS

1. Has your Dog had any previous Infections?

Yes ……………… No…………….

2. What common Infections were found?

………………………………………………………………………………………………………..

3. Who diagnosed the Infection?

Self-Diagnosis……………………

Veterinary Clinic………………..

Others……………………………..

4. What were the signs and symptoms of the Infection?

……………………………………………………………………………………………………….

5. How were the Infections Managed?

Dipping …………………………..

Vaccination ……………………….

Other……………………………..

Vaccination History

1. Has your dog been vaccinated?

Yes………………….. No ………………..

If No, go to the next section

2. When was the last vaccination done?

0-6months …………..

7-12months……………

More than 12months……………

3. How often are the dogs vaccinated?

Once a year…………………..

Twice a year …………………..

More than twice a year……………

4. Which vaccine was used?

Local Vaccine…………….

Rabisin Vaccine……………….

Other…………………

5. Who vaccinated the Dog?

Veterinary Doctor……………….

Veterinary Assistant……………….

Other……………….

6. Have you observed any ticks in your area?

Yes ………… No ……………….

7. Did you use any control measures?

Yes…………. No……………….

8. What control measures did you use?

……………………………………………………………………………………………

9. How often do you use this control measure?

…………………………………………………

Economic Demographics

1. Employment Type

Formal………… Informal ………….

2. How much do you spend on Pets (Vaccines, treatment etc) on average?

Yes………………… No…………….

3. If Yes, how much do you spend on average?

……………………………………………………………………………………………