Species composition of ticks and tick-borne pathogens in domestic ruminants and dogs in Tchicala-Tcholoanga, Huambo Province, Angola

by

GOURGÉLIA DO NASCIMENTO FORTUNA SILI

Submitted in fulfilment of the requirements for the
Master (Veterinary Science) degree

in the

Department of Veterinary Tropical Diseases
Faculty of Veterinary Science

at the

University of Pretoria

Study leader: **Prof Luís Neves**
Co-study leader: **Prof Marinda Oosthuizen**

October, 2017

Declaration

"I declare that the Master's script, which I hereby submit for the degree in Veterinary Science at the University of Pretoria is entirely my own work and supported by those mentioned in the acknowledgments. It has not been previously submitted by me for a degree at another University."

……

Gourgélia D N F Sili

……

Date

Acknowledgments

The thankfulness goes to my study leader Professor Luís Neves to accept the challenge of being my supervisor, has been the architect of this project and has supported with additional funds for the project and co-supervisor Professor Marinda Oosthuizen for all her advice and support.

The first author thanks Dr. Mauro Ivan da Costa for support with tick and blood collection. It was also much appreciated the collaboration of local administration of Tchicala Tcholoanga municipality, community leaders (sobas) and smallholders for their permission and co-operation to do this study in the region. The appreciation also goes to the Ministry of Agriculture of Angola, ''Serviços Nacionais de Veternária de Angola'' for authorising the exportation of DNA samples and ticks from the Republic of Angola to the Republic of South Africa.

I would like to express my sincere gratitude to Professor Ivan Horak for his crucial role on tick identification. I also thank Charles Byaruhanga for the statistical analyses, Mamohale Chaisi for support on *Anaplasma platys* sequencing and qPCR for *Babesia bigemina* and Helena Steyn for assisting on qPCR for *Ehrlichia ruminantium*. It was also appreciated the support of Faculdade de Medicina Veterinária in Huambo for granting me study leave and for providing transport for the sample collection in the field. Likewise, my appreciation goes to the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, particularly to those who warmly welcomed me and gave me all the training and support, particularly Anne Mari Bosman and Milana Troskie. I would like to emphasise and express my gratitude for the first welcome from Rina Serfontein to the department and thank her for introducing me to Professor Luís Neves who further became my supervisor. Finally, I am deeply grateful to my beloved spouse and my kids for their support and understanding for long absences from home and my family in general for their continuous support and encouragement.

Abstract

Species composition of ticks and tick-borne pathogens in domestic ruminants and dogs in Tchicala-Tcholoanga, Huambo Province, Angola

Student: **Gourgélia D N F Sili**

Study leader: **Prof Luís Neves**

Co-study leader: **Prof Marinda Oosthuizen**

Department: **Veterinary Tropical Diseases**

Degree: **Master (Veterinary Science) degree**

This study was conducted to determine the composition of ticks and tick-borne pathogens, circulating in livestock in Tchicala-Tcholoanga Municipality, Huambo Province, Angola. Samples were collected from cattle, goats, sheep and dogs during January 2016. Using stereomicroscopic observation, ticks were identified to species level. A total of 2,963 ticks, including nymphs and adults, belonging to 5 genera and 17 tick species were recorded; i.e. *Amblyomma pomposum, Haemaphysalis leachi, H. paraleachi, Hyalomma truncatum, Ixodes cavipalpus, Rhipicephalus decoloratus, R. microplus, R. compositus, R. evertsi evertsi, R. evertsi mimeticus, R. kochi, R. lunulatus, R. punctatus, R. simus, R. sulcatus, R. turanicus* and *R. tricuspis.* Moreover, using the reverse line blot (RLB) hybridization assay, 340 DNA samples from different animal species (cattle, goats, sheep and dogs) were analysed, targeting the parasite *16S* rRNA gene for *Ehrlichia*/*Anaplasma* and *18S* rRNA gene for *Theileria*/*Babesia*. Fifteen tick-borne pathogens were detected: *Anaplasma centrale, A. marginale, A. bovis, A. platys, Anaplasma* sp. Omatjenne, *Babesia bigemina, B. bovis, B. rossi, B. vogeli, Ehrlichia canis, Theileria* sp. (sable), *T. mutans, T. velifera, T. bicornis* and *T. ovis*. The RLB assay detected low numbers (3.4 %) of *B. bigemina* positive cases and no *E. ruminantium* DNA was detected. Thus, other tools, such as real-time qPCR for *B. bigemina* and *B. bovis* targeting the *18S* rRNA gene, as well as *E. ruminantium*, targeting *pCS20* gene were used to

screen blood samples and *A. pomposum* ticks. Fifty-three samples, corresponding to 66.3% of the cattle blood samples analyzed, were positive for the presence of *B. bigemina* DNA, while no *B. bovis* DNA could be detected. In addition, *E. ruminantium* was detected in 4.3% blood samples and 7% from *A. pomposum* ticks. The parasite *16S* rRNA gene of *A. platys* RLB selected positive cattle and sheep samples were subsequently amplified, cloned and sequenced. Six recombinant sequences were obtained from cattle and five from sheep samples; of these only three near full-length sequences (1249 bp) could be obtained (7f, 26g, 47b). BLASTn homology searches showed that sequence 7f was identical to *Ehrlichia* sp. Bom Pastor (AF318023). As such, sequences 26g and 47b had 99% identity to various *A. platys* sequences available on GenBank (including the type strain sequence M82801), as well as 99% identity to *Ehrlichia* (*Anaplasma*) sp. Bom Pastor (AF318023) and *Anaplasma* sp. Omatjenne (U54806). As for the partial sequences obtained, sequence 255a (337 bp) obtained from a sheep sample showed 99% identity to *A. ovis* (KX579073), whereas two sequences obtained from cattle, 82b (323 bp) and 64c (287 bp), showed 100% identity to *A. marginale* (KT264188).

This study constituted pioneer work in Angola and showed the diversity of ticks and tick-borne pathogens among domestic ruminants and dogs in the Tchicala-Tcholoanga region.

Table of Contents

List of Figures

List of Tables

1. Introduction

Ticks are important vectors of pathogens that negatively impact the health of animal and human populations across the world and are considered a major constraint to livestock production (Jongejan & Uilenberg, 2004). According to Walker et al. (2003), the Ixodidae tick species is of major veterinary and economic importance belong to the genera *Amblyomma*, *Hyalomma* and *Rhipicephalus*. They are commonly associated with the transmission of *Anaplasma, Babesia, Ehrlichia* and *Theileria* species (Young et al., 1988). Domestic ruminants (Jongejan and Uilenberg, 1994) and dogs (Matthee et al., 2010) are the most affected by these haemoparasites, but wild animals have an important role as reservoirs (Eygelaar et al., 2015). Moreover, the direct effects of tick parasitism on their hosts are anaemia, toxicosis, stress, and decrease in weight gain or weight loss, negatively affecting herd production and productivity and consequently affecting community livelihoods (Minjauw & McLeod, 2003). A study conducted by Castro (1997) showed that the annual global costs associated with ticks and tick-borne diseases (TBDs), only in cattle, is estimated in billions of US dollars. Many haemoparasites such as *Anaplasma* spp., *Babesia* spp., *Ehrlichia* spp. and *Theileria* spp., are implicated as aetiological agents of diseases such as anaplasmosis, babesiosis, ehrlichiosis and theileriosis in Southern Africa, ranking amongst the top constraints for farmers in local or national surveys on animal health and production. These diseases constitute a great impediment to the development of productive breeds in Africa. Besides the ruminants, TBDs such as canine babesiosis, hepatozoonosis, ehrlichiosis, and anaplasmosis also affect dogs and are considered important causes of morbidity and mortality (Kaewkong et al., 2014).

In Angola, the existing information on ticks and their distribution is old and somewhat fragmented, requiring an urgent update. The historic thirty years of civil war and consequent displacement of people, animals and goods plus a lack of investment in research are the main reasons for this lack of updated information. However, studies conducted in the last 7 decades (Dias, J.A.T.S., 1983; Dias, V. S., 1948; Gomes, 1993; Gomes et al., 1994; Serrano, 1963) show that tick records from Angola do not differ substantially from the rest of southern Africa. The great diversity of Ixodid fauna is supported by a

suitable tropical climate that comprises three main climatic zones: A wet tropical area in the north; a dry temperate area in the centre and part of the south; and a semi-desert zone in the remaining southern regions of the country, up to the Namibian border. There are two seasons, a dry cool season from May to late September, and a rainy hot season from October to April. The average temperature is 20°C and relative humidity varying between 60% and 80%, with a good vegetation cover. Angola has borders with Congo, Zambia and Namibia that facilitate the spread of tick species from one country to another, due to uncontrolled trade amongst animal smallholders.

In spite of the wide range of molecular tools currently available for the diagnosis of TBDs, in Angola, classical diagnostic assays such as stained blood smears and serological tests, with known limitations of sensitivity and specificity, remain practically the only used diagnostic methods to detect haemoparasites. Therefore, the diagnostic capacity of TBDs is considered an important limiting factor to the current knowledge on diseases transmitted by ticks in Angola.

The aim of this study was to contribute to the scientific knowledge on ticks and tick-borne diseases in Angola by updating the information on spatial distribution and host preference of ticks infesting livestock in Tchicala-Tcholoanga Huambo Province, Angola, through classical taxonomic identification by microscopy and detection of common tick-borne pathogens, using PCR and RLB hybridization assays. This constituted pioneer work in Angola.

2. Literature review

2.1. Important tick species in southern Africa

According to the paper "A world list of valid tick names" Horak and Camicas (2002), the world's tick fauna comprises, 183 Argasid, 683 Ixodid and 1 Nuttalliellid species. In Africa, 40 tick species are recognized as a threat to the health of domestic animals (Walker et al., 2003). In most of southern Africa, the tick burden is particularly intense and perceived as a major challenge to livestock health and production. The epidemiology of tick and tick-borne diseases in southern Africa is regarded as highly complex due to great tick diversity and nature of the tick-borne diseases transmission complexes, where the interaction between domestic and wild animals is considered an important driver of pathogen circulation and distribution (Minjauw & McLeod, 2003). Thus, at interface areas, ticks circulate between domestic animals, wildlife and human populations, causing important zoonoses and representing also a threat to companion animals. Furthermore, this kind of interaction is also important because it may potentially affect the virulence of the pathogen strains and hence the severity of disease (Walker et al., 2003; Simuunza et al., 2011).

Findings from different studies have shown the most abundant and economically important tick species among ruminants and dogs in southern Africa: *R. appendiculatus, R. decoloratus, R. microplus*, *R. e. evertsi, A. hebraeum* and *A. variegatum* are the most common tick species found in the studies mentioned below (de Matos et al., 2008; Fourie et al., 2010; Horak et al., 2009; Isa et al., 1995; Matthee et al., 2010; Mushi et al., 1996; Musuka et al., 2001; Ndhlovu et al., 2009; Nyangiwe et al., 2013; Portillo et al., 2007; Smith & Parker, 2010; Spickett, 2013; Zieger et al., 1998).

In Angola, ticks of both families Ixodidae and Argasidae have been reported in different geographical locations. Consequently, the genera and tick species described below have been reported from various regions of Angola infecting ruminants and dogs and are considered to be the most economically important ticks, in accordance with the current knowledge on the existing Ixodid fauna.

2.1.1. The genus *Amblyomma*

The *Amblyomma* spp. are three-host ticks and are mainly implicated in the transmission of *E. ruminantium,* the causal agent of heartwater. However, some species are also responsible for transmitting the protozoa *T. mutans* and *T. velifera* that cause benign bovine theilerioses (Walker et al., 2003). According to the same author, the *Amblyomma* spp. have as preferential hosts, domestic and wild ruminants. The long mouthparts of *Amblyomma* spp. cause damage to the skin of the host that usually predisposes it to opportunistic infections. These vectors are very well adapted to climates with abundant rainfall. The exuberant ornamentation present on the scutum and conscutum of the ticks belonging to this genus, is considered a major taxonomic feature for inter-specific and intra-specific differentiation (Walker et al., 2003).

Of the twenty-six *Amblyomma* species present in Africa, (Guglielmone et al., 2014), fifteen *Amblyomma* species were recorded in Angola and they occur in 14 out of the 18 provinces: *A. astrion*, *A. compressum, A. hebraeum, A. marmoreum, A. nuttalli, A. paulopunctatum, A. pomposum, A. sparsum, A. splendidum, A. tholloni, A. variegatum* and *A. superbum* (Dias, J.A.T.S., 1983; Dias, V. S., 1948; Gomes, 1993; Gomes et al., 1994; Serrano, 1963). However, Guglielmone et al. (2009) considered the last species to be invalid. *Amblyomma arcanum, A. exornatum* and *A. latum* from the former genus Aponoma, now synonymous of *Amblyomma*, were posteriorly added to the list of *Amblyomma* spp. occurring in Angola (Horak & Camicas, 2002). These *Amblyomma* spp. were collected from domestic animals (cattle, sheep, goats, horses, dogs, pigs and donkeys) and wild animals (buffaloes, elephants, lions, pangolins, tortoises and reptiles). In Angola, the genus *Amblyomma* is abundant in areas with a rainy tropical climate, with an average annual temperatures of 20ºC, relative humidity between 65% – 80% and rainfall varying from 1200 to1600 mm annually (Serrano, 1963).

According to Walker et al. (2003) *A. pomposum* is mostly confined to southern central Africa and Angola is the country of reference for this vector. The *A. pomposum* species is the most widespread in Angola and it was also recorded by Serrano (1963) in Tchicala-Tcholoanga, Huambo Province, where this study was conducted. In addition,

among ruminants, cattle are the main hosts followed by goats and sheep. *A. pomposum* is well established in Angola and has been recorded in almost all of the country, from the north to the south, and west in provinces such as Benguela, Bié, Cuando Cubango, Cuanza Norte, Cuanza Sul, Cunene, Huambo, Huíla, Lunda Norte, Lunda Sul, Malanje, Moxico, Namibe and Uíge (Gomes, 1993; Gomes et al., 1994; Serrano, 1963). The association between *A. pomposum* and the transmission of *E. ruminantium* was experimentally proven by Serrano, (1963), and since then, this species has been considered one of the most economically important *Amblyomma* spp. in Angola, due to its importance as a vector of heartwater. *A. pomposum* is easily distinguishable from other *Amblyomma* spp. by its marked coloration.

A. variegatum is another important vector of *E. ruminantium* in Angola. However, it is confined to the East region and may also be found in the North. The Angolan provinces of Moxico and Cabinda are well known locations for this species (Gomes, 1993). According to the same author, a possible interspecific competition between *A. pomposum* and *A. variegatum* may be the reason for the confinement of *A. variegatum* to these regions, since the climate in all provinces of Angola is favourable for the establishment of both *Amblyomma* species. The reasons for which *A. variegatum* is confined to two provinces of Angola, deserve further investigation. Although *A. variegatum* is known as a vector of *E. ruminantium* and other pathogens (Walker et al., 2003), it was stated by Gomes (1993) that *A. variegatum* is not regarded as a species of major importance in Angola because it mainly exists in regions with insignificant livestock density. In Africa, the distribution of *Amblyomma* spp. varies according to the region. Thus, Kwak et al. (2014) found, among Ixodid ticks, that *A. variegatum*, the vector of *E. ruminantium*, the causative agent of heartwater, is the most widely distributed species in Tanzania. *A. variegatum* has also been reported as an important tick in Zambia, Guine-Bissau and Nigeria (Makala et al., 2003; Zuquete et al., 2017; Lorusso et al., 2013; Lynen et al., 2007).

In Botswana, heartwater transmitted by *A. hebraeum* is also considered to be one of the most important tick-borne diseases (Fanikiso & Ndzinge, 1992). According to Gomes (1993), *A. hebraeum*, a well-known vector of heartwater, has probably been recently introduced into Camabatela plateau (Benguela's province) from Botswana. This tick was identified for the first time in Benguela province, Angola, in 1982. To confirm the establishment of *A. hebraeum* in Angola further investigation is required. In Zimbabwe, Ndhlovu et al. (2009) and Sungirai et al. (2015) found *A. hebraeum* as the main vector of *E. ruminantium*. Similarly, this vector was also found in Mozambique, where it is considered a tick of great economic importance (Horak et al., 2009).

2.1.2. The genus *Rhipicephalus*

Rhipicephalus spp. are the most widespread genus in Angola and its hosts are mainly cattle, goats, sheep and dogs. At least 20 species of the genus *Rhipicephalus* have been recorded in Angola: *R. appendiculatus*, *R. capensis, R. complanatus, R. decoloratus, R. duttoni, R. dux, R. evertsi evertsi, R. evertsi mimeticus, R. longus, R. lunulatus, R. oculatus, R. pravus, R. punctatus, R. sanguineus, R. simus, R. supertritus, R. tricuspis, R. turanicus, R. zambeziensis* and *R. ziemanni* (Dias, J.A.T.S., 1983; Dias, V.S., 1948; Gomes, 1993; Gomes et al., 1994; Serrano, 1963). The specimens of genus *Rhipicephalus* identified in Angola were collected from different animal species, including wild boar, cattle, dogs, leopard, lion, plains zebra, red river hog, impala, giant forest hog, sable antelope and small ruminants.

The species *R. appendiculatus,* vector of *Theileria parva*, causal agent of East Cost fever (ECF) and Corridor disease, was recorded in an unspecified area of Angola in the 1940's and 60's by Dias (1948) and Serrano (1963), respectively. This tick species has not been found since then in subsequent surveys that have been conducted in Angola. This fact, may suggest that there may have been a misidentification in the past. Differentiating *R. appendiculatus* from species such as *R. punctatus, R. pravus, R. zambeziensis* and *R. duttoni* is a very difficult task due to their morphological similarities (Walker et al., 2003).

A study conducted by Makala et al. (2003), "The current status of major tick-borne diseases in Zambia", states that *T. parva*, which causes East Coast fever and Corridor disease, the vector of which is *R. appendiculatus*, is the most important tick pathogen in Zambia, causing significantly more deaths than all the other tick-borne diseases combined. *R. appendiculatus* is widely distributed in the North, Centre and West of Zambia. Its presence in the West may be of the utmost importance to Angola due to the large border that these two countries share.

R. e. evertsi and *R. e. mimeticus* are two-host ticks and have often been identified in sub-Saharan Africa. These species have been found on various hosts, but cattle and sheep are the preferred ones. The yellow bands on the legs of *R. e. mimeticus* make up one of the characteristics that easily differentiates this species from *R. e. evertsi*. Moreover, *R. e. evertsi* transmits the protozoan parasites *Babesia caballi* and *Theileria equi* to horses and is known to release toxins causing paralysis in lambs. This tick is also a vector of *A. marginale*, which causes bovine anaplasmosis in cattle and transmits *T. ovis* to sheep (Walker et al., 2003). Both species, *R. e. evertsi* and *R. e. mimeticus* were recorded in Angola (Dias, J.A.T.S., 1983; Dias, V.S., 1948; Serrano, 1963). Furthermore, Gomes et al. (1994) found *R. e. mimeticus* to be the most abundant cattle tick, in Huíla province, Angola.

In Angola, *R. zambeziensis* was collected from cattle, for the first time, by Gomes et al. (1994). It is considered very important due to its implication in the transmission of *T. parva*, the causative agent of Corridor disease. In cattle, this tick species also transmits *A. bovis* and *T. taurotragi* (Walker et al., 2003).

R. turanicus is a tick species of the group *R. sanguineus* and has a three-host life cycle. It is usually found in dogs and often confused with *R. sanguineus* due to their similarities. Domestic ruminants (cattle, goats and sheep) and wild animals are also hosts of *R. turanicus*. This tick species is widely distributed in Africa (Walker et al., 2003) and was also reported for the first time in Angola, Huíla province by Gomes et al. (1994), which suggests that this tick species was possibly mistakenly reported as *R. sanguineus* in the 40's and 60's. *R. sanguineus* was reported in Angola by Dias, J.A.T.S. (1983); Dias, V.S., (1948) and Serrano (1963). This species has dogs as preferential hosts, whereas *R.*

turanicus has a wide range of hosts (Horak et al., 2009). According to Walker et al. (2003), *E. canis*, *B. canis* and *H. canis* are some of the important haemoparasites transmitted by *R. sanguineus.*

Similar to other ticks from the genus *Rhipicephalus, R. simus* has a three-host life cycle. Cattle, followed by goats and sheep are the main hosts for adult ticks, but this species is also commonly found on dogs. Moreover, immature stages are often found on rodents. This tick is considered to be a competent vector of *A. marginale* (Walker et al., 2003).

R. simus has been found in all tick surveys conducted in Angola. Pigs, leopards and cattle are, so far, the only hosts from which this tick has been collected (Dias, J.A.T.S., 1983; Dias, V.S., 1948; Gomes et al., 1994; Serrano, 1963). In addition, Dias, V.S. (1948) also identified other *Rhipicephalus* spp. such as *R. capensis* and *oculatus,* while Serrano (1963) reported *R. longus, R. pravus* and *R. supertritus.* Moreover, Dias, J.A.T.S. (1983) added *R. dux* and *R. ziemanni* to the Angola Ixodid fauna. The current distribution of these species requires an update.

The tick species, *R. decoloratus* and *R. microplus,* go through every parasitic stage of their life cycle on a single host. Cattle are their preferential hosts, but they may be found feeding on other animal species. They have similar characteristics to those of the members of the *genus Rhipicephalus*. However, *R. decoloratus,* is the only species that presents a different teeth column arrangement (3+3). This tick is responsible for the transmission of *B. bigemina* to cattle, causing bovine babesiosis, commonly known as African redwater. In addition, *A. marginale* is another haemoparasite transmitted by this vector, causing bovine anaplasmosis. Additionally, *R. microplus,* the most widespread cattle tick species is implicated in the transmission of *B. bigemina, B. bovis* and *A. marginale*, making it the most important vector from the genus *Rhipicephalus* (Walker et al., 2003).

In Angola, *R. decoloratus* is the species that has been consecutively reported (Dias, J.A.T.S., 1983; Dias, V.S., 1948; Gomes et al., 1994; Serrano, 1963). It is worth noting that the presence of *R. microplus* has not been confirmed in the country. However, there is strong evidence, based on the frequent import of significant numbers of cattle (A. José

and A. Gomes, personal communication, 2015) from regions where this tick is already established; Brazil and Namibia to Angola, suggesting the relatively recent introduction of this tick species in Angola. The wide distribution of this tick species and its role in disease transmission clearly indicates the importance of this species in Angola.

2.1.3. The genus *Haemaphysalis*

The ticks of this group are primarily dog parasites, but may also be found in ruminants such as cattle due to close contact between dogs and livestock. The genus *Haemaphysalis* has a three-host life cycle and is easily identified by its conical mouthparts shape (Walker et al., 2003).

In Angola, *H. humerosoides* and *H. leachi* are the two species belonging to this group that have already been reported. *H. leachi* was reported feeding on dogs, while *H. humerosoides* was found on lions (Dias, J.A.T.S., 1983; Dias, V.S., 1948; Serrano, 1963). *H. leachi* is implicated in the transmission of *B. rossi*, causing canine babesiosis in dogs (Matjila et al., 2004; Fourie et al., 2010).

Studies on ticks parasitising dogs have never been systematically conducted in Angola. Because dogs were never the main target in previous tick surveys, the existing information on this topic is quite limited and fragmented.

2.1.4. The genus *Hyalomma*

The ticks of this genus have a two-host life cycle and infest domestic as well as a variety of wild animals. These ticks inoculate toxins causing sweating sickness in cattle.

H. impressum, *H. rufipes* and *H. truncatum* are species from the genus *Hyalomma* that have already been collected from cattle in Angola (Dias, J.A.T.S., 1983; Dias, V.S., 1948; Serrano, 1963).

The adult stage of *H. impressum* was collected from cattle, sheep, and horses while the immature stages were found on rabbits. This species was mainly identified in provinces of southern Angola, such as Huambo, Bié, Huíla and Benguela, as well as the province of Luanda in the North. Based on the fact that the only report of *H. impressum* was made by Dias in 1948, further research on this tick species is necessary. However, *H. rufipes* and *H. truncatum* were collected from cattle at Huíla province (Gomes et al., 1994).

2.1.5. The genus *Ixodes*

The presence of *I. cavipalpus* in Angola was recorded by Dias, V.S. (1948); Gomes et al. (1994) and Serrano (1963) on cattle, goats, dogs and monkeys. The impact of this tick species on livestock and the haemoparasites transmitted by it are not currently well-known.

2.1.6. The genus *Ornithodoros*

The species *O. moubata* has, as its preferred hosts, domestic pigs, though humans are also often infested by it. This tick transmits the African swine fever virus and *Borrelia duttoni* (Burrage, 2012). According to Serrano (1963), *O. moubata* is part of the Angolan tick fauna.

2.1.7. The genus *Argas*

The species *A. boueti, A. brumpti, A. persicus* and *A. vespertilionis* have previously been recorded in Angola (Serrano, 1963), but there is no record of the animal species and provinces from which they were collected. According to Walker et al. (2003), the species *A. persicus* mainly parasite domestic birds and transmit the bacteria, *B. anserine* and *Aegyptianella pulorum*.

The genera *Dermacentor*, *Rhipicentor* and species such as *D. rhinocerinus* and *R. bicornis* that exclusively parasite wild animals have also been found in Angola (Serrano, 1963).

2.2. Haemoparasites transmitted by ticks

A great variety of haemoparasite species belonging to the genera, *Anaplasma*, *Babesia*, *Ehrlichia* and *Theileria* have had a great impact as agents of diseases affecting domestic and wild animals worldwide (Minjauw & McLeod, 2003).

In Angola, there is no organized and functional system to collect information on the control of animal diseases. Non-commercial livestock production is considered to be vital not only to the livelihood of rural communities, but also to the sustenance of urban communities. Diseases transmitted by ticks have a strong negative impact on livestock production. Despite this, smallholders generally have poor knowledge of disease transmission and epidemiology, and receive little technical support. Consequently, they have limited tools to deal with regular tick-borne disease outbreaks associated with high mortality and morbidity. However, there are no official reports of such outbreaks. To date, few studies have been published on pathogens transmitted by ticks in Angola. Although there is a lack of information on the haemoparasites circulating in Angola, it is possible to predict the haemoparasite species composition based on the Ixodid fauna that has previously been recorded. Thus, it is certain that *Anaplasma* spp., *Babesia* spp., *Ehrlichia* spp. and *Theileria* spp. are genera of haemoparasites that circulate among ruminants and dogs in Angola.

2.2.1. The genus *Anaplasma*

Anaplasma spp. are rickettsial organisms belonging to the family Anaplasmataceae that lodge themselves inside the erythrocytes of their hosts. A great variety of tick species ocurring in Africa are implicated in its transmission, particularly *Rhipicephalus* spp. However, mechanical and congenital transmission cannot be ignored. Considering that *Anaplasma* spp. exclusively infects red blood cells, its pathogenic effect leads to the destruction of erythrocytes, causing severe anaemia, icterus and fever to their hosts (Kocan et al., 2010). Domestic ruminants such as cattle, goats and sheep are the main hosts of *Anaplasma* spp., but it has also been found in wild animals, which are considered to be reservoirs of these haemoparasites (Aubry & Geale, 2011). The severity of bovine

anaplasmosis is related to age; calves generally have a greater resistance compared to older animals. Similarly, breed plays an important role in resistance to infection, for example, *Bos indicus* have shown greater resistance to anaplasmosis than *Bos taurus*. The host, once infected, may become an asymptomatic carrier of parasites for a long time (Kocan et al., 2010).

There are different species of *Anaplasma* that infect domestic ruminants: *A. marginale* and A. *centrale,* whose main hosts are cattle; *A. ovis* that mostly parasites sheep and goats; *A. platys,* a dog-specific haemoparasite and *A. phagocytophilum*, which is implicated in both dog and human diseases (Zobba et al., 2014). *A. marginale* is the most virulent and its name comes from the marginal position of the haemoparasite inside the erythrocyte, while *A. centrale*, which causes a milder disease, has a more central localization inside the erythrocyte (Minjauw & McLeod, 2003). *R. decoloratus, R. microplus, R. simus, R. e. evertsi* and *H. rufipes,* are the five species that are currently known to be involved in the transmission of *A. marginale* in Africa. However, many other vector species may be implicated in the transmission of haemoparasites of the genus *Anaplasma* worldwide (Kocan et al., 2004). In addition, *A. platys* is an important haemoparasite in which *R. sanguineus* seems to play an important role in its transmission to dogs. For example, Lauzi et al. (2016) detected *A. platys* in more than half of the dogs studied on Cape Verde archipelago, and suggested *R. sanguineus*, the only hard tick existing in the region, to be involved in its transmission. Similarly, Kamani et al. (2013) found *A. platys* in dogs in Nigeria.

A study conducted in Bié Province, involving cattle and goats, indicated the occurrence of *A. ovis* in all sampled goats, while *A. marginale*, *Theileria* sp. and *Babesia* sp. occurred in cattle. Furthermore, the survey revealed that the presence of mixed infections with *Anaplasma* and *Theileria* species were common in cattle, (Kubelova et al., 2012).

2.2.2. The genus *Babesia*

Babesia is an important protozoan parasite genus that causes babesiosis in different mammalian hosts. It occurs across the entire African continent, coincinding with the

distribution of its vectors. The tick species, *R. decoloratus*, *R. microplus* and *R. e. evertsi*, have been identified as the most important vectors of bovine and equine babesiosis throughout southern Africa (Uilenberg, 2006). In the host, the haemoparasite lives inside the erythrocyte, causing cellular lysis. Consequently, anaemia and icterus are the main clinical symptoms. Additionally, haemoglobinuria is a characteristic sign of babesiosis, which is present in the majority of clinical cases (Bock et al., 2004).

There are two economically important species of cattle *Babesia* in southern Africa. *B. bigemina,* transmitted mainly by *R. decoloratus*, *R. microplus* and *R. e. evertsi,* is widely distributed, perhaps due to its vector diversity. Conversely, *B. bovis,* a more virulent species, is transmitted only by *R. microplus* (Bock et al., 2004). Thus, the spread of *R. microplus* in southern Africa is a matter of great importance. Nyangiwe et al. (2013) confirmed the recent establishment of this tick species in Namibia. Additionally, this species is also known to be widely distributed in Zimbabwe (Sungirai et al., 2015), Mozambique and South Africa (Horak et al., 2009). Companion animals are also severely affected by *Babesia* parasites. Pathogens such as *B. canis*, *B. vogeli,* and *B. rossi* are the main etiological agents of canine babesiosis (Matjila et al., 2008).

In Africa, early exposure to ticks and haemoparasites such as *Babesia* spp., often renders animals resistant to the diseases caused by these parasites, leading to an equilibrium between host, vector and haemoparasite, resulting in minimal occurrence of disease (Perry et al., 1985).

In Angola, the impact of babesiosis on livestock is not well known. The only information available comes from a single epidemiological survey on bovine babesiosis, conducted by Gomes et al. (1991) in southern Angola (Huambo, Huíla, Cunene and Namibe), using an indirect fluorescent antibody test technique. This survey indicated endemic stability in most herds and farms investigated. Similarly, information on canine tick-borne pathogens in Angola is extremely scarce. In Huambo province, the only available sources of data on canine babesiosis are non-published records of blood stained smears positive for *Babesia* spp. from private veterinary clinics. In addition, a molecular investigation of tick-borne pathogens, using PCR, was recently carried out in Luanda at

a veterinary medical centre. The results of this study indicated the presence of *A. platys, B. gibsoni, B. vogeli, E. canis* and *H. canis* (Cardoso et al., 2016).

2.2.3. The genus *Ehrlichia*

Ehrlichia spp. are gram negative bacteria belonging to the family Anaplasmataceae. In Africa, *E. ruminantium* is one the most important species within this genus. It causes heartwater (also known as cowdriosis) in ruminants. Cases of this disease are usually fatal (Allsopp, 2015). The most prominent clinical signs of heartwater are related to the effusion of exudate, resulting from increased vascular permeability into body cavities (pericardium, thorax and cranium) and tissues of the body of the host, leading to oedema. Neurological signs are very common during the final stages of disease (Camus & Barre, 1987). The bacteria, *E. ruminantium,* preferentially replicates in cerebral endothelial cells (Mahan, 1995). Cattle, sheep and goats are the main hosts of this bacteria, but small ruminants are considered to be more susceptible (Allsopp, 2015). African buffalo, among other wild animals, have been identified as important reservoirs in southern Africa (Eygelaar et al., 2015).

Amblyomma is the only genus that is implicated in the transmission of *E. ruminantium*. Moreover, records from sub-Saharan Africa show that among *Amblyomma* species, *A. variegatum* and *A. hebraeum* are the most common vectors of *E. ruminantium* (Horak et al., 2009; Musuka et al., 2001; Smith & Park, 2010; Spickett, 2013; Zieger et al., 1998; Ndhlovu et al., 2009; Kwak et al., 2014). According to Walker & Olwage (1987), *A. pomposum,* a tick species that is very well established and widely distributed in Angola, is not considered to be a big concern in relation to the transmission of heartwater. Nevertheless, in an earlier study conducted in Angola, it was experimentally demonstrated that *A. pomposum* is a competent vector of *E. ruminantium* (Serrano, 1963). In addition, *A. variegatum,* another important vector of *E. ruminantium* that is present in Angola, is confined to two provinces (Cabinda and Moxico), and according to Gomes et al. (1994), due to the low density of livestock in these regions, heartwater does not emerge as a major problem. Moreover, the same author stated that *A. hebraeum* has been

introduced recently in Angola from Botswana. This finding requires special attention given the efficiency of this tick species in transmitting heartwater. In Zambia, which neighbours Angola in the East, heartwater, mainly transmitted by *A. variegatum*, is considered to be a major constraint to cattle production and health (Makala et al., 2003). According to the same authors, the presence of this disease is not limited to the exotic or cross-breeds of cattle; it also affects indigenous cattle breeds, which do not commonly undergo regular acaricide treatment. This is particularly problematic in areas where there is interaction between domestic and wild ruminants.

2.2.4. The genus *Theileria*

Among the haemoprotozoans of the genus *Theileria* are *T. annulata, T. buffeli, T. ovis, T. parva, T. separata, T. velifera, T. mutans, T. lestoquardi* and *T. taurotragi.*

T. taurotragi is another haemoparasite that causes theileriosis in cattle and has *R. appendiculatus* as its main vector (de Vos & Roos, 1981). *T. mutans* and *T. velifera* are the only *Theileria* species transmitted by ticks of the genus *Amblyomma*. The diseases they transmit are usually benign to cattle and African buffalo (Norval et al., 1992). *T. separata*, *T. lestoquardi* and *T. ovis* are important etiological agents of theileriosis in sheep and goats. However, *T. lestoquardi* is the most virulent and found in North Africa, while *T. ovis* is known as a non-pathogenic agent (Uilenberg, 1981). Regarding the importance in cattle, *T. parva* is considered to be a major constraint to livestock health and production in sub-Saharan Africa. ECF and Corridor disease are forms of theileriosis. The diseases are caused by *T. parva* or its strains, which have as main vector *R. appendiculatus* (Norval et al., 1992). Corridor disease and ECF mainly affect cattle and have buffalo as reservoir (Minjauw & McLeod, 2003). It has been speculated that buffalo are the original hosts of *T. parva* and later spread to cattle, which thus far remain the only natural domestic host (Norval et al., 1992). These diseases have been diagnosed in central, East and southern Africa, in countries where its vectors are present. Both diseases are usually fatal, causing up to 90% mortality in naive cattle herds (Lawrence, 1992).

In Angola, more attention should be given to the elucidation of the occurrence of *T. parva*, since *R. appendiculatus*, *R. duttoni* and *R. zambeziensis* have been recorded (Dias, V.S., 1948; Gomes et al., 1994; Serrano, 1963) and implicated as vectors. It is suspected that *R. duttoni* might be the main vector in Angola (Lawrence et al., 2004; Norval et al., 1992) and the presence of *R. duttoni* and *R. zambeziensis* was last reported by Gomes et al. (1994) in Huíla province, Angola. Besides, the circulation of *T. parva* among livestock, still requires further investigation.

2.3. Diagnostic tools applied to haemoparasites

The diagnosis of tick-borne diseases (TBDs) is still a challenging issue due to the technical difficulty of developing assays with a desired level of sensitivity, specificity, robustness and suitability for application in the field (Sparagano et al., 1999).

There are two optional and complementary methods to diagnose haemoparasites transmitted by ticks. One allows for the detection of parasites or parasitic material in host or tick tissue, while the other method permits the detection of the presence of molecules associated with a present or past parasite infection. These methods are generally used on an individual or herd basis to determine the presence of parasites in cases of disease, death or for trade purposes. They are also extensively applied in epidemiological studies involving hosts and vectors, normally in a wider geographical dimension (Böse et al., 1995).

2.3.1. Microscopic examination

The traditional method, thin and thick Giemsa stained smears, is arguably the most widespread method for haemoparasite identification (Mahoney & Saal, 1961). Used essentially for clinical diagnostic purposes, the direct microscopic method should always be considered in association with clinical signs of the disease and the presence of its vector in the region (Bell - Sakyi et al., 2004). Although blood is the most common sample type, this method has also been used for parasite detection post-mortem, using

impression smears from kidney, spleen, liver or brain samples. A typical example of this latter use is the case of *E. ruminantium,* where the use of brain smears is the most effective detection method (Allsopp, 2010). Haemoparasites belonging to the genera, *Anaplasma, Ehrlichia, Babesia* and *Theileria* may be detectable using Giemsa stained smears, though species-level diagnosis can be challenging (Bell - Sakyi et al., 2004). *Anaplasma* spp. can be easily identified inside erythrocytes using stained thin blood smears. Depending on the position of the parasite inside the erythrocyte, it is possible to differentiate *A. marginale* from *A. centrale* (Minjauw & McLeod, 2003).

However, the main weaknesses of this technique are its limited ability to detect parasites in cases of low parasitaemia and the difficulty to specifically identify a parasite in the presence of unidentified stained material inside the erythrocytes (Bose et al., 1995).

In order to detect *B. bigemina* and *B. bovis,* thick and thin Giemsa stained blood smears can be used successfully, given that the level of haemoparasites in the blood is sufficient to be detectable. However, *B. bigemina* is commonly found in major blood vessels, while *B. bovis* is more easily found in peripheral circulation (Bell -Sakyi et al., 2004).

Unlike *Babesia* species, *E. ruminantium* is poorly detected in circulating blood. Instead, it is often found in the cytoplasm of cerebral endothelial cells, using Giemsa stained smears. However, the definitive diagnosis of *E. ruminantium* is often made in dead animals, combining the results of brain smears with post-mortem findings, mainly the presence of fluids in the pericardium and thoracic cavity (Allsopp, 2010).

Additionally, smears prepared from peripheral blood or fluid from superficial lymph nodes are the preferred samples used to detect schizonts of *Theileria*.

In general, microscopic examination using Giemsa stained smears is very useful to confirm the presence of pathogens. The thick blood smear requires a greater volume of blood, which increases its sensitivity when compared to Giemsa stained thin blood smears (Mahoney & Saal, 1961). However, according to Bell-Sakyi et al. (2004), the choice of method used; thin or tick smear, should be made taking the specific haemoparasite to be detected into consideration. For better results, thin smear should be applied for

detection of small parasites such as *Anaplasma* spp.; since these microorganisms are difficult to stain, they are not easily visualised in thick smears, except in cases of high parasitaemia. However, larger parasites such *Theileria* spp. and *Babesia* spp. are more readily visualised in thick smears. Moreover, the morphological similarities between parasite species constitute a serious limitation for microscopic examination, particularly when there are mixed infections or low parasitaemias (Irwin, 2009; Manyarara, et al., 2015).

2.3.2. Serological diagnosis

Serological tests play a central role in the epidemiological assessment of tick-borne diseases. Furthermore, the evaluation of efficacy of immunogens and drugs as well as other applied immunological studies are potential applications of these tests (Levings et al., 1993; Weiland & Reiter, 1988). The ability to recognise infection through the detection of antibody titres makes these tests an important complement to indirect techniques. Complement fixation (CF), Indirect haemagglutination (IHA), Latex agglutination (LA), Indirect fluorescent antibody test (IFAT), Enzyme-linked immunosorbent assay (ELISA) and Radio immunoassay (RIA) are some of the serological tests used to detect antigen-antibody complexes. However, the most widely used techniques are IFAT and ELISA. The contribution of IFAT and ELISA tests to epidemiological studies on tick-borne diseases in the field is undoubtedly important. However, as with all serological methods, there are limitations to accurate antibody detection (Bose et al., 1995). These assays are constantly challenged due to the presence of closely-related species that lead to antibody cross-reactions (Salih et al., 2007). Other limiting factors are, for example, that several serological assays do not differentiate carrier animals from symptomatic animals and the continued presence of antibodies after infections can lead to false positive results (Bose et al., 1995). Furthermore, the relatively low specificity and some subjectivity, in the interpretation of IFAT results, are the more frequent limitations associated with these assays (Manyarara et al., 2015; Wright, 1990). Generally, the availability of good quality

antigen is considered essential to achieve good results in serology (Bose et al., 1995; Jongejan, 1991).

2.3.3. DNA probes

This technique consisted of the detection of the nucleotide sequence of a specific parasite by design-specific probes, which were combined with radioactive isotopes, 32P, 35S, 75Se, 125I, 14C, 3H (Viljoen & Luckins, 2012; Shompole et al., 1989; Waghla et al., 1991; Yunker et al., 1993). Among them, the radioactive isotopes 32P and 35S (Viljoen et al., 2012) were preferentially used. Additionally, there were also non-radioactive isotopes (Gigoxigenin), fluorescent molecules (Ambrosio & De Waal, 1990) and enzymes (Shompole et al., 1989). The hybridization was conducted through the use of southern blot, northern blot or dot-blot techniques and the binding probes were visualised by X-ray film. Therefore, using this method, probes were developed to detect various rickettsial organisms. *A. marginale*, *A. centrale*, *A. ovis* DNA probes were developed to detect DNA of these specific pathogens (Visser & Ambrosio, 1987; Aboytes-Torres et al., 1989; Shompole et al., 1989). Further, Eriks et al. (1989) designed an RNA probe to detect *A. marginale* genomic DNA in carrier cattle. Waghela et al. (1991) developed 32P-labeled DNA probe pCS20 and pCR9 DNA probe, which could detect all strains of *E. ruminantium* DNA, although pCR9 DNA probe has been considered to be less specific (Yunker et al., 1993). Meanwhile, protozoa DNA probes were developed by Figueroa et al. (1992), detecting *B. bigemina*, using non-radioactive probes (digoxigenin-labeled probe), while Buening et al. (1990) detected sequence DNA of *B. bigemina*, using 32P labelled DNA probe. Allsopp et al. (1993) detected the DNA sequences of *T. annulata, T. buffeli, T. mutans, T. parva,* and *T. taurotragi*, using 32P oligonucleotide RNA probe.

Although the use of radioactive isotopes is a sensitive method, their use is also associated with environmental hazards, making this the main drawback of the technique. Conversely, non-radioactive probes showed low sensitivity (Ambrosio & de Waal, 1990). Therefore, the DNA probe method fell into disuse and was later replaced by PCR, which yields sufficient DNA molecules from the amplification of a gene.

2.3.4. Polymerase Chain Reaction (PCR)

Presently, a wide range of molecular diagnostic methods are available, thus choosing the ideal one is an increasingly important component of experimental design (Guillemi et al., 2015) as well as the choice of the best genomic region to be targeted for molecular detection (Kent, 2009). In the context of haemoparasite diagnosis, PCR is the method that is traditionally more extensively used. PCR is a technique that allows for specific parasite detection from picograms or even femtograms of target DNA. In fact, at the time of its development, this technique increased the sensitivity limits of the already existing assays by approximately a thousand fold, while retaining specificity values only attainable otherwise by direct microscopy and DNA probes (Viljoen & Luckins, 2012).

There are numerous probes/primers that have been developed and used to detect gram negative bacteria of the order Rickettsia. To date, various probes have been used to detect haemoparasites of the genera *Anaplasma*/*Ehrlichia* from different types of samples. These probes include those designed from the extensively used *16S* rRNA gene to detect, for example, *A. marginale, A. centrale, E. ruminantium* (Bekker et al., 2002), *A. platys* (Inokuma et al., 2000; Nijhof et al., 2003), *E. canis* and *E. chaffeensis* (Schouls et al., 1999). In addition, *groEL*, another targeting gene, has also been used to design probes/primers to detect species of the family Anaplasmataceae such as *A. platys* (Inokuma et al., 2002; Zobba et al., 2014) and *E. canis* (Otranto et al., 2010). In addition, many probes for the identification of the family Anaplasmataceae were designed based on the *gltA* gene; *A. centrale, A. marginale, A. platys E. canis* and *E. ruminantium* (Inokuma et al., 2001). However, even within the family Anaplasmataceae, the molecular diagnostic method of choice and targeting gene depend on the pathogen species of interest (Guillemi et al., 2015). In this regard, due to the limitations in detection of *E. ruminantium* targeting the *16S* rRNA gene, Waghela et al. (1991) developed a new DNA probe from the *pSC20* gene region that was able to recognise many more strains of *E. ruminantium* from different samples; blood, tick or endothelial cells. Hence, for the identification of two important genera, *Anaplasma* and *Ehrlichia*, two genes are frequently targeted; *pCS20* and *msp1α*. For the molecular detection of *E. ruminantium,* the usual gene fragment of choice is the *pCS20*

gene (Steyn et al., 2008; Van Heerden et al., 2004; Waghela et al., 1991), while the *msp1α* gene is often used when the purpose is to detect *A. marginale* (de la Fuente et al., 2007; Mtshali et al., 2007).

For the detection of *Babesia* and *Theileria* spp., the *18S* rRNA gene has been commonly targeted. Based on this gene region, probes and primers have been designed and used for PCR and RLB assays. As a result, a great diversity of piroplasm species such as *B. bigemina, B. bovis* (Gubbles et al., 1999) *B. canis, B. rossi, B. vogeli* (Matjila et al., 2004) have been detected.

Presently, more sensitive PCR-based assays such as nested PCR (nPCR), multiplex PCR and qPCR have been developed (Canever et al., 2014; Decaro et al., 2008; Kim et al., 2007; Mtshali et al., 2014; Steyn et al., 2008). Nested PCR has a higher level of sensitivity due to the use of a different set of primers in each amplification cycle. Multiplex PCR permits the detection of several haemoparasites in a single test (Mtshali et al., 2014). In the case of real-time PCR (qPCR), the detection of haemoparasites is achieved through the use of dyes (SYBR Green) or specific probes (FRET® or TaqMan®) that emit a fluorescent light, allowing for the detection and quantification of the PCR product, which is repeatedly increased in each cycle. This technique does not require prior DNA amplification by conventional PCR or intervention by the operator monitoring the assay at any step of the process, thus avoiding contamination. Therefore, qPCR, using the *pCS20* DNA fragment is considered to be the most suitable detection method for most *E. ruminantium* strains in ruminants and *Amblyomma* spp. (Steyn et al, 2008; Van Heerden et al., 2004). However, the same authors highlight the existence of cross reactions with *E. canis* and *E. chaffeensis* as important disadvantages. The real-time PCR assay using TaqMan probes has also been extensively used to detect *B. bovis* and *B. bigemina,* making it an important tool for epidemiological studies of bovine babesiosis. This technique has been shown to be quick, sensitive and highly specific for the detection of *B. bovis* and *B. bigemina* (Kim et al., 2007). One of the limitations of this technique is the use of expensive equipment, which is often not available in poor countries. Chaisi et al. (2017), compared three different nucleic acid-based methods (RLB, qPCR and nPCR) for the detection of *A.*

marginale and *A. centrale* and concluded that qPCR was more effective as it detected more positive cattle than the other tests.

2.3.5. Reverse Line Blot (RLB) assay

The RLB hybridization assay has considerably improved haemoparasite diagnostic capacity, particularly due to its ability to simultaneously detect over 40 tick-borne pathogen species belonging to the genera, *Theileria*, *Babesia*, *Anaplasma* and *Ehrlichia* (O'Sullivan et al., 2011). This feature makes this assay economically advantageous and has contributed to an increase in the global knowledge on diversity of pathogens among domestic and wild animals. The hybridization of PCR products to specific probes immobilized on a membrane is the basis of the RLB technique. Furthermore, the same membrane can be used up to 20 times, allowing for the running of 800 samples, including controls. The RLB assay employs two sets of primers for specific amplification of the parasite *18S* rRNA gene in the V4 hypervariable region of *Theileria*/*Babesia* (Gubbels et al., 1999) and *16S* rRNA gene in the V1 hypervariable region of *Anaplasma*/*Ehrlichia* (Bekker et al., 2002). This method has been extensively used and provides an alternative for rapid identification of tick-borne pathogens in domestic animals, particularly in endemic areas (Torina & Caracappa, 2012; Aktas et al., 2015; Bolívar, 2013; Gubbels et al., 1999; Irwin, 2010; Kubelova et al., 2011; Krause, 2003; M'ghirbia et al., 2013; Njiiri et al., 2015; Schnittger et al., 2004; Sparagano & Jongejan, 1999). Nevertheless, expensive equipment, acquisition of membranes, probes and other consumables; laborious protocols and well-trained personnel are some of the requirements of this technique that limit its use in many countries.

3. Material and methods

3.1. Study area

The field work performed in the present study was conducted in four communes of Tchicala Tcholoanga Municipality, Huambo province, Angola (Figure 1), namely Mbave, Sambo, Samboto and Sede. Tchicala-Tcholoanga Municipality has an area of 4380 km² and there are two seasons during the year: the rainy season from October to April, with an annual rainfall between 1200 mm and 1600 mm; and a dry season from May to September. The average temperature in the area is 20°C and the temperature occasionally drops below zero in the dry season. It is situated in a tropical region; latitude: S12°37'52'' and longitude: E16°2'37''. The main economic activity is agriculture and livestock production on small traditional farms with indigenous breeds. The animal population is composed mainly of cattle and goats, although sheep and pigs can also be found on some farms. Since 1975, when the colonial period ended, the provision of veterinary assistance to this area dropped dramatically. Consequently, the information on animal disease status is extremely scarce and there is no control of animal movement between provinces. Similarly, records on acaricide dipping and spraying are non-existent, but it is known that acaricides are used by some livestock owners, which may result in the development of resistance.

Dogs constitute another important animal species, which is frequently present in grazing areas in this region. They are considered to be companion animals besides also being used as hunters.

A census conducted in 2014 by the regional administration service showed that the animal population of Tchicala Tcholoanga municipality is composed of a total of 23,756 cattle, 19,208 goats, 2,325 sheep and 2,696 dogs, among other animal species.

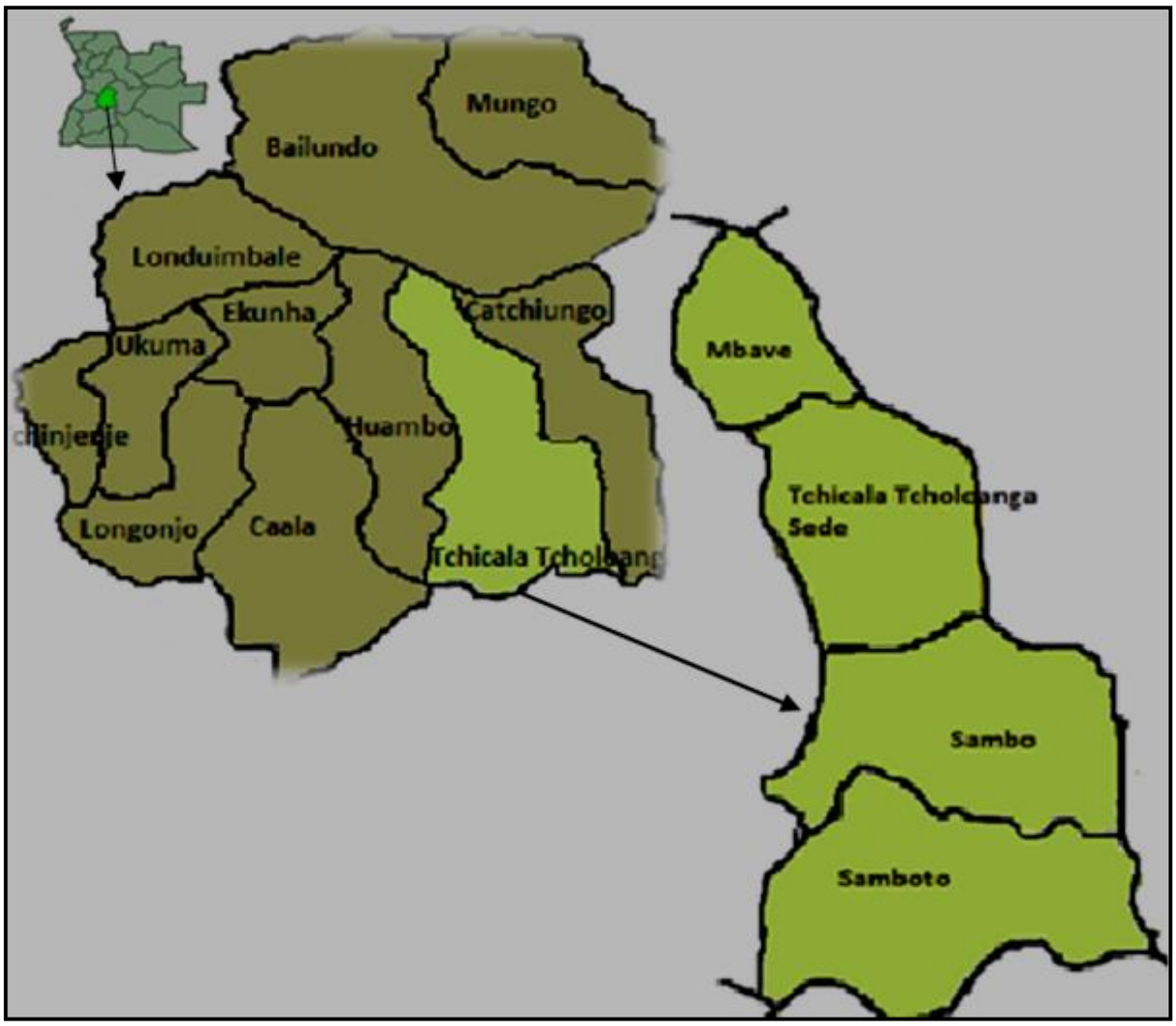

Figure 1 Map of Huambo province, Angola and administrative divisions of Tchicala-Tcholoanga municipality

3.2. Sample collection

In this study, ticks and blood samples were collected from cattle, goats, sheep and dogs in Tchicala-Tcholoanga, Huambo Province, Angola from the 5th to the 28th of January 2016. The target animals were randomly chosen, and included animals of both sexes and of different ages, from indigenous breeds.

3.2.1. Tick collection

The animals were restrained on the ground, and live ticks were manually collected from 20 animals per commune (five animals per commune per species) from half of the body, with special attention to the ears, udder, perineum, and tail. Ticks were preserved in 70% ethanol until their identification.

Ticks collected from cattle, goats, sheep and dogs were stored separately for each animal in 25 ml plastic containers, which were labeled with a number, collection date, commune name, GPS reference, host species, age (< 1 year = young and > 1 year = adult) and sex. A pencil-written label with all the aforementioned identification elements was placed inside each container. The total number of ticks collected from a single animal represented a tick collection.

3.2.2. Blood collection

Three hundred and forty animals; 88 cattle, 82 goats, 85 sheep and 85 dogs were sampled in order to detect at least one positive animal, with a probability of 0.95, assuming 15% as the expected proportion of positive animals and a confidence interval of 95%. Blood samples (4 ml) for tick-borne pathogen detection were collected from the jugular vein in cattle, sheep and goats and from the cephalic vein in dogs, using EDTA vacutainers and 21 G needles. In the field, the blood was stored in cooler boxes without ice and transported to the main laboratory at Huambo. The time from collection to the laboratory was around four to five hours. Once at the laboratory, blood samples from each animal were aliquoted into 2 ml cryopreservation tubes with screw lids; one to be used for the subsequent DNA extraction and the other to be kept as a backup. Tubes were labelled on the outside. The label on each tube had a unique sample code that corresponded to a database entry including collection date, number, commune name, GPS reference, host species, age (young or adult) and gender. The label on each tube was protected by transparent cellophane tape. All blood samples were stored at -20ºC until DNA extraction was performed.

3.3. DNA extraction

In March 2016, 340 DNA samples were extracted from whole blood using the commercial extraction kits, (QIAGEN, Hilden, Germany), QIAamp DNA mini kit, Catalog no. 51306 according to manufacturer's instructions for blood samples. This was carried out at Central Laboratory, Instituto de Investigação Veterinária, Huambo. For each sample, a volume of 200 µl of blood was used for DNA extraction. The final DNA eluted in 200 µl of buffer AE was stored at -20°C until it was transported to the Department of Veterinary Tropical Disease (DVTD) laboratories, Faculty of Veterinary Science, University of Pretoria for further analysis.

3.4. Tick identification

In August 2016, at the DVTD, 80 tick collections from cattle, goats, sheep and dogs were morphologically identified to species level by stereomicroscopic examination. Although the main tick identification keys were based on Walker et al. (2003), other publications (Camicas et al., 1983; Camicas et al., 1973; el Kammah et al., 1992) were used to differentiate between *H. leachi* and *H. paraleachi* found in this study.

3.5. Polymerase Chain Reaction (PCR)

Before the extracted DNA was subjected to PCR, 20 samples were randomly tested on gel electrophoresis to evaluate the DNA integrity.

Two separate PCRs were set up in order to amplify the V4 hypervariable region of the parasite *18S* rRNA gene of *Theileria* and *Babesia* species according to Gubbles et al. (1999) and the V1 hypervariable region of the parasite *16S* rRNA gene for *Anaplasma* and *Ehrlichia* species according to Bekker et al. (2002), using two different sets of primers: RLB-Forward primer (5'-GAC ACA GGG AGG TAG TGA CAA G-3'), RLB-Reverse primer (5'-biotin- CTA AGA ATT TCA CCT CTG ACA GT-3') and *Ehrlichia*-Forward (5'-GGA ATT CAG AGT TGG ATC MTG GYT CAG-3'), *Ehrlichia*-Reverse (5'-biotin-CGG GAT CCC GAG TTT GCC GGG ACT TYT TCT-3'), respectively. The PCR master mix

contained 60 U/ml Platinum Taq DNA polymerase, 40 mM Tris-HCl (pH 8.4), 100 mM KCl, 6 mM MgCl2, 400 µM dGTP, 400 µM dATP, 400 µM dCTP, 400 µM dUTP, 40 U/ml UDG, totalizing 12.5 µl of Platinum ® Quantitative PCR Super Mix-UDG (Life Technology™, South Africa). The final volume of 25 µl was obtained, adding 0.25 µl RLB-Forward primer (8 pmol), 0.25 RLB-Reverse primer (8 pmol), 9.5 µl water and 2.5 µl of DNA template.

The PCR mixture was always prepared one day before the PCR product was subjected to RLB. Known *A. centrale* and *B. bovis* DNA samples (Onderstepoort Biological Products, South Africa) were used as positive controls and water without DNA template used as a negative control. The thermocycler program displayed below was used for *Ehrlichia* and *Anaplasma* and *Theileria* and *Babesia* touchdown PCR. In the first cycle, UDG was activated and inactivated, and the remaining cycles consisted of the denaturation of a double stranded DNA template, annealing of primers, extension of PCR products by Taq polymerase. The last cycle served to promote a final extension. Thermocycler program: 1 cycle: (37°C/3 min and 94°C/10 min), 2 cycles: (94°C/20 sec, 67°C/30 sec, 72°C/30 sec); (94°C/20 sec, 65°C/30 sec, 72°C/30 sec); (94°C/20 sec, 63°C/30 sec, 72°C/30 sec); (94°C/20 sec, 65°C/30 sec, 72°C/30 sec); (94°C/20 sec, 59°C/30 sec, 72°C/30 sec); 40 cycles: (94°C/20 sec, 57°C/30 sec, 72°C/30 sec); 1 cycle: (72°C/7 min).

3.6. Membrane preparation

A Biodyne® C membrane (Separations, South Africa) with genus and species-specific probes for *Anaplasma, Ehrlichia, Theileria* and *Babesia* attached was used for the RLB. The details of the probes can be found in Table 1. In short, the membrane was prepared following three steps: membrane preparation, probe dilution and loading the probes onto the membrane. The size of the membrane was determined by the size of the support cushion. Two membrane corners were marked with ink in order to have a precise notion of the position of the different probes. Then the membrane was placed in a plastic container and was activated, 10 min at room temperature, using 10 ml of 16% EDAC. After the activation period, the membrane was washed with distilled water and placed

on the miniblotter, the remaining fluid from slots was removed by aspiration. The probes were then thawed and numbered according to the order that they would have in the membrane. For probe dilution, 142 µl of 0.5 M NaHC03 (pH 8.4) solution were placed in 200 µl tubes, into which 8 µl of each probe was added. A total of 150 µl of each diluted probe was applied to the respective slot, using different tips. The first and last slots were filled with ink and the membrane was incubated for two minutes at room temperature. After that, the probe fluid was aspirated from the slots and the membrane was inactivated at room temperature during 8 minutes by shaking, using 100 ml of 100 mM NaOH. Finally, the membrane was placed in a plastic container and was washed with 100 ml of 2 x SSPE/0.1% SDS at 60°C/5minutes.

3.7. Reverse Line Blot (RLB)

The RLB hybridization was performed according to Nijhof et al. (2005) and was modified by mixing the PCR products of each primer set (25 µl) before the preparation of the hybridization step.

The *Anaplasma* and *Ehrlichia* PCR product was mixed with the *Theileria* and *Babesia* PCR product, to which 130 µl of 2x SSPE/0.1% SDS were also added. The denatured diluted PCR product was processed in a thermocycler for 10 minutes at 99.9 °C and after that, the PCR product was immediately cooled on ice to prevent the rebinding of DNA molecule strands. The diluted PCR product was added to the membrane in a miniblotter where all residual fluids were removed by aspiration. The miniblotter slots were filled with the diluted PCR product. The membrane used had previously been blotted with 38 species-specific probes and five genus-specific probes (Table 1). Therefore, upon filling the miniblotter slots the PCR product hybridization took place in an incubator at 42°C for 60 minutes on a horizontal surface. The membrane was then washed twice in preheated 2 x SSPE/0.5% SDS for 10 minutes at 50°C in the incubator with moderate shaking, followed by discarding of the remaining 2 x SSPE/0.5% SDS. The following step was to incubate the membrane with 10 ml 2xSSPE/0.5% SDS and 12.5 µl streptavidin (peroxidase labelled) conjugate (1.25 U) for 30 minutes at 42°C under moderate shaking and

discarding the solution at the end. The membrane was then washed twice in preheated 2 x SSPE/0.5% SDS for 10 minutes at 42°C in an incubator under moderate shaking. The second step consisted of two washing processes with 2 x SSPE for 5 minutes at room temperature under moderate shaking. Finally, 6 ml ECL detection fluid was applied to the membrane, which was in a container, keeping it wet for one minute at room temperature by moderate shaking, before the fluid was discarded. The membrane was then laid on an X-ray film inside a cassette. The X-ray film was exposed for 30 seconds in a dark room and was ready to be read after being washed with developer and fixer solutions, rinsed with water and dried. ECL is catalysed by peroxidase and the products of this reaction emit light, which was captured on the X-ray film as small black dots. The RLB membrane was reused nine times after stripping the PCR products each time, using 200 ml of 1% SDS pre- heated at 80°C, followed by another wash with 200 ml 20 mM EDTA. The membrane was then stored at 4°C in 50 ml 20 mM EDTA in a plastic container until subsequent use.

Table 1 List of probes used in the RLB hybridization assay

Oligonucleotide probe identification	Sequence 5' - 3'	Reference
Anaplasma/Ehrlichia genus-specific	GGG GGA AAG ATT TAT CGC TA	Bekker et al., 2002
A. bovis	GTA GCT TGC TAT GRG AAC A	Bekker et al., 2002
A. centrale	TCG AAC GGA CCA TAC GC	Bekker et al., 2002
A. marginale	GAC CGT ATA CGC AGC TTG	Bekker et al., 2002
A. phagocytophilum	TTG CTA TAA AGA ATA ATT AGT GG	Bekker et al., 2002
A. platys	GCT TGC TAT GAT AAA AAT TAG TGG C	Nijhof et al., 2003
Anaplasma sp. Omatjenne	CGG ATT TTT ATC ATA GCT TGC	Bekker et al., 2002
E. canis	TCT GGC TAT AGG AAA TTG TTA	Schouls et al., 1999
E. ruminantium	AGT ATC TGT TAG TGG CAG	Bekker et al., 2002
Babesia/Theileria genus-specific	TAA TGG TTA ATA GGA RCR GTT G	Gubbels et al., 1999
Babesia 1 genus-specific	ATT AGA GTG CTC AAA GCA GGC	Nijhof (unpublished)
Babesia 2 genus-specific	ACT AGA GTG TTT CAA ACA GGC	Nijhof (unpublished)
B. bicornis	TTG GTA AAT CGC CTT GGT C	Nijhof et al., 2003
B. bigemina	CGT TTT TTC CCT TTT GTT GG	Gubbels et al., 1999
B. bovis	CAG GTT TCG CCT GTA TAA TTG AG	Gubbels et al., 1999
B. caballi	GTG TTT ATC GCA GAC TTT TGT	Butler et al., 2008
B. canis	TGC GTT GAC GGT TTG AC	Matjila et al., 2004
B. divergens	ACT RAT GTC GAG ATT GCA C	Nijhof et al., 2003
B. felis	TTA TGC GTT TTC CGA CTG GC	Bosman et al., 2007
B. gibsoni	TAC TTG CCT TGT CTG GTT T	Yisaschar-Mekuzas et al., 2010
B. lengau	CTC CTG ATA GCA TTC	Bosman et al., 2010
B. leo	TTA TGC TTT TCC GAC TGG C	Bosman et al., 2007
B. microti	GRC TTG GCA TCW TCT GGA	Nijhof et al., 2003
B. occultans	CCT CTT TTG GCC CAT CTC G	He et al., 2012
B. rossi	CGG TTT GTT GCC TTT GTG	Matjila et al., 2004
Babesia sp. (sable)	GCG TTG ACT TTG TGT CTT TAG C	Oosthuizen et al., 2008
B. vogeli	AGC GTG TTC GAG TTT GCC	Matjila et al., 2004
Theileria genus-specific	ATT AGA GTG TTT CAA GCA GAC	Nijhof (unpublished)
T. annae	CCG AAC GTA ATT TTA TTG ATT G	Yisaschar-Mekuzas et al., 2010
T. annulata	CCT CTG GGG TCT GTG CA	Georges et al., 2001
T. bicornis	GCG TTG TGG CTT TTT TCT G	Nijhof et al., 2003
T. buffeli	GGC TTA TTT CGG WTT GAT TTT	Gubbels et al., 1999
T. equi	TTC GTT GAC TGC GYT TGG	Butler et al., 2008
T. lestoquardi	CTT GTG TCC CTC CGG G	Schnittger et al., 2004
T. mutans	CTT GCG TCT CCG AAT GTT	Gubbels et al., 1999
T. ovis	TGC GCG CGG CCT TTG CGT T	Bekker et al., 2002
T. parva	GGA CGG AGT TCG CTT TG	Nijhof et al., 2003
T. separata	GGT CGT GGT TTT CCT CGT	Schnittger et al., 2004
Theileria sp.(buffalo)	CAG ACG GAG TTT ACT TTG T	Oura et al., 2004
Theileria sp. (kudu)	CTG CAT TGT TTC TTT CCT TTG	Nijhof et al., 2003
Theileria sp. (sable)	GCT GCA TTG CCT TTT CTC C	Nijhof et al., 2003
T. taurotragi	TCT TGG CAC GTG GCT TTT	Gubbels et al., 1999
T. velifera	CCT ATT CTC CTT TAC GAG T	Gubbels et al., 1999

Symbols for degenerate positions: R = A/G and W = A/T

3.8. Real-time PCR (qPCR) for *B. bigemina* and *B. bovis*

Two qPCR assays were used for the detection of *B. bigemina* and *B. bovis* DNA (Kim et al., 2007), from 80 and 88 DNA samples from cattle, respectively.

For both qPCR assays, each reaction mixture (20 µl) contained 10 µl of TaqMan Universal®, PCR Master Mix (Applied Biosystems), 2 µl of template DNA and 6.5 µl water. Nuclease free water was used as a negative control while DNA samples extracted from *B. bovis* and *B. bigemina* blood vaccines (Onderstepoort Biological Products, Pretoria, South Africa), and confirmed as such by qPCR and species-specific sequence analysis, were included as positive controls.

For the *B. bigemina* qPCR, primers and probes were used in a final concentration of 0.5 µM of forward primer BiF (5′ - AAT AAC AAT ACA GGG CTT TCG TCT - 3′); 0.5 µM of reverse primer BiR (5′- AAC GCG AGG CTG AAA TAC AAC T - 3′), and 0.25 µM BiP probe (VIC – 5′- TTG GAA TGA TGG TGA TGT ACA ACC TCA - 3)′ (Kim et al., 2007). For the *B. bovis* qPCR, the final primer and probe concentrations used were: 0.5 µM of forward primer BoF (5′ - AGC AGG TTT CGC CTG TAT AAT G - 3′), 0.5 µM of reverse primer BoR (5′ - AGT CGT GCG TCA TCG ACA AA - 3′) and 0.25 µM BoP probe (FAM– 5′ - CCT TGT ATG ACC CTG TCG TAC CGT TGG - 3′) (Kim et al., 2007).

For both qPCR assays, the following PCR conditions were applied, using the StepOnePlus real-time PCR system (Applied Biosystems®, Life Technologies™, Johannesburg, South Africa): 1 cycle of 50°C/2 minutes to UDG digest, 1 cycle of 95°C/10 minutes for TaqGold pre-activation, 45 cycles of 95°C/20 seconds for amplification, 55°C/1 minute for annealing, and cooling at 4°C. For the *B. bovis* assay, the annealing temperature was optimized to 57°C/1 minute. The fluorescence data was analysed using the StepOne™ Software v2.3.

3.9. Quantitative real-time PCR (qPCR) assay for *E. ruminantium*

Based on the *pCS20* gene, the TaqMan quantitative real-time PCR as described by Steyn et al., (2008) was used to detect *E. ruminantium* from DNA extracted from 20 cattle, 20

goats, 20 sheep and 10 dog blood samples, and 100 *A. pomposum* ticks (male and female) collected from apparently healthy ruminants. *A. pomposum* ticks were randomly selected among tick populations collected in Tchicala-Tcholoanga, Huambo province, Angola, without considering the geographic area or host. The main reason for this choice was to confirm whether this tick species is involved in the transmission of *E. ruminantium* in Angola, since no other *Amblyomma* spp. were found in the collection. DNA extraction from 100 adult *A. pomposum* ticks (male and female), preserved in 70% ethanol was performed using the commercial extraction kits, QIAGEN, QIAamp DNA mini kit, according to the manufacturer's instructions. Firstly, each tick was washed and cut, using a new blade for each specimen, and placed in an Eppendorf tube, following the kit protocol steps. In addition, 70 DNA extracts from ruminants and dog blood were also analysed.

The CowF forward primer (5' - CAA AAC TAG TAG AAA TTG CAC A - 3') and CowR reverse primer (5' - TGC ATC TTG TGG TGG TAC - 3') were used to amplify a 226 bp fragment of the conserved *pCS20* gene region of *E. ruminantium.* The TaqMan probe Cow™ (5' - 6FAM TCC TCC ATC AAG ATA TAT AGC ACC TAT TA XT – PH-3') was used in the reaction.

Each PCR reaction consisted of a final concentration of 4 mM MgCl2, 1 U Uracil DNA N-Glycosylase, 5 U Taq polymerase, 0.5 μM for both primers, 0.4 μM probe and 2 μl DNA template, which were added to each mixture at the end of the preparation process. The 2 μl of Welgevonden genomic DNA was used as positive control and distilled water was used as negative control. The samples were run on the software version, rotor-Gene 2.0.2.4 4.0 (Qiagen), using thermal cycling under the following conditions: initial incubation at 40°C/10 minutes, denaturation at 95°C/10 minutes, followed by 38 cycles of denaturation at 95°C/10 seconds, annealing at 48°C/10 seconds, extension at 58°C/30 seconds and final cooling at 4°C.

3.10. Cloning and sequencing of *Anaplasma* spp. *16S* rRNA gene

The near full-length parasite *16S* rRNA gene of six cattle and five sheep DNA samples that tested positive for *A. platys* DNA using the RLB assay were selected for *16S* rDNA amplification, cloning and sequencing. The conventional PCR was set up using 12.5 µl Phusion mix (Thermo Fisher Scientific), 0.4 µM of forward fD1 (5′ - AGA GTT TGA TCC TGG CTC AG - 3′) and reverse rP2 primers (5′ - ACG GCT ACC TTG TTA CGA CTT - 3′) (Weisburg et al., 1991), 9 µl of water and 2.5 µl of DNA template. Genomic DNA from the *A. centrale* vaccine (obtained from Onderstepoort Biological Products, Pretoria, South Africa) was used as a positive control and molecular grade water was used as the negative control. The following thermal cycling conditions were applied: initial denaturation at 98°C/10 seconds, followed by 35 cycles of denaturation at 98°C/1 second, 55°C/5 seconds for annealing, 72°C/15 seconds for extension, a final extension at 72°C/1 minute, and cooling at 4°C. The PCR products were analysed on a 2 % agarose gel run at 120 V/30 minutes. The PCR products were purified using the QIAquick PCR purification kit (Qiagen, USA). Cloning was done with the CloneJET cloning kit (Thermofisher Scientific, South Africa). The 20 µl ligation reaction was set up using 10 µl of the 2x ligation buffer, 1 µl pJET cloning vector (50 µg/µl), 1-3 µl (app. 80 ng) PCR product purified, 1 µl T4 DNA ligase and nuclease-free water. The ligation reaction products were directly used for transformation. The recombinants were transformed in competent *E. coli* cells at 42°C, grown in SOC medium (Invitrogen, USA) and incubated for 1.5 hours at 37°C, after which they were plated on LB agar plates (Invitrogen, USA). The plates were incubated at 37°C overnight. The next day, white colonies were picked up and screened by colony PCR. The 20 µl volume was obtained using 10 µl DreamTaq, 0.4 µl pJet-Forward (5′ - CGA CTC ACT ATA GGG AGA GCG GC - 3′) and pJet-Reverse (5′ - AAG AAC ATC GAT TTT CCA TGG CAG - 3′) primers and 9.2 µl of water. The thermocycler was run at 95°C/3 minutes, followed by 25 cycles at 94°C/30 seconds, 60°C/30 seconds, 72°C/1 minute, and 72°C/7 minutes of final extension. Positive colonies were incubated overnight at 37°C (with shaking) in 5 ml liquid broth and plasmid DNA was extracted using the High Pure Plasmid Isolation kit (Roche Life Science, Germany). Ten positive

clones were sequenced at Inqaba Biotechnologies (South Africa) using the vector primers pJet1.2 forward and pJet1.2 reverse.

The forward and reverse sequences were assembled, edited and the consensus sequences were aligned with related sequences using CLC Main Workbench v 7.7.1 (https://www.qiagenbioinformatics.com/products/clc-genomics-workbench/). Sequence identity searches were performed using BLASTn on the National Centre for Biotechnology Information website (http://blast.ncbi.nlm.hih.gov/) (Altschul et al., 1990). The alignment was truncated to the size of the shortest sequence using BioEdit v7 (Hall, 1999). The evolutionary history was inferred by using Neighbour-Joining method (Saitou & Nei, 1987) based on the Jukes-Cantor model (Jukes & Cantor, 1969) in combination with the bootstrap method (Felsenstein, 1985) using 1000 replicates/tree using the Molecular Evolutionary Genetics Analysis version 7.0 (MEGA7) software package (Kumar et al., 2016). The genetic distances between the sequences were estimated by determining the number of base pair differences between sequences using MEGA7 All consensus trees were edited using MEGA7 (Kumar et al., 2016).

3.11. Statistical analysis

The RLB data for tick-borne pathogens were used to detect and identify the population of haemoparasites present in ruminants and dogs, with 95% confidence intervals. A multivariable logistic regression was used to test for significant association between potential risk variables and infection with tick-borne pathogens using the Statistical Package for the Social Sciences (SPSS, 2014) version 23.0 IBM.

The analysis was carried out considering the infection as a binary outcome (negative or positive). The following equation was used to predict the dependent variables (tick-borne species detected by RLB) using the independent variables: sex (female, male), age (< 1 year, > 1 year) and commune (Mbave, Sambo, Samboto, and Sede):

$Y = X_1b_1 + X_2b_2 + X_3b_3 + A$, where Y is the dependent variable (infection status as determined by RLB) that was predicted by the independent variables X_1, X_2 and X_3; b_1, b_2 and b_3 are the coefficients or multipliers that describe the size of the effect that the independent variables have on the dependent variable Y, and A is the value that Y is predicted to have when all the independent variables are equal to zero. We tested if the analytical models fitted the data by measuring the predictive power, i.e., how well the dependent variable is predicted based on the independent variable (NagelKerke R square) and the goodness-of-fit statistic (Pearson chi-square). The data were analysed using SPSS version 23.0 IBM (SPSS, 2014) at 5% level of significance.

The Kruskal-Wallis one-way analysis of variance was used to test for statistical differences in tick infestation among animal species and location. The Mann-Whitney U test was used to test the difference in tick infestation between female and male animals. Post hoc analyses were conducted using the Mann-Whitney U test for pairwise comparisons for animal species and locations, with a Bonferroni correction applied, resulting in a modified significance level of $p<0.0083$.

4. Results

4.1. Tick identification

Ticks were morphologically identified to species level by stereomicroscopic examination, with the aid of publications that included original species descriptions and identification keys (Walker et al., 2003).

A total of 2,963 ticks were counted. These included nymphs and adults belonging to 5 genera and 17 tick species that were collected from cattle, goats, sheep and dogs (n=20 for each animal species) in four communes (Mbave, Sambo, Samboto and Sede) of Tchicala-Tcholoanga, Huambo province, Angola (Table 2). Despite having conducted the tick collection process in a way that would be conducive to obtaining a set of ticks including all growth stages, no larvae were found during collection.

4.1.1. Ticks on cattle

From 20 sampled cattle, 1,535 ticks (51.8%), belonging to 12 tick species (Table. 2) were collected and the three most predominant species were *A. pomposum* (n = 617, 40.2%), *R. decoloratus* (n = 466, 30.4%) and *H. truncatum* (n = 270, 17.6%). The most intense cattle tick infestation was observed at Samboto and Sede communes with 517 and 551 ticks, respectively, collected from the half body of the animals. In these two communes, three cattle were infested with at least nine different tick species each. In Samboto, from just a single animal, 291 ticks, belonging to 9 tick species, were collected from half body. On the contrary, the least infested single cattle was from Sede, with only 12 ticks belonging to two species counted. Ten ticks identified as *R. microplus* were collected from Sede (latitude: S12°49'33.6'' and longitude E016°06'02.5'') in female cattle that were under 1 year of age. However, confirmation of its establishment in the region is required, due to the small number of ticks collected and identified.

4.1.2. Ticks on goats

As for goats, a total of 136 (4.6%) ticks were collected and nine tick species were identified (Table 2). Among these, the majority of ticks belonged to the species *A. pomposum* (n = 52, 38.2%), *R. punctatus* (n = 41, 30.1%) and *R. e. mimeticus* (n = 16, 11.8%).

Sambo was the commune where the highest tick infestations on goats were recorded. However, the most infected goat was found in Samboto, with 15 ticks belonging to 5 tick species: *A. pomposum, R. decoloratus, R. e. mimeticus, R. lunulatus* and *R. punctatus.*

4.1.3. Ticks on sheep

Three hundred and fifty-nine ticks (12.1%) were collected from sheep and nine tick species were identified (Table 2). The three most abundant species were *A. pomposum* (n = 127, 35.4%) *R. punctatus* (n = 91, 25.4%), *R. e. mimeticus* (n = 50, 13.9%).

The highest infestation of ticks on sheep was found at Sede, where one of the animals was infested with 51 ticks of two tick species: *A. pomposum* and *R. e. mimeticus.* It was, however, in Samboto where the greatest tick diversity was found in a single animal: *A. pomposum*, *R. decoloratus, R. compositus, R. evertsi evertsi, R. evertsi mimeticus, R. punctatus* and *H. truncatum.* Mbave was the commune where the lowest infestation was recorded; only 57 ticks were found in the 20 sampled animals.

4.1.4. Ticks on dogs

Out of the four host species used for tick collection in the study, after cattle, dogs were the species from which most ticks were collected. A total of 933 (31.5%) ticks belonging to 11 species were identified (Table 2). *R. turanicus* (n = 427, 45.8 %), *R. tricuspis* (n = 187, 20.0 %), *R. punctatus* (n = 85, 9.1 %), *R. sulcatus* (n = 84, 9.0 %) were the most abundant species.

Dogs from Mbave and Sede were infested with more ticks than other communes. However, the most infested single dog was found in Sambo with 108 ticks belonging to seven species collected from half body. The tick species collected from that single dog are described below in order of abundance (highest to lowest): *R. turanicus, R. tricuspis, R. sulcatus, H. leachi, I. cavipalpus, R. punctatus* and *R. lunulatus.*

Table 2 Tick species composition from domestic ruminants and dogs in a cross-sectional study in Tchicala-Tcholoanga, Huambo province, Angola

Tick species	Number of ticks (percentage of total ticks in parenthesis)			
	Cattle	**Goat**	**Sheep**	**Dog**
A. pomposum	617 (40.19)	52 (38.24)	127 (35.38)	8 (0.85)
H. truncatum	270 (17.59)	2 (1.47)	27 (7.52)	0 (0.0)
H. leachi	0 (0.0)	0 (0.0)	0 (0.0)	10 (1.07)
H. paraleachi	0 (0.0)	0 (0.0)	0 (0.0)	57 (6.11)
I. cavipalpus	7 (0.456)	1 (0.74)	0 (0.0)	13 (1.39)
R. decoloratus	466 (30.36)	3 (2.2)	34 (9.47)	4 (0.43)
R. microplus	10 (0.65)	0 (0.0)	0 (0.0)	0 (0.0)
R. compositus	12 (0.78)	0 (0.0)	1 (0.28)	0 (0.0)
R. evertsi evertsi	2 (0.13)	3 (2.2)	8 (2.23)	0 (0.0)
R. e. mimeticus	61 (3.97)	16 (11.76)	50 (13.93)	0 (0.0)
R. kochi	22 (1.43)	0 (0.0)	0 (0.0)	0 (0.0)
R. lunulatus	11 (0.72)	4 (2.94)	4 (1.11)	52 (5.57)
R. punctatus	22 (1.43)	41 (30.15)	91 (25.35)	85 (9.11)
R. simus	0 (0.0)	0 (0.0)	0 (0.0)	6 (0.64)
R. sulcatus	0 (0.0)	0 (0.0)	0 (0.0)	84 (9.00)
R. turanicus	0 (0.0)	0 (0.0)	0 (0.0)	427 (45.77)
R. tricuspis	35 (2.29)	14 (10.29)	17 (4.74)	187 (20.04)
Total	**1535 (100)**	**136 (100)**	**359 (100)**	**933 (100)**

4.1.5. Ticks distribution across host species and hosts gender

In this study, five tick species *(A. pomposum, R. decoloratus, R. lunulatus, R. punctatus* and *R. tricuspis)* were coincidently found feeding on all the mammal species studied (cattle, goats, sheep and dogs). In addition, in ruminants, besides the tick species mentioned above, *H. truncatum, R. evertsi evertsi* and *R. evertsi mimeticus* were also found feeding on all ruminants studied. However, *R. kochi* was found feeding only on cattle. Moreover, *H. leachi, H. paraleachi, R. simus, R. sulcatus* and *R. turanicus* were found to feed exclusively on dogs.

Tick infestation was not normally distributed among individual animals, as determined by the Shapiro-Wilk test ($p<0.001$). Statistically significant differences in tick infestation were found between the sampled animal species, Kruskal-Wallis Test: $X^2=47.96$, $p<0.001$. The median (range) tick infestation values were 52.5 (12 to 291) for cattle, 5.5 (2 to 15) for goats, 18.5 (2 to 51) for sheep and 45.0 (5 to 108) for dogs. There were no significant differences between cattle and dogs ($Z=-1.38$, $p=0.17$), despite an overall difference in tick infestation *vs* animal species. However, there were statistically significant differences in tick infestation between animal species in the cattle *vs* goats ($Z=-5, 32$, $p<0.001$), cattle *vs* sheep ($Z=-4.11$, $p<0.001$) and sheep *vs* dogs ($Z=-3.76$, $p=0.001$) comparisons. The difference in tick infestation in male and female animals was found to be significant, $Z=-2.62$, $p=0.009$. The median (range) tick infestation values were 15.0 (2,291) for females and 33.0 (2,100) for males.

Table 3 Geographic distribution of tick species from four communes in Tchicala-Tcholoanga, Huambo province, Angola

Tick species	Geographic distribution			
	Mbave	**Sambo**	**Samboto**	**Sede**
A. pomposum	✓	✓	✓	✓
H. truncatum	×	✓	✓	✓
H. leachi	×	✓	✓	✓
H. paraleachi	✓	✓	✓	✓
I. cavipalpus	✓	×	✓	×
R. decoloratus	✓	✓	✓	✓
R. microplus	×	×	×	✓
R. compositus	×	✓	✓	×
R. evertsi evertsi	✓	×	✓	✓
R. e. mimeticus	✓	✓	✓	✓
R. kochi	✓	×	✓	✓
R. lunulatus	✓	✓	✓	✓
R. punctatus	✓	✓	✓	✓
R. simus	✓	×	×	✓
R. sulcatus	✓	✓	✓	✓
R. turanicus	✓	✓	✓	✓
R. tricuspis	✓	✓	✓	✓

✓ found
× not found

Regarding geographic distribution, nine out of 17 tick species (*A. pomposum, H. leachi, R. decoloratus, R. evertsi mimeticus, R. lunulatus, R. punctatus, R. sulcatus, R. turanicus* and *R. tricuspis*) were found in all communes studied, while *R. microplus* was the only tick species found in just one commune (Sede). The diversity of tick species distribution varied between 12 and 15 tick species per commune.

No statistically significant differences in tick infestation were observed between the locations sampled, Kruskal-Wallis Test: $X^2 = 2.81$, p=0.042. There were also no significant differences between locations when pairwise comparisons were used (p>0.0083). The median (range) tick infestation values were 16.0 (2 to 94) for Mbave, 21.5 (5 to 108) for Sambo, 23.5 (3 to 291) for Samboto and 33.0 (2 to 198) for Sede.

4.2. Reverse Line Blot

Three hundred and forty DNA samples from different animal species (cattle, goats, sheep and dogs) were processed using the RLB assay, which was set up, targeting the *16S* rRNA gene of *Ehrlichia* and *Anaplasma* and the *18S* rRNA gene to *Theileria* and *Babesia*. In this assay, five genus-specific probes and 38 species-specific probes were used (Figure. 1) and 15 haemoparasites were detected: *A. centrale, A. marginale, A. bovis, A. platys, Anaplasma* sp. Omatjenne, *B. bigemina, Theileria* sp. (sable), *T. mutans, T. velifera, B. bovis, T. bicornis, T. ovis, E. canis, B. rossi,* and *B. vogeli*.

4.2.1. Tick-borne pathogens in cattle

Eighty-eight PCR products from cattle were hybridized to the RLB membrane and 82 samples reacted positively, whereas six samples tested negative (or below the detection limit of the assay). Nine haemoparasites were identified in cattle and are listed according to their abundance as found by this study: *T. velifera* (78.4%), *T. mutans* (73.9%), *Theileria* sp. (sable) (71.6%), *A. marginale* (28.4%), *Anaplasma* sp. Omatjenne (25.0%), *A. platys* (18.2%), *A. centrale* (12.5%), *B. bigemina* (3.4%) and *A. bovis* (1.1%). From those *T. velifera* (78.4 %) followed by *T. mutans* (73.9 %) and *Theileria* sp. (sable) (71.6%) were the most abundant haemoparasites identified. In addition, PCR products hybridized with the following genus-specific probes: 69 (84.1%) with *Anaplasma*/*Ehrlichia*, 80 (97.6 %) with *Theileria*/*Babesia*, 78 (95.1 %) with *Theileria* spp., 18 (21.9 %) with *Babesia*1 and 2 (2.4%) with *Babesia*2.

The results obtained from cattle revealed a high level of mixed infections. In fact, 80 out of 82 cattle had mixed infections (97.6%), whereas single infections (2.4%) were detected only in two cattle samples. The most frequent mixed infection was observed between *Theileria* sp. *(*sable), *T. mutans* and *T. velifera*, representing 63.4% of multiple infections. In general, most of the signals detected on the RLB assay were strong. However, some faint signals were also observed and had to be sufficiently visible to be considered positives.

Furthermore, 93.8 % (n = 15) of cattle samples that were positive to *A. platys (*n = 16; 18.2 %) by RLB assay were also positive to *Anaplasma* sp. Omatjenne infection.

4.2.2. Tick-borne pathogens in goats

From goats, 82 PCR products were hybridized to the RLB membrane; results are shown in Table 4. A total of 66 (80.5%) of the PCR amplicons reacted to the *Ehrlichia*/*Anaplasma* genus-specific probe, 3 (3.6%) with the *Theileria*/*Babesia* genus-specific probe, 2 (2.4%) with the *Theileria* genus-specific probe and 1 (1.2%) with the *Babesia* genus-specific probe. Two samples tested positive for *A. centrale* DNA (2.4%, 95% CI -0.8, 5.6%). PCR amplicons of these two samples also reacted with the *Ehrlichia*/*Anaplasma* genus-specific probe.

4.2.3. Tick-borne pathogens in sheep

The PCR samples of 85 sheep were hybridized to the RLB membrane and resulted in 83 (97.6%) positive and 2 (2.4%) negative animals. From five genus-specific probes that were hybridized to the membrane 67 (80.7%) *Ehrlichia* and *Anaplasma*, 72 (86.7%) *Theileria* and *Babesia*, 68 (81.9%) *Theileria* spp. and 3 (3.6 %) *Babesia*1 were detected. With the species-specific probes, the following haemoparasites were detected and are listed in order of abundance: *T. ovis* (80%), *Theileria* sp. *(*sable) (54.1%), *A. platys* (5.9%), *Anaplasma* sp. Omatjenne (4.7%), *T. bicornis* (4.7%), *A. centrale* (2.4%), *A. marginale* (1.2%), *B. bovis* (1.2%) and *B. rossi* (1.2%). Mixed infections occurred in 76 (91.6%) animals, whereas single infections were detected in seven (8.4%) sheep. The most common haemoparasite

association was between *Theileria* sp. *(*sable) and *T. ovis* in 43 (51.8%) samples. Curiously, with the exception of three cases, wherever *Theileria* sp. *(*sable) was identified, *T. ovis* was also present. However, 25 out of 68 *T. ovis* positive samples were not associated with *Theileria* sp. *(*sable).

The detection of *A. platys* (5 = 5.9%), *T. bicornis* (4 = 4.7%), *B. bovis* (1= 1.2%) and *B. rossi* (1 = 1.2%) from sheep samples were unexpected findings in this work. Moreover, except in one case, all samples from sheep, in which *A. platys* was detected, *Anaplasma* sp. Omatjenne also gave positive results.

4.2.4. Tick-borne pathogens in dogs

Eighty-five samples from dogs were exposed to RLB hybridization and 75 (88.2%) were positive for one or more pathogens. PCR amplicons of 10 samples did not hybridize with any genus- or species-specific probes. The genus-specific probes used in the RLB membrane captured *Ehrlichia*/*Anaplasma* (13 = 13.3%), *Theileria*/*Babesia* (53 = 70.7%), *Theileria* spp. (5 = 6.7%) and *Babesia* spp. (64 =85.3%). Furthermore, four haemoparasites were identified in dogs by RLB assay and are presented in order of abundance: *B. vogeli* (35.3%), *B. rossi* (23.5%), *T. ovis* (8.2%) and *E. canis* (1.2%). The pathogen, *E. canis,* was identified in only one dog, in which signals of *Ehrlichia*/*Anaplasma*, *Theileria*/*Babesia*, were also observed. A considerable number of co-infections (54 = 72%) were observed. Among them, the association between *B. vogeli* and *B. rossi* was the most common (5 = 9.2%).

The detection of *T. ovis* signals in seven dogs is considered an unusual finding that needs to be clarified by sequencing.

Table 4 Tick-borne pathogens detected by RLB hybridization assay in blood samples from domestic ruminants and dogs

	Number of positive samples	Prevalence of infection (%)	95% confidence interval
Cattle (n=88)			
A. centrale	11	12.5	5.6, 19.4
A. marginale	25	28.4	18.9, 37.8
A. bovis	1	1.1	-1.1, 3.4
A. platys	16	18.2	10.1, 26.2
Anaplasma sp. Omatjenne	22	25	16.0, 34.0
B. bigemina	3	3.4	-0.4, 7.2
Theileria sp. (sable)	63	71.6	62.2, 81.0
T. mutans	65	73.9	64.7, 83.0
T. velifera	69	78.4	69.8, 87.0
Sheep (n=85)			
A. centrale	2	2.4	-0.9, 5.6
A. marginale	1	1.2	-1.1, 3.5
A. platys	5	5.9	0.9, 10.9
Anaplasma sp. Omatjenne	4	4.7	0.2, 9.2
B. bovis	1	1.2	-1.1, 3.5
B. rossi	1	1.2	-1.1, 3.5
Theileria sp. (sable)	46	54.1	43.5, 64.7
T. bicornis	4	4.7	0.2, 9.2
T. ovis	68	80	71.5, 88.5
Goats (n=82)			
A. centrale	2	2.4	0.5, 5.6
Dogs (n=85)			
E. canis	1	1.2	-1.1, 3.5
B. rossi	20	23.5	14.5, 32.5
B. vogelii	30	35.3	25.1, 45.4
T. ovis	7	8.2	2.4, 14.1

4.2.5. Association between exposure variables and tick-borne pathogens

Results of multivariable logistic regression on the RLB hybridization test status of tick-borne infections in various animal species in a cross-sectional study carried out in Tchicala Tcholoanga municipality, Huambo Angola in January 2016 are shown in Tables 5 and 6. The total number of cattle and dogs sampled were 88 and 85, respectively. Only results involving significant associations between tick-borne infection and the variables: age (<1 year and > 1 year) and location (Mbave, Sambo, Samboto and Sede), are presented in this study (Table 5 and Table 6). The variable gender had no influence on infection in the animal species studied. Similarly, there were no significant associations in sheep and goats. Cattle older than one year were significantly more likely to be infected with, *Theileria* sp. (sable) (OR = 5.55, p = 0.015) and *T. velifera* (OR = 7.53, p = 0.004) than cattle of less than a year old (Table 5). However, infection with *B. bigemina* was more likely in younger cattle (< 1 year) than in older cattle (> 1 year) (OR = 25.0, p= 0.033). Significant associations between the variables considered and tick-borne infections were found in dogs for *B. rossi* and *B. vogeli* infections. There was a higher likelihood (OR = 6.28, p = 0.013) of infection with *B. rossi* in dogs younger than one year than those older than one year.

Table 5 Association between tick-borne infections and age of the animals

Animal species	Tick-borne infection	Variable	Categories	No. of samples	No. of animals infected (% in parenthesis)	Odds ratio	p - value
Cattle	*B. bigemina*	Age	> 1 year	75	1 (1.3)	25.0	0.033
			< 1 year	13	2 (15.4)		
	Theileria sp. (sable)	Age	> 1 year	75	57 (76.0)	5.55	0.015
			< 1 year	13	6 (46.2)		
	T. velifera	Age	> 1 year	75	63 (84.0)	7.53	0.004
			< 1 year	13	6 (46.2)		
Dogs	*B. rossi*	Age	> 1 year	71	12 (16.9)		
			< 1 year	14	8 (57.1)	6.28	0.013

Infection with *Theileria* sp. (sable) was higher amongst cattle in Sambo (OR = 4.03, p = 0.059) and Samboto (OR = 5.15, p = 0.043) communes than cattle in Mbave, but equal occurrence of the infection was observed between Mbave and Sede (60%). The likelihood of *T. mutans* infections was higher in Mbave OR = 5.34, p=0.019), Sambo (OR= 4.67, p=0.033) and Samboto (OR=2.95, p=0.12) than in Sede.

Table 6 Association between tick-borne infections and animal locations

Animal species	Tick-borne infection	Variable	Categories	No. of samples	No. of animals infected (% in parenthesis)	Odds ratio	p - value
Cattle	*Theileria* sp. (sable)	Location	Mbave	25	15 (60.0)		
			Sambo	23	19 (82.6)	4.03	0.059
			Samboto	20	17 (85.0)	5.15	0.043
			Sede	20	12 (60.0)	0.96	0.952
	T. mutans	Location	Mbave	25	21 (84.0)	5.34	0.019
			Sambo	23	19 (82.6)	4.66	0.033
			Samboto	20	15 (75.0)	2.95	0.122
			Sede	20	10 (50.0)		
Dogs	*B. rossi*	Location	Mbave	22	7 (31.8)	2.05	0.459
			Sambo	21	2 (9.5)		
			Samboto	21	2 (9.5)	1.03	0.978
			Sede	21	9 (42.9)	6.37	0.038
	B. vogeli	Location	Mbave	22	11 (50.0)	3.75	0.069
			Sambo	21	4 (19.0)	1.13	0.876
			Samboto	21	11 (52.4)	5.48	0.019
			Sede	21	4 (19.0)		

Only *A. centrale* (two samples, 2.4%, 95% CI -0.8, 5.6%) was detected in goat samples by species-specific probes of the RLB assay. For sheep, there was no significant association ($p>0.05$) between the coefficients of all the independent variables (age, gender and location) in the logistic regression model.

4.3. Sequence of the 16S rRNA gene of *Anaplasma* spp. from Angola

Six out of sixteen DNA samples from cattle (7, 26, 47, 64, 82 and 98) and five from sheep (255, 262, 267, 275 and 286) that were positive for *A. platys* DNA using the RLB assay were selected for 16S rDNA analysis. The near full-length of the *16S* rRNA gene (~ 1400 bp) was amplified, cloned and the recombinants sequenced. Figure 2 shows the unpurified PCR products of these samples.

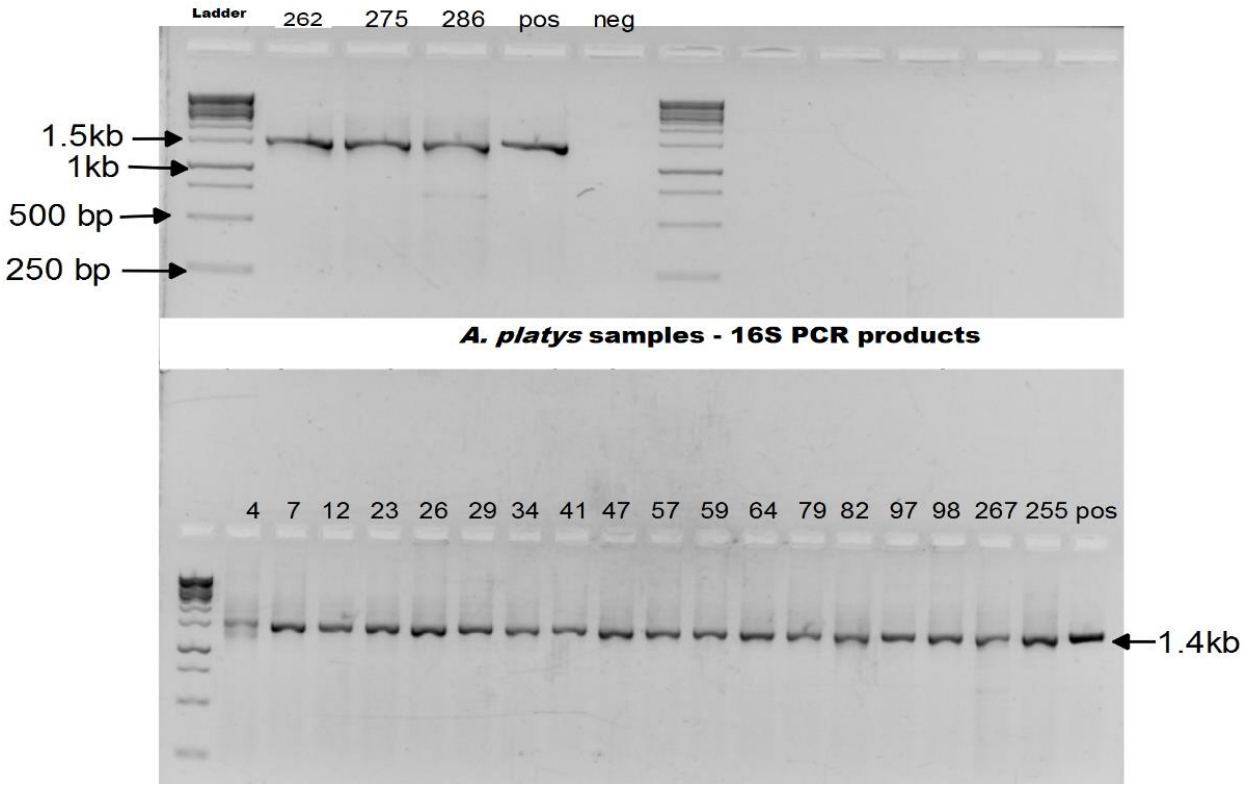

Figure 2 Unpurified *16S* rRNA PCR product of *A. platys*

Six recombinant sequences were obtained from cattle and five from sheep samples; of these only three near full-length sequences (1249 bp) could be obtained (7f, 26g, 47b). BLASTn homology searches showed that sequence 7f was identical to *Ehrlichia* sp. Bom Pastor (AF318023) previously described from a goat in Mozambique. The *Ehrlichia* sp. Bom Pastor sequence was shown to be homologous to *Ehrlichia* sp. strain Omatjenne (Allsopp et al, 1997); now known as *Anaplasma* sp. Omatjenne. As such, sequences 26g and 47b had 99% identity to various *A. platys* sequences available on GenBank (including the type strain sequence M82801), as well as 99% identity to *Ehrlichia* (*Anaplasma*) sp. Bom Pastor (AF318023) and *Anaplasma* sp. Omatjenne (U54806).

As for the partial sequences obtained, sequence 255a (337 bp) obtained from a sheep sample showed 99% identity to *A. ovis* (KX579073), whereas two sequences obtained from cattle, 82b (323 bp) and 64c (287 bp), showed 100% identity to *A. marginale* (KT264188). The remainder of the obtained sequences were of bad quality and subsequently discarded.

A comparison of estimated evolutionary divergence between the observed gene sequences and those of closely related *Anaplasma 16S* rRNA gene sequences was subsequently compared by determining the number of base differences per near full-length *16S* rRNA gene sequence (1245 bp) (Table 7). None of the obtained sequences were identical; they differed from each other by seven to nine nucleotides. Comparisons of the novel sequences to published *Anaplasma 16S* rRNA sequences indicated that sequence 7f was identical to *Ehrlichia* (*Anaplasma*) sp. Bom Pastor (AF318023) and differed by one nucleotide from *Anaplasma* sp. Omatjenne (U54806). Sequence 26g was most closely related (with five nucleotide differences) to *A. platys* (isolate 2ax1) (KJ659044) previously described from Sika deer in China. Sequence 47b differed by nine nucleotides from sequence 7f, *Ehrlichia* (*Anaplasma*) sp. Bom Pastor and an uncultured *Anaplasma* sp. (clone Dog2) (KP006398) described from a dog in the Philippines.

The analysis involved 19 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1245 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Table 7).

Table 7 Estimates of evolutionary divergence between sequences, showing the number of base differences per sequence from analysis between sequences

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1. *A. platys* (Spain)(AY530806)																		
2. *A. platys* (France)(AF303467)	0																	
3. A. platys (Okinawa) (AY077619)	0	0																
4. *A. platys* (Cuba)(KX792089)	0	0	0															
5. *A. platys* (Thailand)(EF139459)	0	0	0	0														
6. *A. platys* (Venezuela)(AF287153)	2	2	2	2	2													
7. *A. platys* (isolate 2ax1, China)(KJ659044)	1	1	1	1	1	3												
8. *A. platys* (type strain)(M82801)	2	2	2	2	2	4	3											
9. **7f**	1	1	1	1	1	3	2	3										
10. *E. (*Anaplasma*)* sp. Bom Pastor (AF318023)	1	1	1	1	1	3	2	3	0									
11. *Anaplasma* sp. Omatjenne (U54806)	2	2	2	2	2	4	3	2	1	1								
12. Anaplasma sp. (clone Dog2)(KP006398)	3	3	3	3	3	5	4	5	2	2	3							
13. **47b**	10	10	10	10	10	12	11	12	9	9	10	9						
14. **26g**	6	6	6	6	6	8	5	8	7	7	8	9	16					
15. *A. phagocytophilum* (HGE2 human)(AF093789)	10	10	10	10	10	12	10	12	11	11	12	11	14	11				
16. *A. phagocytophilum* (HZ human)(NC007797)	10	10	10	10	10	12	10	12	11	11	12	11	14	11	0			
17. *A. phagocytophilum* (Webster human)(U02521)	10	10	10	10	10	12	10	12	11	11	12	11	14	11	0	0		
18. *Anaplasma* sp. (SA dog1076)(AY570539)	19	19	19	19	19	21	20	21	20	20	21	20	24	17	16	16	16	
19. *Anaplasma* sp. (SA dog1108)(AY570538)	18	18	18	18	18	20	19	20	19	19	20	19	25	16	15	15	15	1

The observed sequence similarities were subsequently confirmed by phylogenetic analyses. Neighbour-joining was used to reveal the relationships between the obtained sequences and related *Anaplasma* species previously deposited in GenBank. A representative tree obtained by the neighbour-joining method is shown in Figure 3. All the obtained sequences formed a monophyletic group with published *A. platys, Ehrlichia (Anaplasma)* sp. Bom Pastor and *Anaplasma* sp. Omatjenne.

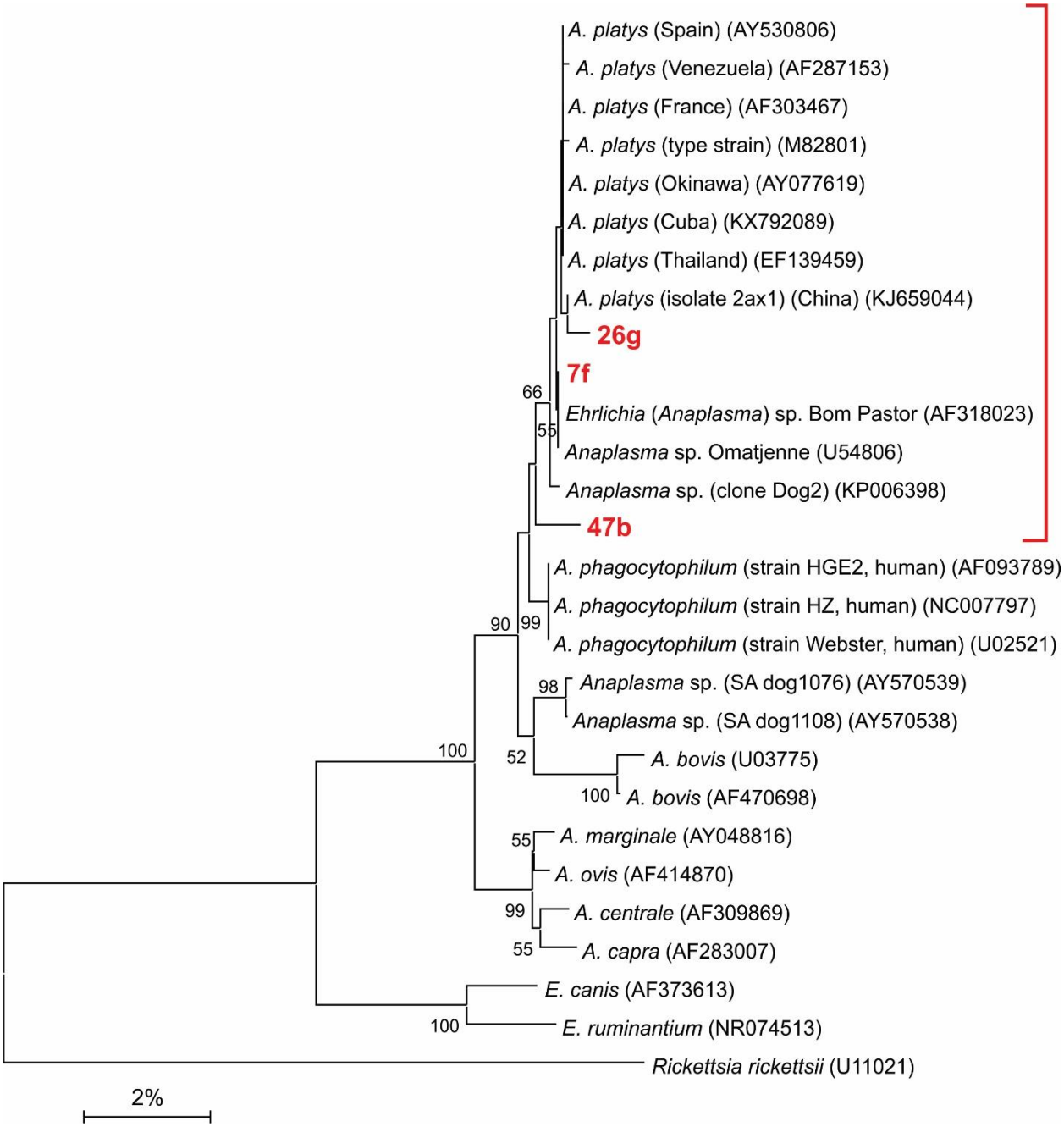

Figure 3 Neighbour-joining tree, with the Jukes-Cantor distance calculation, showing the phylogenetic relationship of the obtained sequences to related *Anaplasma* species based on the near full-length *16S* rRNA gene sequences (1245 bp)

Relationships are presented as an unrooted tree with branch lengths being proportional to the estimated genetic distance between the strains. The scale bar represents the % nucleotide difference. Rickettsia rickettsii (U11021) was used as the outgroup. The GenBank accession numbers are indicated in parentheses.

4.4. Real-time PCR for *B. bigemina* and *B. bovis*

Fifty-three out of eighty DNA samples (66.3%) were positive for the presence of *B. bigemina* DNA by the qPCR assay. However, from 88 cattle samples sujected to RLB assay only three samples (3.4%) were positive for *B. bigemina*, while no *B. bovis* positive samples were detected using qPCR.

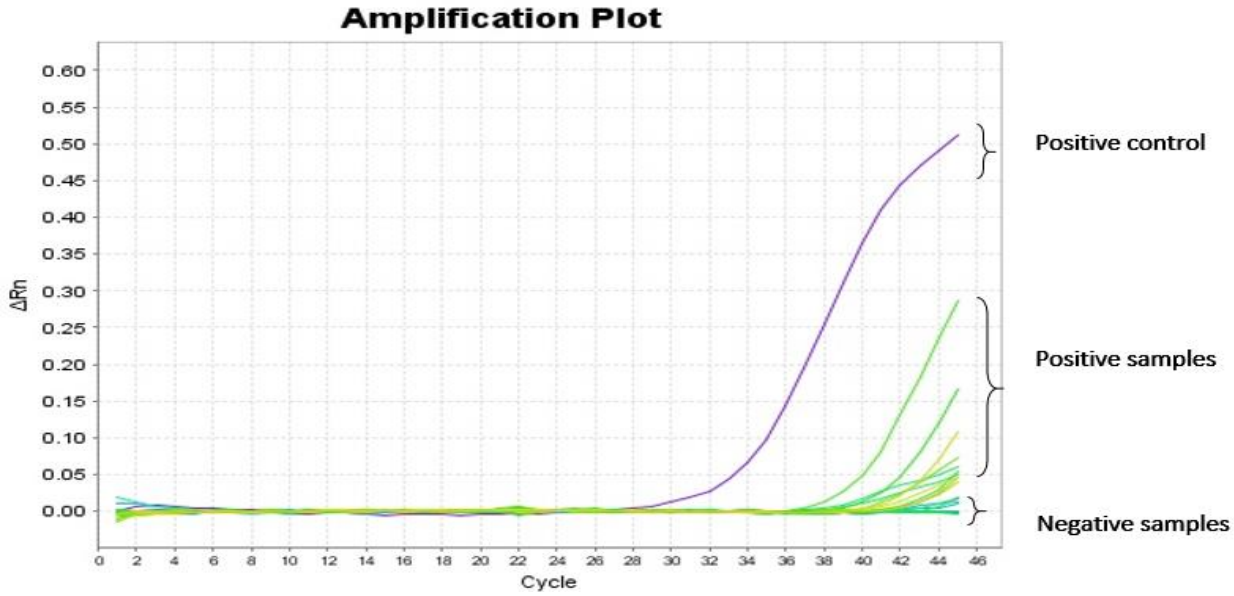

Figure 4 Detection of *Babesia bigemina* by real-time qPCR

4.5. Quantitative real-time PCR for *E. ruminantium* using *pCS20* gene

E. ruminantium was not detected by RLB assay in any of the tested samples. However, seventy DNA samples extracted from cattle, goat, sheep and dog blood samples were subjected to real-time qPCR and three samples (2 cattle and 1 goat) tested positive (4.3%). Furthermore, out of the 100 A. pomposum DNA samples tested, 7 were positive to E. ruminantium (7%).

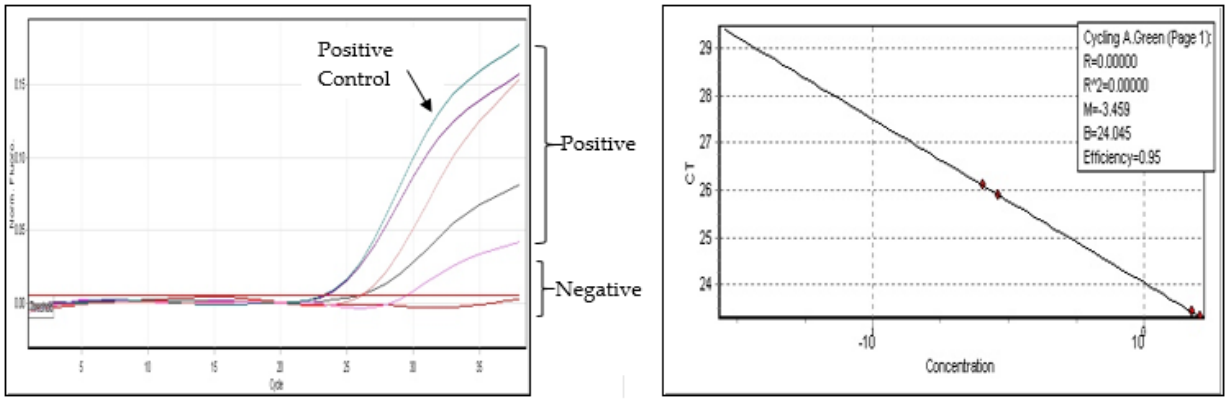

Figure 5 Ct values, showing detection of *E. ruminantium*

5. Discussion

5.1. Tick Identification

In order to understand the current distribution of ticks in Angola, it is important to take the impact of major historical events into consideration, especially that of the lengthy armed conflict that affected the country for over 3 decades. It is thought that the war led to uncontrolled animal dispersion as well as a drastic reduction in livestock. Consequently, animals were imported from neighbouring countries like Botswana (Gomes, 1993), Namibia, Zambia Gomes, A. (personal communication, 2015) and overseas, particularly Brazil José, A. (personal communication, 2015). Studies published in the last 7 decades, showed a great diversity of tick species in Angola (Dias, J.A.T.S., 1983; Dias, V.S., 1948; Gomes, 1993; Gomes et al., 1994 and Serrano, 1963). However, the information pertaining tick distribution in Angola, is fragmented and requires an urgent updating.

The first studies related to ticks were conducted by Dias, V.S. (1948) in various regions of Angola. Cattle, goats, sheep and dogs were the main hosts from which ticks were collected. A substantial number of collections were also made from wildlife species. This survey included Huambo, the province where the present study was conducted. Subsequently, other studies were carried out (Dias, J.A.T.S., 1983; Gomes et al., 1994 and Serrano, 1963), showing an increase in the number of tick species recorded in Angola.

The results of the present study indicate the presence of tick species that were not reported in earlier studies of ticks in Angola. Conversely, some tick species that were previously reported, were not found in this study. This fact strongly suggests the need for a more comprehensive investigation on the distribution of tick species in Angola.

The present study shows that Tchicala Tcholoanga municipality has a great diversity of tick species, with 17 tick species found in just 4.380 km². It is worth mentioning, that Dias, J.A.T.S. (1983) and Serrano (1963) recorded 19 and 34 tick species, respectively, in studies that included various regions of Angola. Later, Gomes et al. (1994)

found 13 tick species in a survey conducted in Huila province. The majority of tick species recorded in the current study were also mentioned in previous studies. These include: *A. pomposum, H. truncatum, H. leachi, I. cavipalpus* and *R. decoloratus* (Dias, J.A.T.S., 1983; Dias, V.S., 1948; Gomes et al., 1994 and Serrano, 1963), *R. evertsi evertsi* (Serrano, 1963; Gomes et al., 1994), *R. evertsi mimeticus* (Dias, J.A.T.S., 1983; Dias, V.S., 1948; Gomes et al., 1994), *R. lunulatus* (Dias, J.A.T.S., 1983; Gomes et al., 1994), *R. punctatus* (Dias, V.S., 1948, Gomes et al., 1994); *R. simus* (Dias, J.A.T.S., 1983; Dias, V.S., 1948; Gomes et al., 1994; Serrano, 1963); *R. turanicus* (Gomes et al., 1994), *R. tricuspis* (Dias, V.S., 1948; Serrano, 1963) and *R. compositus*, which was reported for the first time by Serrano (1973). *H. paraleachi, R. microplus, R. sulcatus* and *R. kochi,* were recorded, for the first time, in the present study. Due to the low number of *R. microplus* found in this study, further investigation is required to ascertain the extent of the establishment of this tick species in Huambo province. Presviously only recorded along western and southern regions, *R. duttoni* a tick species that was recorded in Angola between 1948 and 1994 by all the authors mentioned above, was not found in this study. Similarly, *R. zambeziensis* and *H. rufipes*, found by Gomes et al. (1994), were also not found in the present survey. The same author recorded *R. simus* and *R. turanicus* feeding on cattle, while in this study they were collected exclusively from dogs.

5.1.1. *Amblyomma pomposum*

From 15 *Amblyomma* spp. that have been reported in Angola, *A. pomposum* is the most frequently reported (Dias, V.S., 1948; Gomes et al., 1994 and Serrano, 1963). In the present study, *A. pomposum* was the most recorded species (27.1% of all collected ticks) and was found infesting all sampled host species. Among these, cattle were undoubtedly the main host species followed by goats, sheep and dogs. On this latter host, seven nymphs and 1 adult *A. pomposum* were found. This finding seems unusual, but Adamu et al. (2014), Dias, V.S. (1948), Horak et al. (1987), Kumsa and Mekonnen (2011) and Neves et al. (2004) also found nymphs or adults of *Amblyomma* spp. in dogs in Nigeria, Angola, South Africa, southern Ethiopia and Maputo, Mozambique, respectively. The infestation of dogs by

Amblyomma spp. could be explained by the presence of dogs in close proximity to cattle herds. Furthermore, the wide host range of *Amblyomma* ssp., that includes not only ruminants (Walker et al., 2003), but also birds (Hamer et al., 2012) and small mammals (Oguge et al., 2009), increases the chances of tick survival and hence contributes to its abundance. Gomes et al. (1994) found 26.4% of cattle infested with *A. pomposum* in Huíla Province, situated in southern Angola. The predominance of *Amblyomma* sp. was also verified in studies carried out in Tanzania (Kwak et al., 2014; Lynen et al., 2007), South Africa, Mozambique (Horak et al., 2009) and Zimbabwe (Ndhlovu et al., 2009). *A. pomposum,* the only *Amblyomma* species identified in the present study, is the most important vector of *E. ruminantium* in Angola (Gomes, et al., 1994). According to Walker et al. (2003), in Africa, this tick species is restricted to savanna regions in western Zambia and southern Democratic Republic of Congo, occurinng mainly in highland wet habitats. In the present study, *A. pomposum* was found at the central plateau, with humid climate and abundant vegetation.

5.1.2. *Rhipicephalus decoloratus* vs *Rhipicephalus microplus*

In this study, *R. decoloratus* was the second most abundant species, infesting mainly cattle (30.4%) and sheep (9.5%), but was also found infesting 3 goats and 4 dogs. Its abundance may be favoured by the development of all life stages on a single host (Walker et al., 2003). *R. decoloratus* was previously reported in Angola infesting cattle, goats, sheep, donkeys and dogs (Dias, J.A.T.S., 1983; Dias, V.S., 1948; Serrano, 1963). In a more recent study, *R. decoloratus* was reported as the third most frequent tick species infesting cattle in Huíla, Angola (Gomes at al., 1994). This finding is in agreement with observations from other authors that have reported *R. decoloratus* to be amongst the most predominant tick species in some regions of sub-Saharan Africa. Lorusso et al. (2013) found *R. decoloratus* infesting cattle as the most abundant tick species in Nigeria (41.4%), which is relatively higher than 30.4% found on cattle in the present study. Sungirai et al. (2015) also found the same tick species on cattle (47.1%) in Zimbabwe. According to Nyangiwe et al. (2013) *R. decoloratus* is also well established in Namibia.

R. microplus was also found in this study. However, the ten *R. microplus* ticks found on cattle in this study are not sufficient to assume that this tick species is completely established in Tchicala-Tcholoanga. Notwithstanding, special attention is needed regarding the possible introduction of *R. microplus*, taking into account the massive animal importation from Brazil and neighbouring countries, where this tick species is already established. In Zimbabwe, Sungirai et al. (2015), found more *R. microplus* (58.7%) than *R. decoloratus* (47.1%). Furthermore, *R. microplus* is a well-established species in South Africa (Walker et al., 2003) and was recently reported from Namibia (Nyangiwe et al., 2013). The transmission of *B. bigemina, B. bovis* and *A. marginale* by *R. microplus,* and its tendency to displace *R. decoloratus* makes this tick species an extremely important vector (Horak et al., 2009; Nyangiwe et al., 2013).

5.1.3. *Haemaphysalis leachi* vs *Haemaphysalis paraleachi*

In this study, *H. leachi* (1.0%) and *H. paraleachi* (6.1%) were found feeding exclusively on dogs. *H. leachi* was previously recorded in Angola (Dias, J.A.T.S., 1983; Dias, V.S., 1948; Serrano, 1963), yet, this is the first time that *H. paraleachi* is reported in the country. From six tick species belonging to the *H. leachi* group, *H. paraleachi* is the most widely spread in the African continent, feeding mainly on carnivores, but can also be found on cattle (el Kammah et al., 1992). However, there are many controversies around the *H. leachi* group (Apanaskovich et al., 2007; Fourie et al., 2010; Matthee et al., 2010). According to Apanaskovich et al. (2007) *H. leachi* is not a southern African tick. Consequently, the tick species previously reported as *H. leachi* (Horak et al., 1987) in this region should be considered to be *H. elliptica*. The same author went further, suggesting that this concept should be extended to southern and East Africa. Conversely, Camicas et al. (1998) stated that *H. elliptica*, *H. leachi* and *H. paraleachi* coexist in these geographical regions. Furthermore, the reinstatement of *H. elliptica* as a valid species also changed what was previously accepted in terms of pathogen transmission. Thus, *B. rossi,* the most important etiological agent of canine babesiosis in South Africa, which was thought to be transmitted by *H. leachi* (Fourie et al., 2010) has now *H. elliptica* as the only confirmed

vector. The present study reports the first georeferenced population of *H. leachi* in southern Africa since the reinstatement of *H. elliptica* in 2007. *H. humerosoides*, was previously identified in Angola (Dias, J.A.T.S., 1983; Dias, V.S., 1948). However, Camicas et al. (1998), consider *H. humerosoides* a synonym of *H. leachi.*

5.1.4. *Rhipicephalus turanicus* vs *Rhipicephalus sanguineus*

R. sanguineus, which was previously reported in Angola (Dias, J.A.T.S., 1983; Dias, V.S., 1948; Serrano, 1963), was not found in the current study. According to our results, *R. turanicus* (45.8%) was the most prevalent species on dogs and was found feeding exclusively on this host species. It is worth mentioning that no immature stages of *R. turanicus* were found in this collection. Furthermore, Gomes et al. (1994) found in Huíla province, Angola this tick species feeding in cattle. Contrarily to *R. sanguineus,* which has a single preferential host (dogs), *R. turanicus* has a wide host range (Horak et al., 2009; Walker et al., 2003).

In countries such as South Africa and Namibia, *R. sanguineus* was found to be the most prevalent species in dogs (Matthee et al., 2010). According to Walker et al. (2003), *R. turanicus* has not been incriminated as a pathogen vector. However, considering that while *R. sanguineus* was not found in the present study, *E. canis* and *B. vogeli* were detected in dog blood by RLB assay, strongly suggesting a role for *R. turanicus* in the transmission of the above-mentioned haemoparasites.

5.1.5. *Rhipicephalus lunulatus* vs *Rhipicephalus tricuspis*

The similarities between the species, *R. lunulatus* and *R. tricuspis,* have often been the subject of controversy. In fact, the haemoparasites associated with these vectors are not well known (Walker et al., 2003). *R. lunulatus* was first reported in Angola by Dias, J.A.T.S. (1983) and more recently by Gomes et al. (1994), while *R. tricuspis* was reported by Dias, V.S. (1948) and Serrano (1963). In this study, both species were found

parasitizing all animal species (cattle, goats, sheep and dogs). Dogs and cattle were the hosts harbouring the heaviest infestations.

5.1.6. *Rhipicephalus evertsi evertsi* vs *Rhipicephalus evertsi mimeticus*

R. e. mimeticus was already recorded in Angola (Dias, J.A.T.S., 1983 and Dias, V.S., 1948) while *R. e. evertsi* was reported by Serrano (1963). The first time that both tick species were recorded in the same collection was by Gomes et al. (1994) and in the present study. *R. e. mimeticus* and *R. e. evertsi* are recorded in this study, infesting sheep (13.9% and 2.2%), goats (11.8% and 2.2%) and cattle (4% and 0.1%), respectively. In comparative terms, these ruminants were more infested with *R. e. mimeticus* than *R. e. evertsi*. A study conducted by Gomes at al. (1994) in Huíla presented a similar situation in cattle, where *R. e. mimeticus* (27.1%) were found more frequently than *R. e. evertsi* (only one animal infested). By contrast, the records from sub-Saharan Africa presented *R. e. evertsi* as the most widely distributed tick species. Ndhlovu et al. (2009) and Sungirai et al. (2015) reported on their studies in Zimbabwe, the presence of *R. e. evertsi* (17.7% and 67.8%) respectively, but no *R. e. mimeticus* was recorded in their studies. Likewise, only *R. e. evertsi* was reported in studies conducted by Horak et al. (2009) in South Africa (34.1%) and in Maputo, Mozambique (5.6%) on cattle. The goats from South Africa and Mozambique presented an infestation rate with *R. e. evertsi* (50.2% and 19.9%) respectively. Those rates are considerably higher (Horak et al., 2009) than the ones found on goats in the present study (2.2%). Mushi et al. (1996), found in Botswana 93.1% of the goats infested by *R. e. evertsi* and also a complete absence of *R. e. mimeticus*. According to Walker et al. (2000), *R. e. evertsi* is considered one of the most widely distributed tick species in sub-Saharan Africa.

5.1.7. *Hyalomma truncatum*

H. truncatum is widely distibuted throughout Africa. This tick species was recorded in Angola by Serrano (1963) and Gomes et al. (1994). The latter author found only 3 % of

cattle infested in Huíla province, whereas in this study 17.6 % were reported from cattle. In addition, *H. truncatum* was found in all ruminants species, although cattle was found to be the preferential host followed by sheep.

5.1.8. *Rhipicephalus simus*

In this study, *R. simus* was found in low infestations (1 to 2 ticks per host), parasitizing only four dogs. According to Walker et al. (2003), this tick species is never found in large numbers. The same author stated that *R. simus* is distributed in Angola, in regions with high rainfall and it can also infest dogs, besides cattle to be one of the preferred host. However, *R. simus* in Angola has been reported as a common tick of cattle (Dias, V.S., 1948; Gomes et al., 1994). In different surveys conducted across southern Africa, *R. simus,* has been found to be the predominant tick species infesting dogs (de Matos et al., 2008; Horak et al., 1987 and Horak et al., 2009).

5.1.9. *Ixodes cavipalpus*

I. cavipalpus has been several times reported from Angola (Dias, V.S., 1948; Gomes et al., 1994; Serrano, 1963) and is a tick species that is widely distributed in Africa. As mentioned by de Matos et al. (2008), *I. cavipalpus* is a parasite of cattle, goats and dogs, which corroborates the findings of the current study. It is worth mentioning that in this study, dogs were the most commonly infested host. Sheep was the only animal species from which *I. cavipalpus* was not recovered in the course of this survey.

5.1.10. *Rhipicephalus punctatus*

R. punctatus was previously reported in Angola by Dias, V.S. (1948) and Gomes et al. (1994) in central and southern regions of Angola, corresponding to Bié, Huambo and Huíla provinces. In this study, it was found feeding on all animal species. Goats, followed by sheep, were the most infested, which is in agreement with previous findings that indicated that *R. punctatus* is a common tick of small ruminants (Dias, V.S., 1948; Hove et

al., 2008). Sungirai et al. (2015) found, in his collection, a variant of *R. punctatus* that was infesting cattle.

Three tick species are reported for the first time in Angola, namely *R. compositus, R. kochi,* and *R. sulcatus*. The latter tick species was found only in dogs, whereas *R. compositus* and *R. kochi* were collected only from cattle. *R. kochi* was also reported in Mozambique (Horak et al., 2009), while less than 1% of *R. compositus* were identified in a collection from Zimbabwe (Sungirai et al., 2015).

5.2. Tick-borne pathogens at Tchicala-Tcholoanga, Huambo

Fifteen haemoparasites species were detected, using the RLB assay to analyse cattle, goat, sheep and dog samples. The majority of haemoparasites detected are associated with the 17 tick species identified in this region, during the course of the present study. Njiiri et al. (2015) used the same technique and detected the same diversity of haemoparasites in Kenyan calves. There is still controversy on the role of some tick species as vectors of the haemoparasites detected. As previously mentioned, the presence of both *E. canis* and *B. vogeli* in the absence of *R. sanguineus*, strongly indicates a role for *R. turanicus* in the transmission of the above mentioned haemoparasites. Moreover, the low detection rate of the RLB assay for some parasite species that are expected to occur endemically in the study area suggests a possible lack of hybridisation of some of the species-specific probes with the *16S* and *18S* region of certain species of Angolan pathogens. Furthermore, cross hybridisation was also observed. This is the case of *A. platys* which was detected by the RLB from cattle and sheep DNA samples; *B. bovis*, *B. rossi* and *T. bicornis* found from sheep and *T. ovis* from dogs. Therefore, there was the need to apply other diagnostic tools in order to determine with certainty which species of pathogens were involved.

5.2.1. Tick-borne pathogens in cattle

From 88 cattle samples submitted to the RLB assay, nine haemoparasite species were detected in 82 cattle samples. Moreover, five haemoparasite species were common to both

cattle and sheep (*A. centrale, A. marginale, A. platys, Anaplasma* sp. Omatjenne, and *Theileria* sp. (sable). Co-infections are commonly found in samples subjected to the RLB assay (Berggoetz et al., 2014; Lorusso et al., 2016; Njiiri et al., 2015; Yusufmia et al., 2010), since this technique was specifically designed to allow for multiple detection of pathogens (Bekker et al., 2002; Gubbles et al., 1999). In this study, 93.2% of the cattle samples were positive for a minimum of one and a maximum of six haemoparasite species. Similar rates of positivity (89.6%) were obtained by Njiiri et al. (2015) from calves in Kenya as well as Lorusso et al. (2016) from cattle in Nigeria with 82.6%. In the present study, 97.6% of mixed infections were observed in cattle samples. While single infections were detected only in 2.4% of the cattle. In addition, the genus-specific probe *Ehrlichia*/*Anaplasma* catch all (E/A) and the *Theileria*/*Babesia* catch all (T/B) presented a positive signal in 84.1% and 97.6% samples, respectively. According to Bekker et al. (2002), the hybridization of genus specific probe, generally represents the presence in the sample of pathogen DNA from a species belonging to the genus represented by the probe.

There was no detection of *E. ruminantium* by the RLB assay, although its vector, *A. pomposum,* was the most abundant tick species in this survey. According to Serrano (1963) and Walker and Olwage (1987), this tick species is a competent vector of *E. ruminantium*. This fact, suggests that the species specific probe used on the RLB assay has low sensitivity. However, this lack of detection might be attributable to ricketsiaemias below the detection level of the assay or even absence of *E. ruminantium* from the blood, since the sampled animals were apparently asymptomatic. It is also known that *E. ruminantium* preferentially infects endothelial cells of brain capillaries (Mahan, 1995) and are not circulating in the peripheral blood except in the acute phase (febrile) of the disease (Bekker et al., 2002). The detection of *E. ruminantium* by RLB assay is often very low (Lorusso et al., 2016; Muhanguzi et al., 2010; Njiiri et al., 2015) or absent (Berggoetz et al., 2014; Buling et al., 2007; Byaruhanga et al., 2016; Martins et al., 2009; Ogo et al., 2012; Oura et al., 2004). In order to circumvent this technical drawback and prove the presence of *E. ruminantium* in the region, a quantitative real-time PCR (qPCR) TaqMan probe assay, targeting *pCS20* gene was used (Steyn et al., 2008). In the present study, 7% of 100 *A. pomposum* ticks were qPCR positive to *E. ruminantium*. These results were within the

range of *E. ruminantium* infection rates determined in different *Amblyomma* species from the field. Using PCR *pCS20,* Allsopp et al. (1999) detected *E. ruminantium* in *A. hebraeum* (13%) in South Africa, Muramatsu et al. (2005) in Sudan detected in *A. variegatum* (8.2%), and in *A. lepidum* (1.9%). Peter et al. (1995) detected *E. ruminantium* by PCR (28%) and DNA Probe (1.3%) in *A. hebraeum* ticks from Zimbabwe. According to Allsopp (2010) low *E. ruminantium* infection rates are commonly found in areas known as endemic to heartwater. This is the first time that *E. ruminantium* infection rates were determined in an *A. pomposum* population. Furthermore, seventy DNA samples extracted from blood (cattle, sheep, goats and dogs) were also analysed by qPCR targeting *pCS20* gene. Three positive samples (two cattle and one goat) were identified, which cannot be considered a dramatic increase in detection, when compared to the RLB results. Byaruhanga et al. (2016) using the qPCR method detected only 1.7 % of positive blood samples from cattle infected with *E. ruminantium* in Uganda. However, Njiiri et al. (2015), has detected by RLB two positive cattle samples to *E. ruminantium* and none positive samples were obtained using qPCR. Moreover, six species from the family Anaplasmataceae, *A. bovis*, *A. centrale, A. marginale, A. platys* and *Anaplasma* sp. Omatjenne were found in this study. After *A. marginale*, the other two most frequently detected *Anaplasma* species by RLB were *Anaplasma* sp. Omatjenne and *A. centrale*. Similar results, regarding *Anaplasma* species frequency were reported by Byaruhanga et al. (2016), in Uganda. The percentage of *A. marginale* positive samples (28.4%) detected by the RLB assay from cattle in this study, was lower than the percentage found in Bié (Angola) (38%) by Kubelova et al. (2012), using conventional PCR. Additionally, 1% of *A. bovis* detected in this study was much lower, when compared to 39.9% found in Kenya by Njiiri et al. (2015) using RLB assay. Curiously, in this study RLB assay also detected *A. platys* in cattle (18.2%) and sheep (5.9%) samples. This fact prompted further investigation by sequencing since *A. platys* is considered a specific haemoparasite of dogs and is believed to be primarily transmitted by *R. sanguineus* (Kamani et al., 2013). Similarly, Lorusso et al. (2016) detected 3.9% of positive cattle to *A. platys* in Nigeria. According to Zobba et al. (2014), *A. platys* have been reported mostly from dogs worldwide. The same author in the study on "Molecular investigation and phylogeny of *Anaplasma* spp. in Mediterranean ruminants'' stated that

"ruminants host a number of neutrophil-tropic strains genetically closely related to the canine pathogen *A. platys*". This could explain the detection of this pathogen in cattle and sheep samples. In this study, the near full-length of 16S rRNA gene of positive *A. platys* samples (6 cattle and 5 sheep) detected by RLB were submitted to PCR amplification, cloning and sequencing. BLASTn and subsequent phylogenetic analysis of three near full-length sequences obtained reveal that only one of the sequences was closely related to *A. platys*, while one sequence (7f) was identical to *Ehrlichia* (*Anaplasma*) sp. Bom Pastor (AF318023). Sequence 47b was not identical, but most closely related to *Ehrlichia* (*Anaplasma*) sp. Bom Pastor and an uncultured *Anaplasma* sp. (clone Dog2) (KP006398). Similar results were recently also reported from Algeria by Dahmani et al. (2015), using qPCR targeting *23S* rRNA gene. According to the same author, the sequence of the bovine infected with *A. platys* showed 100 % identity to the sequence of *A. platys* from an infected dog. Thus, the called "aberrant incidental infections" by Njiiri et al. (2015), seems to be commonly detected by RLB assay. The author referred above, using the same technique also found in his studies *T. ovis*, *T. equi* and *E. canis* in cattle.

In terms of protozoan parasites, *T. mutans* and *T. velifera* were the most commonly detected species with 73.9% and 78.4 % of cattle infected. This might be an expected result, since all ruminants in this study were heavily infested with *A. pomposum,* although it is not yet known whether this tick species also transmit these two pathogens. However, *Amblyomma* spp. namely *A. hebraeum*, *A. lepidum* and *A. variegatum* are well known as vectors of both *T. mutans* and *T. velifera* (Walker et al., 2003). Using RLB assay, Oura et al. (2004) also found high infection rates of *T. mutans* and *T. velifera* in indigenous cattle in Uganda (95% and 91%), as well as Lorusso et al. (2016) in Nigeria (66.3% and 52.4%). The mixed infections among *T. mutans, T. velifera* and *Theileria* sp. (sable) were the most commonly found co-infection. A similar triple pathogen association infection were observed in several studies, using the same technique (Byaruhanga et al., 2016; Njiiri et al., 2015; Oura et al., 2004). However, Kubelova et al. (2012) using PCR found in Bié, (Angola) only *T. velifera* in a lower rate (14%) when compared to the present study (78.4%). Nijhof et al. (2003); Njiiri et al. (2015) and Yusufmia et al. (2010), also found commonly in cattle *Theileria* sp. (sable) infections.

It was evident that RLB species specific probes for *B. bigemina* used in this study were not able to efficiently detect the parasite specific DNA from Angolan strains, after verifying only 3.4% of positive samples. The RLB results for *B. bigemina* were not expected, since its vector *R. decoloratus,* was one of the most frequently collected tick species in this survey, with 30.4% of cattle infested by this ectoparasite. Similarly, many authors using RLB assay have found low rates of *B. bigemina* between 0% to 8% in surveys conducted in endemic areas (Byaruhanga et al., 2016; Ceci et al., 2014; Lorusso et al., 2016; Martins et al., 2009; M'ghirbi et al., 2008; Njiiri et al., 2015; Ogo et al., 2012; Oura et al., 2004). It is worth mentioning that, Kubelova et al. (2012) using PCR and sequencing also detected low rates (1.3%) of *B. bigemina.* In order to examine the accuracy of the results in this study, real-time qPCR was also performed for *B. bigemina*.

The use of qPCR for *B. bigemina* indicated 66.3% of *B. bigemina* positive samples, emphasising the extremely low sensitivity of RLB, in its current format, to detect Angolan strains of *B. bigemina*. Similarly, Martins et al. (2009) using Semi-nested hot PCR obtained 88.9% of *B. bigemina* cattle positive in Maputo, Mozambique, while from RLB all samples came negative. It is believed that the level of detection of *B. bigemina* associated to the large number of *R. decoloratus* identified in this study, strongly suggests the existence of an equilibrium among host, parasite and the vector population that concurs to the minimal occurrence of clinical manifestation of disease in the region. Moreover, Gomes et al. (1991) indicated the occurrence of endemic stability, based on the results of a survey conducted in the smallholder sector at the South West of Angola.

In the present study one case of *B. bovis* was detected in sheep and none was found in cattle. It is known that *B. bovis* is a haemoparasite transmitted by tick vector *R. microplus* (Bock et al., 2004) and this tick was found in this study infecting cattle in a number that not allow for the confirmation of its establishment in Angola. The qPCR has not confirmed the presence of *B. bovis* in cattle. This may be indication of a recent establishment of *R. microplus* in the studied region.

5.2.2. Tick-borne pathogens in goats

The RLB assay detected *A. centrale* as the single haemoparasite infecting goats and only two goats were positive, from 82 samples. However, the genus specific oligonucleotide probe designed to catch all species of *Ehrlichia/Anaplasma* gave positive results in 80.5% of goat samples, whereas 3.6% reacted with *Theileria/Babesia* genus probes, 2.4% to *Theileria* and 1.2% to *Babesia* genus specific probes. Kubelova et al. (2012) detected in Bié province, neighbouring the region where this study took place, 100% of positive goats to *A. ovis,* using PCR. Unfortunately, *A. ovis* species specific probe was not available in this RLB assay, but we believe that most of the samples that had cross-reacted with *Ehrlichia/Anaplasma* genus specific probe would be positive to *A. ovis*, if the samples would have been sequenced.

5.2.3. Tick-borne pathogens in sheep

From nine pathogens detected, *T. ovis* (80%) was the most abundant haemoparasite detected in sheep. However, while Uilenberg (1981) considers this haemoparasite of negligible pathogenic importance, Durrani et al. (2011) indicated that *T. ovis* was implicated in massive sheep losses in Pakistan. Furthermore, el Imam et al. (2016) detected in Sudan 88.6% of sheep positive to *T. ovis* by RLB, while M'ghirbi et al. (2013), using the same technique detected in northern Tunisia 28.1% of sheep infected with the same pathogen. Some parasite species detected in sheep on the course of this study were totally unexpected. *T. bicornis,* originally reported from rhinos (Nijhof et al., 2003; Govender et al., 2011) was found in four sheep samples. Strangely, this species was also reported infecting cattle in Uganda (Muhanguzi et al., 2010). Similarly, 2.4% and 1.2% of *A. marginale* and *A. centrale* respectively, detected in the present study in sheep raised some doubts, since Young et al. (1988) stated that they are primarily bovine haemoparasites. In addition, *A. platys* and *B. rossi*, which are specific dog haemoparasites of were also found in sheep, as well as *B. bovis* that usually is exclusively a cattle parasite. These findings strongly indicate the occurrence of undesirable cross reactions that deserve further investigation.

The *16S* rDNA of *A. platys* positive sheep samples were amplified, cloned and sequenced in order to determine which parasites were truly implicated. Only partial sequences could be obtained; of these, sequence 225a obtained from a sheep sample showed 99% identity to *A. ovis*, whereas two sequences obtained from cattle (82b and 64c), showed 100% identity to *A. marginale* (KT264188). The remainder of the obtained sequences were of bad quality and subsequently discarded. However, Berggoetz et al. (2014) found in South Africa a sequence isolated from sheep with 99% homology to *A. platys.*

5.2.4. Tick-borne pathogens in dogs

From the 85 dog samples analysed by RLB assay, 88.2% were positive to one or two different parasite species, being *B. vogeli* the most frequent pathogens. Adamu et al. (2014) using the same technique, found in Nigeria 72% of positivity in dogs to one or more haemoparasites, being *B. rossi* the commonest parasite. While Cardoso et al. (2016), using PCR found in Luanda, Angola, 45% positive dogs and *A. platys* was the most detected haemopathogen. In the present study, the most common mixed infection involved *B. rossi* and *B. vogeli* (9.3%). Although Matjila et al. (2004) have found *B. rossi* and *B. vogeli* in South Africa, no mixed infection of these pathogens were found. In the present survey, four haemoparasites were identified by RLB assay, including *T. ovis,* an unexpected parasite species, found in 5 (8.2 %) dog samples. Adamu et al. (2014) detected by RLB eight species of haemopathogens in dogs, including unusual findings such as *Anaplasma* sp. Omatjenne*, E. ruminantium, T. equi* and *Theileria* sp. (sable).

In the present study, *B. vogeli* was the most abundant following by *B. rossi*. However, in Luanda, the detection of pathogens verified at a veterinary clinic, showed that *A. platys*, and *H. canis* were the most frequent parasites detected in dog blood samples (Cardoso et al., 2016). It is worth mentioning, that none of these pathogens were identified in dogs in the present survey. Moreover, the finding that *B. vogeli*, was the most frequently detected haemoparasite in dogs, and the absence of *R. sanguineus*, an established vector of this parasite (Hauschild and Schein, 1996), from the tick collections

in our survey, triggers the need for further investigation on the possible role of *R. turanicus* in the transmission of this parasite. In this study, *R. turanicus* represented 45.8% of all ticks found in dogs. Attempts to correlate the presence of certain vectors to the presence of pathogens that they transmit, based on RLB results, are often considered an inconsistent exercise. This is illustrated by Adamu et al. (2014) that found very low detection of *B. vogeli* (1 %), whereas *R. sanguineus* (73 %) was reported to be highly abundant in the area. Furthermore, Matjila et al. (2004), found much lower infection rates of *B. vogeli* (4.4%) than the ones found in the present study (35.3%). Using the PCR assay, Cardoso et al. (2016) found only 5.8% of dogs positive to *B. vogeli* in Luanda, Angola. With respect to the *B. vogeli* infection rates, it is worth emphasising the difference of the dog populations screened by Matjila et al. (2004) and Cardoso et al. (2016), that were fundamentally urban, with the rural population of dogs sampled in this study.

The present study indicates that *B. rossi,* found in 23.5% of the dog samples, is transmitted in Tchicala-Tcholoanga by *H. leachi* as previously reported by Hauschild & Schein (1996). This is the first strong indication of transmission of *B. rossi* by *H. leachi* in southern Africa, after the renaming of the latter species in South Africa as *H. elliptica* (Apanaskevich et al., 2007). Moreover, in the present survey, *H. paraleachi* was found to be more abundant (6.1%) than *H. leachi (*1.1%). This observation points to the need to further investigation of a possible role for *H. paraleachi* as vector of *B. rossi.* Similarly, Adamu et al. (2014) identified *B. rossi* (53%) as the most abundant pathogen in Nigerian dogs, while its vector *H. elliptica* was found in only 1% of sampled dog population. The same author speculated that the *H. leachi* that was found in 18% in his study would be also a competent vector of *B. rossi* in Nigeria. Further investigation should be carried out in order to elucidate the role of these three tick species (*H. leachi*, *H. paraleachi* and *H. elliptica*) as vectors of *B. rossi* in Africa.

In the neighboring Republic of Namibia, *E. canis* was considered the most important pathogen for dogs (Manyarara et al., 2015), while in the present study, only one dog was positive to *E. canis.* However, Cardoso et al. (2016) reported 5.8% of dogs positive to *E. canis* using PCR, in Luanda, Angola. According to Matthee et al. (2010) *R.*

sanguineus is the main vector implicated in *E. canis* transmission, however no *R. sanguineus* was identified in the tick collections made from dogs in this survey.

Using the RLB assay, infection rates lower 5 % of *E. canis,* were also reported from Turkey, Cape Verde and South Africa by Aktas et al. (2015); Lauzi et al. (2016) and Matjila et al. (2008), respectively.

Moreover, the detection of *T. ovis* in dogs in the present study was unexpected, but no further investigation was attempted. However, Kamani et al. (2013) found in Nigeria a sequence of *Theileria* sp. in dogs that was 98% similar to *T. ovis* from sheep. Similarly Matjila et al. (2008) reports from the first time in South Africa *Theileria* sp. in dogs. Surprisingly, in the present study, *A. platys* was not detected in dogs, whereas the specie specific probe used on RLB hybridised with samples from cattle and sheep. Contrastingly, Cardoso et al. (2016) found *A. platys* (20.4%) as the most frequently detected pathogen in Luanda, Angola. The discrepancies between the findings of the present study and the one conducted by Cardoso et al. (2016) in Luanda, might be explained by the differences in geographical location of the studies and the different techniques used. Furthermore, in the present study the dogs were from remote rural areas while Cardoso et al. used on his study urban pet dogs.

The diversity and some intriguing results from the RLB assay, illustrates the diversity of haemoparasite strains circulating in this region of Angola, which constitutes a major challenge for this technique.

The results of this study provided information on the occurrence of haemoparasites in Tchicala-Tcholoanga. This is the first and the most comprehensive combined report on ticks and tick-borne diseases of domestic ruminants and dogs from this central region of Angola.

6. Conclusion

Seventeen tick species and 15 haemoparasite species occur in Tchicala-Tcholoanga, Huambo and the exposure of ruminants and dogs to infections caused by species of the genera *Anaplasma, Babesia, Ehrlichia* and *Theileria* are evident and should be considered a threat to livestock. Predominant tick species such as *A. pomposum* and *R. decoloratus* indicate the risk of heartwater and bovine babesiosis outbreaks and are limiting factors for the introduction and improvement of more productive breeds. Furthermore, this study clearly indicates that ticks and tick- borne pathogens affect the health and welfare of dogs. The frequent detection of *B. vogeli* and the presence of *R. turanicus* in this study, together with the absence of *R. sanguineus* strongly suggest the role of the latter tick species in the transmission of *B. vogeli*. Similarly, the detection of *B. rossi* and absence of *H. elliptica* indicate that *H. leachi* and *H. paraleachi* found in the present survey, might be implicated in the transmission of this species. Additionally, the improvement of species-specific probes to detect *B. bigemina* by RLB assay should be considered, due to the lack of hybridisation and result discrepancies between the latter method and qPCR verified in this study. In the present study, the tick pathogens diagnostic was focused mainly on host rather than in vectors. For future studies it is advisable to consisder more molecular analyses in vectors.

The present study contributes to the research and scientific knowledge in Angola, generating and updating geo-referenced information about ticks and tick-borne pathogens, circulating in Tchicala-Tcholoanga, Huambo Province, Angola. In addition, data collected and analysed during the study is relevant information that will be extremely important to the Veterinary Services at Huambo province to design and implement improved control strategies for ticks and tick-borne diseases.

Competing interest

The authors declare that there is no competing interest.

Ethics approval

This study was approved by Animal Ethics Committee of University of Pretoria, Faculty of Veterinary Science project number V053-16.

7. List of references

Adamu, M., Troskie, M., Oshadu, D.O., Malatji, D.P., Penzhorn, B.L. & and Matjila, P.T. 2014, "Occurrence of tick-transmitted pathogens in dogs in Jos, Plateau State, Nigeria", *Parasites & Vectors,* no. 7, pp. 119.

Aktas, M., Özübek, S., Altay, K., Ipek, N.D.S., Balkaya, I., Utuk, A.E., Kırbas, A., Şimsek, S. & Dumanlı, N. 2015, "Molecular detection of tick-borne rickettsial and protozoan pathogens in domestic dogs from Turkey", *Parasites & Vectors,* vol. 8, no. 157.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. 1990, "Basic local alignment search tool", *Journal of Molecular Biology,* vol. 3, no. 215, pp. 403-410.

Allsopp, B.A. 2015, "Heartwater-*Ehrlichia ruminantium* infection", *Revue Scientifique et Technique (International Office of Epizootics),* no. 34, pp. 557–568.

Allsopp, B.A. 2010, "Natural history of *Ehrlichia ruminantium*", *Veterinary Parasitology,* vol. 167, no. 2–4, pp. 123-135.

Allsopp, M.T., Hattingh, C.M., Vogel, S.W. & Allsopp, B.A. 1999, "Evaluation of *16S*, *map1* and *pCS20* probes for detection of Cowdria and *Ehrlichia* species", *Epidemiology & Infection*, no. 122, pp. 323 - 328.

Allsopp, B.A., Baylis, H.A., Allsopp, M.T.E.P., Cavalier-Smith, T., Bishop, R.P., Carrington, D., Sohanpal, M.B. & Spooner, P. 1993, "Discrimination between six species of *Theileria* using oligonucleotide probes which detect small subunit ribosomal RNA sequences", *Parasitology,* no. 107, pp. 157-165.

Allsopp M., Visser E.S., du Plessis J.L., Vogel S.W. & Allsopp B.A. 1997, "Different organisms associated with heartwater as shown by analysis of *16S* ribosomal RNA gene sequences", *Veterinary Parasitology,* 71, pp. 283–300.

Ambrosio, R.E. & de Waal, D.T. 1990, "Diagnosis of parasitic disease", *Revue Scientifique et technique (Office international of Epizootics*), vol. 3, no. 9, pp. 759-778.

Apanaskevich, D.A., Horak, I.G. & Camicas, J. 2007, "Redescription of *Haemaphysalis (Rhipistoma) elliptica* (Koch, 1844), an old taxon of the *Haemaphysalis (Rhipistoma) leachi* group from East and southern Africa, and of *Haemaphysalis (Rhipistoma) leachi* (Audouin, 1826) (Ixodida, Ixodidae)", *Onderstepoort Journal of Veterinary Research,* no. 74, pp. 181–208.

Aubry, P. & Geale, D.W. 2011, "A review of bovine anaplasmosis", *Transboundary Emergency Diseases,* no. 58, pp. 1–30.

Bekker, C.P.J., de Vos, S., Taoufik, A., Sparagano, O.A.E. & Jongejan, F. 2002, "Simultaneous detection of *Anaplasma* and *Ehrlichia* species in ruminants and detection of *Ehrlichia ruminantium* in *Amblyomma variegatum* ticks by reverse line blot hybridization", *Veterinary Microbiology,* vol. 89, no. 2–3, pp. 223-238.

Bell-Sakyi, L., Koney, E.B.M., Dogbey, O. & Walker, A.R. 2004, ''Incidence and Prevalence of tick-borne haemoparasites in domestic ruminants in Ghana'', *Veterinary Parasitology,* 124(1–2), 25-42.

Berggoetz, M., Schmid, M., Ston, D., Wyss, V., Chevillon, C., Pretorius, A. & Gern, L. 2014, "Tick-borne pathogens in the blood of wild and domestic ungulates in South Africa: Interplay of game and livestock", *Ticks and Tick-borne Diseases,* no. 5, pp. 166-175.

Bock, R., Jackson, L., de Vos, A. & Jorgensen, W. 2004, "Babesiosis of cattle", *Parasitology,* no. 129, pp. S247–S269.

Bolívar, A.M. 2013, "Metodología diagnóstica para hemoparásitos dentro de la ganadería bovinacon énfasis en la reacción en cadena de la polimerasa y su variante múltiple", *Revista de la Salud Animal,* vol. 1, no. 35, pp. 1 - 9.

Bose, R., Jorgensen, W.K., Dalgliesh, R.J., Friedhoff, K.T. & de Vos, A.J. 1995, "Current state and future trends in the diagnosis of babesiosis", *Veterinary Parasitology,* no. 57, pp. 61-74.

Bosman, A.M., Oosthuizen, M.C., Peirce, M.A. & Venter, E.H. & Penzhorn, B.L. 2010, "*Babesia lengau* sp., a novel *Babesia* species in cheetah (*Acinonyx jubatus*, Schreber, 1775) populations in South Africa", *Journal of Clinical Microbiology,* no. 48, pp. 2703–2708.

Bosman, A.M., Venter, E.H. & Penzhorn, B.L. 2007, "Occurrence of *Babesia felis* and *Babesia leo* in various wild felid species and domestic cats in Southern Africa, based on reverse line blot analysis", *Veterinary Parasitology,* no. 144, pp. 33-38.

Buening, G.M., Barbet, A., Myler, P., Mahan, S., Nene, V. & McGuire, T.C. 1990, "Characterization of a repetitive DNA probe for *Babesia bigemina*", *Veterinary Parasitology,* no. 36, pp. 11-20.

Buling, A., Criado-Fornelio, A., Asenzo, G., Benitez, D., Barba-Carretero, J.C. & Florin-Christensen, M. 2007, "A quantitative PCR assay for the detection and quantification of *Babesia bovis* and *B. bigemina*", *Veterinary Parasitology,* no. 147, pp. 16-25.

Burrage, T.G. 2012, "African swine fever virus infection in *Ornithodoros* ticks, correctedcopy", *Virus Research*, vol. 173-1, pp. 131-139.

Butler, C.M., Nijhof, A.M., Jongejan, F. & Van der Kolk, J.H. 2008, "*Anaplasma phagocytophilum* infection in horses in the Netherlands", *Veterinary Record,* no. 162, pp. 216–218.

Byaruhanga, C., Collins, N.E., Knobel, D., Chaisi, M.E., Vorster, I., Steyn, H.C. & Oosthuizen, M.C. 2016, "Molecular investigation of tick-borne haemoparasite infections among transhumant zebu cattle in Karamoja Region, Uganda", *Veterinary Parasitology: Regional Studies and Reports,* vol. 3-4, pp. 27-35.

Camicas, J., Hervy, J., Adam, F. & Morel, P. 1998, " Les tiques du monde (Acarida, Ixodida): nomenclature, stades décrits, hôtes, répartition (espèces décritesavant le 1/01/96)". *ORSTOM.*

Camicas, J.L., Hoogstraal, H. & el Kammah, K.M. 1983, "Notes on African *Haemaphysalis* ticks. XIV. Description of adults of *H. (Rhipistoma) paraleachi* sp., a carnivore parasite of the *H. (R.) leachi* group (Ixodoidea: Ixodidae)", *Journal of Parasitology,* no. 69, pp. 400–404.

Camicas, J.L., Hoogstraal, H. & el Kammah, K.M. 1973, "Notes on African *Haemaphysalis* ticks. XI. *H. (Rhipistoma) punctaleachi* sp., a parasite of West African forest carnivores (Ixodoidea: Ixodidae)", *Journal of Parasitology,* no. 59, pp. 563–568.

Camus, E. & Barre, N. 1987, "Diagnosis of heartwater in the live animal: experiences with goats in Guadeloupe'', *Onderstepoort Journal Veterinary Research,* no. 54, pp. 291–294.

Canever, M.F., Vieira, L.L., Reck, C., Richter, L. & Miletti, L.C. 2014, "First evaluation of an outbreak of bovine babesiosis and anaplasmosis in Southern Brazil using Multiplex PCR", *Korean Journal Parasitology,* vol. 52, no. 5, pp. 507-511.

Cardoso, L., Oliveira, A.C., Granada, S., Nachum-Biala, Y., Gilad, M., Lopes, A.P., Sousa, S.R., Vilhena, H. & Baneth, G. 2016, "Molecular investigation of tick-borne pathogens in dogs from Luanda, Angola", *Parasites & Vectors*, no. 9, pp. 252.

Castro, J.J. 1997, "Sustainable tick and tick-borne disease control in livestock improvement in developing countries", *Veterinary Parasitology,* no. 71, pp. 77 - 97.

Ceci, L., Iarussi, F., Greco, B., Lacinio, R., Fornelli, S. & Carelli, G. 2014, "Retrospective study of hemoparasites in cattle in southern Italy by reverse line blot hybridization'', *The Journal of Veterinary Medical Science*, no. 76, pp. 869–875.

Chaisi, M.E., Baxter, J.R., Hove, P., Choopa, C.N., Oosthuizen, M.C., Brayton, K.A., Khumalo, Z.T.H., Mutshembele, A.M., Mtshali, M.S. & Collins, N.E. 2017, "Comparison of three nucleic acid-based tests for detecting *Anaplasma marginale* and *Anaplasma centrale* in cattle", *Onderstepoort Journal of Veterinary Research,* vol. 1, no. 84, pp. 1262.

Dahmani, M., Davoust, B., Benterki, M.S., Fenollar, F., Raoult, D. & Mediannikov, O. 2015, "Development of a new PCR-based assay to detect Anaplasmataceae and the first report of *Anaplasma phagocytophilum* and *Anaplasma platys* in cattle from Algeria", *Comparative immunology, microbiology and infectious diseases,* vol. 39, pp. 39-45.

Decaro, N., Carelli, G., Lorusso, E., Lucente, M.S., Greco, G., Lorusso, A., Radogna, A., Ceci, L. & Buonavoglia, C. 2008, "Duplex real-time polymerase chain reaction for simultaneous detection and quantification of *Anaplasma marginale* and *Anaplasma centrale*", *Journal of Veterinary Diagnostic Investigation,* vol. 5, no. 20, pp. 606–611.

de la Fuente, J., Ruybal, P., Mtshali, M.S., Naranjo, V., Shuqing, L. & Mangold, A.J. 2007, "Analysis of world strains of *Anaplasma marginale* using major surface protein 1a repeat sequences", *Veterinary Microbiology,* no. 119, pp. 382–390.

de Matos, C., Sitoe, C., Neves, L., Bryson, N.R. & Horak, I.G. 2008, "Ixodid ticks on dogs belonging to people in rural communities and villages in Maputo Province, Mozambique", *Onderstepoort Journal of Veterinary Research*, no. 75, pp. 103–108.

de Vos, A.J., Bessenger, R. & Banting, L.F. 1981, "*Theileria? Taurotragi*: a probable agent of bovine cerebral theileriosis", *Onderstepoort Journal of Veterinary Research*, no. 48, pp. 149–153.

Dias, J.A.T.S., 1983, Subsídios para o conhecimento da fauna ixodológica de Angola, série Zoologia 11(1-2), 57-69.

Dias, V.S., 1948, Subsídios para o estudo dos ixodídeos de Angola, animais domésticos. Anais dos serviços de Veterinária.

Durrani, A.Z., Younus, M., Kamal, N., Mehmood, N. & Shakoori, A.R. 2011, "Prevalence of ovine *Theileria* species in district Lahore", *Pakistan Journal Zoology.,* no. 43, pp. 57-60.

el Imam, A.H., Hassan, S.M., Gameel, A.A., El Hussein, A.M., Taha, K.M. & Oosthuizen, M.C. 2016, "Molecular identification of different *Theileria* and *Babesia* species infecting sheep in Sudan", *Annals of Parasitology,* vol. 1, no. 62, pp. 47–54.

eI Kammah, K.M., Hoogstraal, H. & Camicas, J. 1992, "Notes on African *Haemaphysalis* ticks: XI. *H. (Rhipistoma) paraleachi* (Ixodoidea: Ixodidae) Distribution and hosts of adults", *International Journal of Acarology*, vol. 18, no. 3.

Eriks, I., Palmer, G., Mcguire, T., Allred, D. & Barbet, T. 1989, "Detection and quantitation of *Anaplasma marginale* in carrier cattle using a nucleic acid probe", *Journal of clinical Microbiology*, no. 27, pp. 206-212.

Eygelaar, D., Jori, F., Mokopasetso, M., Sibeko, K.P., Collins, N.E., Vorster, I., Troskie, M. & Oosthuizen, M.C. 2015, "Tick-borne haemoparasites in African buffalo (*Syncerus caffer*) from two wildlife areas in Northern Botswana", *Parasites & Vectors*, pp. 8:26.

Fanikiso, M. & Ndzinge, I.T. 1992, "Situation report on tick infestation of livestock tick-borne disease and tick control methods in Botswana", *Insect Science and its Application*, vol. 4, no. 13, pp. 645-648.

Felsenstein, J. 1985, "Confidence limits on phylogenies: An approach using the bootstrap", *Evolution* no. 39, pp. 783-791.

Figueroa, J.V., Cheives, L.P., Johnson, G.S. & Buening, G.M. 1993, "Multiplex polymerase chain reaction based assay for the detection of *Babesia bigemina*, *Babesia bovis* and *Anaplasma marginale* DNA in bovine blood", *Veterinary Parasitology* no. 50, pp. 69–81.

Fourie, J.J., Fourie, L.J., Horak, I.G. & Snyman, M.G. 2010, "The efficacy of a topically applied combination of cyphenothrin and pyriproxyfen against the southern African yellow dog tick *Haemaphysalis elliptica* and the cat flea *Ctenocephalides felis* on dogs", *Journal of the South African Veterinary Association* vol. 1, no. 81, pp. 33 - 36.

Georges, K., Loria, G.R., Riili, S., Greco, A., Caracappa, S., Jongejan, F. & Sparagano, O. 2001, "Detection of haemoparasites in cattle by reverse line blot hybridisation with a note on the distribution of ticks in Sicily", *Veterinary Parasitology* no. 99, pp. 273–286.

Gomes, A.F., Pombal, A.M. & Venturi, L. 1994, "Observations on cattle ticks in Huíla Province (Angola)", *Veterinary Parasitology* vol. 51, no. 3-4, pp. 333-336.

Gomes, A.F. 1993, "The tick vectors of cowdriosis in Angola", *Revue d'élevage et de Médecine Vétérinaire des Pays Tropicaux* vol. 1-2, no. 46, pp. 237-243.

Gomes, A.F., Kageruka, P. & Brandt, J. 1991, "Epidemiology of bovine babesiosis in South-western Angola", *Revue d'élevage et de Médecine Vétérinaire des Pays Tropicaux* vol. 4, no. 44, pp. 429-435.

Govender, D., Oosthuisen, M.C. & Penzhorn, B.L. 2011, "Piroplasm parasites of white rhinoceroses (*Ceratotherium simum*) in the Kruger National Park and their relation to anaemia", *Journal South African Veterinary Association* no. 82, pp. 36–40.

Gubbels, J.M., de Vos, A.P., Van Der Weide, M., Viseras, J., Schouls, L.M., de Vries, E. & Jongejan, F. 1999, "Simultaneous detection of bovine *Theileria* and *Babesia* species by reverse line blot hybridization", *Journal of Clinical Microbiology* vol. 37, no. 6, pp. 1782–1789.

Guglielmone, A.A., Robbins, R.G., Apanaskevich, D.A., Petney, T.N., Estrada-Pena, A. & Horak, I.G. 2009. "Comments on controversial tick (Acari: Ixodida) species names and species described or resurrected from 2003 to 2008". *Experimental and Applied Acarology* no. 48, pp. 311-327.

Guglielmone, A.A., Robbins, R.G., Apanaskevich, D.A., Petney, T.N., Estrada-Pena, A. & Horak, I.G. 2014, "The hard ticks of the world", *Springer Science*.

Guillemi, E.C., Tomassone, L. & Farber, M.D. 2015, "Tick-borne Rickettsiales: Molecular tools for the study of an emergent group of pathogens", *Journal of Microbiological Methods* no. 119, pp. 87-97.

Hall, T.A. 1999, "BioEdit: A User-Friendly Biological Sequence Alignment Editor and Analysis Program for Windows 95/98/NT. Nucleic Acids Symposium Series", *Oxoford university press* vol. 41, pp. 95-98.

Hamer, S.A., Goldberg, T.L., Kitron, U.D., Brawn, J.D., Anderson, T.K., Loss, S.R., Walker, E.D. & Hamer, G.L. 2012, "Wild Birds and Urban Ecology of Ticks and Tick-borne Pathogens, Chicago, Illinois, USA, 2005–2010", *Emerging Infectious Diseases,* vol. 18 no. 10.

Hauschild, S. &, Schein, E. 1996, "The subspecies specificity of *Babesia canis*", *Berl Munch Tierarztl Wochenschr* no. 109(6-7), pp. 216-9.

He, L., Feng, H.H., Zhang, Q.L., Zhang, W.J., Khan, M.K., Hu, M., Zhou, Y.Q. & Zhaq, J.L. 2012, "Development and evaluation of real time PCR assay for the detection of *Babesia orientalis* in water buffalo (*Bubalus bubalis*, Linnaeus, 1758)", *Journal of Parasitology* no. 97, pp. 1166-1169.

Horak, I.G., Nyangiwe, N., de Matos, C. & Neves, L. 2009, "Species composition and geographic distribution of ticks infesting cattle, goats and dogs in a temperate and in a subtropical region of South-East Africa", *Onderstepoort Journal of Veterinary Research* no. 76, pp. 263–276.

Horak, I.G. & Camicas, J.L. 2002, "The Argasidae, Ixodidae and Nuttlalliellidae (Acari: Ixodida): A world list of valid tick names", *Experimental and Applied Acarology* no. 28, pp. 27-54.

Horak, I.G., Guillarmod, A.J., Moolman, L.C. & de Vos, V. 1987, "Parasites of domestic and wild animals in South Africa. XXII. Ixodid ticks on domestic dogs and on wild carnivores", *Onderstepoort Journal of Veterinary Research* no. 54, pp. 573-580.

Hove, T., Mukandi, R., Bere, M., Horak, I.G. & Latif, A.A. 2008, "Ixodid ticks infesting domestic goats in communal land areas of Zimbabwe", *Journal of South Africa Veterinary Association* no. 79, pp. 116-120.

IBM SPSS, 2014. IBM SPSS Statistics for Windows, Version 23.0. IBM Corp., Armonk, N.Y.

Inokuma, H., Fujii, K., Okuda, M., Onishi, T., Beaufils, J.P., Raoult, D. & Brouqui, P. 2002, "Determination of the nucleotide sequences of heat shock operon *groESL* and the citrate synthase gene (*gltA*) of *Anaplasma* (*Ehrlichia*) *platys* for phylogenetic and diagnostic studies", *Clinical and Diagnostic Laboratory Immunology* vol. 5, no. 9, pp. 1132–1136.

Inokuma, H., Ohno, K., Onishi, T., Raoult, D. & Brouqui, P. 2001, "Detection of ehrlichial infection by PCR in dogs from Yamaguchi and Okinawa prefectures, Japan", *Journal of Veterinary Medical Science* vol. 7, no. 63, pp. 815–817

Inokuma, H., Raoult, D. & Brouqui, P. 2000, "Detection of *Ehrlichia platys* DNA in brown dog ticks (*Rhipicephalus sanguineus)* in Okinawa island, Japan", *Journal of Clinical Microbiology* vol. 11, no. 38, pp. 4219–4221.

Irwin, P.J. 2010, "Canine babesiosis", *Veterinary Clinic Small Animals* no. 40, pp. 1141-1156.

Irwin, P.J. 2009, "Canine babesiosis: from molecular taxonomy to control", *Parasites & Vectors* no. 2(S1):S4.

Isa, J.F.W. & Mushi, E.Z. & Modo, J.M. 1995, "Ticks in goats at Oodi, Kgatleng District, Botswana", *Bulletin of Animal Health and Production in Africa,* vol. 1, no. 43, pp. 17-20.

Jongejan, F. & Uilenberg, G. 2004, "The global importance of ticks", *Parasitology* no. 129: (S3-S14).

Jongejan, F. & Uilenberg, G. 1994, "Ticks and control methods", *Revue scientifique et techinique (Office international of Epizootics)* vol. 4, no. 13, pp. 1201-1226.

Jongejan, F. 1991, "Serodiagnosis of *Cowdria ruminantium*: Current status. Recent development in the control of anaplasmosis, babesiosis and cowdriosis. Proceedings of a workshop held at ILRAD, 13-15 May, 1991. ed (Dolan, T.T)", *The international Laboratory for research on Animal Diseases*.

Jukes, T.H. & Cantor, C.R. 1969, "Evolution of protein molecules. In Munro HN, editor, Mammalian Protein Metabolism", *Academic Press, New York* vol. 21, pp. 132.

Kaewkong, W., Intapan, P.M., Sanpool, O., Janwan, P., Thanchomnang, T., Kongklieng, A., Tantrawatpan, C., Boonmars, T., Lulitanond, V., Taweethavonsawat, P., Chungpivat, S. & Maleewong, W. 2014, "High throughput pyrosequencing technology for molecular differential detection of *Babesia vogeli*, *Hepatozoon canis*, *Ehrlichia canis* and *Anaplasma platys* in canine blood samples", *Ticks and Tick-Borne Diseases* vol. 4, no. 5, pp. 381-5.

Kamani, J., Baneth, G., Mumcuoglu, K.Y., Waziri, N.E., Eyal, O. & Guthmann, J. 2013, "Molecular Detection and Characterization of Tick-borne Pathogens in Dogs and Ticks from Nigeria", *PLoS Neglected Tropical Diseases* vol. 7, no. 3.

Kent, R.J. 2009, "Molecular methods for arthropod blood meal identification and applications to ecological and vector-borne disease studies", *Molecular Ecology Resources* vol. 1, no. 9, pp. 4–18.

Kim, C., Iseki, H., Herbas, M.S., Yokoyama, N., Suzuki, H., Xuan, X., Fujisaki, K. & Igarashi, I. 2007, "Development of Taqman-Based Real-Time PCR Assays for Diagnostic Detection of *Babesia bovis* and *Babesia bigemina*", *American Journal of Tropical Medicine and Hygiene* vol. 5, no. 77, pp. 837-841.

Kocan, K.M., de la Fuente, J., Blouin, E.F., Coetzee, J.F. & Ewing, S.A. 2010, "The natural history of *Anaplasma marginale*",*Veterinary Parasitology* vol. 167, no. 2-4, pp. 95-107.

Kocan, K.M., de la Fuente, J., Blouin, E.F. & Garcia-Garcia, J.C. 2004, "*Anaplasma marginale* (Rickettsiales: Anaplasmataceae): recent advances in defining host-pathogen adaptations of a tick-borne rickettsia", *Parasitology* no. 129, pp. 285-300.

Krause, P.J. 2003, "Babesiosis diagnosis and treatment", *Vector-Borne Zoonotic Disease* no. 3, pp. 45-51.

Kubelova, M., Mazancova, J. & Siroky, P. 2012, "*Theileria, Babesia,* and *Anaplasma* Detected by PCR in Ruminant Herds at Bie Province, Angola", *Parasite-Journal De La Societe Francaise De Parasitologie* vol. 19, no. 4, pp. 417-422.

Kubelova, M., Tkadlec, E., Bednar, M. & Roubalova, E. & Siroky, P. 2011, "West to east differences of *Babesia canis canis* prevalence in *Dermacentor reticulatus* ticks in Slovakia", *Veterinary Parasitology* no. 180, pp. 191-196.

Kumar, S., Stecher, G. & Tamura, K. 2016, "MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets", *Molecular Biology and Evolution* vol. 33, pp. 1870-1874.

Kumsa, B.E. & Mekonnen, S. 2011, "Ixodid ticks, fleas and lice infesting dogs and cats in Hawassa, southern Ethiopia'', *Onderstepoort Journal of Veterinary Research* vol. 78, pp. 14.

Kwak, Y.S., Kim, T.Y., Nam, S., Lee, I., Kim, H., Mduma, S., Keyyu, J. & Fyumagwa, R. & Yong, T-S. 2014, "Ixodid Tick Infestation in Cattle and Wild Animals in Maswa and Iringa, Tanzania", *Korean Journal Parasitology* vol. 5, no. 52, pp. 565-568.

Lauzi, S., Maia, J.P., Episa, S., Marcos, R., Pereira, C., Luzzago, C., Santos, M., Puente-Payo, P., Giordano, A., Pajoro, M., Sironi, G. & Faustino, A. 2016, " Molecular detection of *Anaplasma platys*, *Ehrlichia canis*, *Hepatozoon canis* and *Rickettsia monacensis* in dogs from Maio Island of Cape Verde archipelago", *Ticks and tick-borne diseases* no. 7, pp. 964–969.

Lawrence, J.A., Perry, B.D. & Williamson, S.M. (eds) 2004, *Corridor disease, in Infectious diseases of livestock*, 2nd ed. edn, edited by J.A.W. Coetzer & R.C. Tustin, Cape Town: Oxford University Press Southern Africa.

Lawrence, J.A. (ed) 1992, *History Of Bovine Theileriosis In Southern Africa. The Epidemiology of Theileriosis in Africa.* In: Norval, R.A.I., Perry, B.D. & Young, A.S. (eds). edn, Academic Press, London.

Levings, R.L. & Henderson, L.M. & Metz, C.A. 1993, "In vitro potency assays for no replicating veterinary vaccines: comparison to in vivo assays and considerations in assay development", *Veterinary Microbiology* vol. 3-4, no. 37, pp. 201-219.

Lorusso, V., Wijnveld, M., Majekodunmi, A.O., Dongkum, C., Fajinmi, A., Dogo, A.G., Thrusfield, M., Mugenyi, A., Vaumourin, E., Igweh, A.C., Jongejan, F., Welburn, S.C. & Picozzi, K. 2016, "Tick-borne pathogens of zoonotic and veterinary importance in Nigerian cattle", *Parasites & Vectors* no. 9, pp. 217.

Lorusso, V., Picozzi, K., de Bronsvoort, B.M.C., Majekodunmi, A., Dongkum, C., Balak, G., Igweh, A. & Welburn, S.C. 2013, "Ixodid ticks of traditionally managed cattle in central Nigeria: where *Rhipicephalus (Boophilus) microplus* does not dare (yet?)", *Parasites & Vectors*, pp. 6:171.

Lynen, G., Zeman, P., Bakuname, C., Di Giulio, G., Mtui, P. & Sanka P. & Jongejan, F. 2007, "Cattle ticks of the genera *Rhipicephalus* and *Amblyomma* of economic importance in Tanzania: Distribution assessed with GIS based on an extensive field survey", *Experimental & Applied Acarology* no. 43 (4), pp. 303-319.

M'ghirbi, Y., Ros-García, A., Iribar, P., Rhaim, A., Hurtado, A. & Bouattour, A. 2013, "A molecular study of tick-borne haemoprotozoan parasites (*Theileria* and *Babesia*) in small ruminants in northern Tunisia", *Veterinary Parasitology* no. 198, pp. 72 - 77.

M'ghirbi, Y., Hurtado, A., Brandika, J., Khlif, K., Ketata, Z. & Bouattour, A. 2008, "A molecular survey of *Theileria* and *Babesia* parasites in cattle, with a note on the distribution of ticks in Tunisia", *Parasitology Research* no. 103, pp. 435–442.

Mahan, S.M. 1995, "Review of the molecular biology of *Cowdria ruminantium*", *Veterinary Parasitology* no. 57, pp. 51-56.

Mahoney, D.F. & Saal, J.R. 1961, "Bovine babesiosis: thick blood films for the detection of parasitaemia", *Australian Veterinary Journal* no. 37, pp. 44-47.

Makala, L.H., Mangani, P., Fujisaki, K. & Nagasawa, H. 2003, "The current status of major tick-borne diseases in Zambia", *Veterinary Research* vol. 34, no. 1, pp. 27-45.

Manyarara, R., Tubbesing, U. & Soni, M. & Noden, B.H. 2015, "Serodetection of *Ehrlichia canis* amongst dogs in central Namibia'', *Journal of the South* African *Veterinary Association* vol. 86 (1).

Martins, T.M., Neves, L., Pedro, O.C., Fafetine, J.M., Rosario, V.E & Domingos, A. 2009, "Molecular detection of *Babesia* spp. and other haemoparasitic infections of cattle in Maputo Province, Mozambique", *Parasitology*, pp. 1-8.

Matjila, P.T., Leisewitz, A.L., Jongejan, F. & Penzhorn, B.L. 2008, "Molecular detection of tick-borne protozoal and ehrlichial infections in domestic dogs in South Africa", *Veterinary Parasitology* vol. 155, no. 1–2, pp. 152-157.

Matjila, P.T., Penzhorn, B.L., Bekker, C.P.J., Nijhof, A.M. & Jongejan, F. 2004, "Confirmation of occurrence of *Babesia canis vogeli* in domestic dogs in South Africa", *Veterinary Parasitology* vol. 122, no. 2, pp. 119-125.

Matthee, S., Lovely, C., Gaugler, A., Beeker, R., Venter, H.R. & Horak, I.G. 2010, "Ixodid ticks on domestic dogs in the northern Cape Province of South Africa and in Namibia", *Journal of the South African Veterinary Association-Tydskrif Van Die Suid-Afrikaanse Veterinere Vereniging* vol. 81, no. 2, pp. 126-128.

Minjauw, B. & McLeod, A. 2003, "Tick-borne diseases and poverty. The impact of ticks and tick-borne diseases on the livelihood of small-scale and marginal livestock owners in India and eastern and southern Africa", Research report, DFID Animal Health Programme. UK: *Centre for Tropical Veterinary Medicine*, University of Edinburgh.

Mtshali, P.S., Tsotetsi, A.M., Thekisoe, M.M.O. & Mtshali, M.S. 2014, "Nested PCR Detection and Phylogenetic Analysis of *Babesia bovis* and *Babesia bigemina* in Cattle from Peri-Urban Localities in Gauteng Province, South Africa", *Journal Veterinary Medicine Science* vol. 1, no. 76, pp. 145–150.

Mtshali, M.S., de la Fuente, J., Ruybal, P., Kocan, K.M., Vicente, J. & Mbati, P.A. 2007, "Prevalence and genetic diversity of *Anaplasma marginale* strains in cattle in South Africa", *Zoonoses and Public Health* no. 54, pp. 30.

Muhanguzi, D., Ikwap, K., Picozzi, K., & Waiswa, C. 2010, "Molecular Characterization of *Anaplasma* and *Ehrlichia* species in Different Cattle Breeds and Age Groups in Mbarara District (Western Uganda)", *International Journal of Animal and Veterinary Advance* no. 2(3), pp. 76-88.

Muramatsu, Y., Ukegawa, S., El Hussein, A.R.M., Rahman, M.B.A., Gabbar, K.M.A.A., Chitambo, A.M., Komiya, T., Mwase, E.T., Morita, C. & Tamura, Y. 2005, "*Ehrlichia ruminantium*, Sudan'', *Emerging Infectious Diseases* vol. 11, no. 11.

Mushi, E.Z. & Isa, J.F.W. & Sakia, R.M. 1996, "Monthly fluctuation of Ixodid tick infestation on indigenous goats in Botswana", *Small Ruminant Research* no. 21, pp. 277-279.

Musuka, G.N., Mafika, M. & Molathwa, E. & Nuru, H. 2001, "New distribution records of *Amblyomma hebraeum* in the sandveld region of Botswana", *Journal of The South African Veterinary Association* vol. 1, no. 72, pp. 3-3.

Neves, L., Afonso, S. & Horak, I.G. 2004, "Ixodid ticks on dogs in southern Mozambique", *Onderstepoort Journal of Veterinary Research* no. 71, pp. 279-283.

Ndhlovu, D.N. & Makaya, P.V. & Penzhorn, B.L. 2009, "Tick infestation, and udder and teat damage in selected cattle herds of Matabeleland South, Zimbabwe", *Onderstepoort Journal of Veterinary Research* no. 76, pp. 235–248.

Nijhof, A.M., Pillay, V., Steyl, J., Prozesky, L., Stoltsz, W.H., Lawrence, J.A., Penzhorn, B.L. & Jongejan, F. 2005, "Molecular characterization of *Theileria* species associated with mortality in four species of African antelopes", *Journal Clinical Microbiology* no. 43, pp. 5907-5911.

Nijhof, A.M., Penzhorn, B.L., Lynen, G., Mollel, J.O., Morkel, P., Bekker, C.P.J. & Jongejan, F. 2003, " *Babesia bicornis* sp. novel and *Theileria bicornis* sp. novel: tick-borne parasites associated with mortality in the black rhinoceros (*Diceros bicornis*)", *Journal Clinical Microbiology* no. 41, pp. 2249–2254.

Njiiri, N.E., Bronsvoort, B.M.D., Collins, N.E., Steyn, H.C., Troskie, M., Vorster, I., Thumbi, S.M., Sibeko, K.P., Jennings, A., van Wyk, I.C., Mbole-Kariuki, M., Kiara, H., Poole, E.J., Hanotte, O., Coetzer, K., Oosthuizen, M.C., Woolhouse, M. & Toye, P. 2015, "The epidemiology of tick-borne haemoparasites as determined by the reverse line blot hybridization assay in an intensively studied cohort of calves in western Kenya", *Veterinary Parasitology* vol. 210, no. 1–2, pp. 69-76.

Norval, R.A.I., Perry, B.D., Young, A., Lawrence, J.A., Mukhebi, A.W., Bishop, R.P., McKeever, D.J. & Shaw, M.K. 1992, "The Epidemiology of Theileriosis in Africa", *Academic Press Limited, London NWI 7DX, UK*, pp. 481.

Nyangiwe, N., Matthee, C., Horak, I. & Matthee, S. 2013, "First record of the pantropical blue tick *Rhipicephalus microplus* in Namibia", *Experimental and Applied Acarology* vol. 61, no. 4, pp. 503-507.

Ogo, N.I., Mera, I.G.F., Galindo, R.C., Okubanjo, O.O., Inuwa, H.M., Agbede, R.I.S., Torina, A., Alongie, A., Vicente, J., Gortázar, C. & Fuente, J. 2012, "Molecular identification of tick-borne pathogens in Nigerian ticks", *Veterinary Parasitology* no. 187, pp. 572– 577.

Oguge, N.O., Durden, L.A., Keirans, J.E., Balami, H.D. & Schwan, T.G. 2009, "Ectoparasites (sucking lice, fleas and ticks) of small mammals in southern Kenya", *Medicine of Veterinary Entomology* vol. 4, no. 23, pp. 387–392.

Oosthuizen, M.C., Zweygarth, E., Collins, N.E., Troskie, M. & Penzhorn, B.L. 2008, "Identification of a novel *Babesia* sp. from a sable antelope (*Hippotragus niger*) Harris, (1838)", *Journal Clinical Microbiology* no. 46, pp. 2247–2251.

O'Sullivan, M.V.N., Zhou, F., Sintchenko, V., Kong, F. & Gilbert, G.L. 2011, "Multiplex PCR and reverse line blot Hybridization Assay (mPCR/RLB)", *Journal of Visualized Experiments* no. 54.

Otranto, D., Testini, G., Dantas-Torres, F., Latrofa, M.S., Diniz, P.P., de Caprariis, D., Lia, R.P., Mencke, N., Stanneck, D., Capelli, G. & Breitschwerdt, E.B. 2010, "Diagnosis of canine vector-borne diseases in young dogs: a longitudinal study", *Journal of Clinical Microbiology* vol. 9, no. 48, pp. 3316–3324.

Oura, C.A.L., Bishop, R.P., Wampandea, E.M., Lubegaa, G.W. & Taitb, A. 2004, "Application of a reverse line blot assay to the study of haemoparasites in cattle in Uganda", *International Journal for Parasitology* no. 34, pp. 603–613.

Perry, B.D., Musisi, F.L., Pegram, R.G., Schels, E.L.F. 1985, "Assessment of endemic stability to tick-borne diseases'', *World Animal Review* no. 56, pp. 24–32.

Peter, T.F., Deem, S.L., Barbet, A.F., Norval, R.A.I., Simbi, B.H. & Kelly, P.J. 1995, "Development and evaluation of PCR assay for detection of low levels of *Cowdria ruminantium* infection in *Amblyomma* ticks not detected by DNA probe'', *Journal of Clinical Microbiology* no. 33, pp. 166–72.

Portillo, A., Perez-Martinez, L., Santibanez, S., Blanco, J.R. & Ibarra, V. & Oteo, J.A. 2007, "Short report: Detection of *Rickettsia africae* in *Rhipicephalus (Boophilus) decoloratus* - Ticks from the Republic of Botswana, South Africa", *American Journal of Tropical Medicine and Hygiene* vol. 2, no. 77, pp. 376-377.

Saitou, N. & Nei, M. 1987, "The neighbour-joining method: A new method for reconstructing phylogenetic trees'', *Molecular Biology and Evolution* vol. 4, pp. 406-425.

Salih, D.A., el Hussein, A.M., Seitzer, U. & Ahmed, J.S. 2007, "Epidemiological studies on tick-borne diseases of cattle in Central Equatoria State, Southern Sudan", *Parasitology Research* no. 101, pp. 1035-1044.

Schnittger, L., Yin, H., Qi, B., Gubbels, M.J., Beyer, D., Niemann, S., Jongejan, F. & Ahmed, J.S. 2004, "Simultaneous detection and differentiation of *Theileria* and *Babesia* parasites infecting small ruminants by reverse line blotting", *Parasitology Research* vol. 92, no. 3, pp. 189-196.

Schouls, L.M., Van De Pol, I., Rijpkema, S.G. & Schot, C.S. 1999, "Detection and identification of *Ehrlichia*, *Borrelia burgdorferi* sensu lato, and *Bartonella* species in Dutch Ixodes ricinus Ticks", *Journal of Clinical Microbiology* no. 37, pp. 2215–2222.

Serrano, F.M.H. 1973, Luta contra as carraças. Instituto de Investigaçao Veterinária de Angola, Luanda.

Serrano, F.M.H. 1963, Considerações sobre morfologia, ecologia e biologia dos ixodideos dos generos *Amblyomma* e d*ermacentor* assinalados em Angola, Revista Ciência Veterinária. Vol. LVIII. Nº 386/7.

Shompole, S., Waghela, S.D., Rurangirwa, F.R. & McGuire, T.C. 1989, "Cloned DNA probes identify *Anaplasma ovis* in goats and reveal a high prevalence of infection", *Journal of Clinical Microbiology* no. 27, pp. 2730–2735.

Simuunza, M., Bilgic, H., Karagenc, T., Syakalima, M., Shiels, B., Tait, A. & Weir, W. 2011, "Population genetic analysis and sub-structuring in *Babesia bovis*", *Molecular and Biochemical Parasitology* no. 177(2), pp. 106-115.

Smith, E.R. & Parker, D.M. 2010, "Tick communities at the expanding wildlife/cattle interface in the Eastern Cape Province, South Africa: implications for Corridor disease", *Journal of the South African Veterinary Association* no. 81(4), pp. 237–240.

Sparagano, O. & Jongejan, F. 1999, "Molecular characterization of ticks and tick-borne pathogens", *Parasitology* no. 41 Suppl. 1, pp. 101-5.

Sparagano, O.A.E., Allsopp, M.T.E.P., Mank, R.A., Rijpkema, S.G.T., Figueroa, J.V. & Jongejan, F. 1999, "Molecular detection of pathogen DNA in ticks (Acari: Ixodidae): A review", *Experimental and Applied Acarology* no. 23, pp. 929-960.

Spickett, A. 2013, "Ixodid ticks of major economic importance and their distribution in South Africa. Ticks and Tick-borne Diseases", *Afrivet and Agriculture Research Council*.

Steyn, H.C., Pretorius, A., McCrindle, C.M.E., Steinmann, C.M.L. & Van Kleef, M. 2008, "A real-time PCR assay for *Ehrlichia ruminantium* using pCS20", *Veterinary Microbiology* vol. 131, no. 3–4, pp. 258-265.

Sungirai, M., Madder, M., Moyo, D.Z., de Clercq, P. & Abatih, E.N. 2015, "An update on the ecological distribution of the Ixodidae ticks in Zimbabwe", *Experimental and Applied Acarology* no.66, pp. 269–280.

Torina, A. & Caracappa, S. 2012, "Tick-borne diseases in sheep and goats: Clinical and diagnostic aspects", *Small Ruminant Research* vol. 106, Supplement, pp. S6-S11

Uilenberg, G. 2006, "*Babesia* — a historical overview", *Veterinary Parasitology*, no. 138, pp. 3–10.

Uilenberg, G., 1981, "Theilerial species of domestic livestock. In: Irvin, A.D., Cunningham, M.P., Young, A.S. (Eds.), Advances in the Control of Theileriosis", *Martinus Nijhoff Publishers, The Hague*, pp. 4–37.

Van Heerden, H., Steyn, H.C., Allsopp, M.T.E.P., Zweygarth, E., Josemans, A.I. & Allsopp, B.A. 2004, "Characterization of the *pCS20* region of different *Ehrlichia ruminantium* isolates", *Veterinary Microbiology* vol. 101, no. 4, pp. 279-291.

Viljoen, G.J. & Luckins, A.G. 2012, "The role of nuclear technologies in the diagnosis and control of livestock diseases—a review", *Tropical Animal Health and Production,* no. 44, pp. 1341–1366.

Visser, E.S. & Ambrosio, R.E. 1987, "DNA probes for the detection of *Anaplasma centrale* and *Anaplasma marginale*", *Onderstepoort Journal of Veterinary Research* no. 54, pp. 623-627.

Waghela, S.D., Rurangirwa, F.R., Mahan, S.M., Yunker, C.E., Crawfers, T.B., Barbet, A.F., Burridge, M.J. & Mc Guire, T.C. 1991, "A cloned DNA probe identifies *C. ruminantium* in *Amblyomma variegatum* ticks", *Journal of Clinical Microbiology* no. 29, pp. 2571-2577.

Walker, A.R., Bouattour, A., Camicas, J., Estrada-Peña, A., Horak, I.G., Latif, A.A., Pegram, R.G. & Preston, P.M. 2003, "Ticks of Domestic Animals in Africa: a Guide to Identification of Species".

Walker, J.B., Keirans, J.E. & Horak, I.G. 2000, "The genus Rhipicephalus (Acari: Ixodidae): a guide to the brown ticks of the world", *Cambridge University Press, Cambridge, United Kingdom,* pp. 382–391.

Walker, J.B. & Olwage, A. 1987, "The tick vectors of *Cowdria ruminantium* (Ixodoidea, Ixodidae, genus *Amblyomma*) and their distribution", *Onderstepoort Journal of Veterinary Research* no. 54, pp. 353–379.

Weiland, G. & Reiter, I. 1988, "Methods for the measurement of the serological response to *Babesia*. In: Ristic M, editor. Babesiosis of domestic animals and man", *Boca Raton, Fla: CRC Press, Inc.*, pp. 143–162.

Weisburg, W.G., Barns, S.M., Pelletier, D.A. & Lane, D.J. 1991, "*16S* ribosomal DNA amplification for phylogenetic study", *Journal of Bacteriology* no. 173, pp. 697–703.

Wright, I.G. 1990, "Immunodiagnosis of and immunoprophylaxis against the haemoparasites *Babesia* sp. and *Anaplasma* sp. in domestic animals", *Revue scientifique et technique (International Office of Epizootics)* vol. 2, no. 9, pp. 345-356.

Yisaschar-Mekuzas, Y., Jaffe, C.L., Pastor, J., Cardoso, L. & Baneth, G. 2010, "Identification of *Babesia* species infecting dogs using reverse line blot hybridization for six canine piroplasms, and evaluation of co-infection by other vector-borne pathogens", *Veterinary Parasitology* no. 191, pp. 367– 373.

Young, A.S., Groocock, C.M. & Kariuki, D.P. 1988, "Integrated control of ticks and tick-borne diseases of cattle in Africa", *Parasitology* no. 96, pp. 403–432.

Yunker, C.E., Mahan, S.M., Waghela, S.D., Mc Guire, T.C., Rurangirwa, F.R., Barbet, A.F. & Wassink, L.A. 1993, "Detection of *C. ruminantium* by means of a DNA probe in infected bont ticks, *Amblyomma hebraeum,* the major vector of heartwater in South Africa", *Epidemiology and Infection* no. 110, pp. 95-104.

Yusufmia, S.B.A.S., Collins, N.E., Nkuna, R., Troskie, M., Van den Bossche, P. & Penzhorn, B.L. 2010, "Occurrence of *Theileria parva* and other haemoprotozoa in cattle at the edge of Hluhluwe-iMfolozi Park, KwaZulu-Natal, South Africa", *Journal of South African Veterinary Association* no. 81, pp. 45–49.

Zieger, U., Horak, I.H. & Cauldwell, A.E. 1998, "Dynamics of free-living ixodid ticks on a game ranch in the Central Province, Zambia", *Onderstepoort Journal of Veterinary Research* no. 65, pp. 49-59.

Zobba, R., Anfossi, A.G., Pinna Parpaglia, M.L., Dore, G.M., Chessa, B. & Spezzigu, A. 2014, "Molecular investigation and phylogeny of *Anaplasma* spp. in Mediterranean ruminants reveal the presence of neutrophil-tropic strains closely related to *A. platys*", *Applied and Environmental Microbiology* no. 80, pp. 271–80.

Zúquete, S.T., Coelho, J., Rosa, F., Vaz, Y., Cassamá, B., Padre, L., Santos, D., Basto, A.P. & Leitão, A. 2017, "Tick (Acari: Ixodidae) infestations in cattle along Geba River basin in Guinea-Bissau.", *Ticks and Tick-borne Diseases* no. 8, pp. 161–169.

8. Animal Ethics Committee Approval

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Special composition and distribution of ticks and tick-borne pathogens in ruminants and dogs in Tchicala-Tcholoanga, Angola**
PROJECT NUMBER	**V053-16**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr. G Sili**

STUDENT NUMBER (where applicable)	**UP_15393438**
DISSERTATION/THESIS SUBMITTED FOR	**MSc**

ANIMAL SPECIES	**Cattle, Goats**	**Sheep, Dogs**
NUMBER OF ANIMALS	**80 per species**	**80 per species**
Approval period to use animals for research/testing purposes		**July 2016 – July 2017**
SUPERVISOR	**Prof. L Neves**	

Conditions: The AEC has noted that this project will be completed in a facility outside of South Africa. Since the AEC has not inspected the facility, please note that we cannot comment on the quality of the facility other than what was provided in the study questionnaire

KINDLY NOTE:

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

APPROVED	Date 25 July 2016
CHAIRMAN: UP Animal Ethics Committee	Signature

9. Import permit and DAFF section 20 approval

agriculture, forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate of Animal Health
Import-Export Policy Unit
Private Bag X138
Pretoria, 0001
Republic of South Africa
Tel: (27)-012-319 7514
Fax: (27)-012-329 8292

DEPARTMENT OF AGRICULTURE
DIRECTORATE: ANIMAL HEALTH
PRIVATE BAG X 138
2016 -06- 29
PRETORIA 0001
FORESTRY & FISHERIES

IMPORTER:
PROFESSOR LUIS NEVES
DEPARTMENT OF VETERINARY TROPICAL DISEASES
FACULTY OF VETERINARY SCIENCE
UNIVERSITY OF PRETORIA

PERMIT NO: 13/1/1/30/2/0-201606005178
Valid from: 2016-06-28
Expiry date: 2016-09-28
Valid for 3 months

VETERINARY IMPORT PERMIT FOR TICKS AND DNA TO BE INACTIVATED AT THE TAD-P LABORATORY

[Issued in terms of the Animal Diseases Act, 1984 (Act No. 35 of 1984)]

Authority is hereby granted for you to import 80 TICK COLLECTION PRESERVED IN 70% ETHANOL/ 320 X 1ml EPPENDORF TUBES EACH CONTAINING 250µl OF DNA EXTRACTED FROM BLOOD OF CATTLE, SHEEP, GOAT AND DOG into Republic of South Africa:

From: ANGOLA
subject to the following conditions:

1. The consignment must be accompanied by this original permit and an original veterinary health certificate, complying with the conditions stipulated overleaf, duly completed and signed by an official veterinarian, authorised thereto by the Veterinary Authority of the exporting country.
2. The specimens are to be securely packed and transported in leakproof containers, sealed by an authorised official of the Veterinary Authority of the exporting country;
3. The consignment must be airfreighted through port of entry O R TAMBO INTERNATIONAL AIRPORT. **Samples may only be imported as manifest cargo under an airwaybill number and may not be imported as personal luggage.**
4. The consignment must be accompanied by this permit and its arrival reported immediately to the inspecting veterinary official: KEMPTON PARK Tel: 011 973 2827, and may not be released without his/her written permission.
5. Upon arrival the inspecting veterinary official will inspect the consignment and release it to the importer only after he/she is satisfied that all the import conditions have been complied with in full.
6. **The samples as stipulated above must proceed from the port of entry directly to The Foot and Mouth Disease Laboratory, TAD-P, Onderstepoort Veterinary Institute, Old Soutpan Road, Onderstepoort, 0110, RSA under guidance of a red cross permit. Dr Livio Heath must be informed of the details regarding the dispatch and estimated time of arrival of the consignment (email: HeathL@arc.agric.za; tel no. 012-5299272) well in advance of the arrival in South Africa. The treatments will be at the expense of the importer.**
7. At the **Foot and Mouth Disease Laboratory, TAD-P, Onderstepoort Veterinary Institute** the extracted animal DNA must be treated as per the specifications of TAD to render the DNA non-infectious;
8. The ticks, stored in 70% ethanol, must be placed in a water bath and subjected to a heat treatment of 57°C for 30 minutes OR any other process prescribed by TAD to render the ticks non-infectious

SIGNATURE: pp Abrahams

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

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

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

Professor Luis Neves
Faculty of Veterinary Science
Department of Veterinary Tropical Diseases
Private bag x04,
Onderstepoort,
0110

RE: DISPENSATION ON SECTION 20 APPROVAL IN TERMS OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984) FOR: "SPECIES COMPOSITION AND DISTRIBUTION OF TICKS AND TICK-BORNE PATHOGENS IN RUMINANTS AND DOGS IN TCHICALA-TCHOLOANGA, HUAMBO ANGOLA"

A dispensation is hereby granted on Point 2 of the Section 20 approval that was issued for the above mentioned study (attached):

i) DNA samples will be stored in a -80 freezer located at the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria and will be used for PCR and hybridization techniques in the Molecular Biology Laboratories in the same department.

ii) Stored samples may not be outsourced or used for further research without prior written approval from DAFF

iii) Upon completion of the study, stored samples must be destroyed by incineration with a registered waste disposal/treatment company

Kind regards,

MMaja.

DR. MPHO MAJA
DIRECTOR: ANIMAL HEALTH
Date: 2016 -05- 13

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

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

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

Professor Luis Neves
Faculty of Veterinary Science
Department of Veterinary Tropical Diseases
Private bag x04,
Onderstepoort,
0110

RE: DISPENSATION ON SECTION 20 APPROVAL IN TERMS OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984) FOR: "SPECIES COMPOSITION AND DISTRIBUTION OF TICKS AND TICK-BORNE PATHOGENS IN RUMINANTS AND DOGS IN TCHICALA-TCHOLOANGA, HUAMBO ANGOLA"

A dispensation is hereby granted on Point 2 of the Section 20 approval that was issued for the above mentioned study (attached):

i) DNA samples will be stored in a -80 freezer located at the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria and will be used for PCR and hybridization techniques in the Molecular Biology Laboratories in the same department.

ii) Stored samples may not be outsourced or used for further research without prior written approval from DAFF

iii) Upon completion of the study, stored samples must be destroyed by incineration with a registered waste disposal/treatment company

Kind regards,

DR. MPHO MAJA
DIRECTOR: ANIMAL HEALTH
Date: 2016 -05- 13

8. The ticks and extracted animal DNA may only be released to the researcher once they have both been rendered non-infectious by inactivation processes conducted by TAD.

Title of research/study: Species composition and distribution of ticks and tick-borne pathogens in ruminants and dogs in Tchicala-Tcholoanga, Huambo Angola.
Researcher (s): Prof Luis Neves.
Institution: Department of Veterinary Tropical Diseases
Your Ref./ Project Number:
Our ref Number: 12/11/1/1/9

Kind regards,

DR. MPHO MAJA

DIRECTOR OF ANIMAL HEALTH

Date: 2016 -05- 13

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

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

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

Professor Luis Neves
Faculty of Veterinary Science
Department of Veterinary Tropical Diseases
Private bag x04,
Onderstepoort,
0110

Dear Prof Neves,

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

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

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

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. All potentially infectious material utilised or collected during the study is to be destroyed at the completion of the study. Records must be kept for five years for audit purposes. A dispensation application may be made to the Director Animal Health in the event that any of the above is to be stored or distributed;
3. A veterinary import permit will be required for the importation of ticks from Angola;
4. A veterinary import permit will be required for the importation of extracted animal DNA from Angola;
5. The imported ticks and extracted animal DNA are to be transported from the port of entry directly to the ARC-OVI Transboundary Animal Disease (TAD) Facility under a Red Cross Permit where the following inactivation processes will be conducted;
6. The extracted animal DNA will be heat treated as per the specifications of TAD to render the DNA non-infectious;
7. The ticks, stored in 70% ethanol, will be placed in a water bath and subjected to a thermic treatment of 57°C for 30 minutes or any other process prescribed by TAD to render the ticks non-infectious;