ECOLOGY AND MOLECULAR EPIDEMIOLOGY OF TICK-BORNE INFECTION IN KACHIA GRAZING RESERVE, NIGERIA

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By

BABAGANA MOHAMMED ADAM

School of Environment and Life Sciences, College of Science and Technology University of Salford, Manchester, UK

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Declaration of Originality

I declare that the research described within this thesis is my own work and that this thesis is my own composition and I certify that it has never been submitted for any other degree or professional qualification.

Babagana Mohammed Adam

PhD – The University of Salford – 2019

Abstract

Ticks and tick-borne infections (TBIs) are serious veterinary and medical concerns in much of sub-Saharan Africa (SSA). Despite their economic importance, information on the epidemiology of these infections in many SSA countries, including Nigeria, is lacking or inadequate, resulting in potentially inappropriate disease control strategies being implemented. Therefore, the aim of this thesis was to investigate the diversity and burden of ticks, and the epidemiology of several TBI-causing pathogens (*Anaplasma* spp*., Ehrlichia* spp., *Babesia* spp. *Theileria* spp., *Rickettsia* spp*.,* and *Bartonella* spp.) in Nigerian livestock.

Firstly, the thesis was undertaken by critically reviewing and updating the existent knowledge of the veterinary and medical importance of TBIs, with particular focus on those ticks and pathogens present in SSA. Secondly, two cross-sectional surveys of livestock (cattle, sheep and goats) inhabiting the Kachia Grazing Reserve (KGR) in North Western Nigeria were carried out in which animals were systematically surveyed for TBIs in ticks and blood samples. Thirdly, ticks were identified and TBIs in ticks and livestock blood were detected and identified using molecular methods. Finally, laboratory and field data were collated and analysed using statistical models to quantify the epidemiology of each TBI and clarify epidemiological determinants.

A total of 478 domestic ruminants were randomly selected and surveyed for the presence of ticks. 172 ticks were collected and identified of which most of these ticks 137 (80 %) were identified as *Ambloymma variegatum*, 20 (8.7 %) were *Rhipicephalus* species and 15 (11.6%) *Hyalomma* species. The prevalence of tick infestation was significantly higher on goats than either sheep or cattle. Male animals were found to be statistically less infested by ticks than female ($p<0.006$). In addition, young animals less than (<24 months) were found to be significantly less parasitised than adults animals. Interestingly, ticks were significantly more frequently found on animals with a “good” body condition compared to animals with either a poor or medium body condition. No significant variation in tick infestation rates was observed between KGR blocks. The infection rate of ticks occurred in 20.3% of *Theileria/Babesia* species (*T. ovis, T. velifera* and *B. caballi*), 84% in *Rickettsia* species (*R. africae* and *R. massilae*) and 55.8% for *Ehrlichia/Anaplasma* species (*E. canis, A. platys* and *Anapalsma* spp.).

In cattle, a total of 225/268 (84.0 %) animals were found infected, with 201 (75.0 %) of them being infected by two or more microorganisms, with up to 92 possible combinations of pathogens detected. *Theileria mutans* and *Theileria velifera* was the most prevalent microorganism (69.4% and 69.4%) respectively, followed by (52.4 %), *Theileria equi*-like (56.0 %), *Anaplasma marginale* (54.9 %), *Ehrlichia* spp. (Omatjenne) (50.4 %), *Theileria* spp. MSD4 (36.2 %), *Babesia bovis* (22.8 %), *Babesia bigemina* (16.0 %), *Anaplasma centrale* (7.5 %), *Bartonella* species (3.4 %), *Babesia caballi* (3.0 %), *Rickettsia* species (2.6 %), *Anaplasma phagocytophilum* (1.9 %), and *Ehrlichia ruminantium* (1.1 %). The prevalence of infection was greater in older animals than younger animals, but this difference was not statistically significant ($\chi2 = 3.39$, $P = 0.065$).

In sheep, a total of 178/257 (69.3 %) animals were found infected, with 101 (38.2 %) of them being infected by two or more microorganisms, with up to 36 possible combinations of pathogens detected. *Theileria equi*-like was the most prevalent microorganism (42.4 %), followed by *Rickettsia* species (19.8 %), *Anaplasma centrale* (17.5 %), *Theileria velifera* (12.1 %), *Ehrlichia* spp. (Omatjenne) (10.1 %), *Theileria mutans* (8.9 %), *Theileria* spp. MSD4 (7.0 %), *Babesia bovis* (2.7 %), *Bartonella* species (0.8 %), *Babesia caballi* (0.4 %), *Ehrlichia ruminantium* (0.4 %) and Typhus group (0.4 %). The prevalence of infection was greater in older animals than younger animals, but this difference was not statistically significant ($\chi2 = 2.00$, $P = 0.156$).

In goats, a total of 50/196 (22.5 %) animals were found infected, with 28 (14.2 %) of them being infected by two or more microorganisms, with up to 14 possible combinations of pathogens detected. *Theileria equi*-like was the most prevalent microorganism (14.3 %), followed by *Anaplasma centrale* (13.3 %), *Theileria mutans* (13.3 %), *Theileria velifera* (13.3 %), *Theileria* spp. MSD4 (13.3 %), *Anaplasma marginale* (6.6 %), *Babesia bovis* (5.6 %), *Rickettsia* species (3.1 %), *Babesia bigemina* (2.0 %), *Ehrlichia* spp. Omatjenne (1.5 %), *Bartonella* species (1.0 %) and *Babesia caballi* (1.0 %). The prevalence of infection was greater in older animals than younger animals, a significant difference was observed ($\chi2 = 3.89$, $P = 0.048$)

In conclusion, the outcome of the present study has contributed to the knowledge on the epidemiology of tick-borne diseases in the country and the information will serve as a data baseline to policymakers in making an informed decision in tick control programmes and surveillance of diseases in the study area and the country at large.

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List of abbreviations

ATBF – African tick bite fever

AU-IBAR – African Union-Interafrican Bureau for Animal Resources

BCS – Body condition score

BLAST – Basic local alignment search tool

DNA – Deoxyribonucleic acid

ECF – East coast fever

EDTA – Ethylene diamine tetraacetic acid

ELISA - Enzyme-linked immunosorbent assay

EPNs – Entomopathogenic nematodes

FTA card – Flinders Technology Associates cards

GDP – Gross domestic product

HCT – Haematocrit

HGE – Human granulocytic ehrlichiosis

IFAT – Indirect fluorescent antibody test

IPM – Integrated pest management

KGR – Kachia Grazing Reserve

ml - Millilitres

mPCR – Multiplex polymerase chain reaction

PCR – Polymerase chain reaction

PCV – Packed cell volume

qPCR – Quantitative polymerase chain reaction

RBC – Red blood cell

RLB – Reverse line blot

rRNA – Ribosomal ribonucleic acid

SDS - Sodium dodecyl sulfate

SFG – Spotted fever group

SSPE – Saline-sodium phosphate-EDTA

TBIs – Tick-borne infections

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Chapter 1: Introduction and Literature Review

1.1 Introduction

Ticks and tick-borne infections (TBIs) are the major constraints to the development of livestock sector in sub-Saharan Africa (Minjauw & Mcleod, 2003). Ticks are second only to mosquitoes as vectors of human and animal diseases (Parola & Raoult, 2001). They transmit a considerable array of pathogens, which include protozoa, bacteria, nematodes, and viruses (Parola & Raoult, 2001). Ticks of the genera *Amblyomma, Hyalomma and Rhipicephalus*, sub-genus *Boophilus* are the ticks of greatest medical and veterinary importance (Lorusso et al., 2013). In Nigeria, livestock production plays a significant role in the economy by upgrading the socioeconomic status of small-scale farmers and alleviating poverty (Lawal-Adebowale, 2012). The population of livestock comprises of about 23 million sheep, 28 million goats and 15.2 million cattle (Aregheore, 2009). A significant proportion of this population is farmed in the Northern part of the country. The livestock sector serves as a source of income, employment, food security, protein and nutrients to the country with over 80% of the population engaged in subsistence farming and animal husbandry (Lawal-Adebowale, 2012). In 2009, Aregheore reported that livestock production contributes about 12.7% of the total agricultural Gross Domestic Product (GDP) of Nigeria. Despite its impact on the economy, livestock production has been affected by inadequate food supply, poor animal husbandry practice and ubiquitous parasitism by a wide range of microorganisms including numerous tick-borne haemoparasites (Minjauw & Mcleod, 2003). Tick-associated manifestations include anaemia, stress, loss of productivity, depreciation of hide and skin value, drop in milk and meat yield, toxicosis and hypersensitivity which can predispose animals to secondary infections (Lorusso et al., 2013; Nejash, 2016).

In Nigeria, TBIs pose a significant threat to highly productive animals, especially during the raining season when ticks are most active. Concerning the upgrade of local stock, livestock such as cattle, sheep and goat are highly susceptible when moved from areas that are free from parasites to areas where they are endemic (Allsopp, 2010). Furthermore, the occurrence of TBIs is often neglected even in endemic areas (Bruno Minjauw & Mcleod, 2003). Hence, it is hard to accurately quantify the economic impact of parasitism. Nonetheless, approximations suggest that this impact is huge. In endemic areas, morbidity and mortalities attributed to TBIs were greater by threefold compared to those attributed to other diseases. According to a review by de Castro, (1997), the estimated annual global cost of TBIs in cattle amounted between US$ 13.9 to US$ 18.7 billion. In another report by African Union-Interafrican Bureau for Animal Resources [AU-IBAR], (2013), in Mozambique, Malawi,

South Africa, Botswana, Tanzania, Swaziland and Zimbabwe, the financial cost of heartwater disease alone was estimated around US$ 47.6 million per annum. The greatest elements of the losses in the economy were treatment expenses (5%), milk losses (18%) and acaricide costs (76%) (Mukhebi et al., 1999). In Nigeria, the likely economic losses resulting from TBIs are not yet documented, and existing information on their incidence and prevalence are outdated and mostly derived from research carried out from the southern part of the country (Lorusso et al., 2013; Musa et al., 2014).

Efforts to enhance local breeds via crossing with more productive dairy cattle or small ruminants from regions where TBIs are not endemic have led to increased death rates associated with TBI (Minjauw & Mcleod, 2003). Presently, as there are no registered vaccines for TBIs, their control is dependent on acaricides to hinder their transmission, and/or antibiotics/antiparasitic to hinder their exploitation of reservoir hosts (Manjunathachar et al., 2014). To date, there is very little data on the epidemiology of TBIs in Nigeria. Research focusing on the prevalence and incidence of infections has been hindered by the absence of sensitive and specific tools for diagnosis that is relevant for application in the country, a limitation that is particularly acute for small ruminants. The efficient management of TBIs in Nigeria requires rapid and effective diagnostic tests, which can also serve to monitor the effectiveness of therapeutic and prophylactic measures. The traditional method of TBI diagnosis is based on clinical signs, microscopic examination of blood smears and/or lymph node biopsies and serological methods for the detection of antibodies (Leeflang, 1977; Ajayi & Dipeolu, 1986; Kamani et al., 2010). However, these approaches lack sensitivity and specificity (Salih et al., 2015). Direct cytological diagnosis can be affected by the difficulty of differentiating morphologically several pathogens within the host's biological samples (e.g. blood, lymph node, bone marrow, etc.) via microscopic examination (Salih et al., 2015). Moreover, serological techniques such as the fluorescent antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA), rapid card agglutination test, capillary tube agglutination test can be affected by false negative results, due to cross-reactions among several species of the same taxonomical genus, and also by the inability to differentiate between active and previous infection (Papadopoulos et al., 1996; Salih et al., 2015). A molecular approach to diagnosis is widely considered as a means of circumventing these shortfalls thereby offering a specific and sensitive way of determining an animal's current infection status. Numerous PCR-based methodologies have been described, some species-specific, others more generic (Roux & Raoult, 2000; Bock et al., 2004; Salih et al., 2015).

In summary, controlling TBI is likely to lead to a significant improvement in the productivity of livestock in Nigeria. However, the nature of this control cannot be determined until a reliable and comprehensive understanding of the epidemiology of TBIs in the country has been achieved. Accurate diagnosis of TBIs is essential for this goal and a molecular approach currently appears to offer the best alternative for this.

1.2 Literature review

1.2.1 Tick taxonomy

Ticks are obligate blood-sucking ectoparasites of vertebrates (including humans and livestock) throughout the world. Ticks are contained with the order Ixodida, which is taxonomically delineated into three families; *Ixodidae* (hard ticks), which contains 14 genera and about 700 species; *Argasidae* (soft ticks), which contains five genera and about 200 species; *Nuttalliellidae*, which contains only a single species (Walker et al., 2003). Ixodida is one order within the class Arachnid, which also includes spiders, scorpions and mites.

1.2.2 Morphology

Ticks can be easily distinguished from adult insects because the tick body is not divided into three sections; rather there is a strong fusion of the body segments giving the tick body a sack-like appearance. In common with most arachnids, the adult and intermediate nymphal life stage have eight legs, although larvae have six legs like adult insects. Tick mouthparts and the basis capituli form the capitulum. All nymphal and adult ticks have a pair of spiracles located latero-ventrally on the abdomen. Depending on the species, a pair of simple eyes may be present (Walker et al., 2003) (see Figure 1:1).

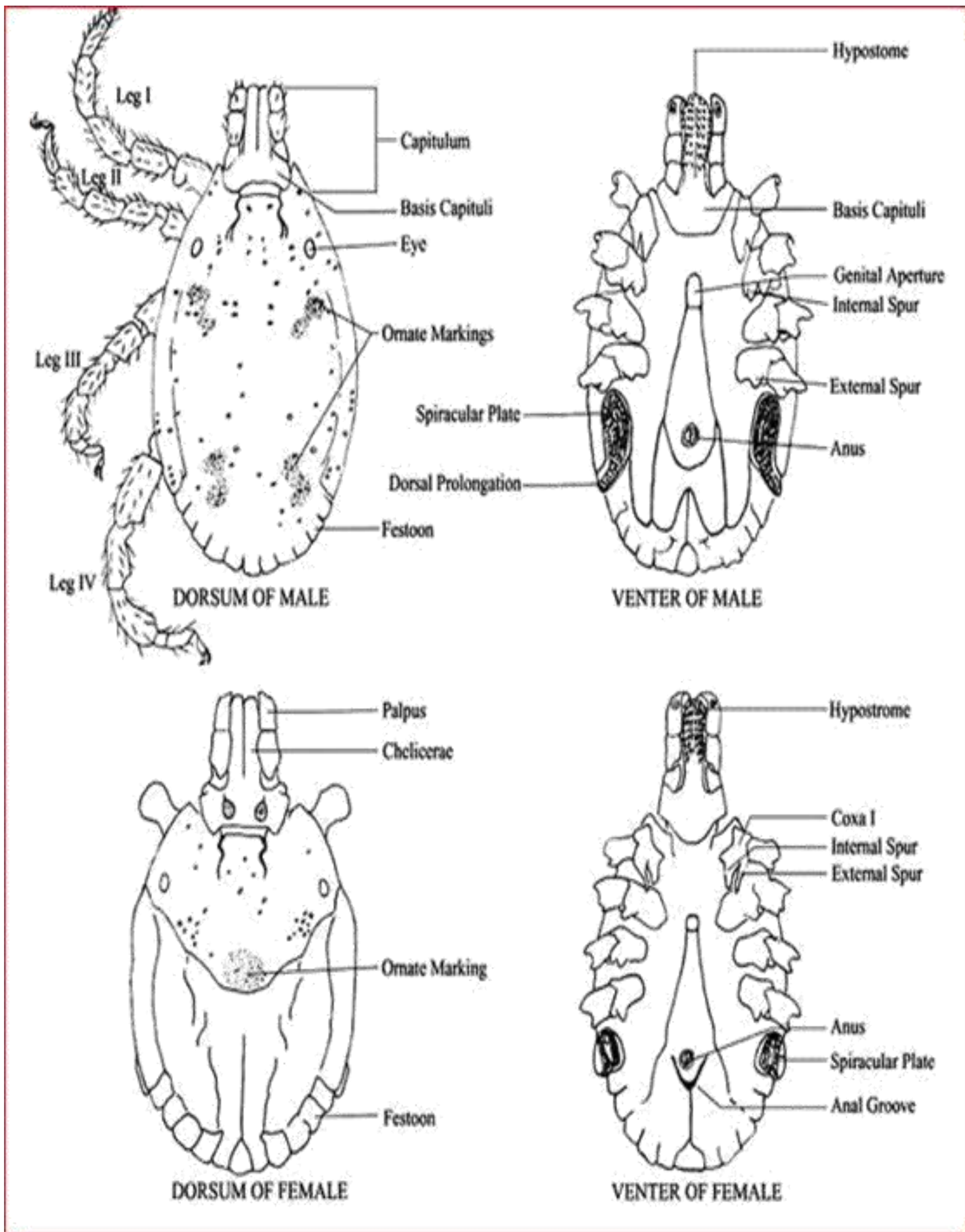


Figure 1:1: Key morphological features of male and female hard ticks. Source: Walker et al., (2003)

1.2.3 Family *Argasidae* (soft ticks)

Members of the family *Argasidae* occur throughout most of the tropical and subtropical and some of the temperate areas of the world. Three genera, *Argas, Ornithodoros* and *Otobius*, which contain 183 species and subspecies are of medical and veterinary importance (Klompen & Oliver, 1993). *Argasidae* is characterised by the absence of scutum; the mouthparts are situated anteriorly on the ventral surface and are not visible from the dorsal side. Eyes are present or absent. There are two pairs of eyes situated laterally in supra coxal folds in some species of *Ornithodoros*. There is one pair of spiracles located between the third and the fourth coxa. Sexual dimorphism is not marked; however, this can be differentiated by the size of the genital opening; being large in females and small in the males. Male and female usually mate outside the host. Eggs are laid in several batches of hundreds and the females brood the eggs until they hatch into larvae. There are usually two or more nymphal stages. Larvae, nymphs and adults, depending on different genera, feed repeatedly. The adult females usually lay eggs after each blood meal except in the case of female *Otobius*, which does not feed on blood. They usually infest nests burrows and buildings and attach to sleeping hosts (Sonenshine & Roe, 2014).

1.2.4 Family *Ixodidae* (hard ticks)

Members of the family *Ixodidae* are also encountered in tropical, sub-tropical and temperate regions of the world. Unlike *Argasidae*, *Ixodidae* possess a scutum which covers either the entire (males) or most of the (females) dorsal surface. The mouthparts (capitulum) project forwards and are visible from the dorsal view. Larvae have three pairs of legs and nymphs have four pairs of legs and are lacking porous areas, scutum and genital openings (Fischer, 2009). Scutum size remains constant during engorgement of females and thus covers a progressively smaller proportion of the dorsum. Eggs are laid in a single batch of thousands. There is only one nymphal stage. Larvae, nymphs and adults feed only once in each stage and require as long as several days to complete engorgement (Fischer, 2009).

1.2.5 Life cycles of hard ticks

The life cycles of the *Ixodidae* can be divided into three types according to their feeding behaviours.

One host tick: Where all the three instars (larva, nymph and adult) feed on one host to complete their life cycles. Male and female copulate on the host during the feeding process. The engorged female then drops to the ground to lay eggs. Eggs are laid as one batch of large quantity and the female afterwards die a natural physiological death. Examples of this type are the species of *Boophilus* (Walker et al., 2003; Sonenshine & Roe, 2014) (see Figure 1:2)

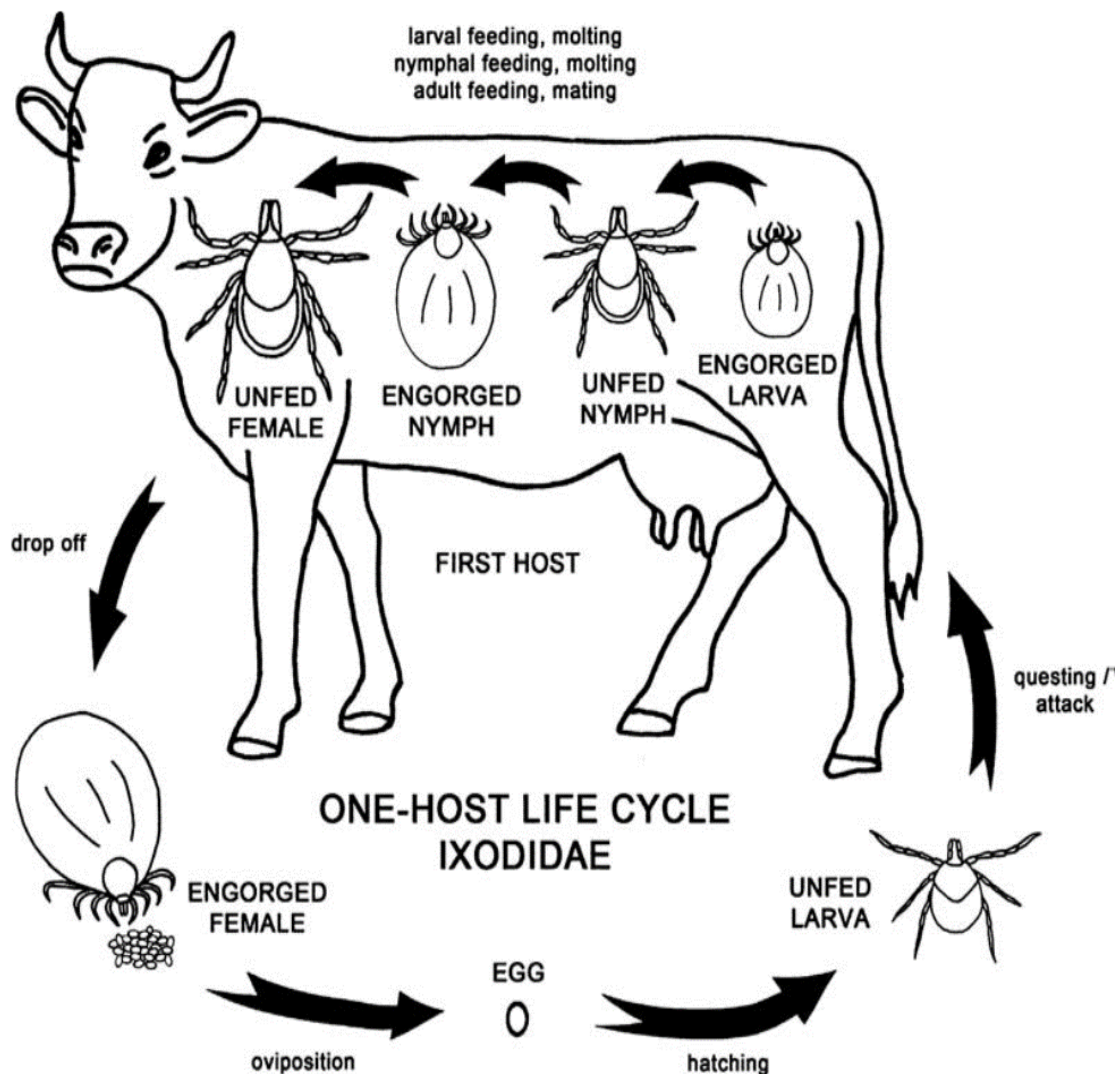


Figure 1:2: Life cycle of one host Ixodidae ticks

Two host tick: In this type, the larvae attach to the host, feed, engorge and moult into nymphs. The nymphs feed on the same host, engorge and drop to the ground to moult into adults. The adult male and female attach to a different host and copulate during the feeding process. The engorged female drops to the ground to lay eggs and after that dies a natural death. An example of this type is *Rhipicephalus evertsi* and *R. bursa* (Walker et al., 2003) (see Figure 1:3).

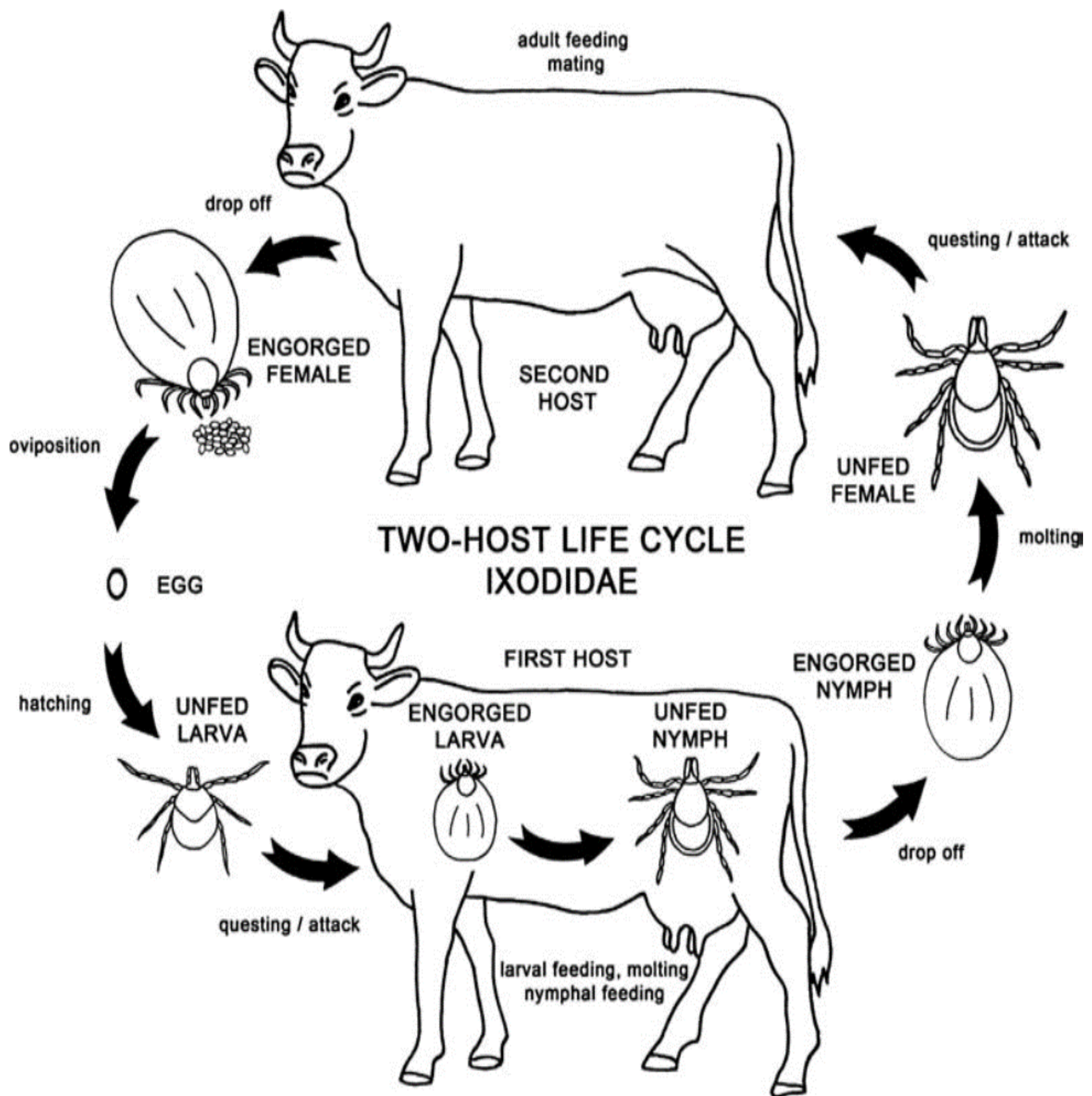


Figure 1:3: Life cycle of two host Ixodidae ticks

Three host ticks: In this type, the larvae attach to the host feed, engorge and drop to the ground to moult into nymphs. The nymphs attach to a second host feed, engorge and drop to the ground to moult into adults. The adult male and female ticks attach to the third host and copulate during the feeding process. The engorged female drops to the ground to lay eggs. The females after laying eggs die a natural physiological death. Examples of this type of tick are all *Amblyomma* species, *Haemaphysalis* and most *Rhipicephalus* and *Hyalomma* species (Sonenshine & Roe, 2014) (see Figure 1:4).

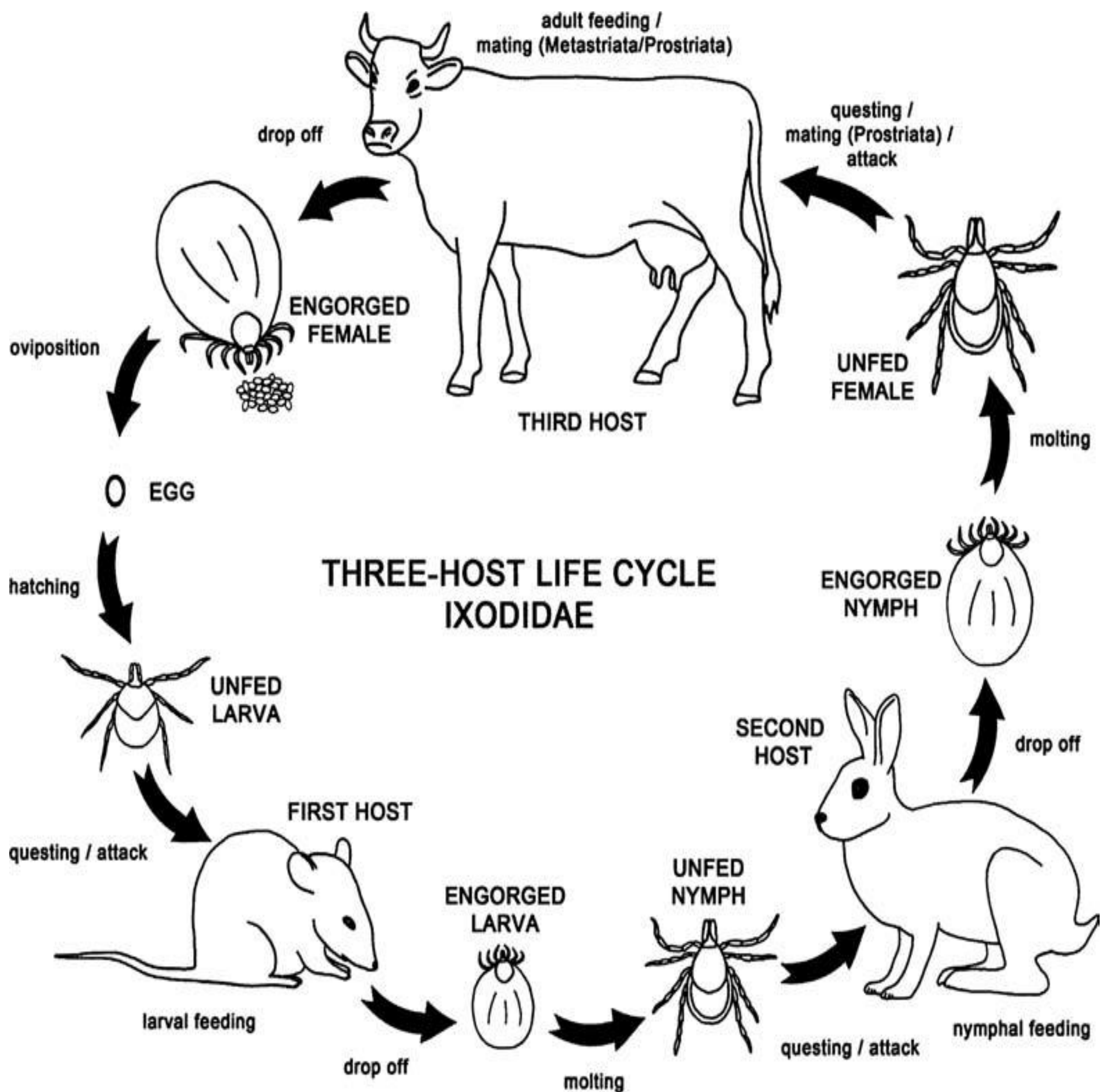


Figure 1:4: Life cycle of three host Ixodidae ticks

1.2.6 Tick ecology in sub-Saharan Africa

Several factors influence the spread and distribution of ticks in sub-Saharan Africa these include climate changes, changes in land use, movement of animals and importation of exotic and wildlife species. The climatic variations are the main factors, which determine the ecological and geographical distribution of ticks. Different species of tick are adapted to a certain range of temperature and moisture. Some ticks occur only in warm regions with a fair degree of humidity, while ticks in temperate areas become more active during the summer season. Changes in the climatic situation may considerably cause variations in the geographical distribution of ticks (Kovats et al., 2001). However, the activity of ticks within their habitats is influenced by local environmental factors such as temperature, relative humidity and rainfall.

Temperature and relative humidity are important factors in regulating the life cycle of ticks. The attachment of *Boophilus microplus* larvae onto a host has been shown to be promoted at temperatures between 31-38°C (Doube & Kemp, 1979). However, a decrease in temperature prolonged egg incubation, larval and nymph pre-moulting periods and female pre-oviposition periods of *Hyalomma dromedarii* (Hagras & Khalil, 1988). It means that the temperature has a strong correlation with tick activities by initiation and termination of host-seeking by an individual tick. As a result, the maximum and minimum temperatures directly influence the development of ticks.

The relative humidity, on the other hand, is an important determinant for the survival of ticks by regulating the water balance and preventing dehydration (Parola & Raoult, 2001). High humidity is particularly important for the survival of Ixodid ticks rather than Argasid ticks. Ixodid ticks quickly die of dehydration when conditions drop below the relative humidity at which ticks can absorb water from the air (critical equilibrium humidity 75–95%, depending on life stage), ticks cannot absorb water vapour and begin to dehydrate (Yoder et al., 2012). According to Schulze, Jordan, & Hung (2001), *Ixodes scapularis* tended to quest earlier and later in the day when temperatures were low and the relative humidity higher. Hence, humidity plays an essential role in the tick’s activities and survival. Moreover, Meyer-König, Zahler, & Gothe (2001) found that *Dermacentor reticulates* and *D. marginatus* ticks compensated their water losses during the subsequent incubation at 95% relative humidity. Similarly, different stages of *H. anatolicum* were able to take up water vapour at higher humidity and steadily lose it at lower humidity (Walker et al., 2014).

Rainfall is another factor which has a significant role in tick ecology and distribution throughout the world. The effect of the rainfall on tick challenge to their hosts was investigated at Kyle Recreational Park in Zimbabwe. Mooring, Mazhowu, & Scott (1994) found that *R. appendiculatus* adult infestation on the host was 2-3 times greater during the high rainfall. They concluded that the tick burden on hosts is high during the wet season due to high rainfall. Many species of ticks are adapted to seasonal variations in climate within their geographical range. In the tropics, this is usually to overcome the adverse effects of prolonged dry seasons (Walker et al., 2003). Dry environmental conditions are a serious danger to ticks, particularly to the questing larvae which are very susceptible to drying out fatally (Ikpeze et al., 2015). The survival of many species is improved if they have a seasonal cycle which reduces these risks. For example, *A. variegatum* is generally recorded during the wet season (Petney et al., 1987). Previous studies report the occurrence of only one generation of *A. variegatum* per year in Nigeria, with adults being most abundant between May and June, and nymphs between December and February (Bayer & Maina, 1984). Adults of both *H. rufipes* and *H. truncatum* are most numerous during the late wet season, whereas the immature stages are most prevalent in the dry months (Walker et al., 2003).

1.2.7 The veterinary and medical importance of ticks

Ticks infestation is a significant burden to "the livestock sector" in sub-Saharan Africa. They may be detrimental to animal health in a variety of ways:

1.2.7.1 Direct effect

Feeding by large numbers of ticks retards weight gain and can compromise milk production in domesticated animals (Peter et al., 2005), while tick bites also reduce the quality of hides (Walker, 1996). Very heavy tick infestations can cause severe dermatitis.

Tick-bite paralysis: It is characterized by an acute ascending flaccid motor paralysis caused by the injection of a toxin by certain ticks while feeding. Examples are paralysis caused by the feeding of *D. andersoni*, sweating sickness caused by *H. truncatum*, and tick toxicosis caused by *Rhipicephalus* species (Rajput et al., 2006). Tick paralysis is most common when the adult ticks are active, but it can occur at any time if the weather is warm and humid (Viljoen et al., 1986).

1.2.7.2 Indirect effect – vector of parasites

Ticks act as vectors for parasites that they transmit from host to host during blood feeding. A wide variety of protozoa, bacteria and parasites are tick-transmitted and those of greatest veterinary importance in sub-Saharan Africa include babesiosis, anaplasmosis, theileriosis (East Coast fever) and heart-water (Jongejan & Uilenberg, 2004). The presence, dynamics and intensity of parasite in ticks exert a major influence on the kinetics of transmission of tick-borne diseases (Morel, 1980). Generally, ticks become infected with parasites by feeding on infected animals. The parasite is then maintained transstadially by the tick and is transmitted to a susceptible host when the next life stage takes a blood meal. In some cases, parasites are passed from infected adult female ticks to their eggs, thus larvae can be infectious (Rajput et al., 2006).

1.2.8 Livestock ticks in Nigeria

In Nigeria, three genera of ticks are usually found infesting livestock (cattle, sheep and goats), namely *Amblyomma, Hyalomma* and *Rhipicephalus* spp. (*Boophilus* sub-genus included) (Bayer & Maina, 1984; Walker et al., 2000). An infestation of the same host by several species occurs commonly, although each species load of infestation varies according to the seasons (Bayer & Maina, 1984). Usually low in the dry season (i.e. November to April), tick loads tend to increase after the first scattered rains, reaching the highest abundance one month after the heavy rains (i.e. between July and September) (Bayer & Maina, 1984).

1.2.9 *Amblyomma variegatum*

A. variegatum is a member of the family Ixodidae (hard ticks). Hard ticks have a dorsal shield (scutum) and their mouthparts (capitulum) protrude forward when they are seen from above (Walker et al., 2003). *Amblyomma* ticks are large ticks with long, strong mouthparts. The palps are long; the second segment is twice as long as it is wide. Eyes are present, and the festoons are well developed (Walker et al., 2003). The males have no adanal shields, accessory shields or subanal shield. Female *A. variegatum* are brown, but the males are brightly ornamented with orange. When they are engorged, the adult female ticks are about the size of a nutmeg (approximately 2 to 3 centimetres long) (Walker et al., 2003) (see Figure 1:5).

Figure 1:5: Adult *Amblyomma variegatum* (a) male and (b) female. Bar = 1mm

A. variegatum is a three-host tick. Immature ticks feed on small mammals, ground-feeding birds and reptiles, as well as cattle, sheep and goats (Walker, 1996). Adult ticks prefer cattle but can also be found on other livestock including camels, as well as dogs and some wildlife (Hoogstraal, 1956). The adult ticks are usually found on the relatively hairless parts of the body; most are located on the ventral body surface and the genitalia, or under the tail (Hoogstraal, 1956; MacLeod et al., 1977). Adult *A. variegatum* feeds mainly in the "rainy season" (May and June), while the immature ticks feed primarily during the dry season (December and February) (Bayer & Maina, 1984; Petney et al., 1987). *Amblyomma variegatum* occurs in areas with a wide variety of climates, from the rain forest, temperate (Highland), savanna through to steppe. It is widely distributed through West, Central and East Africa and in southern Africa extends into Zambia, North Eastern Botswana, the Caprivi Strip of Namibia, North Western Zimbabwe and Central and Northern Mozambique. Its spread southward appears to be limited by interspecific competition with *A. hebraeum* with which it shares similar habitats, hosts and sites of attachment. On cattle transportations, it has spread to most of the Caribbean islands and to Madagascar (Walker et al., 2003).

The long mouthparts of *A. variegatum* leave large wounds and make this tick difficult to remove manually (Walker et al., 2003), and its bite is severe and painful and can result in significant damage to the skin (Walker, 1996). Secondary infections can cause septic wounds or abscesses, and inflammation on the teats of cows may affect milk production (Bruno Minjauw & Mcleod, 2003). In some regions, *A. variegatum* bite wounds may become infested by *Chrysomya bezziana* (screwworms). *A. variegatum* is the vector of a range of parasites, including the bacteria *Ehrlichia ruminantium* (formerly *Cowdria ruminantium*) (Oberem & Bezuidenhout, 1987; Walker & Olwage, 1987; Deem et al., 1996), the agent of

heartwater, and *Rickettsia africae* (Socolovschi et al., 2009), the agent of African tick bite fever, which is an emerging zoonosis in rural sub-Saharan Africa and the Caribbean. *A. variegatum* has been found to be naturally infected with Crimean Congo haemorrhagic fever (CCHF) virus worldwide (Hoogstraal, 1979; Shepherd et al., 1989) and experimental transmission of the yellow fever virus was also demonstrated for the first time by (Cornet et al., 1982). Jos virus originally isolated from cow serum in Nigeria in 1967, and was then isolated from *Amblyomma* and *Rhipicephalus* (*Boophilus*) ticks in sub-Saharan Africa which are characterized by acute cell necrosis in the liver, lymph nodes, bone marrow and spleen (Lee et al., 1974; Bussetti et al., 2012). Immature ticks feed on small mammals, ground–feeding birds, reptiles, cattle, sheep and goats. Adult ticks prefer cattle, but can also be found on sheep, goats, horses, camels, dogs and some large wildlife including antelope (Walker et al., 2003).

1.2.10 *Hyalomma* species

The hard tick genus *Hyalomma* is a complex of a few species that exhibit an almost endless variation of morphology. For identification purposes, there is a need to consider only the differential features not subject to change on an individual basis; these are mainly seen in replete ticks (Hoogstraal, 1956). Adult *Hyalomma* ticks share the following common features: a basis capituli that is usually rectangular, clearly wider than long; a long hypostome with a rounded tip and 3/3 denticles; long palps with the 2nd article longer than the 3rd; shiny eyes with a surrounding orbit (a circular groove surrounding the eye), which are often convex and placed in the confluence between the anterior and the posterior edges of the scutum in females and in the corresponding place in males; a dark scutum rich in punctations and grooves; festoons that are more evident in unfed ticks; long, strong and banded legs with two large spurs on the first pair of coxae; and adanal, subanal and accessory plates in the male. In most cases, it is not possible to identify immature stages to the species level using only morphology (Walker et al., 2003).

There are about 30 species of *Hyalomma* most of which have a 3-host life cycle, but some have either a 1-host or 2-host life cycle (Magano et al., 2000). They may be the most abundant tick parasites of livestock in warm arid and semi-arid habitats. Several are of considerable veterinary or public health importance (Walker et al., 2003). Two *Hyalomma* species (*H. rufipes* see Figure 1:6 and *H. truncatum* Figure 1:7) have been reported in

Nigeria (Bayer & Maina, 1984; Lorusso et al., 2013).

Figure 1:6: Adult *Hyalomma rufipes* (a) male and (b) female. Bar = 1mm

H. rufipes is a two-host tick. The larva attaches to the first host (a bird), feeds, and then moults to a nymph while on the host. After engorgement at the nymphal stage, the ticks drop off from the bird and moult on the ground before attaching to their second and final host which is usually a large mammal (Walker et al., 2003). It transmits the bacterium *A. marginale* to cattle causing bovine anaplasmosis (Potgieter, 1979), the bacterium *R. conorii* causing tick typhus in humans (Parola, 2006) and the protozoan *Babesia occultans* to cattle (Blouin & van Rensburg, 1988). The feeding of adults on cattle causes large lesions at the attachment sites, leading to the formation of severe abscesses

Figure 1:7: Adult *Hyalomma truncatum* (a) male and (b) female. Bar = 1mm

H. rufipes is widely distributed in much of sub-Saharan Africa and has been recorded from every climatic region from desert to rain forest. However, the distribution is patchy, and it is probably commoner in the drier areas. The infestation of birds by the immature stages of this tick contributes to its extensive distribution. It also occurs in southern Europe and extends eastwards to central Asia (Walker et al., 2003). While *H. truncatum* this species of tick is adapted to dry habitats and is commonest in the desert, steppe and savanna climatic regions, but is also recorded from temperate (Highland) climates. This tick is endemic to the Afrotropical zoogeographical region and thus is generally restricted to areas south of the Sahara although it has been recorded from Northern Sudan and from Egypt. In sub-Saharan Africa, *H. truncatum* is very widespread and often common but at a local level, its abundance may be influenced by the abundance of hares that are the preferred hosts of the immature stages. *Hyalomma albiparmatum* occurs in southern Kenya and Northern Tanzania.
H. truncatum contains a toxin in their saliva that causes sweating sickness, acute dermatitis in cattle, particularly calves. The condition can be reversed when the tick, often attached to the tip of the tail, is removed. Its attachment to the lower legs and feet of lambs can lead to lameness (Fourie & Kok, 1995). Both *Hyalomma truncatum* and *H. rufipes* can transmit Crimean Congo Haemorrhagic fever virus to human (Salman et al., 2012) and *R. conorii* to humans (Parola, 2006). *H. truncatum* transmits *Babesia caballi* the cause of equine piroplasmosis. Because the immature stages of *H. truncatum* do not feed on horses the transmission of *B. caballi* by this tick has of necessity to take place both transstadially and transovarially and multiply in ticks for multiple generations (Kaufmann, 1996).

1.2.11 *Rhipicephalus* (*Boophilus*) species

The genus *Rhipicephalus* comprises 70 species (excluding *Boophilus* spp.) and is a member of the family Ixodidae (hard ticks). These small to medium sized ticks with short, broad palps are usually inornate with eyes and festoons (Walker et al., 2003). Most *Rhipicephalus* spp. are found on mammals on the African continent.

These are usually three-host ticks, although some have a two-host cycle (e.g. *R. evertsi)* (Jongejan & Uilenberg, 2004). *R. appendiculatus,* the brown ear tick, is the most important rhipicephalid tick where it occurs on a wide variety of domestic and wild ruminants. This tick prefers to feed on the ears of the hosts in the adult stage (Walker et al., 2003). *Boophilus* are one-host ticks, which take about three weeks to complete their blood meal, preferably on cattle (Jongejan & Uilenberg, 1994). Although *Boophilus* ticks have short mouthparts,

damage to hides is considerable as the preferred feeding sites are often of good leather potential. *B. microplus* is the most important species (Walker et al., 2003). Apart from the direct damages, the veterinary importance of *Boophilus* ticks is mainly associated with their capacity to act as vectors of *Anaplasma* spp. and *Babesia* spp. (Hodgson, 1992; Samish et al., 1993).

In Nigeria, thus far three *Boophilus* species have been found infesting livestock, namely *Rhipicephalus* (*Boophilus*) *decoloratus* (see Figure 1:8), *Rhipicephalus* (*Boophilus*) *annulatus* and *Rhipicephalus* (*Boophilus*) *geigyi* (Lorusso et al., 2013). For all three species, cattle are considered as the host species responsible for the maintenance of their population in a given geographic area (Walker et al., 2003). Nevertheless, these boophilids may also successfully accomplish their life cycles on small ruminants and wild ungulates (Walker et al., 2003).

Both *Rh.* (*Bo.*) *decoloratus* and *Rh.* (*Bo.*) *annulatus* are known as vectors of *Anaplasma marginale*, *Anaplasma centrale* and *Babesia bigemina* (Bock et al., 2004; Samish et al., 1993). *Rhipicephalus* (*Bo*.) *decoloratus* transmits the protozoan *B. bigemina*, causing bovine babesiosis (redwater) in cattle. The *Babesia* is transmitted only by the nymph and adult after it has passed transovarially from the previous generation of ticks. Once established in the tick host, *B. bigemina* can be transmitted by many successive generations without their acquiring new infections. This tick transmits the bacteria *Anaplasma marginale,* the cause of bovine anaplasmosis and *Borrelia theileri*, the cause of spirochaetosis in cattle, sheep, goats and horses.

Figure 1:8: Adult specimens of *Rhipicephalus* (*Boophilus*) *decoloratus:* male (a) female (b). Bar = 1mm

1.2.12 Economic impact of TBIs

Globally, TBIs continue to be a major constraint on profitable livestock production and productivity. Recently, TBIs were again ranked high globally in terms of their impact on the livelihood of resource-poor farming communities in developing countries (Minjauw et al., 2000). TBI affects 80% of world cattle population, but are of particular threat in tropical and sub-tropical countries (FAO, 2011). De Castro estimated that the annual global costs associated with TBIs in cattle amounted between US$ 13.9 to US$ 18.7 billion (De Castro, 1997). In Australia alone, losses due to cattle tick *B. microplus* were estimated to be US$ 62 million and in Brazil, losses were around US$ 2 billion per year (Manjunathachar et al., 2014). In Africa, TBIs are considered to be the most important problems in animal production (Minjauw & Mcleod, 2003). In India, the economic losses due to TBIs in animals were calculated as US$ 498.7 million per annum (Singh et al., 2014). Bovine tropical theileriosis caused by the protozoan parasite *T. annulata* is transmitted by the tick species of the genus *Hyalomma* worldwide, putting about 250 million cattle at risk to this important protozoan disease (Gharbi et al., 2006). The estimated loss due to *T. annulata* and tick worry worldwide was US$ 384.3 million (Mondal et al., 2013). TBIs, directly or indirectly affect the growth of the livestock industry, which is of fundamental importance to rural people in Nigeria. Livestock production are the source of income to small hold/landless farmers and ensure food supply and income during the quiescent period the agriculture (Ghosh et al., 2007). Accurate estimation of losses due to TBIs is very difficult, but they significantly affect the farm income. In cattle, tick infestation alone can cause anaemia, stress, reduction in weight gain and milk yields, depreciation of hide value, hypersensitivity and toxicosis, leading also to secondary infections (Jongejan & Uilenberg, 2004). In crossbred Holstein-Zebu cows when infested with an average of 105 ticks, a reduction in 23% of milk yield/day was observed, losing about a quarter of income through milk loss (Singh et al., 2014). Frisch et al., (2000) reported that animals with an average of 40 ticks/day could lose weight of up to 20 kg/year and also reduce hide value by 20%-30%.

1.2.13 Public health impact of ticks

Tick-borne diseases of humans represent a public health burden worldwide. Although some human TBIs have long been recognised, awareness of them has increased dramatically since the discovery of Lyme borreliosis (LB) in the early 1980s (Burgdorfer et al., 1982). Each year, approximately 30,000 cases of LB are reported to the centre for disease control (CDC) by state health departments (CDC, 2019), as well as human ehrlichiosis and babesiosis (Jongejan & Uilenberg, 2004). Since there are no ticks specific for humans, there is normally no human to human transmission. However, an interesting exception is relapsing fever in Africa, caused by *Borrelia duttoni* and transmitted by soft ticks of the *Ornithodoros moubata* group in human dwellings. In fact, in large areas of Africa and elsewhere, soft ticks transmit *Borrelia* spirochetes to humans inducing relapsing fevers, which may be confused with malaria and these are certainly much underestimated human tick-borne diseases in terms of morbidity and impact on public health (Dworkin et al., 2008).

Lyme disease (LD) or Lyme borreliosis is a multisystemic disease caused by spirochetes belonging to the *Borrelia burgdorferi sensu lato* (*sl*) complex. In Europe, these spirochetes are transmitted by *Ixodes ricinus* (Gern et al., 2010). *B. burgdorferi s.l.* complex constitutes a group of 15 different *Borrelia* species and among them, 7 species like *B. burgdorferi sensu strict* (ss), *B. garinii, B. afzelii, B. valaisiana, B. lusitaniae, B. spielmanii* and *B. bissettii* have been identified in the ticks in Europe (Gern et al., 2010). Infection usually occurs when the ticks finish the feeding (not earlier than 24 hours after penetration into the skin), usually on the third day (Wesołowski et al., 2014). Considering the risk of the transmission of the pathogens in forestry workers, Cisak et al., (2012) evaluated the exposure to infection with tick-borne pathogens. They based their results on epidemiological investigation and serological tests. The result of their study clearly indicated that the wood-cutters are at the greatest risk of tick bites and transmission of tick-borne pathogens, including *Borrelia burgdorferi* spirochetes (Cisak et al., 2012). Bartosik et al., (2011) performed research in south-eastern Poland (Lublin Provence) in areas of high agricultural and recreational value and they revealed a high rate of infection of *I. ricinus* ticks with different pathogens. Furthermore, they found that the probability of infection with *Borrelia burgdorferi* spirochetes is higher in humid mixed forests than in coniferous forests (Bartosik et al., 2011).

Human granulocytic anaplasmosis (formerly known as ehrlichiosis) is a febrile illness, caused by *Anaplasma phagocytophilum* Gram-negative intracellular bacterium (Wesołowski et al., 2014). *A. phagocytophilum* is an emerging tick-borne pathogen with veterinary and public health significance (Yang et al., 2017). Since the first description of the agent in 1932, many wild and domestic animals have been considered as the reservoir hosts for *A. phagocytophilum* (Rar & Golovljova, 2011; Yang et al., 2017). This disease was first identified due to a Wisconsin patient who died with a severe febrile illness two weeks after a tick bite. During the last stage of the infection, a group of small bacteria were seen within the neutrophils in the blood. Other symptoms include fever,
headache, leucopenia, thrombocytopenia and mild injury to the liver (Dumler et al., 2005). This organism causes lameness which can be confused with symptoms of Lyme disease. *A. phagocytophilum* is a vector-borne zoonotic disease whose morula can be visualized within neutrophils (a type of white blood cell) from the peripheral blood and synovial fluid. It can cause lethargy, ataxia, loss of appetite, and weak or painful limbs. So far, no reports of clinical cases associated with *A. phagocytophilum* have been published in Nigeria. Very few studies have reported its occurrence in animals and ticks within the Africa continent. *A. phagocytophilum* has also been detected in ticks in Africa: in questing *Rhipicephalus pulchelus* in Ethiopia (Teshale et al., 2016) and in *I. ricinus* in Tunisia (Sarin et al., 2005).

Babesiosis is a human disease caused by parasitic protozoa of the genus *Babesia*. This disease is an example of a world-wide emerging zoonosis (Gray et al., 2010). The manifestation of babesiosis ranges from asymptomatic infection to malaria-like disease, resulting in severe haemolysis and occasionally death (Welc-Falêciak et al., 2012). The disease is known to be especially dangerous for immunocompromised individuals (Welc-Falêciak et al., 2012). *Babesia* spp. multiply in red blood cells by asynchronous binary fission, resulting in considerable pleomorphism (Gray et al., 2010). Asymptomatic *Babesia* invasion in humans may persist for months or even years. In this form of diseases, the parasites may be transmitted during a blood transfusion. The first human case of babesiosis was identified in 1957 near Zagreb, Croatia (Skrabalo & Deanovic, 1957). A young farmer had been grazing cattle on tick-infested pastures and presented with fever, anaemia and haemoglobinuria. He was asplenic and died of renal insufficiency during the second week of illness. In a functioning spleen, blood is filtered, removing old red blood cells, and phagocytoses infected erythrocytes, which can defend against intraerythrocytic parasites. Thus, parasitic infections such as babesiosis and malaria are more common in patients with

asplenia. Further, babesiosis puts asplenic patients at risk due to autoimmune haemolytic anaemia after the infection resolves. Initially reported as *Babesia bovis*, the agent most likely was *Babesia divergens*, another pathogen of cattle. In 1968, *B. divergens* was confirmed as the etiologic agent in an asplenic person infected while vacationing in the Irish countryside (Fitzpatrick et al., 1968). While these asplenic cases were attracting the attention of physicians in Europe, babesiosis was diagnosed in several residents of Nantucket Island, off the coast of Massachusetts. The causative agent was determined to be *Babesia microti*, which typically infects mice and other small rodents (Western et al., 2010). Spielman and colleagues subsequently identified the vector as *Ixodes dammini* (also known as *I. scapularis*) and recognized the white-tailed deer (*Odocoileus virginianus*) as an important natural host (Spielman, 2002). In the 1990s, *Babesia duncani* (WA1) was identified in human cases reported from the Northern Pacific coast, (Persing et al., 2002) and a *B. divergens*-like organism in a case from Missouri (MO1) (Herwaldt et al., 1996). Another *Babesia* spp. was identified in asplenic patients from the Tyrol region of Austria and the Alpine region of Italy in 2003 (Herwaldt et al., 2003). They experienced a severe illness caused by *B. venatorum*, a species closely related to *B. odocoilei* and known to infect white-tailed deer. Additional babesial species infecting humans have been identified in Taiwan (TW1) (Shih et al., 1997) and Korea (KO1) (Kim et al., 2007). Initially diagnosed in Europe and North America, human babesiosis is now reported from around the world

Borrelia species are tick-borne, Gram-negative, spiral-shaped bacteria that causes several diseases across the world, grouped into Lyme borreliosis (LB) and tick-borne relapsing fever (TBRF) (Socolovschi et al., 2013). TBRF is a bacterial febrile illness caused by the spirochaete *Borrelia* (Vial et al., 2006). Tick-borne relapsing fever is endemic and an important public health problem in some parts of the world. In Western Africa, the incidence of human TBRF is high, accounting for about 13% of febrile illnesses (Parola et al., 2011). In endemic regions of East Africa, TBRF borreliosis is one of the highest ranked causes of mortality among children (Talbert et al., 1998). *Borrelia theileri* transmitted by hard-bodied ticks including *Rhipicephalus (Boophilus)* is the causative agent of bovine borreliosis. The disease has also been reported from cattle, sheep and horses from several countries in Africa, South America, Europe and Australia (Uilenberg et al., 1988; McCoy et al., 2014). It is often associated with babesiosis. The clinical signs observed include fever, haemoglobinuria, loss of appetite, diarrhoea, pale mucous membranes, enlarged superficial lymph nodes and rough

hair coats (Sharma et al., 2000). Clinically infection is usually benign hence underreported especially if there is a mixed infection.

African tick-bite fever (ATBF) is an emerging infectious disease endemic in sub-Saharan Africa and the most commonly encountered rickettsiosis in travel medicine (Raoult et al., 2001). It is an acute, influenza-like syndrome that includes symptoms such as fever, nausea, headache, and myalgia (Jensenius et al., 2003), and is caused by the spotted fever group *Rickettsia*, *Rickettsia africae. R. africae* is a gram-negative obligately intracellular bacterium that is transmitted to humans through the bite of an infected tick. The first human case of ATBF was reported in 1992 (Kelly et al., 1992). Since then, *R. africae* has been detected or isolated from ticks or humans in 15 countries in Africa (Jensenius et al., 2003; Ndip et al., 2004). In rural central Africa, *R. africae* is transmitted by *Amblyomma variegatum* ticks, which serve as vectors and reservoirs (Kelly et al., 1996). *R. africae* infection in *Amblyomma* ticks is frequently high with a prevalence of up to 100% reported in ticks obtained in some disease-endemic countries (Parola & Raoult, 2001). Almost all case reports of *R. africae* come from industrialised countries and the true burden and diversity of TBIs in humans in SSA is entirely unknown – the recent work looking at *R. africae* exposure rates is the first to specifically attempt to address this shortfall.

1.3 Tick-borne pathogens of veterinary importance in sub-Saharan African

The tick-borne diseases of importance to livestock in sub-Saharan Africa reviewed in this thesis are caused by bacterial (ehrlichiosis, anaplasmosis, and spotted fever group rickettsiosis and bartonellosis) and protozoan (theileriosis and babesiosis) parasites (Minjauw & Mcleod, 2003; Jongejan & Uilenberg, 2004; Kamani et al., 2013).

1.3.1 *Ehrlichia ruminantium*

Ehrlichia ruminantium (formerly called *Cowdria ruminantium*) is an obligate intracellular bacterium that parasitizes vascular endothelial cells, neutrophils and macrophages of the mammalian host and causes disease in ruminants called heartwater (or cowdriosis) (Peter et al., 1995). The genus *Ehrlichia* belongs to the Order Rickettsiales and was recently placed within the family *Anaplasmataceae,* together with the genera *Anaplasma*, *Neorickettsia* and *Wolbachia* (Dumler et al., 2001). Heartwater is found throughout most of sub-Saharan Africa and neighbouring islands, and at least three islands in the Caribbean (O'Callaghan et al., 1998; Uilenberg, 1996).

1.3.1.1 Vectors of *E. ruminantium*

E. ruminantium is transmitted transtadially by ticks of the genus *Amblyomma* of which twelve species are known to be able to transmit the pathogen, although the most important vectors are *A. variegatum* and *A. hebraeum* (Uilenberg, 1996; Walker & Olwage, 1987). Only nymphs and adults are able to transmit the pathogen after being infected as larvae and nymphs, respectively (O'Callaghan et al., 1998). Transtadial transmission can occur via adults infected as larvae without reinfection as nymphs (Norval et al. 1990). Vertical transmission of *E. ruminantium* in livestock, from the dam to the calf, has been reported (Deem et al., 1996).

1.3.1.2 Life cycle of *E. ruminantium*

Information on the development of *E. ruminantium* in both mammalian and tick hosts is limited. *E. ruminantium* occurs within membrane-bound colonies in both vertebrate and tick host cells. The major site of development of *E. ruminantium* in ticks appears to be midgut epithelial cells (Kocan et al., 1987). Subsequent stages invade and develop in the salivary gland acini cells of the vector. The development of the stages of the organism that are transmitted seems to be coordinated with the feeding cycle of the ticks (Kocan et al., 1987). In the vertebrate host, the spread of the parasite from the infection site is poorly understood. It has been proposed that the initial development of the organism occurs mainly, but not exclusively, in reticuloendothelial cells. Then the parasitized reticuloendothelial cells rupture and the organism is released into the general circulation where it invades endothelial cells (Du Plessis, 1970; Prozesky & Du Plessis, 1987). Depending on the host, the organism seems to have a predilection for endothelial cells in certain organs. In ruminants, the highest concentration of bacterial organisms is found in the brain, followed by kidneys (Prozesky & Du Plessis, 1987).

1.3.1.3 Epidemiology of heartwater

Endemic stability is a common epidemiological state of heartwater in sub-Saharan Africa. This is due to the occurrence of the long-term carrier state in ruminants (Andrew & Norval, 1989) and a high *E. ruminantium* infection rate in *Amblyomma* ticks in the field (Norval et al., 1990). It had been known for a long time that new-born lambs and calves possessed an age-related resistance to heartwater which was independent of the dam's immune status and that this resistance was of short duration lasting about three to four weeks (O'Callaghan et al.,1998). However, it has been demonstrated that colostrum plays a significant role in calf-hood immunity to heartwater and that in endemic areas, this immunity lasts beyond the

previously reported age of four weeks (Deem et al., 1996; Norval et al., 1995). In addition to the transplacental route, there is also evidence that infected dams are able to pass on *E. ruminantium* infections to their offspring early in life through infected colostrum, and that vector ticks feeding on these calves are able to pick up the infection and transmit it to susceptible hosts (Deem et al., 1996).

1.3.1.4 Pathogenesis and clinical signs of heartwater

The pathophysiology of heartwater is poorly understood. Post-mortem findings associated with heartwater include hydrothorax, pulmonary oedema, ascites, hydropericardium, cerebral oedema, oedema of the lymph nodes and splenomegaly (Van Amstel et al., 1987). The transudate and the oedema are presumed to be caused by increased capillary permeability, although the mechanisms are not known (Deem, 1998). Damage to the endothelial cells and alveolar capillaries is limited and the often-mild cytopathic changes seen in parasitized endothelial cells suggest that the organism itself may not be the cause of the increased vascular permeability (van Amstel et al. 1988). Frequently observed clinical pathological changes in heartwater include progressive anaemia, marked decline in thrombocytes, fluctuations in total and differential white blood cell counts, increased total bilirubin, and an increase in total serum proteins (Van Amstel et al., 1987). Clinical signs of heartwater range from mild to transient fever in subclinical cases, to death without premonitory signs in peracute cases. The acute form of the disease is characterised by sudden onset of fever, tachycardia, inappetence and neurological signs (hyperaesthesia, high-stepping gait, twitching eyelids, chewing, abnormal tongue movement and individual muscle tremors). Haemorrhagic diarrhoea is commonly reported (Van de Pypekamp & Prozesky, 1987).

1.3.2 *Anaplasma* species

Anaplasmosis is a tick-borne disease caused by obligate intracellular organisms of the genus *Anaplasma* with a wide range of eukaryotic hosts (cattle, sheep, goat and some wild ruminants). The genus *Anaplasma* is one of four genera in the family *Anaplasmataceae*, which also includes *Ehrlichia*, *Wolbachia* and *Neorickettsia* (Dumler et al., 2001). Several species of the genus *Anaplasma* pose severe threats to livestock and human health. The main *Anaplasma* species responsible for animal infections in sub-Saharan Africa are *A. marginale, A. centrale, A. bovis,* and *A. ovis*

In Nigeria, anaplasmosis was formerly known as gall sickness, a disease of ruminants caused by intraerythrocytic bacteria. As a result of a taxonomic reorganization of the order *Rickettsiales* (Dumler et al., 2001), some species of the genus *Ehrlichia* (*E. equi*, *E. phagocytophila* and *Ehrlichia* spp. causing human granulocytic ehrlichiosis (HGE) were renamed as *A. phagocytophilum* in the genus *Anaplasma*. This species is the aetiological agent of granulocytic anaplasmosis in humans and livestock. Similarly, *E. bovis* and *E. platys* are now known as *A. bovis* and *A. platys*. This latter, recently added, species all invade blood cells other than erythrocytes in their respective mammalian hosts.

1.3.2.1 Vectors of *Anaplasma*

Transmission of *Anaplasma* species can occur mechanically by biting flies and blood-contaminated fomites or biologically by ticks (Lankester et al., 2007). Biological transmission of anaplasmosis is affected by ticks and approximately 20 species of ticks have been implicated throughout the world, including *Boophilus* spp, *Rhipicephalus* spp, *Hyalomma* spp, *Dermacentor* spp and *Ixodes* spp (Uilenberg, 1995; Jongejan & Uilenberg, 2004; Katherine et al., 2004; De Waal, 2006). Intrastadial transmission (a processes of transferring infection from one host to another by the same tick in the same stage) of *A. marginale* is mainly affected by male ticks (Kocan et al., 2004). Transovarial transmission of *A. marginale* has only been reported for *D. andersoni* (Lankester et al., 2007). Mechanical transmission frequently occurs via blood-contaminated fomites, including contaminated needles, dehorning saws, nose tongs, tattooing instruments, ear tags devices and castration instruments. In addition to mechanical and biological transmission, *A. marginale* can also be transmitted from cow to calf transplacentally (Kocan et al., 2003).

1.3.2.1 Life cycle of *Anaplasma*

Infected erythrocytes taken into the tick with the blood meal provide the source of *A. marginale* infection in the gut cells. After the development of *A. marginale* in the gut cells, many other tissues become infected, including the salivary glands from where the bacteria are transmitted to vertebrates during tick feeding (Kocan et al., 2003). At each site of infection in ticks, *A. marginale* develops within membrane-bound vacuoles or colonies. The first form of *A. marginale* seen within the colony is the reticulated (vegetative) form that divides by binary fission forming large colonies that contain hundreds of organisms. The reticulated form then changes into the dense form, which is the infective form and can survive extracellularly (see Figure 1:9). Cattle become infected with *A. marginale* when the dense form is transmitted during tick feeding via the salivary glands (Kocan et al., 2004). In the vertebrate host, the

only known site of replication is the bovine erythrocytes. Within the erythrocytes, membrane-bound inclusion bodies contain from 4 to 8 bacteria, and as many as 70 % or more erythrocytes may become infected during acute infection or disease (Kocan et al., 2004).

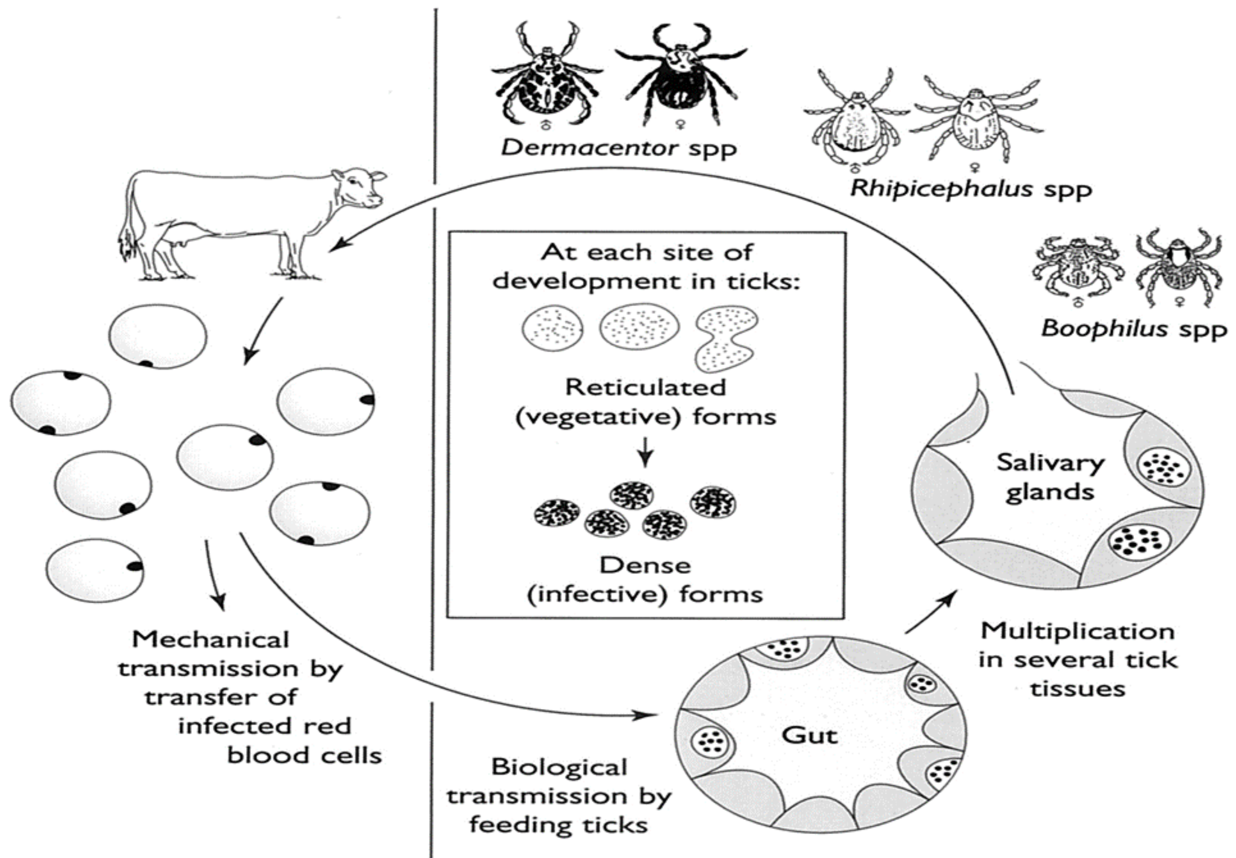


Figure 1:9: Life cycle of *A. marginale* in cattle. Source Kocan et al., (2004)

1.3.2.1 Epidemiology of anaplasmosis

Bovine anaplasmosis, caused by *A. marginale*, is a disease that affects domestic and wild ruminants. *A. marginale* is present in tropical and subtropical regions (Naranjo et al., 2006; Torina et al., 2008). In cattle, the disease causes considerable losses to dairy and beef industries worldwide. Calves under the age of 6 months have innate resistance and will not develop clinical anaplasmosis, no matter the immune status of the mothers. Thereafter, the risk for serious diseases increases with age, unless sufficient contact in the first months of life allowed for the development of immunity. Hence, cattle reared in endemic regions develop a naturally acquired immunity, quite often without passing through a stage of clinical disease, as endemic stability means that all calves need to come into contact with the disease, reservoirs and stable vector populations (Salman et al., 2012). It appears that *A. marginale* is

often introduced into a herd by ticks, but subsequently, mechanical transmission (transmission by insects or by veterinary interventions) may become more important (Kocan et al., 2004). Mechanical transmission occurs via the contaminated mouthparts of biting flies, but can only be achieved within a few minutes after the initial bite, although the pathogen can remain viable and infective in arthropods for several days after ingestion (Hornok et al., 2008). Horseflies (*Tabanus* spp.), stable flies (*Stomoxys* spp.), and to a lesser extent mosquitoes (*Psorophora* spp.), transmit *A. marginale* (Salman et al., 2012).

A. centrale is also an intraerythrocytic tick-borne pathogen that causes mild infections in cattle. A cross-immunity between *A. centrale* exists, and because of its mild virulence, this naturally attenuated strain has been used for more than 100 years in live-blood vaccines to protect cattle from the more virulent *A. marginale* (Minjauw & Mcleod, 2003). These vaccines are mainly used in Africa (Rymaszewska & Grenda, 2008). Not much is known about the epidemiology of *A. centrale*; only a few strains have been characterized.

A. bovis is the aetiological agent of bovine mononuclear or agranulocytic anaplasmosis, a disease occurring mainly in cattle and small mammals (Goethert & Telford, 2003). Goats appear to be resistant. *A. bovis* infects the monocytes of the peripheral blood and the macrophages of the reticuloendothelial system. Infection may occur with limited or no clinical signs. This disease has been reported in West, Central and Southern Africa (Leeflang, 1977; Dumler et al., 2001).

1.3.2.2 Pathogenesis and clinical signs of anaplasmosis

Once infected, the number of infected erythrocytes increases logarithmically, and clinical disease associated with anaemia is consistently observed when 40 to 50 per cent of the erythrocytes have been removed. Destruction of erythrocytes by the reticuloendothelial system results in the development of icterus without haemoglobinaemia and haemoglobinuria (Kocan et al., 2000). Infection of domestic and wild animals and humans with these organisms may lead to a clinical disease collectively called anaplasmosis that manifests as a febrile systemic illness with haematological abnormalities and lymphadenopathy (Rikihisa, 2006). Acute anaplasmosis, caused by *A. marginale*, is characterised by a progressive haemolytic anaemia associated with fever, weight loss, abortion, decreased milk production and in some cases death of the infected cattle (Rymaszewska & Grenda, 2008; Kocan et al, 2010).

1.3.3 *Theileria* species

Theileria is a genus of organisms belonging to the phylum Apicomplexa (order Piroplasmida). *Theileria* species are tick-borne intracellular protozoan haemoparasites, many of which are well-established as being of veterinary and economic importance in different parts of the world (Mans et al., 2015). Ticks can only transmit these haemoparasites transstadially. There is no transovarial transmission because *Theileria* does not passage the ovaries and the eggs of the vectors. Nymphs and adults become infective only if they were infected in the previous developmental stage. The transmission of parasites takes place by the injection of infected saliva of ticks, but it only occurs a few days after the tick has attached; the parasites have first to mature before they become infective (Mehlhorn & Schein, 1985). There are a number of *Theileria* spp. that infect cattle; the two most pathogenic and economically important are *T. parva* and *T. annulata* (Gul et al., 2015). *Theileria parva* occurs in 14 countries in sub-Saharan Africa causing East Coast fever (ECF) and still ranks first among the TBIs of cattle in sub-Saharan Africa, whilst *T. annulata* occurs in Southern Europe as well as North Africa and Asia (Nejash, 2016). Other *Theileria* spp. including *T. mutans*, *T. ovis, T. taurotragi,* and *T. velifera* cause asymptomatic transient infections in ruminants (Norval et al., 1992; Lorusso et al., 2016).

1.3.3.1 Vectors of *Theileria*

T. mutans and *T. velifera* are less pathogenic for cattle and are found in much of sub-Saharan Africa. *T. mutans* and *T. velifera* may apparently cause latent infections in sheep and is transmitted by *Amblyomma* ticks such as *Amblyomma* spp. (*A. gemma, A. cohaerens, A. lepidum A. hebraeum* and *A. variegatum*). Thus, *A. variegatum* is the only vector reported in Nigeria (Walker et al., 2003; Lorusso et al., 2013). Ticks in the genus *Rhipicephalus* spread *T. taurotragi* (Spickler et al., 2010). *R. appendiculatus* is the most important vector for *T. parva* responsible for ECF in parts of Africa (Spickler et al., 2010). *T. annulata* and *T. ovis* is transmitted by ticks in the genus *Hyalomma* spp. (Saeed et al., 2016).

1.3.3.2 Life cycle of *Theileria*

Theileria species have a complex life cycle that involves several morphologically distinct developmental stages in the tick and mammalian host (Fawcett et al., 1982; Shaw & Tilney, 1992; Gul et al., 2015) (see Figure 1:10). The infectious stage of the parasite, the sporozoite, is introduced into the bovine host in the saliva of the ticks feeding as nymphs or adults. They enter lymphoid cells (thus, not all species are associated with lymphoid cells) where they develop into schizonts, which induce the host cell to proliferate and are then disseminated

throughout the body by the normal circulation of the host's lymphoid cells (Shahnawaz et al., 2011). After a period of growth and division in the host lymphoid cells, the schizont gives rise to numerous uninucleate merozoites which leave the lymphoid cells to invade erythrocytes (Khattak et al., 2012). In non-lymphoid transforming species of *Theileria* (e.g. *T. mutans*), there is no intralymphocytic multiplication, and the parasites multiply almost exclusively in the erythrocytes. The intraerythrocytic stages of the parasite, the piroplasms, are ingested in the blood meal by the feeding ticks and released in the gut lumen. There, they divide into macro- and microgametes (Schein et al., 1977) which fuse to form a zygote. The resulting zygotes enter the lining of the gut epithelium where they develop into motile kinetes. Following the moult of the tick stage, the kinetes migrate through the gut wall into the haemocoel and make their way to the tick salivary glands where they become intracellular and transform into sporoblasts (Fawcett et al., 1982; Mans et al., 2015).

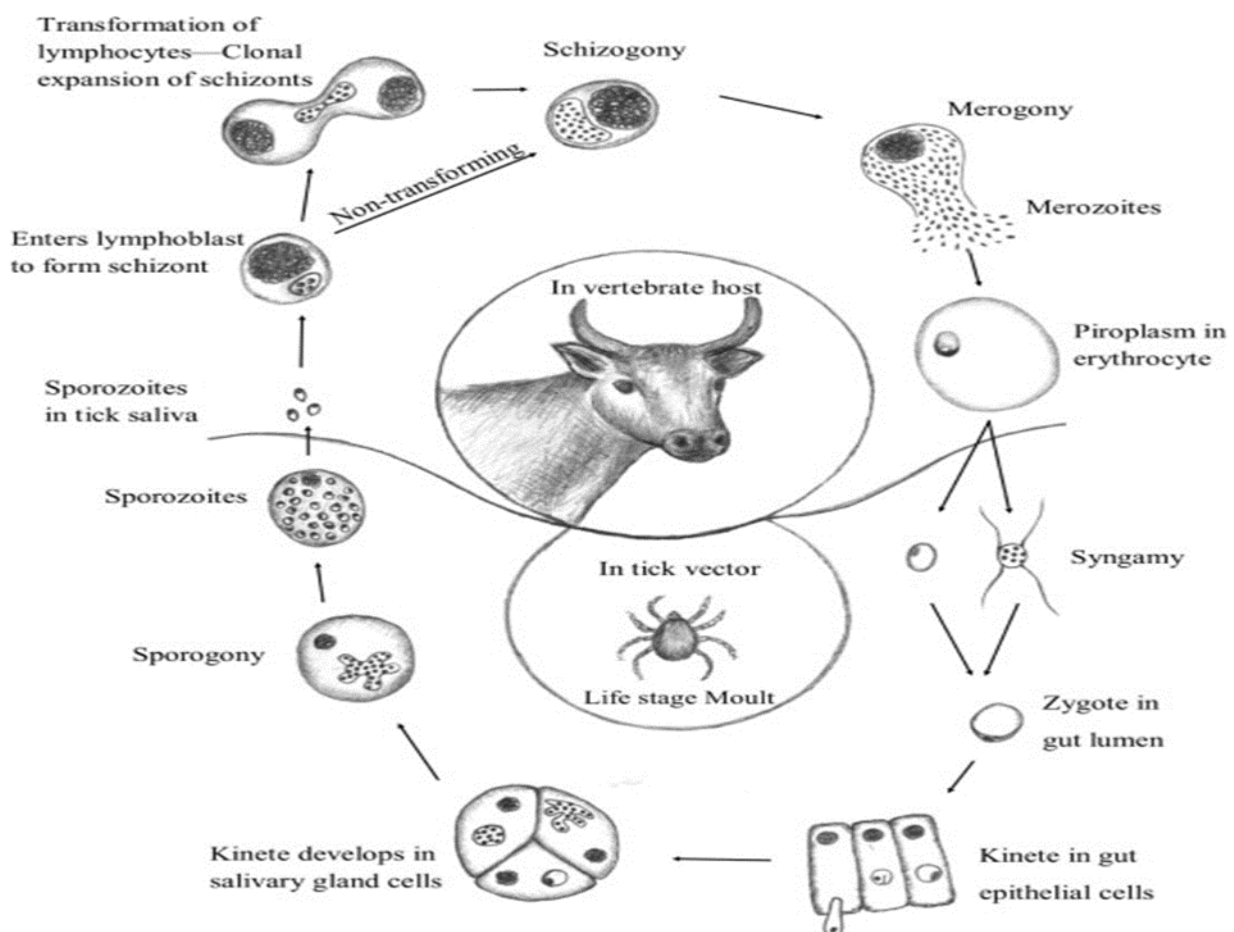


Figure 1:10: Life cycle of the *Theileria* parasite. Source Mans et al., (2015)

1.3.3.3 Epidemiology of theilerioses

Theileria epidemiology considers parasite and vectors distribution, mortality and morbidity of disease outbreaks, risk factors and, host range which includes host resistance and susceptibility (Gachohi., 2012). The geographical distribution of tropical theileriosis is mainly determined by the location and biology of its vector, ticks of the genus *Hyalomma* (Walker et al., 2003). Therefore, the incidence of the disease has a seasonal occurrence, which is modulated by the ecology of its vectors (Nejash, 2016). *Theileria annulata* (tropical theileriosis) occurs from southern Europe and the Mediterranean coast through the Middle East and North Africa and into parts of Asia (Spickler et al., 2010). The disease is prevalent in South Eastern Europe, Southern Europe (Portugal, Spain, Italy, Bulgaria, Greece and Turkey) the near and Middle East, India, China and Central Asia (Nejash, 2016). Tropical theileriosis has also been reported in Ethiopian cattle by Gebrekidan et al., (2014). It is important to emphasize that endemic regions of *T. annulata* and *T. parva* do not overlap however, there were reports of coexistence in southern Sudan (Spickler et al., 2010).

Theileria parva (East Coast fever) is found in sub-Saharan Africa and is prevalent in Southern, Central and Eastern Africa. The affected countries are Kenya, southern Sudan, Burundi, Tanzania, Malawi, Rwanda, Zaire, Mozambique, Zambia, Uganda and Zimbabwe (Gachohi et al., 2012). *Theileria mutans* has been found in Africa and on some Caribbean islands and was reported from the U.S. in 1950 and 1975. *Theileria velifera* and *T. taurotragi* occur in Africa (Spickler et al., 2010). *Theileria orientalis/buffeli* is widespread throughout the world. Infection is generally subclinical; however, the disease can occur in cattle depending on a number of epidemiological factors including previous exposure to theileriae, stress or health status and variations in the species pathogenicity (Nejash, 2016).

Theileria species infect a wide range of both domestic and wild animals (Mans et al., 2015) *Theileria parva* can infect cattle, African buffalo (*Syncerus caffer*), water buffalo and waterbucks. Clinical signs of infections are common only in cattle and water buffalo. *Theileria parva* is highly virulent for European dairy cattle, however, the indigenous cattle breeds and African buffaloes in endemic areas have a natural resistance to this *Theileria* species [30]. The introduction of *T. parva* infection into a previously unexposed cattle population results in an epidemic situation with mortality up to 95% in all age categories of cattle [5]. *T. annulata* occurs in cattle (*Bos taurus* and *Bos indicus*), water buffalo and camels (Nejash, 2016). Mildly pathogenic and non-pathogenic species found in cattle include *T. mutans, T. buffeli, T. velifera, T. taurotragi* and *T. sergenti* have also been recognized.

Theileria spp. has also been found in most wild *Bovidae* in Africa and reported in wild animals in other continents. *T. lestoquardi, T. separata, T. ovis* and other species occur in sheep and goats (Spickler et al., 2010). *T. annulata* sporozoites can be transmitted to goat and sheep and cause a mild febrile response, however, limited experimental studies indicate that schizonts and piroplasms are not produced in these host species (Nejash, 2016).

The prevalence of theileriosis depends upon the geographical region and several other factors like tick density, climatic conditions, age, gender, management practices and immunity (Gul et al., 2015). Prevalence is also influenced by cattle breed as cattle usually differ in tick resistance and innate susceptibility to infection (Muhammad et al., 2008). Tropical theileriosis is more severe in exotic and cross-bred cattle (*Bos taurus*) than indigenous animals (e.g., *Bos indicus*). Mostly, the disease occurs in its subclinical form, leading to significant economic losses; without treatment or control, case fatality rates can reach 80 % in exotic breeds, compared with ~ 20 % in the indigenous breed (Nejash, 2016). Age is one risk factor for example in the recent study by Saeed et al., (2016) the prevalence of tropical theileriosis in young animals (23.4%) showed a higher prevalence than did adults (15%). Innate immunity in calves is not developed enough to combat *T. annulata*. Furthermore, in the result of the reviewed study prevalence was found to be higher in females (24.6%) than male (13.1%).

Environmental Factors are also a risk factor for bovine theileriosis. The disease occurs when there is much tick activity, mainly during summer but a single tick can cause fatal infection (Nejash, 2016). The presence of ticks on animals is an important risk factor for the spread of theileriosis (Khattak et al., 2012). Saeed et al., (2016) reported as there is a higher prevalence of *T. annulata* in hot dry summer. High ambient temperature in this season provides an environment conducive to growth and multiplication of ticks and ultimately increases the transmission of theileriosis.

1.3.3.4 Pathogenesis and clinical signs theileriosis

The *Theileria* spp. can be grouped into schizont “transforming” and “non-transforming” species. Non-transforming *Theileria* are regarded as being benign but still able to cause disease as a result of anaemia induced by the piroplasm stage (Mans et al., 2015). Pathogenesis of various forms of theileriosis is dependent on the production of schizonts in leukocytes and piroplasms in erythrocytes (Morrison, 2015). The severity of infection depends upon the virulence of the causative strain, the quantum of infection, the

susceptibility status, age and health of the host (Mohammed & Elshahawy, 2017). Thus, *T. parva, T. annulata* and *T. hirci* produce numerous schizonts and piroplasms and are very pathogenic; *T. mutans, T. buffeli* and *T. ovis* rarely produce schizonts but may cause varying degrees of anaemia when piroplasms are many in red blood cells; and with *T. velifera* and *T. separata,* no schizonts have been described, the parasitaemia is usually scanty and the infection is mild or subclinical (Morrison, 2015). The occurrence of the disease varies depending on the parasite strain, the host's susceptibility. Furthermore the quantity of sporozoites inoculated and the severity of the disease is directly proportional to the initial inoculum of sporozoites injected (Nejash, 2016). *T. annulata* infection (tropical theileriosis) is characterized by high fever, weakness, weight loss, inappropriate appetite, conjunctival petechia, enlarged lymph nodes and anaemia. Lateral recumbency, diarrhoea and dysentery are also associated with later stages of infection (Gul et al., 2015). Unlike *T. parva*, which causes only a small reduction in circulating erythrocytes, mild to moderate anaemia is observed in tropical theileriosis, although pathology produced by the schizont stage is usually the primary cause of mortality (Morrison, 2015). ECF is characterized by marked peripheral lymphadenopathy, fever, anorexia and respiratory distress. Small numbers of infected cattle develop neurologic signs (turning sickness), due to blockage of cerebral vessels by clusters of parasitized cells. Clinicopathologic changes described in end-stage disease include severe leukopenia, thrombocytopenia, lymphocytolysis in lymphoid tissues and haemorrhage (Fry et al., 2016). The causes of leukopenia, which involves both lymphoid and myeloid cells, are unclear but likely include rupture of infected lymphocytes during merozoite production, and lysis of uninfected cells in lymphoid tissues (Fry et al., 2016). *T. mutans* infection can result in mild clinical signs, but pathogenic strains in eastern Africa cause severe anaemia, icterus and sometimes death (Tomassone et al., 2012). In general, benign theileriosis is characterized by moderate to severe anaemia in heavily parasitized cattle and moderate enlargement of lymph nodes (Morrison, 2015).

1.3.4 *Babesia* species

Babesiosis, also known as piroplasmosis, tick fever, red water or splenic fever, is a tick-borne protozoan disease, caused by an obligate intraerythrocytic organism of genus *Babesia* (Phylum: Apicomplexa Order: Piroplasmida Family: *Babesiidae*) affecting a wide range of domestic and wild animals and occasionally humans (Bock et al., 2004). In Nigeria, so far two species, *B. bigemina* and *B. bovis* have a considerable impact on cattle health and productivity (Leeflang & Ilemobade, 1977; Akinboade & Dipeolu, 1984; Lorusso et al.,

2016). The first written report of the presence of *B. bigemina* in Nigeria was documented in 1914 in cattle presented to a slaughterhouse (reviewed in Leeflang & Ilemobade, 1977). Already in the early 1920s, babesiosis was known to be endemic in most regions of Nigeria (Leeflang & Ilemobade, 1977). At the time, increased mortality attributed to babesiosis was reported in young stock as a consequence of rinderpest immunization, temporarily challenging the immune system of calves, thus favouring the onset of clinical piroplasmosis (Leeflang & Ilemobade, 1977). The first infections distinctively caused by *B. bovis* were diagnosed in 1956 just in the Plateau province when the detection of this parasite was mentioned in two reports (Leeflang & Ilemobade, 1977). Afterwards, a few other reports identified this pathogenic babesia in Nigerian cattle by means of cytological examination of blood smears (Akinboade & Dipeolu, 1984; Kamani et al., 2010), indirect fluorescent antibody test (IFAT) (Ajayi & Dipeolu, 1986) and reverse line blot (Lorusso et al., 2016).

1.3.4.1 Vectors of *Babesia*

All species of *Babesia* are naturally transmitted from animal to animal through the bites of ticks and within ticks' transovarian transmission, and stage-to-stage transmission occurs. Ticks are widely distributed throughout the world particularly in tropical and subtropical countries, and 80% of the world’s cattle are affected with ticks and ticks borne diseases (Homer et al., 2000).

The ticks of genera *Boophilus, Rhipicephalus, Hemaphysalis, Hyalomma*, and *Ixodes* acts as a vector for transmission of *B. bigemina* and ticks of genera *Boophilus, Rhipicephalus*, and *Ixodes* are responsible for the transmission of *B. bovis*(Bock et al., 2004; Leeflang & Ilemobade, 1977).

1.3.4.2 Life cycle of *Babesia*

Cattle are infected by feeding ticks, which inoculates sporozoites that invade erythrocytes where they transform into trophozoites that divide by binary fission (merogony). The erythrocyte membrane breaks down and the released merozoites invade new cells resulting in an intra-erythrocytic cycle. Following a tick blood meal, gametocytes develop in the tick gut, which fuse to form diploid zygotes. Zygotes invade the digestive cells and probably basophilic cells where they undergo a successive round of multiplication before emerging as haploid kinetes. The kinetes migrate to many other organs including the ovaries where further division occurs. After egg hatching, the kinetes migrate to the salivary gland where they transform into multi-nucleated stages (sporogony) which later form sporozoites (see Figure 1:11).

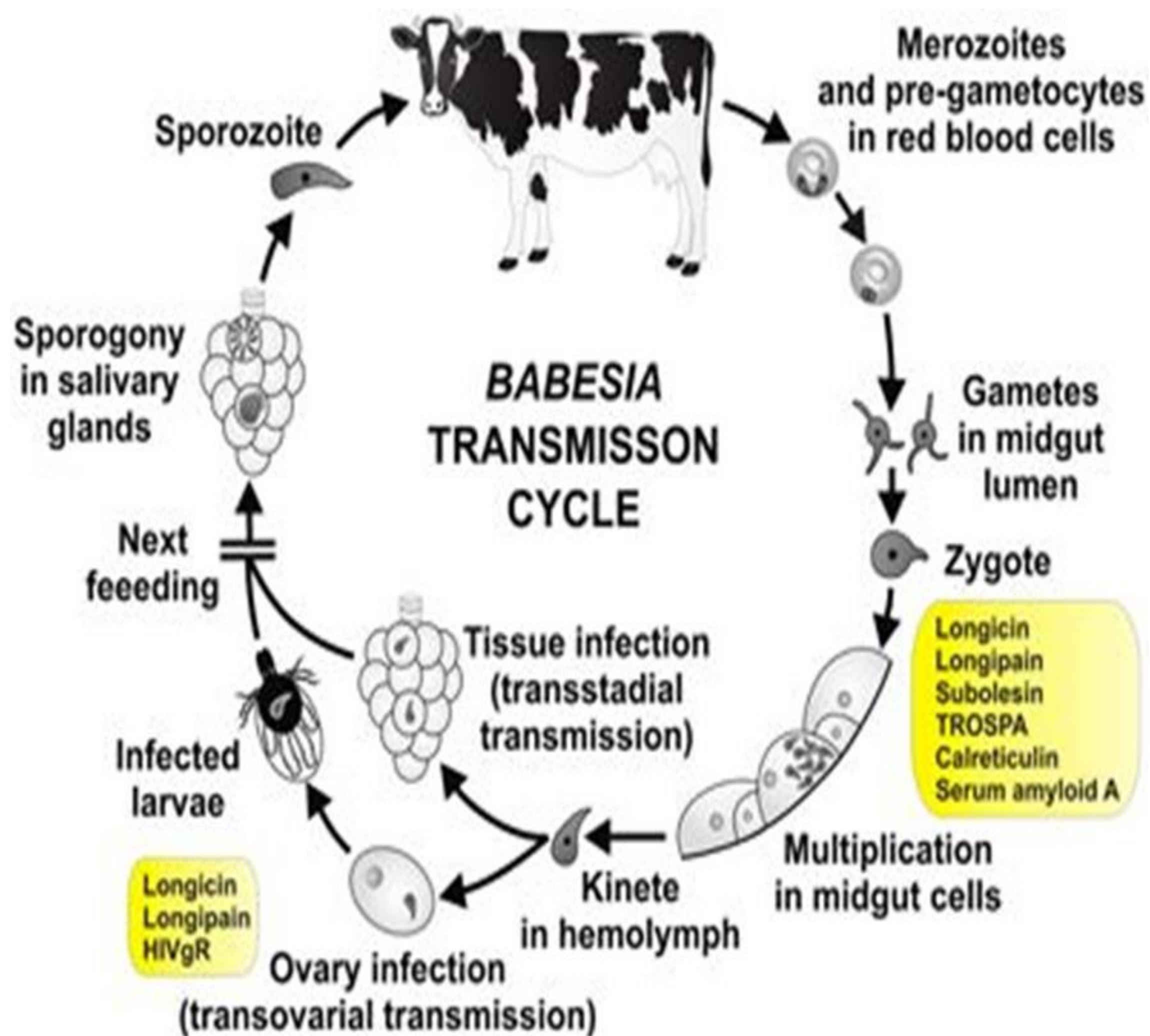


Figure 1:11: Life cycle of *Babesia* parasites. Source: Kocan et al., (2008)

1.3.4.3 Epidemiology of babesiosis

Bovine babesiosis can be found wherever the tick vectors exist, but it is most common in tropical and subtropical areas (Homer et al., 2000). *Babesia bovis* and *B. bigemina* are present in most areas of the world, with the greatest incidence where *Rhipicephalus* (formerly *Boophilus* spp.) tick vector commonly occurs (Lorusso et al., 2013). They are particularly important in sub-Saharan Africa, Central and South America, parts of southern Europe and Australia. Although *B. bovis* is usually found in the same general geographic area as *B. bigemina*, slightly different groups of ticks spread these two species and some differences in their distribution can be seen. For example, *B. bigemina* is more widely distributed than *B. bovis* in Africa (Spickler et al., 2010). Generally, both parasites, *B. bovis* and *B. bigemina*, have the same distribution, but in Africa, *B. bigemina* is more widespread than *B. bovis* because of the ability of *Rhipicephalus* (*Boophilus*) *decoloratus* and *Rhipicephalus evertsi* to also act as vectors for this species (Lorusso et al., 2013). More than 100 known *Babesia* spp. have been identified which infect many types of mammalian host, out of these, 18 spp. cause disease in domestic animals. Babesiosis commonly infects cattle, sheep, goats, horses, pigs, dogs and cats and occasionally man (Uilenberg, 2006). *Babesia bovis* and *B. bigemina* are found in cattle which are the main reservoir hosts. They also affect water buffalo (*Bubalus bubalis*) and African buffalo (*Syncerus caffer*). *Babesia bovis* and *B. bigemina* were recently discovered in white-tailed deer (*Odocoileus virginianus*) in Mexico. Host factors associated with the disease include age, breed and immune status. Because of natural selection pressure, indigenous populations, having lived for a long time with local ticks and tick-borne diseases, have developed either an innate resistance or an innate ability to develop a good immune response to the tick or tick-borne hemoparasitic disease in question. Sheep are more susceptible to *B. ovis* than goats. It is frequently stated that there is an inverse age resistance to *Babesia* infection in that young animals are less susceptible to Babesiosis than older animals; the possible reason is a passive transfer of maternal antibody via colostrum (Derso & Demessie, 2015). The severity of the clinical Babesiosis increases with age so adults are more infected by Babesiosis as compared with calves (Mohamed et al., 2014).

1.3.4.4 Pathogenesis and clinical signs of babesiosis

In *B. bovis* infections, pathology can result from either over-production of pro-inflammatory cytokines and the direct effect of red blood cell destruction by the parasite (Abdela & Jilo, 2016). During an acute infection, macrophages activated by the parasite produce pro-inflammatory cytokines and parasitocidal molecules (Brown & Palmer, 1999). The outcome of infection is related to the timing and quantity of production of these substances. Over-production of inflammatory cytokines results in severe pathology leading to vasodilatation, hypotension, increased capillary permeability, oedema, vascular collapse, coagulation disorders, endothelial damage and circulatory stasis (Ahmed, 2002). Although stasis is induced in the microcirculation by aggregation of infected erythrocytes in capillary beds, probably, the most deleterious pathophysiological lesions occur from the sequestration of parasitized erythrocytes in microcapillaries of the lungs and brain. This results in cerebral babesiosis and a respiratory distress syndrome associated with infiltration of neutrophils, vascular permeability and oedema (Brown & Palmer, 1999; Bock et al., 2004). Progressive haemolytic anaemia develops during the course of *B. bovis* infection. While this is not a major factor during the acute phase of the disease, it contributes to the disease process in more protracted cases. The clinical signs associated with *B. bovis* infections are fever, inappetence, depression, increased respiratory rate, weakness and a reluctance to move. Haemoglobinuria is often present (red water). Anaemia and jaundice develop, especially in more protracted cases. Cerebral babesiosis is manifested by a variety of signs of central nervous system involvement and the outcome is almost invariably fatal (Bock et al., 2004).

In *B. bigemina* infections, pathogenesis is almost entirely related to rapid, and sometimes massive, intravascular haemolysis. Coagulation disorders, cytoadherence and the hypotensive state seen in *B. bovis* are not features of *B. bigemina* infections (Bock et al., 2004). The pathology relates more directly to the destruction of RBC. Haemoglobinuria is present earlier and is more consistent than in *B. bovis* infection. There is no cerebral involvement and recovery in non-fatal cases is usually rapid and complete. However, in some cases, the disease can develop very rapidly with sudden and severe anaemia, jaundice and death, which may occur with little warning (Bock et al., 2004).

In *B. caballi* infection, the disease is characterised by high fever, dyspnoea, and oedema (Hanafusa et al., 1998). The disease is usually run as an acute course although the parasitaemia remains very low, even less than 1.0%. the underlying mechanism by which this parasitaemia remains very low is not well understood and there is a scarcity of report in the

literature on this aspect. Hanafusa et al., (1998) investigated the role of cytokines in the pathogenesis of *B. caballi* in experimentally infected horses. The results suggested that nitric oxide may be a critical effector molecule of immune defence against the parasite. TNF-alpha and nitric oxide might be contributing to the pathogenesis in *B. caballi* infection.

1.3.5 *Rickettsia* species

Rickettsioses are a group of tick-transmitted diseases caused by members of the genus *Rickettsia* (Parola & Raoult, 2001). Evidence suggests that members of the genus *Rickettsia* are primarily obligate intracellular have evolved and survived as obligate intracellular bacteria, cultivating long and well-established relationships with arthropods such as lice, mites, fleas and ticks and vertebrate hosts (Azad & Beard, 1998). *Rickettsia* species are traditionally divided into two antigenically distinct groups based on their lipopolysaccharide (LPS): The typhus group (TG) and the spotted fever group (SFG). The typhus group includes two species, *R. prowazekii*, the agent of epidemic typhus transmitted by the human body louse (*Pediculus humanus corporis*), and *R. typhi*, the agent of murine typhus transmitted by the rat flea (*Xenopsylla cheopis*). The spotted fever group (SFG) includes the majority of rickettsial species, which are transmitted by different arthropod vectors. Of these groups, spotted fever group is the most important in sub-Saharan Africa and the species of greatest concern is *R. africae.* This species has been incriminated in the aetiology of the Africa tick-bite fever (ATBF) in humans in sub-Saharan Africa (Parola & Raoult, 2001). While *R. conorii* the etiologic agent of Mediterranean spotted fever, is maintained in *Rhipicephalus* ticks, which occasionally feed on humans, *R. africae*, the causative agent of ATBF is associated with *Amblyomma* ticks that readily feed on human (Macaluso et al., 2003). Recently, *Rickettsia felis* has been shown to be a common agent of bloodstream infections in among humans in Senegal and Kenya, identified in 7% of the population evaluated (Richards et al., 2010; Socolovschi et al., 2010). However, the epidemiology (including vectors and reservoirs) and clinical picture of this emerging infection in the rest of Africa is largely unknown (Mediannikov et al., 2013; Roucher et al., 2012). During 2011, possibly primary infection with *R. felis,* named “yaaf,” was hypothesized in the case of an 8-month-old girl in Senegal with polymorphous skin lesions (Mediannikov et al., 2013).

1.3.5.1 Vectors of *Rickettsia*

In South Africa, *A. hebraeum*, known as the South African bont tick, is a recognized vector and reservoir for *R. africae*. Elsewhere in sub-Saharan Africa, *A. variegatum* (Beati et al., 2012), the tropical bont tick, is a documented vector of *R. africae* (Parola & Raoult, 2005). Since 2005, *R. africae* has also been detected in *Amblyomma lepidum* ticks in Djibouti (Socolovschi et al., 2007); *Rhipicephalus annulatus* ticks in Guinea (Mediannikov et al., 2010), Senegal (Mediannikov et al., 2010) and Nigeria (Reye, 2012); *Rhipicephalus evertsi evertsi* ticks in Senegal and Nigeria (Mediannikov et al., 2010; Reye et al., 2012); *Rhipicephalus decoloratus* ticks in Nigeria and Botswana (Ogo et al., 2012; Santibáñez et al., 2007); *Rhipicephalus sanguineus* and *Hyalomma impeltatum* ticks in Nigeria (Ogo et al., 2012); *Rhipicephalus geigyi* ticks in Liberia (Ogo et al., 2012); and *Amblyomma compressum* ticks in the Democratic Republic of the Congo (Mediannikov et al., 2012) and Liberia (Mediannikov et al., 2012).

1.3.5.2 Life cycle of *Rickettsia*

Blue arrows indicate main steps of tick natural cycle: (1) oviposition by engorged female; (2) eggs hatched into larvae; (3) larvae feed on small animals; (4) engorged larvae hatch into nymphs; (5) nymphs feed on large or small animals; and (6) nymphs moult into adult ticks that feed on large animals or bite humans. Broken red arrows indicate transovarial (7) and transstadial transmission (8) of rickettsiae and solid red arrows indicate the transmission of rickettsiae to humans through a bite of a nymph (9) or an adult tick (10) Figure 1:12.

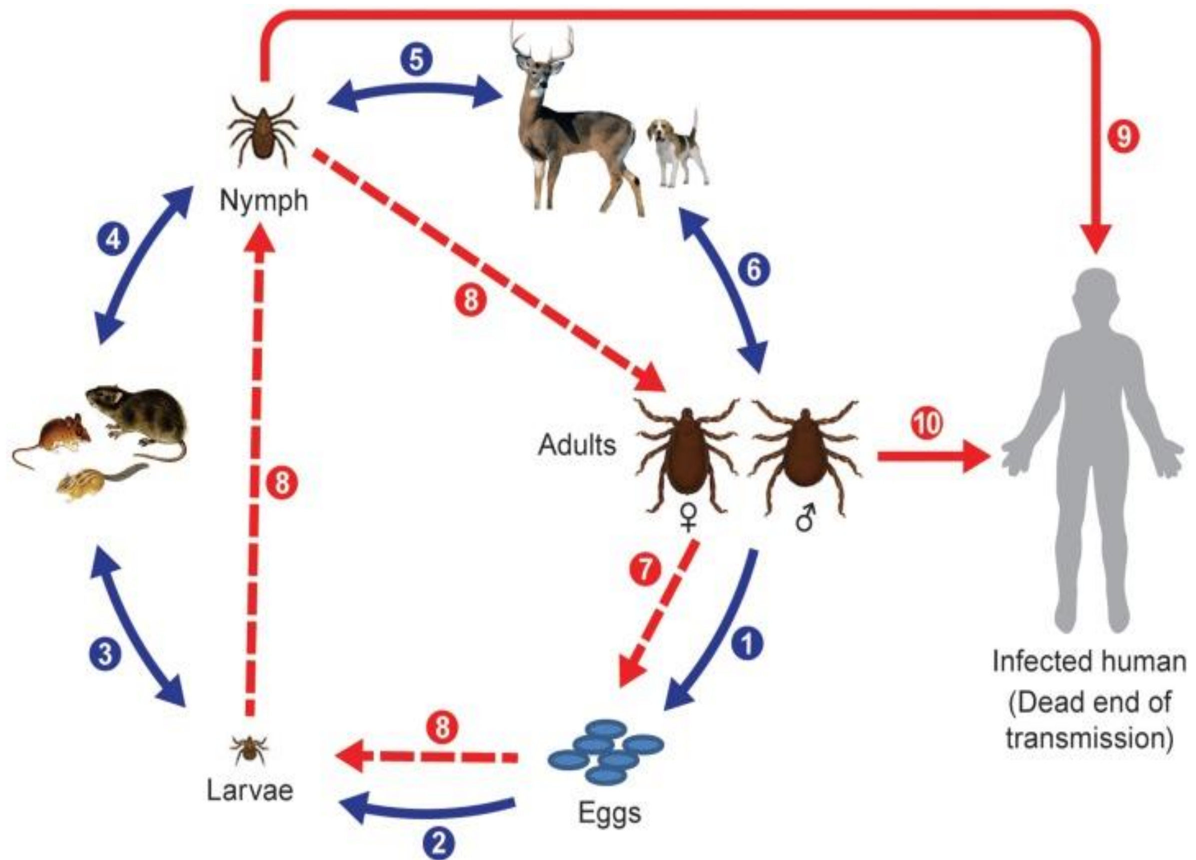


Figure 1:12: Life cycle of Ixodid ticks and natural transmission of rickettsiae. Source: Eremeeva & Dasch, (2015)

1.3.5.3 Epidemiology of rickettsioses

In general, rickettsioses are global emerging and re-emerging vector-borne infectious diseases (Parola et al., 2005; 2013). Moreover, SFG rickettsioses tend to be geographically limited to the range of their specific vectors (Parola et al., 2005; LetaiÌˆef, 2006). Recently, many SFG rickettsiae were recognised and detected on different continents (Parola et al., 2013). For example, *R. africae* was restricted in central and southern Africa and its vector was *Amblyomma* spp. (Kelly et al., 1996; Macaluso et al., 2003; Socolovschi et al., 2007), but it was recently isolated from *Hyalomma* spp. in Egypt (Abdel-Shafy et al., 2012; Abdullah et al., 2016). Furthermore, other *Rickettsia* species have been detected in either animal hosts or tick vectors in many countries such as Nigeria (Lorusso, et al., 2013; Kamani et al., 2018), Mongolia (von Fricken et al., 2018), and Cameroon (Vanegas et al., 2018).

Rickettsioses are transmitted to vertebrate hosts by bite of infected ticks through salivary secretions and blood transfusion (Raoult & Roux, 1997). The salivary glands of ticks are propagation sites for rickettsiae (Santos et al., 2002; Socolovschi et al., 2009). Hard ticks

acquire rickettsiae through different sources. The initial tick infection with rickettsiae occurs by feeding of non-infected ticks on rickettsaemic hosts. Sufficient blood levels of rickettsiae in vertebrate hosts are required for infection (Reháçek, 1989). Also, ticks become infected with rickettsiae through transovarial transmission (Parola & Raoult, 2001; Anderson & Magnarelli, 2008; Socolovschi et al., 2009). The other transmission route is transstadial transmission (Parola & Raoult, 2001). Sexual transmission may also occur, from infected male ticks to non-infected females (Parola & Raoult, 2001). In addition, co-feeding, when several ticks concurrently feed in close proximity on the same host, can also mediate the transmission of rickettsiae directly from an infected tick to an uninfected neighbour (Abdullah et al., 2018).

SFG rickettsiae circulate between wild vertebrates and arthropods vectors (Abdullah et al., 2018). Wild mammals and birds play an important role in amplifying and keeping of rickettsiae in nature (Movila et al., 2013; Ionita et al., 2016). Other studies suggested some other potential animal reservoirs of *Rickettsia* spp.; cattle and camels for *R. africae* (Reye et al., 2012). Initially, it was thought that *R. africae* was associated only with *Amblyomma* spp. (Parola & Raoult, 2001; Parola et al., 2005), but more recently *Hyalomma* spp. and *Rh. sanguineus* have also been implicated in its transmission.

1.3.5.4 Pathogenesis and clinical signs of rickettsioses

Rickettsiae are transmitted to humans by the bite of infected ticks and mites and by the faeces of infected lice and fleas. They enter via the skin and spread through the bloodstream to infect vascular endothelium in the skin, brain, lungs, heart, kidneys, liver, gastrointestinal tract, and other organs (Walker, 1996). Rickettsial attachment to the endothelial cell membrane induces phagocytosis, soon followed by an escape from the phagosome into the cytosol (Sahni et al., 2013). Rickettsiae divide inside the cell. *R. prowazekii* remains inside the apparently healthy host cell until massive quantities of intracellular rickettsiae accumulate and the host cell bursts, releasing the organisms (Sahni et al., 2013). In contrast, *R. rickettsii* leaves the host cell via long, thin cell projections (filopodia) after a few cycles of binary fission. Hence, relatively few *R. rickettsii* organisms accumulate inside any particular cell, and the rickettsial infection spreads rapidly to involve many other cells (Walker, 1996). Perhaps because of the numerous times, the host cell membrane is traversed, there is an influx of water that is initially sequestered in cisternae of cytopathically dilated rough endoplasmic reticulum in the cells more heavily infected with *R. rickettsii*. The main clinical signs of rickettsiosis vary depending on the *Rickettsia* species involved, but typically

manifest 6-10 days after a tick bite. These signs include fever, headache, muscle pain, rash, local lymphadenopathy, and one or more eschars at the site of the tick bite. Thrombocytopenia and leukocyte count abnormalities are common, and hepatic enzyme levels are often elevated (Parola & Raoult, 2001).

1.3.6 *Bartonella* species

Bartonella is a genus of gram-negative bacteria that are transmitted by a range of arthropod vectors that include fleas, lice, sandflies and possibly ticks. *Bartonella* species are obligate haemoparasites, establishing chronic infections in the vasculature of a wide range of mammalian reservoir hosts, parasitising both endothelial cells and erythrocytes (Billeter et al., 2008). At least 20 species are known to cause host-specific intraerythrocytic infections in their specific mammalian reservoir hosts, including the human-specific pathogens *Bartonella quintana* and *Bartonella bacilliformis,* the agents of trench fever and Oroya fever, respectively. A secondary tissue phase can be associated with the development of vasculoproliferative lesions, e.g. bacillary angiomatosis (*Bartonella henselae, B. quintana*) or verruga peruana (*B. bacilliformis*) and may play a role in various other dermal conditions (Anderson & Neuman, 1997; Maurin et al., 1997; Dehio, 2005; Kaiser et al., 2011; Rossi et al., 2015).

1.3.6.1 Vectors of *Bartonella*

Several species of flea have been demonstrated to serve as vectors for some *Bartonella* species, including the most commonly encountered zoonotic species, *B. henselae*. Other species are transmitted by other insects including which exploit humans as reservoirs, namely *B. quintana*, which is transmitted by body lice (*Pediculus humanus humanus*) and *Bartonella bacilliformis*, which is transmitted by *Lutzomyia* sandflies (Billeter et al., 2008). The role of ticks as vectors for *Bartonella* species is unclear. *Bartonella* DNA has been in several tick species, including *I. ricinus*, *I. scapularis*, *I. persulcatus*, *D. reticulatus*, *Rh. sanguineus* and *Carios kelleyi* and there is epidemiological evidence for their involvement (Billeter et al., 2008). Several studies have detected the presence of *Bartonella* spp. in various tick species from around the world (Schouls et al., 1999; Breitschwerdt & Kordick, 2000; Chang et al., 2000; Eskow et al., 2001; Sanogo et al., 2003; Adelson et al., 2004; Halos et al., 2005; Morozova et al., 2005; Kim et al., 2005; Edyta Podsiadly et al., 2007; Hercík et al., 2007; Angelakis et al., 2010; Dietrich et al., 2010; Janecek et al., 2012; Vayssier-Taussat et al., 2013; Rynkiewicz et al., 2015; Rynkiewicz et al., 2015; Moutailler et al., 2016). Vector competence has been demonstrated experimentally by the use of artificial tick-feeding

procedures for *B. henselae* (Cotté et al., 2008) and a murine *Bartonella birtlesii* infection model (Reis et al., 2011). *Bartonella* DNA has been detected in hard ticks removed from dogs. However, as DNA was detectable in only some but not all ticks removed from one particular dog, the infection of the tick may have been acquired from another source previously (Podsiadly et al., 2009). Furthermore, several studies indicate co-transmission of *Bartonella* with other tick-borne pathogens (e.g. *Ehrlichia, Babesia*) in dogs (Chomel et al., 2001; Honadel et al., 2001; Kordick et al., 1999). Several case reports of *B. henselae* infections of humans have been published where no or very limited cat contact was reported, limiting the possibility of transmission via cats or cat fleas. Authors concluded that transmission via arthropod vectors (e.g. ticks) may provide an alternative explanation (Lucey et al., 1992; Billeter et al., 2008). The most important reservoir hosts for tick-borne pathogens are small rodents as they are the preferred hosts of tick larvae and nymphs.
Several *Bartonella* spp. have been detected in these small mammals further supporting the possibility that ticks may represent a vector for *Bartonella* transmission (Chang et al., 2000; Ellis et al., 2002; Rynkiewicz et al., 2015; Benson et al., 2004; Kim et al., 2005; Keesing et al., 2006; Steiner et al., 2008). *Bartonella* spp. have also been isolated from cattle and mule deer in North America. As ruminants are rarely infested with fleas, ticks seem to be more likely to transmit these pathogens to these animals (Chang et al., 2000).

1.3.6.2 Life cycle of *Bartonella*

The infection cycle of *Bartonella* is initiated with the inoculation of a mammal reservoir host from an infected bloodsucking arthropod. Transmission may occur during feeding or via contaminated arthropod faeces that is subsequently scratched into the skin. Following this inoculation, Bartonella migrates to the vascular endothelium, which is considered their primary niche in the reservoir host. From the primary niche, bartonellae are repeatedly seeded into the bloodstream and infect erythrocytes in a sequence of steps ranging from adhesion, invasion, replication then intracellular persistence that facilitates their uptake in the bloodmeal of their vectors (Harms & Dehio, 2012) (see Figure 1:13).

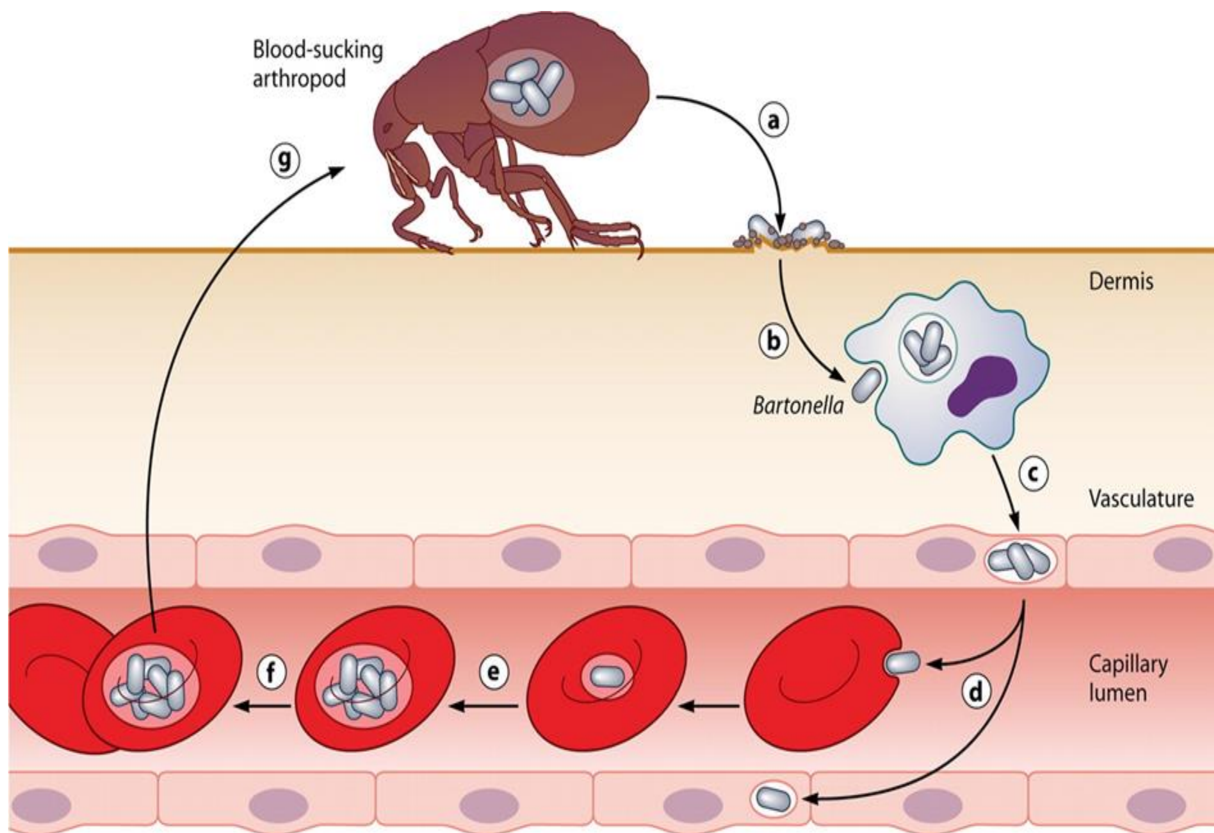


Figure 1:13: Life cycle *Bartonella* parasites; Source: Harms & Dehio, (2012).

1.3.6.3 Epidemiology of Bartonellosis

Bartonella species have adapted to exploit a wide range of wild-living and domesticated mammal species, including humans, as reservoir hosts, including livestock (Chang et al., 2000; Bermond et al., 2002). Infection of reservoirs is usually asymptomatic. However, spill-over into non-reservoir hosts can occur when these hosts are exposed to infected vectors, and this can result in overt disease. Spill-over events are most obvious when they occur in humans, thus several *Bartonella* species are well-recognised zoonotic pathogens. By far the most important of these is *B. henselae*, which exploits cats as its reservoir host. *B. henselae* infection in humans most frequently manifests as cat scratch disease (CSD), characterised by regional lymphadenopathy. CSD is easily treatable and may ultimately be self-limiting but is nonetheless problematic as its clinical presentation resembles those caused by a number of other, more sinister aetiologies. The prevalence of *Bartonella* DNA in hard ticks in Europe has been shown to be as high as 40 % (Dietrich et al., 2010). While *Bartonella* DNA was detected in ~2 % of ticks collected in a recent study from Austria (Müller et al., 2016). Molecular epidemiology techniques have revealed a remarkable diversity within the genus *Bartonella*. A wide variety of *Bartonella* spp. specialized to various mammalian hosts

and transferred by specific arthropod vectors have been identified over the years and the prevalence of infection appears to be widespread across species and geographic regions. At least 13 species of *Bartonella* have been identified as pathogenic to humans with three species responsible for most of the clinically relevant infections in humans: *B. bacilliformis*, *B. quintana* and *B. henselae* (Kaiser et al., 2011). The effect of *Bartonella* infections in cattle is unknown. Because *B. bovis* is very common in some herds, it is difficult to attribute clinical signs to this organism. *B. bovis* was suggested as the cause of endocarditis in two older cows, these animals had high antibody titers to this organism, and DNA was detected in the lesions (Maillard et al., 2007). One study of a dairy herd suggested that adverse effects on health and reproductive success are uncommon. In this herd, there was no correlation between bacteraemia and milk yield, milk cell count or various parameters of reproductive success. Fewer bacteraemic cows retained the placenta, and the interval from calving to first artificial insemination was shorter (Maillard et al., 2006).

1.3.6.4 Pathogenesis and clinical signs of *Bartonella* infections

Bartonella causes clinical diseases in the acute phase which are characterised by haemolytic anaemia, fever, and mortality of 44-88% if left untreated. The subacute phase is characterised by the eruption of mular, crops miliary, or nodular skin lesions, or verrugas (“warts”), containing a serosanguinous fluid which exudes on contact. It then becomes noticeable weeks, or after months of the acute phase. Complication is mostly common in the acute phase of the infection particularly with *Salmonella* species, Histoplasma, Toxoplasma. Gastrointestinal, Haematological, neurological and cardiovascular complications also occur, in pregnancy. Infection can lead to premature labour, miscarriage and maternal death. Children are the most vulnerable in an endemic communities (Sanchez et al., 2012).

1.4 Haematology

Assessing haematological parameters is an important strand of veterinary disease diagnosis. The most widely adopted approach to doing so is the microscopic examination of stained blood smears. This straight-forward, low-tech method is well suited to resource-poor settings.

1.4.1 Anaemia in livestock

Anaemia is a condition in which there is a decrease in red blood cell (RBC) density or haemoglobin concentration in the blood (Hgb) and/or packed cell volume (PCV) (Pugh & Baird, 2012). It can develop from loss, destruction, or lack of production of RBCs. Anaemia is classified as regenerative or non-regenerative. With regenerative anaemia, the bone

marrow responds appropriately to the decreased red cell mass by increasing RBC production and releasing reticulocytes. With non-regenerative anaemia, the bone marrow responds inadequately to the increased need for RBCs. Anaemia caused by haemorrhage or haemolysis is typically regenerative. Anaemia caused by decreased erythropoietin or an abnormality in the bone marrow is non-regenerative (Pugh & Baird, 2012). The RBC in ruminants has a long lifespan, of between 125 and 160 days (Pugh & Baird, 2012). The goat's erythrocytes are smaller, have a high osmotic fragility and are more prone to haemolysis than the RBC of the sheep (Pugh & Baird, 2012).

1.4.2 Regenerative anaemia

The majority of the anaemia in livestock is regenerative and caused either by blood loss or haemolysis of the erythrocytes (Pugh & Baird, 2012). Goats have in general a relatively mild regenerative response, even in severe cases of regenerative anaemia (Smith & Sherman, 2009). Gastrointestinal parasites, primarily *Haemonchus contortus*, are the most common causes of blood loss in small ruminants but also ectoparasites are a possible aetiology (Pugh & Baird, 2012). Haemolysis, on the other hand, is often induced either by intra-erythrocytic parasites, toxins or chronic diseases (Pugh & Baird, 2012). Immune-mediated haemolysis (IMHA) is not common in goats and sheep but could occur due to parasitaemia, antibiotic administration and in lambs or kids fed with bovine colostrum (Pugh & Baird, 2012).

Haemolysis is either intravascular (lysis of RBC within the blood vessels) or extravascular (removal of RBC by phagocytes, foremost in the liver and the spleen). Intravascular haemolysis in goats and sheep, with signs as haemoglobinaemia and haemoglobinuria, is often caused by bacterial toxins, copper toxicosis, or rapid reduction of plasma osmolarity (Pugh & Baird, 2012). *Clostridium perfringens* type A, *Clostridium haemolyticum*, and *Leptospira interrogans* are some of the bacteria capable of producing these type of toxins (Pugh & Baird, 2012). The most common cause of extravascular haemolysis is parasites/bacteria in the RBC, but opsonisation, or ingestion of toxic plants like kale and rapeseed, are other possible aetiologies (Pugh & Baird, 2012). Excessive intake of nitrates, nitrites or copper can also cause extravascular haemolysis (Pugh & Baird, 2012). The most commonly occurring parasites and bacteria within the RBC of livestock are *Anaplasma* spp., *Mycoplasma ovis*, *Theileria* spp and *Babesia* spp (Pugh & Baird, 2012). Extravascular haemolysis may result in icterus and dark urine (Pugh & Baird, 2012).

1.4.3 Non-regenerative anaemia

A less common cause of anaemia in livestock is a decline in the production of erythrocytes, leading to a non-regenerative anaemia. The most common aetiology is a chronic disease which makes the body store the iron in the bone marrow in an unusable form, restraining the erythropoiesis. Iron deficiency, as well as selenium, copper or zinc deficiencies, can also result in a mild non-regenerative anaemia (Pugh & Baird, 2012).

The fact that chronic disease can generate anaemia results in a long list of differential diagnosis when anaemia is detected. Conditions as pneumonia, foot rot and malnutrition can be enough to cause anaemia if they have been going on during a longer time (Pugh & Baird, 2012). Acute renal failure, which decreases the erythropoietin production in the kidneys, is another a less common cause of such severe non-regenerative anaemia in small ruminants (Pugh & Baird, 2012).

Table 1:1: Normal erythrocyte parameters for cattle, sheep and goats

Parameters	**Conventional Units**	**SI Units**	**Cattle**	**Sheep**	**Goat**
PCV	%	$\times 10^{-2}$L/L	24–46	27–45	22–38
Hgb	g/dL	× 10 g/L	8–15	9–15	8–12
RBCs	$\times 10^{6}$/μL	$\times 10^{12}$/L	5.0–10.0	9–15	8–18

1.5 BCS-scoring of livestock

Assessment of body condition score (BCS) is a hands-on method to estimate the deposition of fat and muscle in the animals. The BCS varies with nutritional and physiologic status and works as a general indicator of the condition of the animal (Smith & Sherman, 2009). Lack of suitable protein and lipid reserves affects the health as well as the milk production and wool quality of the animals. Studies have described a positive correlation between BCS and HCT, suggesting that the BCS can be helpful in the search for anaemic animals (Yilmaz et al., 2014). Furthermore, a high negative correlation has been found between BCS and FAffa MAlan CHArt (FAMACHA©) – a system developed in South Africa that uses anaemia, determined on the basis of the colour of the lower eyelid mucous membrane in small ruminants, as a morbidity (Malan et al., 2001; Vatta et al., 2001), showing that decreased BCS was significantly related to paler mucus membranes (Yilmaz et al., 2014a).

In sheep as well as for cattle, a lumbar system is used for BCS, where the size and shape of the fat and muscles covering the lumbar region, between the dorsal and transverse spinous processes, are evaluated (Pugh & Baird, 2012). This assessment is not suitable in goats since they (especially the milking goats) store the majority of their fat in the omentum and the perirenal tissues (Smith & Sherman, 2009). Not even obese goats store much of their fat subcutaneously which contributes to the risk of underestimation of the BCS if only the lumbar score was evaluated (Harwood, 2016). Therefore, both lumbar and sternal scores should be evaluated to estimate the BCS in goats. The lumbar score, which is determined over the second to fifth lumbar vertebrae, better reflects the body protein of the goat, while the sternal score is a better measurement of the amount of adipose tissue (Morand-Fehr et al., 1992). The final BCS of goats is an average of these two scores (Smith & Sherman, 2009). Both sheep and goats are scored on a scale from 1.0 to 5.0, where 1.0 represents emaciation and 5.0 represents extreme obesity (Pugh & Baird, 2012). The ideal BCS for goats and sheep varies between 2.5 and 4.0 depending on where the animal is in the reproductive and production cycles (Pugh & Baird, 2012; Yilmaz et al., 2014). The BCS charts used for goats in this study can be seen in Figure 1:14.

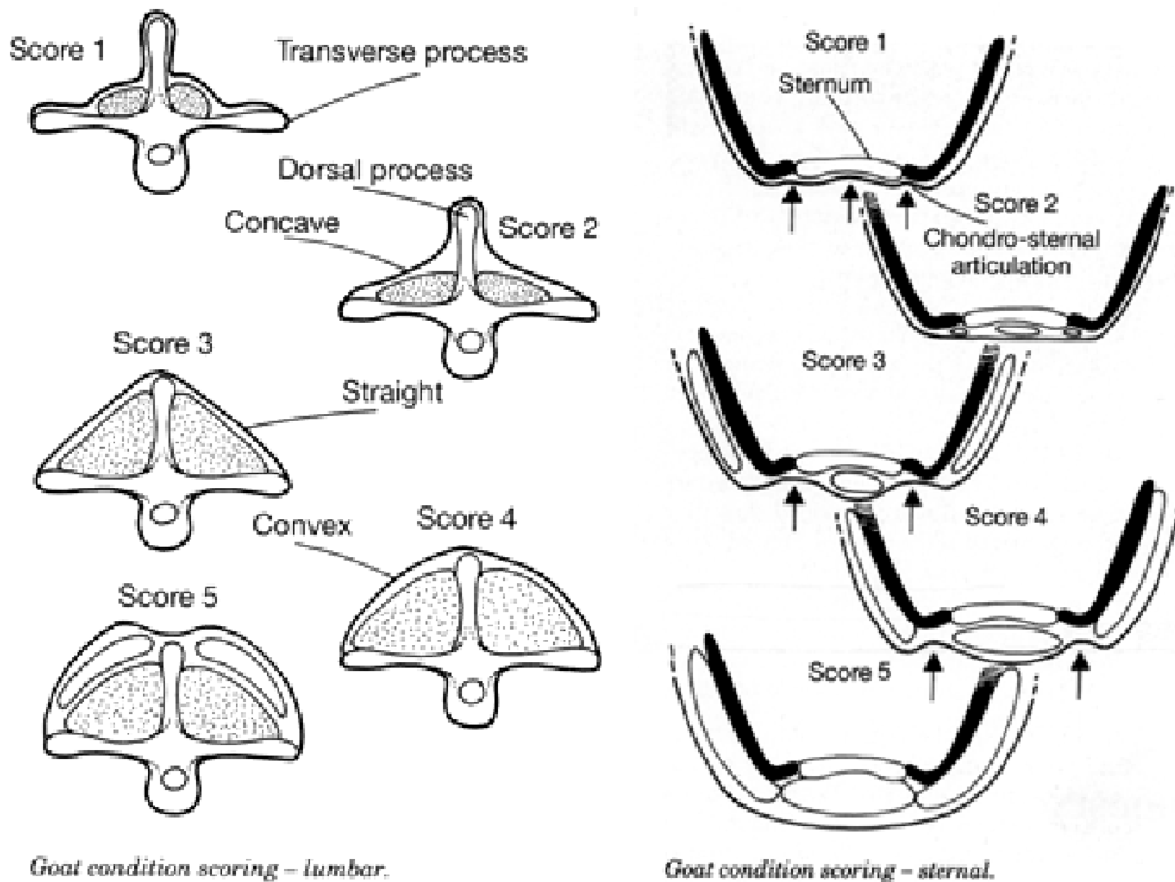


Figure 1:14: The body condition scoring system used for goats. Source: Harwood, (2016)

1.6 Diagnosis of tick-borne infections

Diagnostics provide a cluster of objective measures directed toward identifying the cause of a disease. After scientists discover the causative agent of an emerging infectious disease, they develop, evaluate, and refine diagnostic tests over time. Diagnosis, by contrast, rests on history and clinical signs and observed physical and laboratory findings. Ultimately, accurate diagnosis requires knowledge of epidemiology, clinical manifestations, and diagnostic tests of a disease.

TBIs presents a significant challenge to this standard approach. The presentation of clinical signs may not align directly with the diagnostic laboratory test results. Necessary and sufficient conditions for the diagnosis may not be met, and yet the constellation of findings might lead one to make a diagnosis. At the time of acute presentation to a health professional, serologies may not be definitive. Conversely, serology may be positive, but clinical signs may not match the serological picture. This suggests opportunities to develop laboratory measures that are reliable, valid, and sensitive to change and that may help to define the phases/stages of TBIs, such as acute, post-acute, chronic, and recurrent. In this section,

researchers explored the limitations of existing TBIs tests and suggested promising new approaches to diagnostics that can improve the diagnosis of those diseases.

1.6.1 Microscopic examination

Microscopic techniques for the diagnosis of TBIs are still considered as the "gold standard" technique in many parts of the world as they are quick and cheap. Smears of blood or other clinical material can be stained with a variety of non-specific stains then observed using a light microscope to demonstrate the presence of a parasite/pathogen.

Theileriosis is diagnosed by microscopic examination of Giemsa stained, thin blood or lymph node smears for the presence of piroplasms in red blood cells and macroschizonts in lymphocytes (Chauhan et al., 2015). Microscopic method of diagnosis of theileriosis demands expertise in slide reading for sub-clinical or chronic infections cases, as parasitemias in such cases are often extremely low and piroplasm may be difficult to find in stained blood smears (Chauhan et al., 2015). This conventional method is difficult to perform and time consuming to identify piroplasmic form within the erythrocytes from carrier animals. Which ultimately increase the chances of getting false negative or misdiagnosed results (Chauhan et al., 2015).

Babesiosis is diagnosed by examination of blood or organ smears stained with Romanowsky stain (Böse et al., 1995). The direct method involves identifying the parasite in the stained blood smears; however, this technique shows low sensitivity in the subclinical and chronic phase of the infection (Terkawi et al., 2011). Although different *Babesia* species may have different intra-erythrocyte morphologies, distinguishing between them requires great expertise (Homer et al., 2000). Staining of concentrated buffy coats (quantitative buffy coat) with acridine orange has also been proposed for *Babesia* diagnosis (Levine et al., 1989) and may be more valuable detecting low levels of parasitaemia.

The most commonly used method for microscopic diagnosis of *Anaplasma* infection is the examination of Giemsa-stained thin blood smears. This approach is most useful in the clinical acute form of the disease, with a reported sensitivity of 10^6 infected erythrocytes per millilitre of blood (Gale et al., 1996). However, due to a low-intensity parasitaemia in many carrier state animals, and difficulties associated with differentiating *Anaplasma* from other structures, this approach is not recommended for the characterization of persistently infected cattle (Carelli et al., 2007).

1.6.2 Serological methods

Many serological methods standardised for the diagnosis of TBIs have been employed in epidemiological field studies (Swai et al., 2005; Mans et al., 2015). Among the various serological tests, the most important ones include complement fixation test (CFT), indirect fluorescent antibody technique (IFAT) and enzyme-linked immunosorbent assay (ELISA). CFT has been used for diagnosis of *Babesia*, *Theileria*, *Toxoplasma*, and *Trypanosoma* infections (Herr et al., 1985; Böse et al., 1995). IFAT has been used for the diagnosis of parasites like *Babesia* (Morzaria et al., 1977; Anderson et al., 1980), and *Theileria* (Morzaria et al., 1977; Darghouth et al., 2004). ELISA is increasingly being used for detection of parasite-specific antibodies, antigens and immune complexes (Kachani et al., 1992).

On the other hand, ELISA for the diagnosis of *Theileria* spp. infection in sheep using piroplasm antigen obtained from experimentally infected sheep with parasitaemia reaching 30% was developed (Gao et al., 2002). However, cross-reaction was found with *B. ovis*. This result may be questionable because it could not be excluded that these sheep were already infected with both *Theileria* spp. and *B. ovis*. In spite of relatively good performances of crude antigens, the disadvantages of this approach include the requirement for experimental animals for piroplasm antigen production and the batch-to-batch variation, besides the need to standardize protocols to obtain antigen from crude parasite material (Gubbels, 2000). These problems have been circumvented by the use of several recombinant parasite antigens in ELISA. Two ELISAs based on recombinant proteins have been developed. Firstly, the sporozoite antigen (SPAG-1) has been demonstrated to detect exposure to *T. annulata*, but sensitivity and specificity of this ELISA have not been evaluated (Boulter et al., 1998). Secondly, a merozoite surface antigen, Tams-1 has been tested as a candidate antigen for a diagnostic ELISA (Ilhan et al., 1998). Moreover, an ELISA for detection of *T. annulata* infection was established and validated and applied for epidemiological studies in the field (Schnittger et al., 2002; Bakheit et al., 2004; Salih et al.,2007). An advance in serological diagnosis was achieved with the development of a competitive ELISA applying the TaSP antigen and using a monoclonal antibody (1C7) that was found to bind to TaSP antigen (Renneker et al., 2008). ELISA is widely used as the basis for epidemiological surveys (Passos et al., 1998) and for evaluation of vaccination programmes (Guglielmone et al., 1997). Serological tests have the disadvantage of relying on the presence of specific antibodies, which may take days or weeks to develop in an infected animal or may persist for months after the infection has been cleared (Mosquedaetal.,2012). Mboloi et al., (1999)

investigated the major antigenic protein 1 fragment B (MAP1-B) in ELISA for the diagnosis of *E. ruminantium* infection, concluding that it is a useful test for the diagnosis of *E. ruminantium* infection in small ruminants. However, this test is known to be less sensitive in cattle than in small ruminants (Knopf et al., 2002).

1.6.3 Nucleic Acid-Based Techniques

Molecular nucleic acid-based diagnostic techniques have been developed for a number of haemoparasites. They have proven to be sensitive and easy to use, and allow simultaneous analysis of a large number of samples and detection of parasites directly in clinical and environmental samples without culture (Dey & Singh, 2009). The first introduced diagnostics was nucleic acid hybridization technique, however, in the recent years, PCR and allied techniques along with genomic sequencing have become a driving force for the development of rapid, sensitive and specific assays capable of genomic detection. They are widely used because of being highly sensitive and can be performed rapidly in a cost-effective manner. Various molecular diagnostic techniques have been developed for the diagnosis of parasites including conventional PCR, Restriction Fragment Length Polymorphism- PCR (RFLP-PCR), real-time PCR (RT-PCR), PCR-Reverse Line Blot Assay, loop-mediated isothermal amplification (LAMP), etc.

1.6.3.1 Standard PCR

Most of the DNA based methods employed in the diagnosis of TBIs are based on standard PCR techniques. The sensitivity of a PCR assay can be increased several folds by performing a nested PCR, where two sets of amplification primers are used (Haqqi et al., 1988; Wanger et al., 2017). One set of primers is used for the first round of amplification, then sites located within the first product are subjected to a second round of amplification with another set of primers that are specific for an internal sequence amplified by the first primer set. This process not only improves the sensitivity of the assay but also confirms the specificity of the first round product (Tang et al., 1997). Unlike serological tests, PCR is able to detect current infections. Another advantage of PCR is that assays which are able to detect more than one tick-borne pathogen simultaneously can be developed (Figueroa et al., 1993; Bekker et al., 2002). Such tests have the advantage in that they not only reduce the cost and time of performing the assay, but also allow the study of the epidemiology of several TBIs that can occur concurrently in the same animal for example nested PCR, quantitative PCR, and multiplex PCR

1.6.3.2 Real-time PCR (RT-PCR)

Real-time PCR (qPCR) is based on the detection of fluorescence produced by a reporter molecule which increases as the reaction proceeds. This occurs due to the accumulation of the PCR product with each amplification cycle. These fluorescent reporter molecules include dyes that bind to double-stranded DNA (e.g. SYBR® Green) or sequence-specific probes (e.g. Molecular beacons or FRET® and TaqMan® probes). qPCR facilitates the monitoring of the reaction as it progresses. The advantages of real-time PCR assay over conventional PCR are that it is relatively rapid and convenient because there is no need to perform gel electrophoresis to visualize the PCR products. Real-time PCR is a simple, fast, closed and automatized amplification system responsible for decreasing the risk of cross-contamination. This technique has been used for the detection of a number of parasites including *Anaplasma* spp., *Ehrlichia* spp., *Rickettsia* spp. and *Borrelia* spp. (Shen et al., 2018) in various regions of the world. Real-time PCR has engendered wider acceptance of PCR due to its improved rapidity, sensitivity, reproducibility and the reduced risk of carryover contamination (Mackay, 2004).

1.6.3.3 Reverse line blotting

The reverse line blot (RLB) was initially developed in the mid-1990s, for the identification of *Streptococcus* serotypes (Kaufhold et al., 1994), and, further on, for the differentiation of *Mycobacterium tubercolosis* strains (Kamerbeek et al., 1997), the RLB permits the screening of multiple samples for multiple microorganisms. The novelty of this approach resided in the hybridization of PCR products with species-specific probes immobilized on a blotting membrane. The first application of the RLB technique for the diagnosis of tick-borne infections (TBIs) was developed in 1995 in a laboratory at the National Institute of Public Health and the Environment, in Bilthoven, The Netherlands (Rijpkema et al., 1995). The initial assay could detect up to four *Borrelia* species at the same time using the 5S–23S rDNA intergenic spacer as a target region, with a nested PCR protocol being performed prior to the RLB (Rijpkema et al., 1995). Afterwards, a major push forward in the development of this technique for the diagnosis of TBIs in livestock was represented by the implementation of an assay allowing detection and differentiation of all known *Theileria* (*T. annulata*, *Theileria parva*, *T. mutans*, *T. taurotragi*, *T. velifera* and *Theileria orientalis* group) and *Babesia* (i.e. *B. bigemina*, *B. bovis*, *B. divergens*) species of importance to cattle health in tropical and sub-tropical settings (Gubbels et al., 1999). In 2002, another RLB method was implemented to detect and differentiate *Anaplasma* and *Ehrlichia* species known to occur in ruminants and *A.*

variegatum ticks in the "(sub) tropics" (Bekker et al., 2002). Only one year after, another study (Christova et al., 2003) added an RLB for the detection of *Rickettsia* spp., to the protocol set up by Schouls et al., (1999).

The development and progressive improvement of the RLB assay have played a major contribution to the understanding of the epidemiology of TBIs as well as the competence of their respective vectors across several African countries. Improving the diagnostics of TBIs in tropical and sub-tropical settings, was indeed one of the ideals with which this methodology was developed (Gubbels et al., 1999). While tests enabling the detection only of *Anaplasma* and *Babesia* species are most suited for Central and Southern America where bovids do not harbour theileridae (Criado-Fornelio et al., 2009), diagnostic tools dealing with cattle samples from SSA should certainly take into account *Theileria* species. The great suitability of this technique within this context can be essentially attributed to two reasons: a) the frequent scenario of coinfections, from which the need for an 'integrated' diagnostic approach arises, and b) the endemicity of numerous TBIs characterised by chronically infected/sub-clinical/carrier animals, for which high sensitivity tools are needed given their rather low parasitaemia. Therefore, the assay developed a few years later by (Bekker et al., 2002), included *Ehrlichia*/*Anaplasma* species of importance in SSA (i.e. *E. ruminantium*).

To date, these two RLB assays (Gubbels et al., 1999; Bekker et al., 2002), have been employed, either alone, i.e. *Theileria*/*Babesia* spp. (Gubbels et al., 2000; Nijhof et al., 2003; Matjila et al., 2004, 2008; Ali et al., 2006; Salih et al., 2007, 2010; Oosthuizen et al., 2008, 2009; Köster et al., 2009; Bhoora et al., 2009, 2010; Bosman et al., 2010, 2013; Schoeman & Herrtage, 2008; Penzhorn et al., 2008; Muhanguzi et al., 2010; Yusufmia et al., 2010; Brothers et al., 2011; Chaisi et al., 2011a; Govender et al., 2011a; Oura et al., 2011; Githaka et al., 2013; Goddard et al., 2013) or i.e. *Ehrlichia/Anaplasma* spp. (Faburay et al., 2007; Muhanguzi et al., 2011) or in combination (i.e. *Theileria*/*Babesia* spp. + *Ehrlichia/Anaplasma* spp.) (Oura et al., 2004, Tomassone et al., 2005; Matjila et al., 2008; Berggoetz et al., 2009; Martins et al., 2010; Oura et al., 2011; Pfitzer et al., 2011; Asiimwe et al., 2013; Adamu et al., 2014; Berggoetz et al., 2014b, 2014a) for epidemiological and diagnostic purposes in a number of SSA countries including Uganda (Tait & Oura, 2004; Muhanguzi et al., 2010; Oura et al., 2011 Asiimwe et al., 2013), the Republic of Guinea (Tomassone et al., 2005), The Gambia (Faburay et al., 2007), Sudan (Gubbels et al., 2000; Ali et al., 2006) and South Sudan (Salih et al., 2007; 2010), Ethiopia (Tomassone et al.,

2012), Kenya (Githaka et al., 2013), Mozambique (Martins et al., 2010), Namibia (Penzhorn et al., 2008), South Africa (Nijhof et al., 2003; Matjila et al., 2004; Matjila et al.,2008; Yusufmia et al., 2010; Berggoetz et al., 2014a), and Nigeria (Ogo et al., 2012; Adamu et al., 2014).

Work carried out in the African continent, not only focused on the detection of TBIs in livestock such as cattle (Gubbels et al., 2000; Oura et al., 2004; Ali et al., 2006; Salih et al., 2010; Yusufmia et al., 2010; Muhanguzi et al., 2011; Asiimwe et al., 2013), small ruminants (Tomassone et al., 2012), but also in wildlife and game animals such as the African buffalo (*Syncerus caffer*) (Chaisi et al., 2011), black (*Dicero bicornis*) (Nijhof et al., 2003; Penzhorn et al., 2008) and white rhinoceros (*Ceratotherium simum*) (Govender et al., 2011), nyala (*Tragelaphus angasii*) (Oura et al., 2011; Pfitzer et al., 2011; Berggoetz et al., 2014a;), roan (*Hippotragus equinus*) (Nijhof et al., 2005; Oosthuizen et al., 2009), antelope, greater kudu (*Tragelaphus strepsiceros*) and gray duiker (*Sylvicapra grimmia*) (Nijhof et al., 2005). Moreover, a few other studies also focused on the detection of microorganisms in ticks, to assess pathogen detection (Tomassone et al., 2005; Faburay et al., 2007) and vectorial competence (Berggoetz et al., 2014b). In South Africa, the application of the RLB has been largely employed in epidemiological surveys focusing on wildlife species, enabling the identification of novel species (i.e. *Babesia bicornis* and *Theileria bicornis*) (Nijhof et al., 2003) as well as contributing to the better understanding of epidemiological relationships occurring at the game/livestock interface (Yusufmia et al., 2010; Berggoetz et al., 2014). In 2007, the RLB method was employed for the first time for the longitudinal monitoring of animals in a six-month-long field trial designed to evaluate the prophylactic efficacy of 9% amitraz-impregnated collars (Preventic, Virbac, Carros, France) against canine babesiosis (*Babesia canis rossi*) in South Africa (Last et al., 2007).

With regards to West Africa, to date the RLB has only been employed on studies focusing on ticks collected from cattle in Guinea (Tomassone et al., 2005), The Gambia (Faburay et al., 2007) and Nigeria (Ogo et al., 2012; Adamu et al., 2014a; Lorusso et al., 2016) The former two studies focused specifically on *A. variegatum* ticks, against *Ehrlichia*/*Anaplasma* and *Theileria*/*Babesia* probes (Tomassone et al., 2005), and to assess their vectorial capacity and suitability for xenodiagnosis of *E. ruminantium* infection (Faburay et al., 2007).

The two studies carried out in Nigeria focused on ticks from three species [i.e. *A. variegatum*, *Rh.* (*Bo.*) *decoloratus* and *Rh. sanguineus*] (Ogo et al., 2012) and dog's blood samples (Adamu et al., 2014), screened against *Theileria*/*Babesia* and *Ehrlichia*/*Anaplasma* probes. Both studies included samples collected in the Plateau State. The present work highlights the versatility of the RLB method as an epidemiological and diagnostic tool, to detect both clinically apparent and subclinical infections and for the monitoring of the post-therapeutic progression of infections (Nagore *et al*., 2004a).

The advantages deriving from the employment of the RLB has proved it to be a very sensitive test for a number of microorganisms, being able to detect parasitaemia beyond the detection limit of standard PCR (Gubbels et al., 1999; Schnittger et al., 2004; Tait & Oura, 2004; Molad et al., 2006). By using species-specific oligonucleotide probes, RLB enables the detection and differentiation of species of tick-borne microorganisms infecting pets, livestock and humans. Absence of cross-reactions has been well demonstrated (Gubbels et al., 1999; Schouls et al., 1999; Bekker et al., 2002; Schnittger et al., 2004). Theoretically, competition for the primer volumes available in the PCR master mix can occur among species of the same genus. However, this was shown to be prevented by including a sufficiently high volume of primers in the PCR mixture (Gubbels et al., 1999; Schnittger et al., 2004).

1.7 Control of ticks

Tick control programmes in many countries aim at reduction of the tick burden on both humans and animals and have stimulated intense interest for more than a century. Complete eradication of ticks is difficult. However, there is a variety of strategies for the control of ticks and TBIs (Sonenshine et al., 2006; Willadsen, 2006). Tick prophylaxis should cover the entire period during which ticks are active. Treatment with synthetic chemicals known as acaricides is still the most widely used means to control or prevent tick attacks and has the aim of preventing pathogen transmission (Polar et al., 2005). Control of ticks with acaricides can either be directed against the ticks on the host or against the free-living stages of those ticks in the environment.

The conventional methods of controlling ticks is by the application of chemical acaricides which include several groups of pesticides: organophosphates (e.g. coumaphos, diazinon), carbamates (e.g. propoxur), pyrethroids (e.g. permethrin, deltamethrin, flumethrin), formamidines (e.g. amitraz), two classes (avermectins and milbemycins) of macrocyclic lactones (e.g. ivermectin, doramectin, moxidectin, eprinomectin), phenylpyrazoles (e.g.

fipronil) and natural acaricides such as botanical materials (e.g. extracts of the neem tree containing azadirachtin) (Salman et al., 2012). The use of acaricides has been successful; this is possible through the correct mixing of the acaricides and strategic application of tick control measures, considering seasonal variations (Latif & Walker, 2004). As a matter of fact, acaricides are essential in short-term but do not offer a permanent solution to tick control (Frisch, 1999). The use of cattle dip involves the animals plunging into and swimming through dip tanks or vats containing an aqueous emulsion, suspension or solution of acaricides (Norval et al., 1992).

Several other methods are used for the application of acaricides to animals, such as dips, sprays using manual or motorised high-pressure sprayers, dust, pour-ons, spot-ons, the intraruminal bolus and injections. Dipping vats have been used extensively for tick control on livestock including cattle and sheep. When these are used for several animals over extended periods of time, dipping vats need to be maintained with the proper solution levels and percentage of the active ingredient. Small numbers of livestock can be treated with hand-held sprayers, but manual spraying depends on the skill of the person applying it. Retreatment may be needed several times during a season when sprays and dips are used. Pour-on formulations of acaricides contain high-quality oil that spreads through the greasy hair coat of livestock, and these products can also be used with applicators to treat wild ungulates in game reserves (Latif & Walker, 2004). Thus, pour-on formulations are relatively expensive, but there is little wastage of acaricide, and they may be cheaper in the long term. The other topical application methods are often employed owing to their advantage in limiting the amount of acaricide used; the cattle pass through a heavy low-pressure spray and become soaked with acaricidal fluid, but body parts such as the ears and groin may not be effectively treated by this method (Latif & Walker, 2004). To achieve long-lasting efficacy, acaricides can be incorporated into a plastic or other suitable matrix that provide a slow release of the toxicant over a period of weeks or months. Systemic acaricides offer another means of providing long-lasting and effective tick control. These preparations can be divided into injectable, oral and topically applied products, all of which are delivered to the tick during its feeding activity on the skin. Each application method has its advantages and disadvantages.

The chemical control of ticks on livestock poses several problems. Besides leaving residues in meat and milk, the use of many acaricides (e.g. organophosphates, carbamates) is associated with risks of side effects or poisoning resulting from overdoses on specific species or breed sensitivity (Monteiro et al., 2010). Environmental contamination and effects on non-

target animals have also been well documented for organochlorines, organophosphates, carbamates and pyrethroids (Graf et al., 2004). The development of acaricide resistance by some tick species is an increasing and continuing concern in tropical and subtropical areas, where these ticks have been found to be resistant to chlorinated hydrocarbons, organophosphorus insecticides, pyrethroids and formamidines (Graf et al., 2004).

Traditional methods of tick control are known to be widely used in Africa which includes grass burning, hand removal of the ticks, extracts of leaves and cattle dusting with Ash. The practice of hand removal of ticks has been a method of tick control in most of the sub-Saharan African countries. Its regularity is, however, not uniform, being undertaken almost daily by pastoralist households and irregular in agro-pastoral farming where the practice can be neglected totally during crop planting and harvesting seasons. Habitat modification such as vegetation management as well as burning and heavy grazing can likely contribute to ticks control by reducing ticks population (Sonenshine et al., 2006). In western Ethiopia juices of crushed leaves of *Phytolacca dodecahedra, Vernonia amygdalina,* and the crushed seed of *Lepidium sativum* mixed with fresh cattle faeces were used to control ticks (Regassa, 2000). The variety of traditional practices maintained within different ethnic groups provides an indication of the potential usefulness of this neglected knowledge for livestock husbandry (Mesfin & Obsa, 1994).

The Fulani pastoralists in Nigeria have historically preferred traditional methods of control, such as the manual removal of ticks (Bayer & Maina, 1984), carried out three times a week during the wet season (i.e. April to October) and twice a week during the dry months (i.e. November to March) (Maina, 1986). This method, however, is far from effective, as usually only adult ticks are removed, especially the larger sized *Amblyomma* and *Hyalomma* spp., while most immature stages and smaller sized *Rhipicephalus* and *Boophilus* spp. still remain attached (Maina, 1986). In particular, the Fulani recognize the most conspicuous *Amblyomma* ticks as ‘Koti’ (‘hazardous’), whereas other smaller species (e.g. boophilid ticks) as ‘miri’ (‘less harmful’) (Bayer & Maina, 1984)**.** Furthermore, as the collection is usually performed while the animals are standing, the least easily accessible body parts (e.g. ear canals, axillae, groin, tail base, etc) get less accurately cleared of ticks (Bayer & Maina, 1984). In addition, although it keeps animals free from ‘tick worry’, hand removal of ticks may not help prevent the transmission of TBIs when not performed on a daily basis, as the transmission of pathogens may occur within 24–36 hours after the attachment of these arthropods to their hosts (Bezuidenhout & Bigalke, 1987; Azad & Beard, 1998). Besides adopting the ‘de-

ticking' practice, the Fulani cattle keepers try to minimize the risk of tick infestation in their cattle by reducing the time spent at watering points where they suspect vectors (especially tsetse flies) are most likely to occur. Moreover, in the wet season, they delay the grazing of their herds until late in the morning, as they consider tick infestation on the grass to be the highest in the early hours (Maina, 1986). Fires are also burnt next to the cattle pens to keep away ticks and biting insects at night (Maina, 1986).

1.8 Thesis aims

1. To critically review and update the existing knowledge of veterinary and zoonotic importance of tick-borne infections in Nigeria.
2. To quantify the diversity and prevalence of infestation of ticks parasitizing domestic ruminants (cattle, sheep and goats) in the Kachia Grazing Reserve, Nigeria.
3. To delineate the epidemiology of tick-borne infections of domestic ruminants in the Kachia Grazing Reserve.
4. To identify significant epidemiological determinants of tick-borne infections of domestic ruminants in the Kachia Grazing Reserve.

Chapter 2: Materials and Methods

2.1 Study area

Livestock sampling was carried out in Kachia Grazing Reserve (KGR) also known as 'Ladunga' in Kaduna State, North Western Nigeria (see Figure 2.1). The region falls within Guinea Savannah ecological zone and is situated between latitudes 10°03′ and 10°13′N and longitudes 7°55′ and 8°06′E with a land area of 33,411 hectares. The grazing reserve is positioned around 700–790 meters above sea level and is drained by the Kaduna river system and several streams that feed into it. Within the grazing reserve, a mixed farming system is practised. The reserve contains 777 farmers (heads of households) and over 10,000 people. The population of livestock within the reserve are 40,000 cattle, 10,000 sheep and 5,000 goats (Ducrotoy et al., 2016). The reserve is divided into six 'blocks' which are geographically distinct administrative regions. On the basis of water availability, climate, physiography and land use, Kachia grazing reserve falls in the Sub-Humid Minna-Kaduna-Kafanchan high plain (Figure 2:1) which has significant implications for the spatial and temporal patterns of livestock diseases (Aregheore, 2009). The region has two seasons; the rainy season is lasting 4-5 months (May to September), and a long dry season lasting 6-7 months (October to April). Initially, the dry season (October to April) is cold and dry but later (May to September) it becomes more humid and hot. The ambient temperature is about 28°C by August but could be as high as 40°C or more between April and May. The relative humidity is about 45% during the rainy season but declines to 5% between December and February. The environmental conditions are favourable for ticks leading to high infestation on a wide variety of hosts, which in turn results in the transmission of diseases to livestock, humans, and companion animals. The socio-economy activities of the region are mainly characterised by informal sector with agriculture as the major economic driver.

In general, livestock are poorly managed in KGR mostly managed on free range/extensive system and semi-intensive system. These management systems are basically influenced by cheap means of feeding the stock all year round. Based on this, the animals are thus allowed to roam the grazing reserve and neighbourhood to fend for themselves with little or no special or conscientious provision of supplements for the animals.

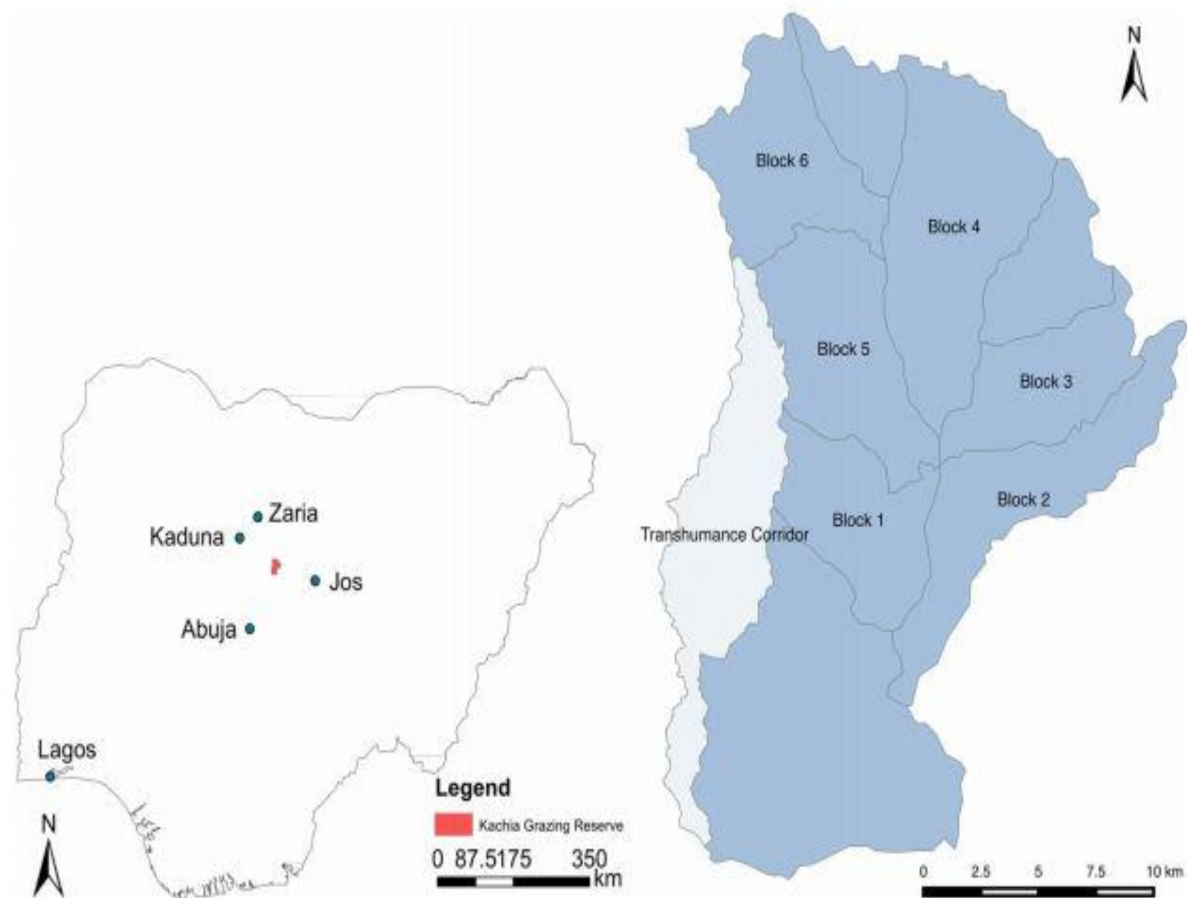


Figure 2:1: Map of Nigeria showing Kachia Grazing Reserve in Kaduna State, North Western Nigeria reproduced from (Ducrotoy et al., 2016)

2.2 Ethical approval

The study protocol was read and approved by the University of Salford Manchester UK and Veterinary Council of Nigeria. The research was conducted by strictly adhering to the Institutional guidelines. The aims of the project, together with its intended methods, were effectively disseminated through the KGR farming community and verbal consent was obtained from the District Head and livestock owners before the project began.

2.3 Study design

Two cross-sectional surveys of livestock in the KGR were conducted between May and August in 2015 and between June and August in 2016. Animals in all six blocks within the KGR were recruited into the study. For each block of KGR, livestock herders were informed by the block head to gather all the available livestock at a particular site. After agreeing on the gathering site, ticks and blood samples were collected between 7-10am daily

for a period of two weeks starting from block one to six. For each sampled animal, information such as age, sex, breed, and body condition score (BCS) was evaluated.

The age of the animals were grouped into two categories based on dentition and the information given by the herdsmen, namely: calves (0-24 months of age), and adults (older than 24 months) in cattle, this difference in age range where based on cattle attainment to slaughter in the country, while in sheep and goat (0-8 months) where grouped as young and (older than 8 months) as adult.

Table 2:1: Census population of the livestock in each block of KGR

Block	Total population of livestock	Animals surveyed		
		cattle	sheep	goats
1	1,123	61	56	25
2	1,524	54	43	15
3	2,063	24	68	10
4	3,287	81	6	14
5	702	48	79	0
6	838	0	5	132
Total	**9,537**	**268**	**257**	**196**

2.4 Sample size determination

The approximate sample size required to estimate the prevalence of ticks and haemoparasites on livestock in the KGR was determined for a defined precision of 5% and at 95% level of the confidence interval as described by Thrusfield and colleagues (2005). The minimum sample size was calculated as 384 where 'N' is the required number of samples, 'P' is the expected prevalence of tick-borne pathogens, 'd' is the desired absolute precision 5% (0.05) and 'Z' is the appropriate value from the desired confidence (1.96)

Where,

$$N = \frac{Z^2 P(1-P)}{d^2}$$

$$= \frac{(1.96)^2 \times 0.50 \times (1-0.50)}{(0.05)^2}$$

$$= \frac{3.8416 \times 0.50 \times 0.5}{0.0025} = 384$$

2.5 Blood sample collection

About five millilitres of blood was collected aseptically from the jugular vein of each animal using 10 ml hypodermic syringe with 21G needle for sheep and goats or 18G needle for cattle. The blood samples were collected into a plastic specimen bottle containing ethylene diamine tetraacetic acid (EDTA). The blood samples were labelled and transported in refrigerated cool boxes to Ahmadu Bello University Zaria Kaduna State Nigeria for haematology analysis. In addition, several drops of blood were applied on Flinders Technology Associates (FTA™) cards (Whatman BioScience Ltd, Cambridge, UK) and allowed to air-dry for 3 hours at room temperature. Each card was then placed into an FTA™ card pouch before transporting the samples to the University of Salford for molecular analysis.

2.6 DNA extraction and elution from blood on FTA card

For each of the blood samples spotted on FTA® cards, five discs of 3 mm of diameter were punched using a Harris Micro-Punch™ (Whatman BioScience Ltd, Cambridge, UK) (see Figure 2:2) and placed into a 1.5 ml Eppendorf tube. To avoid cross-contamination among samples, 5-10 punches were taken from a blank paper between two consecutive samples. Negative controls for the DNA extraction and elution phases were represented by five blank

discs of paper, punched every 10 samples, according to the following sequence:- the first negative control, named as 'Wash 0' ('W_0'), was obtained before starting to punch the first sample of a set of 20; the second, 'Wash 1' ('W_1'), was obtained after the 10th samples; the third, 'Wash 2' ('W_2'), was obtained after the 20th sample ('W_2') and so on. After punching a set of 40 samples, the Harris Micro-Punch™ was disinfected with 70% ethanol, before being wiped and treated with UV light for a minimum of 20 minutes.

The five discs in each 1.5 ml Eppendorf tube were washed twice for 15 minutes by adding 1 ml of FTA™ purification reagent (Whatman BioScience Ltd, Cambridge, UK), in order to remove any potential PCR inhibitor from the sample. Each washing step took place under gentle shaking and was followed by a thorough aspiration of the purification reagent using disposable plastic Pastette/pipette. Discs were then washed twice for 15 minutes with gentle shaking in 1 ml of 1x Tris-EDTA buffer (Sigma-Aldrich, Dorset, UK) to remove traces of the FTA™ purification reagent previously used. The discs were then transferred into a sterile 1.5ml Eppendorf tube and placed in an incubator at 37°C for 30-40 minutes or allowed to air dry at room temperature overnight. DNA was eluted by adding 100 µl of 5% (w/v) Chelex® 100 (Sigma-Aldrich Ltd, UK) solution to each PCR tube and incubating at 90°C for 30 minutes in a thermal cycler then stored at -20°C until used.

Figure 2:2: Picture of FTA™ card spotted with blood sample after taking five-disc punches in a circle format

2.7 Agarose gel electrophoresis

The success of the PCR reaction was determined by observation of agarose gel stained with gel-red on which PCR products had been electrophoretically resolved. 1.5% (w/v) agarose gels was created by dissolving 1.5g of agarose (Bioline, London) in 100ml of 1 x Tris/Borate/EDTA (TBE) buffer (Seven Biotech Ltd). An aliquot (100µl) of GelRed (Biotium, Cambridge) was added to the cooling molten agarose, which was then poured into a mould and allowed to set with the combs inserted. The solidified gel was placed into the electrophoresis tank containing 1XTBE buffer and 5 µl of PCR product was added to each well. At least one well on each gel contained 3 µl Hyper Ladder 1 (Bioline, London) to allow the size of PCR products to be estimated. The conditions for electrophoresis were 110V for 45 mins, after which gels were visualised on a UV transilluminator.

2.8 Reverse line blotting (RLB)

2.8.1 PCR for haemoparasites

The RLB used was made up of 4 group specific PCRs then probes to delineate species within each group. Following DNA extraction and elution, each sample were subjected to four simultaneous PCRs targeting, respectively:

(i) a 460–520 bp long fragment from the V1 hypervariable region of the 16S SSU rRNA gene for *Ehrlichia* and *Anaplasma* spp. (Schouls et al., 1999; Bekker et al., 2002),

(ii) A 460–540 bp long fragment from the V4 hypervariable region of the 18S ribosomal RNA (rRNA) gene for *Theileria* and *Babesia* spp. (Georges et al., 2001),

(iii) a 350–400 bp variable region in the 16S rRNA gene for *Rickettsia* spp. (Christova et al., 2003), and

(iv) a 296 bp long fragment of 16S rRNA gene for *Bartonella* (see also Table 2:2).

Table 2:2: Primer sets used during PCR amplification

Target taxa	Primer type	Primer name	Sequence (5 '-3 ')	Tm*	Reference
Ehrlichia / Anaplasma Spp	Fwd	Ehr-F	GGAATTCAGAGTTGGATC MTGGYTCAG	61.0	(Schouls et al., 1999)
	Rev	Ehr-R	Biotin-CGGGATCCCGAGTTTGCC GGGACTTYTTCT	69.5	(Bekker et al., 2002)
Theileria / Babesia spp.	Fwd	RLB-F2	GACACAGGGAGGTAGTGA CAAG	57.9	(Georges et al., 2001)
	Rev	RLB-R2	Biotin-CTAAGAATTTCACCTCTG ACAGT	53.7	
Rickettsia spp.	Fwd	RCK/2 3-5-F	GATAGGTCRGRTGTGGAA GCAC	69.0 - 74.9	(Christova et al., 2003)
	Rev	RCK/2 3-5-R	Biotin-TCGGGAYGGGATCGTGTG TTTC	78.2 - 80.1	
Bartonella spp.	Fwd	P24E	GGAATTCCCTCCTTCAGT TAGGCTGG	76.6	(Bergmans et al., 1995)
	Rev	P128	Biotin-CGGGATCCCGAGATGGCT TTTGGAGATTA	82.2	

*Tm = melting temperature. [R = A or G; Y = C or T].

PCR amplification was carried out in a total volume of 25 µl, by adding 0.5 µl of 10 mM dNTPs (Qiagen, UK), 5 µl of 5× Phire reaction buffer (Thermo Scientific, UK), 0.5 µl of 20 pmol/µl of each forward and reverse primer (Eurofins, UK), 15.875 µl of water, 0.25 units of Phire Hot Start II DNA Polymerase (Fisher Scientific, UK), and 2.5 µl of template DNA. Positive controls included 2.5 µl of DNA from *Ehrlichia ruminantium*, *Theileria mutans*, *Rickettsia conorii*, and *Bartonella henselea* for each PCR respectively (*Ehrlichia*

ruminantium DNA kindly provided by Dr Lesley Bell-Sakyi, *Theileria mutans* by Prof Jonjegena *Rickettsia conorii* by Prof PE Fournier, VITROME, IHU Mediterranee-Infection, Aix-Marseille University, Marseille, France and *Bartonella henselae* by Prof Richard Birtles University of Salford). Negative controls consisted of 2.5 µl of water and 5 % Chelex® 100 (Sigma-Aldrich Ltd, Dorset, UK) eluted blank white paper (i.e. 'wash controls', as indicated). The 5' of each reverse primer was labelled with a biotin ligand, in order to allow the correct accomplishment of the blotting protocol, to be carried out following each PCR reaction. To enhance binding capacities of the primers on different target pathogen DNA, primer sequences were designed with mixed bases (also known as degenerate bases). Equal amounts of the designated bases are delivered by the synthesiser. To increase PCR productivity, a touchdown PCR program was used (see Table 2:3). DNA amplification was carried out in a Prime Elite thermal cycler© UK.

Table 2:3: PCR conditions

PCR step	Temperature (ºC)	Duration (seconds)	Number of cycles
Initial Denaturation & Hot-start Polymerase Activation	98	30	1
Denaturation	98	5	10*
Annealing	67–57	5	
Extension	72	7	
Denaturation	98	5	40
Annealing	57	5	
Extension	72	7	
Final extension	72	60	1

*Temperature decreasing of 1°C at each cycle.

The outcome of PCR was determined following the electrophoretic run of the positive and negative controls through a 1.5% (w/v) agarose gel stained with GelRed© (Biotium, USA), and 1 kb Hyper Ladder (Bioline, London) which indicated the size of the product (base pairs) (see section 2.1). The migration of positive controls was expected to yield a band (of variable size, depending on the sample and the PCR used), while no bands were expected to be found for the negative controls, in absence of contamination.

2.8.2 Design of membrane

'Catch-all' and species-specific oligonucleotide probes (see Table 2:4) containing a N terminal *N*-(trifluoroacetamidohexyl-cyanoethyle, *N*,*N*-diisopropyl phosphoramidite [TFA])-C6 amino linker (Isogen, The Netherlands) were covalently linked onto a Biodyne C blotting membrane (Pall Biosupport, Ann Arbor, Mich.) as previously described (Lorusso et al., 2016).

Briefly, catch-all probes were included (i) to ensure that the hybridization of positive samples took place correctly (i.e. all samples turning positive for one of more species are expected to be positive for the 'catch-all' probe of their respective genus), but also (ii) to indicate the presence of an unknown species or strain of a certain parasite or the presence of a known species, for which a specific oligonucleotide probe was not included in the assay.
For each catch-all and species-specific probe employed, the amount of oligonucleotide applied on the membrane was optimised to 400 μM, enabling the visualisation, in the presence of the same amount of DNA, of equally intense signals. In order to obtain the desired aforementioned working concentration (i.e. 400 μM), oligonucleotide probes were diluted in 150 μl of $NaHCO_3$ at a pH of 8.4. Prepared dilutions were then applied on the blotting membrane, previously 'activated' via a 10-minute-long incubation step in 16% 1-(3-Dimethylaminopropyl)-ethyl carbodiimide hydrochloride (EDAC) at room temperature, through the use of a Miniblotter MN45 (Immunetics, MA, USA). Afterwards, the membrane was left to incubate for one minute, after which the loaded volumes of $NaHCO_3$ were removed by aspiration, in the same order as they were applied. The Biodyne C blotting membrane was then washed for 8–10 minutes at room temperature in a tray containing 100 ml of 100 mM Sodium hydroxide (NaOH) in order to lose its EDAC due to reactiveness. A washing step in a buffer (2x SSPE/0.1%) Sodium dodecyl sulfate solution (SDS) at 60ºC for 10 minutes concluded the process of membrane preparation.

Table 2:4: 'Catch-all' and species-specific oligonucleotide probes used in RLB

	Tick-borne Microorganism's Genera/Species	**Probe Sequence (from 5'–3')**	**Tm (ºC)**	**Reference**
1	*Ehrlichia/ Anaplasma* catch-all	GGGGGAAAGATTTATCGCTA	49.7	Bekker et al., (2002)
2	*Anaplasma bovis*	GTAGCTTGCTATGRGAACA	46.8 - 48.9	Georges et al., (2001)
3	*Anaplasma centrale*	TCGAACGGACCATACGC	49.5	Bekker et al., (2002)
4	*Anaplasma marginale*	GACCGTATACGCAGCTTG	50.3	Bekker et al., (2002)
5	*Anaplsma* spp. Omatjenne	CGGATTTTTATCATAGCTTGC	48.5	Bekker et al., (2002)
6	*Anaplasma phagocytophilum* – 1	TTGCTATAAAGAATAATTAGTGG	46.4	Schouls et al., (1999)
7	*Anaplasma phagocytophilum* – 2	TTGCTATGAAGAATAATTAGTGG	48.1	Schouls et al., (1999)
8	*Anaplasma phagocytophilum* – 3	TTGCTATAAAGAATAGTTAGTGG	48.1	Schouls et al., (1999)
9	*Anaplasma phagocytophilum* – 4	TTGCTATAGAGAATAGTTAGTGG	49.9	Schouls et al., (1999)
10	*Anaplasma platys*	GTCGTAGCTTGCTATGATA	46.8	Unpublished
11	*Ehrlichia ruminantium* – 1	AGTATCTGTTAGTGGCAG	45.8	Bekker et al., (2002)

12	*Ehrlichia ruminantium* – 2 (i.e. BAA015)	ATTTCTAATAGCTATTCCAT	41.5	Allsopp et al., (1999)
13	*Ehrlichia chaffeensis*	ACCTTTTGGTTATAAATAATTGTT	45.4	Schouls et al., (1999)
14	*Theileria/Babesia* catch-all	TAATGGTTAATAGGA(A/G)C(A/G)GTTG	47.4 - 51.1	Matjila et al., (2008)
15	*Babesia bigemina*	CGTTTTTTCCCTTTTGTTGG	47.7	Gubbels et al., (1999)
16	*Babesia bovis*	CAGGTTTCGCCTGTATAATTGAG	53.5	Gubbels et al., (1999)
17	*Babesia caballi* – 1	GTGTTTATCGCAGACTTTTGT	48.5	Butler et al., (2008)
18	*Babesia caballi* – 2	GTTGCGTT G/TTTCTTGCTTTT	45.6 - 47.7	Govender et al., (2011)
19	*Babesia divergens*	ACTRATGTCGAGATTGCAC	46.8- 48.9	Oosthuize et al.,(2009)
20	*Theileria* catch-all	ATTAGAGTGCTCAAAGCAGGC	52.4	Matjila et al., (2008)
21	*Theileria annulata*	CCTCTGGGGTCTGTGCA	51.9	Georges et al., (2001)
22	*Theileria buffeli*	GGCTTATTTCGGATTGATTTT	46.5	Gubbels et al., (1999)
23	*Theileria equi*	TTCGTTGACTGCYTTGG	44.6 - 47.1	Butler et al., (2008)
24	*Theileria equi*-like	TTCGTTGTGGCTTAGTTGGG	51.8	Unpublished
25	*Theileria mutans*	CTTGCGTCTCCGAATGTT	48	Gubbels et al., (1999)

26	*Theileria parva*	GGACGGAGTTCGCTTTG	49.5	Nijhof et al., (2003)
27	*Theileria taurotragi*	TCTTGGCACGTGGCTTTT	48	Gubbels et al., (1999)
28	*Theileria velifera*	CCTATTCTCCTTTACGAGT	46.8	Gubbels et al., (1999)
29	*Theileria* spp. MSD4	GCTTATTTCGGCGACCTC	50.3	Unpublished
30	*Theileria* spp. (duiker)	CATTTTGGTTATTGCATTG TGG	49.2	Nijhof et al., (2005)
31	*Rickettsia* generic (GP-RICK)	TAGCTCGATTGRTTTACTT TG	46.5 - 48.5	Jado et al., (2006)
32	SFG *Rickettsia* spp. (SFG-R)	ACTCACAARGTTATCAGGT	44.6 - 46.8	Jado et al., (2006)
33	Typhus Group *Rickettsia* spp. (GP-TG)	GTTATTCTATCGTTTTATG TCACG	48.8 - 50.6	Jado et al., (2006)
34	*Bartonella* catch-all	GTTGGGCACTCTAA/GGG	45.9 - 48.5	Schouls et al., (1999)
35	*Bartonella henselae*	TGCCAGCATTTGGTTGG	47.1	Schouls et al., (1999)
36	*Bartonella quintana*	TTGCCATCATTAAGTTGGG	46.8	Schouls et al., (1999)

[R = A/G; K = G/T; W = A or T; Y = C or T].
*Tm = basic melting temperature (calculated on: http://biotools.nubic.northwestern.edu/OligoCalc.html).

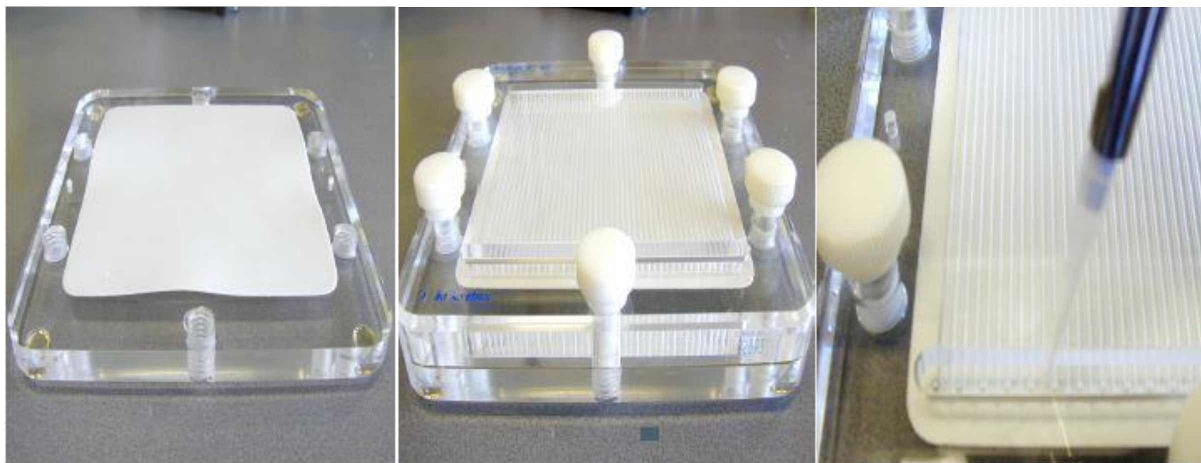
2.8.3 RLB hybridization of PCR product

Hybridization of PCR products to catch-all and species-specific probes was performed as previously described (Lorusso et al., 2016). Briefly, 10 µl of four PCR negative and wash controls was diluted in 120 µl of 2x SSPE/0.1% SDS (for a total of 160 µl of solution) in a sterile 1.5 ml Eppendorf tube. For each tested sample, 10 µl of each PCR product (obtained from the *Anaplasma*/*Ehrlichia* spp., *Babesia*/*Theileria* spp., *Rickettsia* spp. and *Bartonella* spp. PCRs, respectively) were diluted into 120 µl of 2x SSPE/0.1% SDS (for a total of 160 µl of solution) in sterile 1.5 ml Eppendorf tubes. 10 µl of amplified positive controls was diluted in 150 µl of 2x SSPE/0.1% SDS. The diluted PCR products and controls were denatured for 10 minutes at 100°C in a heating block and cooled on ice immediately (see Figure 2:3). Heat denaturation was carried out for the amplified DNA to single-strand and therefore be potentially reactive to complementary oligonucleotide sequences fixed on the Biodyne C membrane.

Figure 2:3: Diluted PCR products placed on ice after denaturation carried out for 10 minutes at 100°C on a heating block to separate the DNA in two single strands

After the samples had cooled down, a short spin centrifuge of 30 seconds at 13,000 g was carried out, in order to remove the condensation formed on the internal surface of the Eppendorf tubes' lids. The samples were then put back and kept on ice until the time they were loaded on the blotting membrane, to prevent DNA from re-double stranding. The Biodyne C membrane was then activated by incubating it for 5 minutes in 100 ml of 2x SSPE/0.1% SDS kept in a plastic tray, at room temperature under gentle shaking. Afterwards, the membrane was placed in a Miniblotter MN45 (Immunetics, MA, USA) on a support

cushion, in a way that the blotter's lanes would be orientated perpendicularly to pattern according to which the probes were fixed on the membrane. Screws were applied two by two, from opposite directions, so to fix firmly the membrane within the two sides of the blotter and the support cushion (placed between the lower surface of the membrane and the bottom side of the blotter, see Figure 2:4. Following membrane activation and prior to the loading of the samples, the residual 2x SSPE/0.1% SDS fluid was then removed from the membrane by aspirating it from blotter's lanes through a vacuum maker. At this point, each blotter's slot was filled with the diluted PCR products of 160 μl of volume (including positive and negative controls), by avoiding the formation of air bubbles using a 200 μl or 1,000 μl, depending on the diameter of the entry of the blotter lanes. The first and last lane, #1 and #45, respectively, were filled with 2x SSPE/0.1% SDS solution, in order to avoid false negative results, in case the loading and fixation of the oligonucleotide probes had not reached the edges of the Biodyne C membranes.



A **B** **C**

Figure 2:4: Illustration of the loading procedure of denatured PCR amplicons onto a Biodyne C membrane onto which genus- (i.e. 'catch-all') and species-specific probes had previously been covalently linked.

(A) The membrane is first placed on the top of a support cushion, lying on the bottom side of the blotting apparatus. Supplied with the Miniblotter, the support cushion prevents the loaded samples from spreading or leaking out of the blotter's lanes, once they have been loaded and the membrane has been firmly secured within the blotter, through its four screws.
(B) Assembly of the mini blotter by closing and screwing its two sides together.
(C) Application of the denatured PCR amplicons and control to the membrane using a micropipette.

Following sample loading, the blotter was placed in an incubator for hybridization to occur for 60 minutes at 42°C, with no shaking. Afterwards, the loaded samples and controls were removed by aspiration using vacuum maker to which a 200 µl or 1,000 µl pipette tips were connected, depending on the diameter of the entry of the blotter's lanes. The membrane was then removed from the blotter and washed twice, for 10 minutes, in pre-heated 2x SSPE/0.5% SDS at 50 °C (approximately 100 ml), in a shaker incubator under gentle shaking.
The membrane was then incubated with 50 ml of 2x SSPE/0.5% SDS preheated at 42 °C + 5 µl of streptavidin (1 unit/µl), for 30 minutes at 42°C in the hybridization thermostat, under gentle shaking. Afterwards, the membrane was washed twice in approximately 100 ml of 2x SSPE/0.5% SDS for 10 minutes at 42°C in the hybridization thermostat under gentle shaking. Lastly, the membrane was washed twice with approximately 100 ml of 2x SSPE for 5 minutes at room temperature, under gentle shaking. The purpose of this step was to remove any residual volume of SDS possibly remaining in contact with the membrane after the washing steps previously described.

2.8.4 X-ray film development

After the series of washing steps illustrated above, 5ml of solutions ECL1 and ECL2 (Amersham, UK) were spread sequentially over the membrane, which was then incubated at room temperature for a minimum of 1 minute. The membrane was then placed in between two stretched layers of a clean, transparent cling film, secured within an exposure cassette and taken into the dark room where the development of the X-ray film would take place, enabling the reading of the results of the experiment. Once in the dark room, the X-ray hyperfilm (Amersham, UK) was 'exposed' (by being put in close contact) to the membrane (secured within the cling film layers) for 7 minutes, within the exposure cassette, for detection of any possible chemiluminescence signal originating as a reaction between streptavidine-conjugated horseradish peroxidase and ECL, wherever a sample's DNA hybridized with a complementary oligonucleotide sequence. At that point, the X-ray film was removed from the cassette and developed using an (Ecomax X-ray film processor, Germany).

An example of the results obtained from the screening of a set of samples collected from cattle at KGR is shown in Figure 2:5 below. Once each hyperfilm X-ray was developed, results would be read by juxtaposing the film on a grid printed on an A4 sheet, designed using Adobe Photoshop CS 5.1, specifically for the membranes used in this study.
To do so, the 'lanes' of the Miniblotter MN45, in which the samples were loaded, had to be

oriented perpendicularly compared to the disposition of the catch-all and species-specific probes (therefore, ‘vertically’, compared to the ‘horizontal’ sense of orientation of the probes in Figure 2:4). For each sample, results were read by examining the presence of squared spots. In particular, the presence of squared spots, in correspondence either with catch-all or species-specific probes, indicates a positive result, regardless of the intensity (i.e. several shades of black-grey). In fact, in presence of a uniform concentration of oligonucleotide probes (which in the case of the membranes here used were of 400 μM), the intensity of a positive signal depends on the concentration of the DNA of the respective target microorganism within the tested sample.

Importantly, in order to be considered as reliable, all species-specific positive signals need to be accompanied by a spot in correspondence of the ‘catch-all probe’ pertaining to their respective genus (e.g. *Theileria mutans*-specific signals need to be accompanied by ‘*Babesia*/*Theileria* catch-all’ and ‘*Theileria* catch-all signals’). Results characterized by a signal at the catch-all probe without a signal at any species-specific probes of the same genus are indicative of the detection of new species or of a different or novel strain compared to those for which the oligonucleotide probes were fixed on the membranes. Samples yielding this type of results (i.e. ‘catch all’-only positive for any of the tested genus of tick-borne microorganisms, not accompanied by a species-specific positivity) were therefore subjected to DNA purification and sequencing. At the same time, no spot of any intensity of black-grey should be detected within the vertical lanes corresponding to the negative and wash controls. Contrarily, the presence of signals within these lanes is indicative of contamination. Results characterized by species-specific signals (of variable intensity), not accompanied by a catch-all signal for the respective genus, are to be considered as ‘doubtful’ and warrant a replication of the RLB protocol as described above.

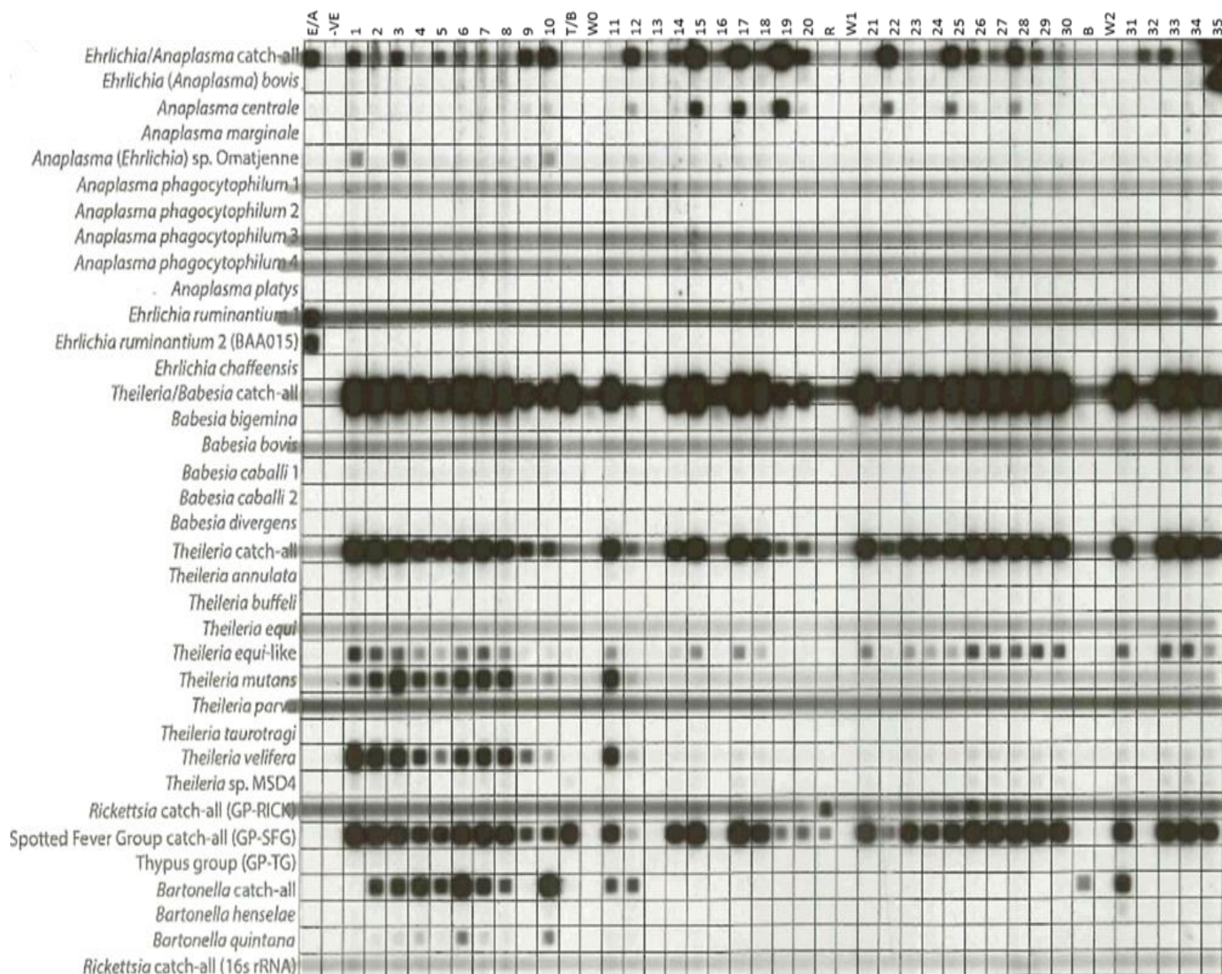


Figure 2:5: RLB results after X-ray development of hyperfilm for cattle samples 1-35 [E/A = *Ehrlichia/Anaplasma* positive control (i.e *Ehrlichia ruminantium*); T/B = *Theileria/ Babesia* positive control (i.e *Theileria mutans*). R = *Rickettsia* positive control (i.e *Rickettsia conorii*). B = *Bartonella* positive control (i.e *Bartonella hensele*). - VE = (MilliQ water control) and W0, W1, and W2 are blank white paper negative control]

2.8.5 Membrane stripping for re-use

Following the development, the 'stripping' of the membrane was carried out at the end of the RLB hybridization protocol, to remove DNA attached to the probes, enabling the re-use of the blotting membrane. Before starting, the temperature of the shaker incubator was set on 70°C with at least 500 ml of 1% SDS for not less than 30 minutes. The membrane to be stripped was then put in a plastic tray, adding approximately 100 ml for the 1% SDS solution pre-heated at 70°C, and incubating for 30 minutes in the shaker incubator at 70°C under energetic shaking. Following these 30 minutes, the 1% SDS solution was discarded and the same washing procedure was repeated for other 30 minutes, using the same conditions

aforementioned.

Following the removal of the second volume of 1% SDS, the membrane was then incubated with 80 ml of 20 mM Ethylenediaminetetraacetic acid (EDTA) solution, for 15 minutes, at room temperature under gentle shaking. The membrane was then placed in a storage plastic seal bag, containing approximately 50 ml of 20 mM EDTA, facilitating its preservation, and stored in a fridge at 4 °C until further use. The plastic seal bag was always signed after usage to identify the number of times each membrane was used. On average, each membrane can be used up to 20 times (Gubbels et al., 1999).

2.9 PCR product purification

PCR products were purified using an Isolate II PCR and gel kit according to the manufacturer's instructions (Qiagen). Four volumes of binding buffer were mixed with one volume of PCR product (typically 20 µl) then the mixture was added into the spin column. The spin column was centrifuged at 10000 x g for 1 minute and the flow-through was discarded. The DNA that bound to the column, was washed by the addition of 650 µl of wash buffer followed by centrifugation at 10000 x g for 1 minute. Again, the flow through was discarded and any residual wash buffer was removed by centrifugation at 10000 x g for 2 min. After that, the DNA was eluted from the column into a 1.7 ml Eppendorf tube by the addition of 15 µl of sterile distilled water into the centre of the column, incubated at room temperature for 1 min, then centrifuged at 10000 x g for 2 min. Purified PCR products were stored at -20°C.

2.10 Sequencing and phylogenetic analysis

Sequencing was performed commercially (Macrogen, Netherlands). Both strands of each PCR product were sequenced using the same primers as for a single round PCR while for a nested PCR the second round primers were for their amplification. The chromatograms (.ab1files) were visualised using ChromasPro software (version 2.1.4). Sequences were verified and, if necessary edited, and primer sequences were removed. Verified sequences were exported into the Molecular Evolutionary Genetic Analysis (MEGA) version 7.0 programme suite (Kumar, Stecher, & Tamura, 2016).

In addition to sequences generated in this study, other relevant data were imported into MEGA7 from GenBank to generate personal databases of relevant sequence data. Sequences drawn from these databases were assembled into multiple sequence alignments using ClustalW (Tamura et al., 2011). Phylogenetic relationships were inferred from these

alignments using the neighbour-joining method (Saitou & Nei, 1987), and visualised in the form of dendrograms using TreeView. The stability of branching orders proposed in these dendrograms was assessed using bootstrapping (1000 replicates) (Felsenstein, 1985).

2.11 Epidemiological statistical analysis

2.11.1 Univariant analysis

Field data and results generated in the laboratory were entered and managed in Microsoft-excel. SPSS version 20.0 software program was initially employed for the data analysis. The overall prevalence of tick and tick-borne haemoparasites was determined by dividing the number of positive animals by total sample size and was expressed as a percentage. Similarly, the exact binomial 95% confidence interval (CI) were determined. Chi-square (χ^2) test was used to assess the association of tick infestation between different variables and to test the null hypothesis for significant difference between age classes (i.e. young and adults) and sex classes (male and female) with regards to overall and individual haemoparasites infection. Effects were reported as statistically significant in all cases if the value is less than 5% ($p>0.05$).

2.11.2 Multivariant analysis

To investigate those factors that influence an individual's probability of testing positive for infection with tick-borne haemoparasites, generalized linear mixed models (GLMMs) were used that assumed a binomial error term and a logit link. In all of these models, the owner of the cattle was used as a random effect to account for potential non-independence due to different owners employing different methods of control. Individual level-factors considered included coinfection with other haemoparasites, age of the individual (categorised as 0-24 months as young, while 24 months and above as adults), gender, haematological parameters including packed cell volume (PCV).

All analyses were carried out using R 3.4 software (R. Development Core Team, 2016) using either the glmer function from the lme4 package. Model selection was based on a backward stepwise model selection with variables dropped according to P-value, with only those variables significant at the $p<0.05$ level being retained in the final model.

Chapter 3: Prevalence of Tick Infestation and Molecular Characterization of Tick-Borne Pathogens in Ticks in KGR, Nigeria

3.1 Introduction

Ticks are excellent vectors for parasite transmission; they are second only to mosquitoes as vectors of human and animal pathogens. They transmit a considerable array of pathogens, which include protozoa, bacteria and viruses, (Parola & Raoult, 2001). According to Walker et al., (2003) ticks in Africa with veterinary importance comprise about more than forty species. Among these the most important tick species in Nigeria domestic ruminants are *Amblyomma, Hyalomma and Rhipicephalus*, sub-genus *Boophilus* are the ticks of greatest medical and veterinary importance (Lorusso et al., 2013; Musa et al., 2014; Ikpeze et al., 2015). The environmental conditions and vegetation found across much of Nigeria are highly conducive for ticks and tick-borne parasite maintenance. The abundance of ticks is usually low in the dry season but will increase dramatically after the first scattered rains, reaching the highest abundance one month after the heavy rains (i.e., from July to September), when all tick species are expected to be present (Bayer & Maina, 1984; Maina, 1986; Lorusso, et al., 2013).

Tick infestation is known to cause a great deal of loss or reduction of productivity by influencing the performance and qualities of the animal yield in the area which in turn leads to a reduction of this sector contribution towards the country's development (Mukhebi et al., 1999). Nevertheless, the Fulani pastoralists of Northern Nigeria do not usually employ chemicals to control ticks in their cattle, merely relying on traditional "picking" methods (i.e. manual removal three times a week during the wet season and twice a week during the dry season (Lorusso, et al., 2013). Neither dip tanks nor acaricides have ever been used in Northern Nigeria (Lorusso, et al., 2013). Picking is of limited effectiveness as it does not prevent immediate re-infestation.

Knowledge of tick distribution is an essential pre-requisite for devising any effective control of these arthropods and the infections they transmit (De Castro, 1997). A survey was conducted by Anthony & Maikai, (2017) to assess the knowledge of farmers about trypanosomosis in KGR revealed that almost 46% of the respondents had knowledge of the arthropod infection as a result of a bite by the tsetse fly. In addition, the respondents reported that infection of animals with the disease was associated with the migration of the animals during the rainy season into the forested areas. However, knowledge of TBI was limited (Majekodunmi et al., 2018). In another survey, the Fulani pastoralists at KGR displayed high levels of ethnoveterinary knowledge and good clinical diagnostic abilities. Diseases considered important by pastoralists at KGR included: *hanta* (Contagious Bovine

Pleuropneumonia [CBPP]); *sammore* (trypanosomiasis); *boro* (foot and mouth disease), *gortowel (liver fluke)*, *dauda* (parasitic gastro-enteritis with bloody diarrhoea) and *susa* (parasitic gastro-enteritis). The parasitology survey supported the participatory epidemiology results but also showed a high prevalence of TBIs that were not mentioned by pastoralists in the study (Majekodunmi et al., 2018). Existing information on tick infestation of domestic ruminants in Nigeria is rather out-dated (Dipeolu, 1975; Bayer & Maina, 1984; Iwuala & Okpala, 1978), mostly derived from studies carried out in the south of the country (Dipeolu, 1975; Iwuala & Okpala, 1978).

To date, there is very little information on the prevalence of ticks and tick-borne pathogens in grazing reserves in Nigeria where a significant number of the livestock are kept, especially in the Northern part of the country. Therefore, this study was carried out to determine the prevalence of tick infestation in relation to age, sex, species, favourite sites of attachment, and body condition score. Also, to determine the genetic diversity of piroplasmosis (*Theileria* and *Babesia*) and *Ehrlichia*/ *Anaplasma* spp. and *Rickettsia* infections by molecular characterization of the ticks collected from domestic ruminants at KGR. The data generated will be of significant relevance to policymakers in making an informed decision in tick control programmes and surveillance of TBIs in the study area and the country at large.

3.2 Materials and Methods

3.2.1 Study design

A cross-sectional study was conducted on the prevalence of tick infestation on domestic ruminants (cattle, sheep and goats) of both sex and different age groups, and body condition in KGR, between May and August 2015. A simple random sampling method was used to select study animals in all six blocks within the grazing reserve as described in Chapter 2 section 2.3.

Body condition scores were determined according to procedures documented by Thompson & Meyer (1994); a visual and tactile evaluation of body fat reserves was performed using a 3-point scale. A score of 1 denotes a poor BCS animal, 2 denotes medium BCS animal, and 3 is good BCS. A poor body condition score was given for cattle, sheep and goats which were extremely thin, having prominent spinous and transverse processes into which a finger could be easily pushed, and had less depth of loin muscle. In contrast, a good body condition score was given when the spinous and transverse processes were smoothing, rounded, and well covered and with full loin muscle (Shirzeyli et al., 2013).

3.2.2 Tick sample collection from host and preservation

Each animal was then surveyed for tick infestation in a standardised manner. During a 15 minute period, five areas of the animal's body were examined, as depicted in Figure 3:1: Body areas of cattle selected for tick survey (squares represent their lateral projection) Original Photo, ©V. Lorusso). Encountered ticks were carefully removed using blunt steel forceps ensuring entire ticks, complete with mouth parts, were collected. Ticks were immediately placed in tubes containing 70% ethanol for preservation.

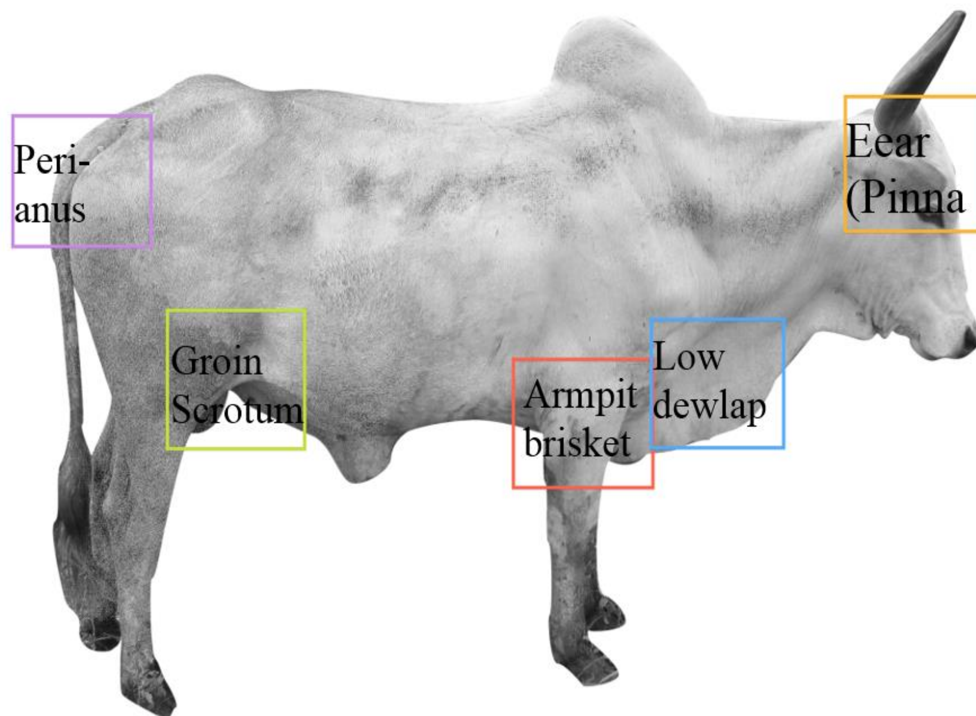


Figure 3:1: Body areas of cattle selected for tick survey (squares represent their lateral projection) Original Photo, ©V. Lorusso

3.2.3 Tick identification

Ticks were identified on the basis of morphological features using a stereomicroscope at a magnification of x40-100 by reference to a tick identification key (Walker et al., 2003). The *Ixodidae* (hard ticks) were characterized by the presence of a tough sclerotised plate on the dorsal body surface, the scutum, covering the entire dorsal body surface in males (sometimes named conscutum), and limited to the anterior approximately one-third of the dorsal body region in unfed females, nymphs and larvae. The folded cuticle posterior to the scutum constitutes the alloscutum. Both scutum and alloscutum are covered with numerous small setae. Sexual dimorphism is apparent only in the adult stage. The scutum is the site of attachment of various dorso-ventral body muscles, cheliceral retractor muscles, and many other muscle groups in the *Ixodidae*. Eyes, if present, are located on the lateral margins of the scutum (see Figure 3:2).

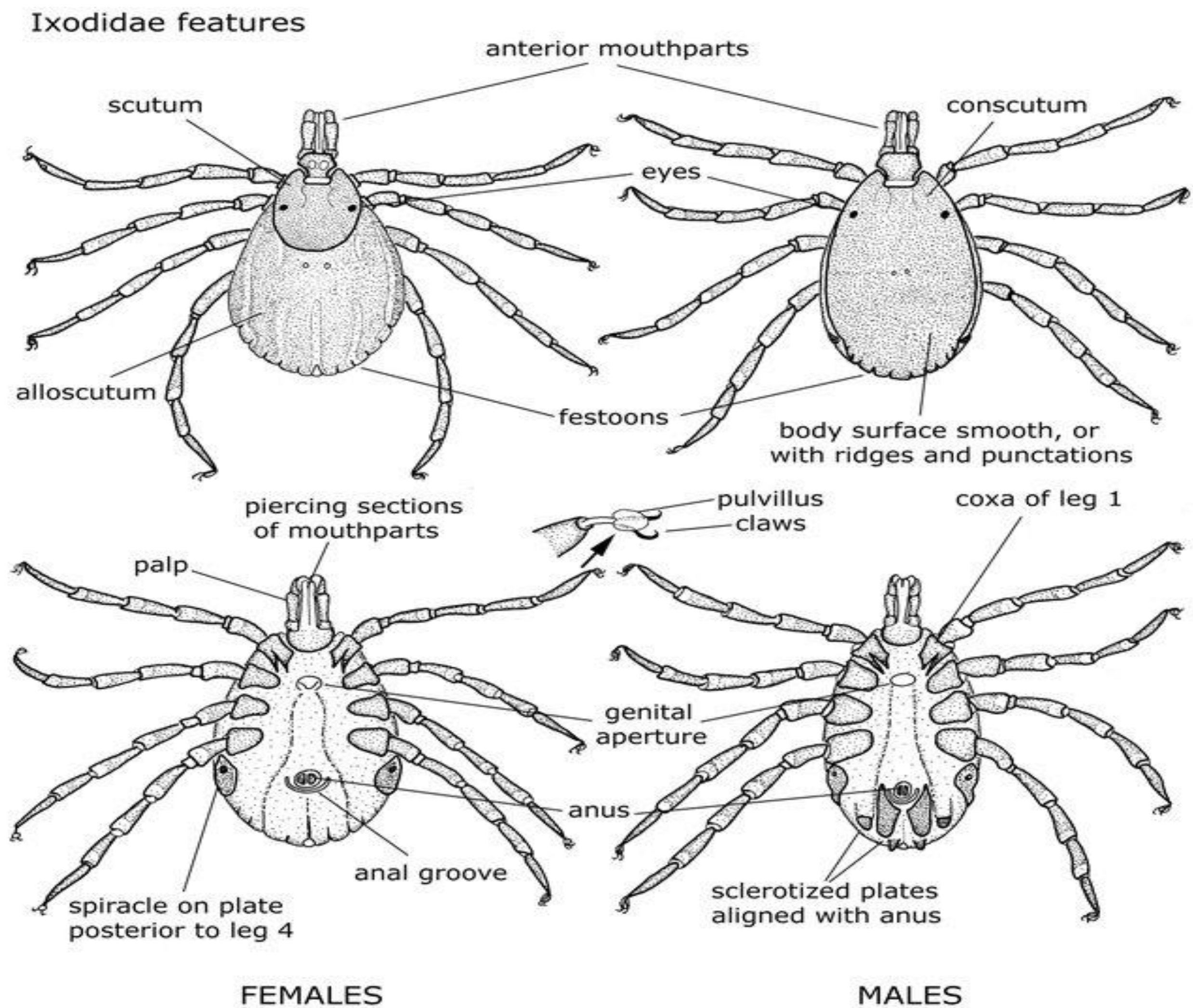


Figure 3:2: General anatomy of male and female hard ticks. From "Ticks of Domestic Animals in Africa" (Walker et al., 2003)

Table 3:1: Morphological features of the different genera of hard ticks found in sub-Saharan Africa

Tick species	Morphological features
Amblyomma variegatum	• Mouthparts very long, elongate second segment of palps • Conscutum and scutum ornate • Eyes present • Festoons present • Adanal plates on males absent, or when present very small • Banded legs
Rhipicephalus spp.	• Mouthparts short to medium length. • Basis capituli generally hexagonal in shape. • Scutum usually uniformly brown, but four species have ivory-coloured ornamentation. • Eyes present. • Festoons present. • Adanal plates, and usually also accessory adanal plates, present on males. • Coxae of first pair of legs with long, prominent posteriorly directed spurs.
Hyalomma spp.	• Mouthparts long, second segment of palps elongate • Scutum pale to dark brown • Eyes present and convex • Festoons present • Adanal, sub-anal, and accessory anal plates present on males • Coxae of first pair of legs with long, prominent posteriorly directed spurs • Banded legs

3.2.4 DNA extraction from ticks

After microscopic identification, DNA extraction was carried out as described in Herrmann & Gern (2010). Each tick was placed into an individual 1.5 ml Surelock microcentrifuge tube containing 100 µl of 1.25 % ammonium hydroxide in nymphs and 500 µl in adults then crushed using a sterile pipette tip. The tube was closed, locked and placed onto a heating block at 100°C for 20 min. Next, each tube was briefly centrifuged at 10,000 x g, then returned to the heating block for a further 25 min, with the lid open. Finally, each tube was closed, relocked and stored at -20°C until required. In order to assess if cross-contamination between samples had occurred during processing, cross-contamination controls were included every time DNA extractions were prepared. These comprised of tubes containing only 100 µl of 1.25 % ammonium hydroxide which was co-processed with tubes containing ticks. One control was used for every four ticks processed.

3.2.5 Conventional PCR for detection of *Ehrlichia* and *Anaplasma* in ticks

The presence of *Ehrlichia/Anaplasma* DNA was determined using a previously described PCR targeting a 247 base pair 16S rRNA-encoding gene fragment (Pancholi et al., 1995). The amplification reaction was performed in a volume of 20 µl containing 1 µl of 10 pmol /µl Ehr521 primer (5'-TGTAGGCGGTTCGGTAAGTTAAAG-3'), 1µl of 10 pmol /µl Ehr747 primer (5'-GCACTCATCGTTTACAGCGTG-3'), 10 µl of MyTaq™ Red Mix (Bioline, London), 6 µl sterile DNA-free water and 2 µl of DNA template. Each assay included a reagent-only negative control and positive control, which was a DNA extract prepared from the blood of an *Anaplasma phagocytophilum*-infected field vole (*Microtus agrestis*) in the UK. The PCR amplification reaction was carried out in a (Prime Elite thermal cycler© UK) with three-step cycle programs under the following conditions: initial denaturation at 93°C for 1 min; 35 cycles including denaturation at 93°C for 1 min, annealing at 55°C for 1min, and extension at 72°C for 30 s. The preparation of PCR mixes with the exception of DNA was done in a dedicated clean room, DNA was added to these mixes and PCRs run in a separate laboratory, located away from the PCR preparation room, and electrophoresis of PCR products was carried out in another separate laboratory. The success of the PCR was determined by observation of agarose gel stained with gel-red on which PCR products had been electrophoretically resolved, as described in Chapter 2 section 2.1.

3.2.6 Nested PCR for detection of *Theileria/Babesia* in ticks

The presence of *Theileria/Babesia* DNA was determined using a previously described nested PCR targeting an approximately 600 base pair 18S rRNA-encoding gene fragment (Simpson et al., 2005). The amplification reaction was performed in a volume of 25 µl containing 1µl of a 10 pmol /µl solution of BmF1 primer (GCGATGTATCATTCAAGTTTCTG), 1µl of a 10 pmol /µl solution BmR1 primer (TGTTATTGCCTTACACTTCCTTGC), 12.5 µl of MyTaq™ Red Mix (Bioline, London), 8.5 µl of ddH2O and 2 µl of DNA template. Each assay included a reagent-only (negative) control and a positive control, which was a DNA extract prepared from the blood of a *Babesia microti*-infected field vole (*Microtus agrestis*) in the UK. The mix was subjected to 39 cycles at 96°C for 20 seconds, 55°C for 20 seconds and 72°C for 50 seconds. Next, the second round was conducted in 25 µl reaction volume containing 1µl of a 10 pmol /µl solution of BmF2 primer (ACGGCT ACCACATCTAAGGAAGGC), 1µl of a 10 pmol /µl solution BmR2 primer (TCTCTCAAGGTGCTGAAGGA), 12.5 µl of Taq red mix (Bioline, London), 9.5 µl ddH_2O and 1 µl of the PCR product from the first PCR reaction as DNA template. The second amplification was programmed to the same cycling condition as above. The preparation of PCR mixes with the exception of DNA was done in a dedicated clean room, DNA was added to these mixes and PCRs run in a separate laboratory, located away from the PCR preparation room, and electrophoresis of PCR products was carried out in another separate laboratory. The success of the PCR was determined by observation of agarose gel stained with gel-red on which PCR products had been electrophoretically resolved, as described in 2.1.

3.2.7 Real-time PCR for detection of *Rickettsia* species in ticks

The presence of *rickettsia* DNA was determined using a previously described Real-Time PCR targeting a fragment of the citrate synthase gene (*gltA*) (Socolovschi et al., 2010). The reaction involved the amplification of 20 µl reaction volume containing 0.5µl of 10pmol /µl solution of RKND03 Reverse primer (GTATCTTAGCAATCATTCTAATAGC), 0.5µl of a 10 pmol /µl solution RKND03 Forward primer (GTGAATGAAAGATTACACTATTTAT), 2 µl of a 2pmol /µl solution of the dual labelled TaqMan probe (6FAMCTATTATGCTTGCGGCTGTCGGTTCTAMRA), 10 µl of MyTaq 2x (clear) (Bioline, London), 2 µl of ddH2O and 5 µl of DNA template. In each amplification, a negative control sterile water, a positive control (*Rickettsia conorii* DNA kindly provided by Prof Pierre-Eduoard Fournier, VITROME, IHU Mediterranee-Infection, Aix-Marseille University, Marseille, France) and cross-contamination controls were included. The

preparation of PCR mixes with the exception of DNA was carried out on a 96 well plate in a dedicated clean room, DNA was added to these mixes and PCRs run in a separate laboratory, located away from the PCR preparation room, and exposed to the following thermal cycle condition; 95ºC for 3 minutes, then 40 cycles of 92ºC for 1 second, 60ºC for 35 seconds and 45ºC for 30 seconds on MJ Research Opticon 2 machine (BioRad, Hemel Hempstead, UK).

3.2.8 Conventional PCR for detection of *Rickettsia* species in ticks

The presence of rickettsia DNA was confirmed using a previously described conventional PCR targeting a fragment of the citrate synthase gene (*gltA*) (Tijsse-Klasen et al., 2011). This assay was used to generate PCR products suitable for sequencing that would allow delineation of Rickettsia species detected using the real-time assay described in 3.2.9. The reaction involved the amplification of 20 µl reaction volume containing 1µl of a 10 pmol /µl solution of CS409d primer (CCTATGGCTATTATGCTTGC), 1µl of a 10 pmol /µl solution Rp1258n primer (ATTGCAAAAAGTACAGTGAACA), 10 µl of MyTaq™ Red Mix (Bioline, London), 6 µl of ddH_2O and 2 µl of DNA template. Each assay included a reagent-only (negative) control and positive control (*Rickettsia conorii*) and cross-contamination controls were included. The mix was subjected to 40 cycles at 95ºC for 15 minutes, 94ºC for 30 seconds, 54ºC for 30 seconds, 72ºC for 55 seconds and 72ºC for 7 minutes (BioRad, Hemel Hempstead). The preparation of PCR mixes with the exception of DNA was done in a dedicated clean room, DNA was added to these mixes and PCRs run in a separate laboratory, located away from the PCR prep room, and electrophoresis of PCR products was carried out in another separate laboratory. The success of the PCR was determined by observation of agarose gel stained with gel-red on which PCR products had been electrophoretically resolved, as described in Chapter 2 section 2.1.

3.2.9 PCR product purification

PCR products were purified using an Isolate II PCR and gel kit according to the manufacturer's instructions (Qiagen). Four volumes of binding buffer were mixed with one volume of PCR product (typically 20 µl) then the mixture was added into the spin column. The spin column was centrifuged at 10000 x g for 1 minute and the flow-through was discarded. The DNA that bound to the column, was washed by the addition of 650 µl of wash buffer followed by centrifugation at 10000 x g for 1 min. Again, the flow through was discarded and any residual wash buffer was removed by centrifugation at 10000 x g for 2 min. After that, the DNA was eluted from the column into a 1.7 ml Eppendorf tube by the addition of 15 µl of sterile distilled water into the centre of the column, incubated at room

temperature for 1 min, then centrifuged at 10000 x g for 2 min. Purified PCR products were stored at -20°C.

3.2.10 Sequencing and phylogenetic analysis

Sequencing was performed commercially (Macrogen, Netherlands) as described in Chapter 2 section 2.4.

3.3 Statistical analysis

Field data and results generated in the laboratory were entered and managed in Microsoft-excel. SPSS version 20.0 software program was initially employed for the data analysis as described in Chapter 2 section 2.5.2.

3.4 Results

3.4.1 Prevalence and distribution of tick infestation on domestic ruminants

A total of 478 domestic ruminants were surveyed in this study. These included 156 cattle, 152 sheep and 170 goats (Table 3:2). Most (387) animals were female, thus only 91 were male. Only (42, 8.7%) were less than 2 years old, whereas 225 (47%) were classified as adults. Most (337, 71%) animals were of "medium" body condition, with (43, 9%) assessed as having a good body condition and the remaining (98, 20%) having a poor body condition. Between 41 and 114 animals were sampled in each of the six blocks of the KGR. The prevalence of tick infestation was significantly higher on goats than either sheep or cattle (Table 3:2). Male animals were found to be statistically less infested by ticks than female ($p<0.006$) (Table 3:2). In addition, young animals less than (<24 months) were found to be significantly less parasitised than adults animals (Table 3:2). Interestingly, ticks were significantly more frequently found on animals with a "good" body condition compared to animals with either a poor or medium body condition. No significant variation in tick infestation rates was observed between KGR blocks (Table 3:2).

Table 3:2: Prevalence of tick infestation in domestic ruminants with different risk factors

Variable	Category	Number examined	Number (%) found to be infested	Number (%) found to be non-infested	Chi-square test
Species	Cattle	156	43 (27.5)	113 (72.4)	χ^2=5.996 P=0.050
	Sheep	152	51 (33.5)	101 (66.4)	
	Goat	170	78 (45.9)	92 (54.1)	
Sex	Female	387	128 (33.1)	259 (66.9)	χ^2=7.464 P=0.006
	Male	91	44 (48.3)	47 (51.6)	
Age	0-24	200	57 (28.5)	143 (71.5)	χ^2=8.359 P=0.003
	>24	278	115 (41.3)	163 (58.6)	
BCS	Good	43	22 (51.2)	21 (48.8)	χ^2=8.957 P=0.011
	Medium	337	116 (34.4)	221 (65.5)	
	Poor	98	47 (48.0)	51 (52.0)	
KGR Block	Block 1	117	34 (29.0)	83 (70.9)	χ^2=9.712 P=0.084
	Block 2	91	26 (28.5)	65 (71.4)	
	Block 3	102	37 (36.2)	65 (63.7)	
	Block 4	53	20 (37.7)	33 (62.2)	
	Block 5	41	14 (34.1)	27 (65.8)	
	Block 6	74	41 (55.4)	33 (44.5)	

A total of 478 animals were checked for tick infestation in 6 blocks of KGR. A total of 172 ticks were identified, most of these ticks (137, 80%) were identified as *A. variegatum* (137, 79.6%), but *Rhipicephalus* species (20, 8.7%) and *Hyalomma* species (15, 11.6%) species were also encountered (Table 3:3). Ticks were found widely distributed across the body (Table 3:3).

Table 3:3: Tick distribution pattern and predilection site

Tick identity	Total count (%)	Predilection sites
A. variegatum	137 (79.6)	udder, external genitalia, inner thighs, groin scrotum, armpit brisket, dewlap, peri anus
Hyalomma spp.	15 (8.7)	scrotum, peri anus, tail
Rhipicephalus spp.	20 (11.6)	Neck/lower dewlap, ear, armpit brisket, groin scrotum, peri anus

Both male and females of all tick taxa were encountered. No significant difference in the numbers of each sex was detected for any tick taxon (Table 3:4).

Table 3:4: Total and average tick burden on the domestic ruminants

Tick Species	Male Tick Count	Female Tick Count	Male: Female Ratio
A. variegatum	71	66	0.929
Hyalomma spp.	8	7	0.875
Rhipicephalus spp.	15	5	0.333

The median of the recorded tick burden varied among the examined animal species. It was highest in goats ranging between (1– 6), followed by sheep (1– 4) and cattle (1– 8) (Figure 3:3).

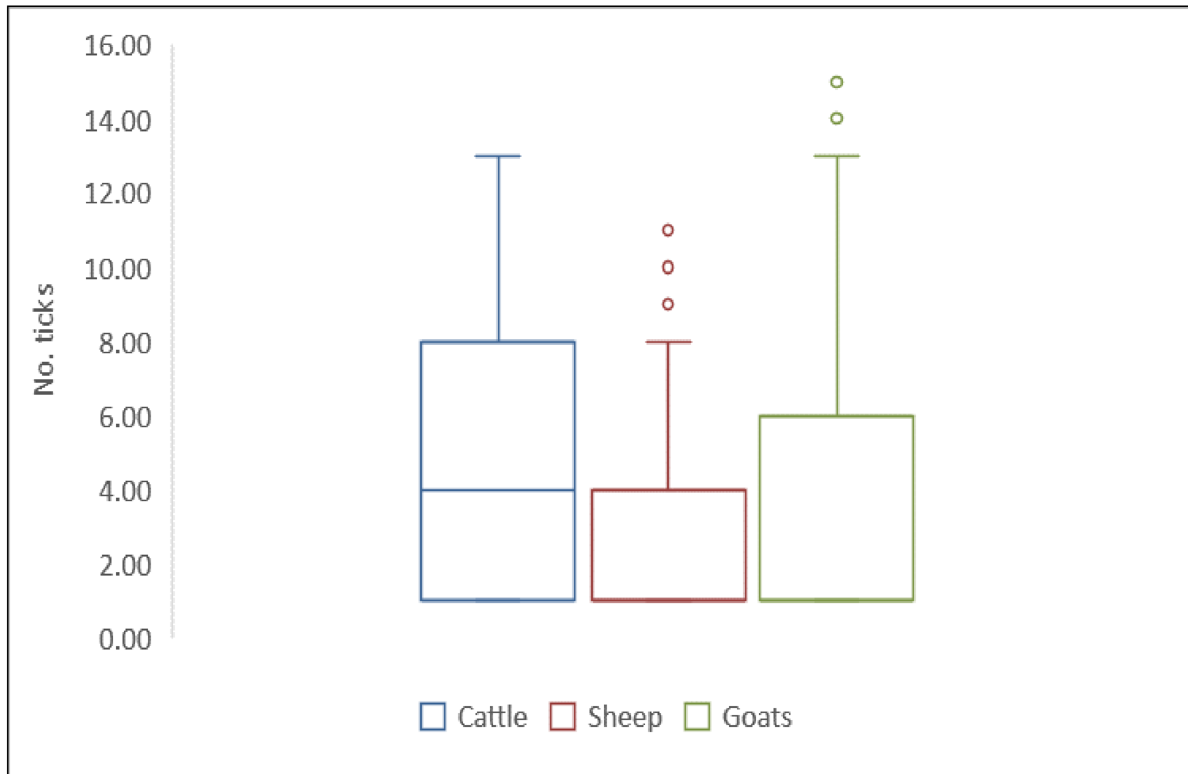


Figure 3:3: Box-and-whisker plots for the tick burden recorded in different animal species

A. variegatum infestations recorded a higher prevalence on goats (38.2%) than on sheep (26.3%) or cattle (20.5). *Rhipicephalus* spp. were slightly higher in goats (4.7%) than cattle (4.4%) or sheep (3.2%). While *Hyalomma* spp. were significantly higher in sheep (3.9%) than goats (2.9%) or cattle (2.6%) (see Table 3:5).

Table 3:5: Species-level prevalence of tick infestation in domestic ruminants

Tick Species	Cattle (n = 156) Positive (%)	Sheep (n =152) Positive (%)	Goat (n =170) Positive (%)	Overall Prevalence (%)
A. variegatum	32/156 (20.5)	40/152 (26.3)	65/170 (38.2)	137/478 (85)
Rhipicephalus spp.	7/156 (4.4)	5/152 (3.2)	8/170 (4.7)	20/478 (12.3)
Hyalomma spp.	4/156 (2.6)	6/152 (3.9)	5/170 (2.9)	15/478 (9.4)
All ticks	43	51	78	172

3.4.1 Single infection

73 single infections were detected, amongst 107 positive microorganisms (68.2 %), of which 29 were in young animals (39.7 % of positive animals) and 44 in adult (60.2 % of positive animals). Cases of single infections were mostly represented by *Rickettsia* spp. ($n = 55$), *Theileria/ Babesia* spp. ($n = 12$), *Ehrlichia/Anaplasma* spp. ($n = 6$).

3.4.1 Coinfections

35/172 (19.7 %) of ticks sampled were positive for two or more microorganisms simultaneously. Overall 4 different combinations of microorganisms were found. The largest variety of coinfections was recorded in (*Theileria/Babesia* species + *Ehrlichia/Anaplasma* species + *Rickettsia* species). On the whole, the most frequent combinations included (i.e. *Ehrlichia/Anaplasma* + *Rickettsia*).

Table 3:6 reports only the 4 most frequent coinfections. Out of 35 ticks that were positive for two or more microorganism the largest variety of coinfection differed between young 9 (25.7%) and adult's animals 26 (74.2%). Coinfection prevalence was significantly higher in adults' animals than younger animals (χ^2=1.6, p=0.193), while sex recorded 13 (38.2%) in males and 21 (61.7%) in females. Age wise prevalence recorded no significance difference while a significant difference was observed in sex (χ^2=3.91 p=0.047).

Table 3:6: Four most frequent multiple infections by tick-borne haemoparasites according to sex, age classes and overall number of ticks.

(*E/A* = *Ehrlichia/Anaplasma* species; *R* = *Rickettsia* spp.; *T/B* = *Theileria/Babesia* species;).

	Tick-borne haemoparasites species combinations	Frequency				Totals
		Ticks		Animals		
		Males	Females	Young	Adults	
1	*E/A* + *R*	2	12	2	12	14
2	*T/B* + *R*	6	6	4	8	12
3	*T/B* + *E/A* + *R*	1	4	2	3	5
4	*T/B* + *E/A*	2	2	1	3	4

3.4.2 Molecular detection of apicomplexa

All 35/172 (20.3%) ticks were examined for the presence of apicomplexa DNA, of which 23 yielded an amplicon of the expected size (c650 base pairs). Unambiguous sequence data were obtained from all amplicons. Data from both strands were obtained from all bar three of these. The amount of sequence data obtained from each amplicon ranged from 529 to 617 (full length) base pairs (Table 3:7). Comparative sequence analysis allowed all bar three sequences to be assigned to specific apicomplexan species. *T. ovis* was detected in eight samples, from *Rhipicephalus* and *Amblyomma* ticks, all of which were collected of either sheep or goats. *T. velifera* was detected in four samples, from *Amblyomma* ticks collected off cattle, sheep and goats. *B. caballi* was detected in eight samples, all from *Amblyomma* ticks off cattle. Three samples yielded sequence data that did not share specific similarity with recognised haemoparasitic apicomplexans. The sequence data from these samples were found to be most similar to uncultured eukaryotic clones obtained in metagenomic environmental surveys (Table 3:7).

Table 3:7: Results of BLAST analysis of 18S rDNA sequences obtained

Tick	Tick/host identity	Closest match	Similarity (bp) (%)	GenBank reference of most similar sequence
T24	*Hyalomma*/cow	*B. caballi*	532/535 (99.4%)	MH424325
T29	*Rhipicephalus*/sheep	*T. ovis*	529/529 (100%)	MG738321
T34	*Amblyomma*/cow	*B. caballi*	523/525 (99.6%)	MH424325
T39	*Amblyomma*/goat	*T. velifera*	584/584 (100%)	LC431550
T40	*Hyalomma*/cow	*B. caballi*	534/536 (99.6%)	MH424325
T46	*Amblyomma*/goat	*T. ovis*	560/560 (100%)	MG738321
T61	*Amblyomma*/sheep	*T. velifera*	588/588 (100%)	LC431550
T68*	*Amblyomma*/cow	*B. caballi*	536/542 (98.9%)	MH424325
T76	*Amblyomma*/sheep	uncultured eukaryote clone	580/599 (96.8%)	HQ259046
T86	*Amblyomma*/sheep	*T. ovis*	563/563 (100%)	MG738321
T88	*Rhipicephalus*/cow	*B. caballi*	528/530 (99.6%)	MH424325
T93	*Amblyomma*/goat	*T. ovis*	572/572 (100%)	MG738321
T99	*Amblyomma*/goat	*T. ovis*	576/576 (100%)	MG738321
T103	*Amblyomma*/cow	*B. caballi*	529/531 (99.6%)	MH424325
T105	*Amblyomma*/goat	*T. ovis*	613/613 (100%)	MG738321
T107*	*Amblyomma*/goat	*T. ovis*	590/590 (100%)	MG738321

T108	*Amblyomma*/goat	*T. velifera*	559/559 (100%)	LC431550
T112†	*Amblyomma*/sheep	*T. ovis*	613/613 (100%)	MG738321
T118†	*Amblyomma*/cow	*B. caballi*	580/582 (99.7%)	MH424325
T125	*Amblyomma*/cow	*B. caballi*	538/540 (99.6%)	MH424325
T127	Amblyomma/cow	uncultured eukaryote clone	575/617 (93.2%)	EF100235
T131	*Amblyomma*/cow	*T. velifera*	547/547 (100%)	LC431550
T133*	*Amblyomma*/goat	uncultured eukaryote clone	519/557 (93.2%)	EF100235

*sequence data derived from single strand (BmR2) only.

†full length sequence

Alignment of the eight sequences tentatively assigned to *T. ovis* indicated that they were indistinguishable from one another, thus one sequence T112 (Table 3:7) presented as KGR-TO4 (Figure 3:4), was selected as a representative for further analysis. Alignment of the four sequences tentatively assigned to *T. velifera* (T131) also revealed they were indistinguishable from one another and thus they were represented in subsequent analyses by KGR-TV1 (Figure 3:4). Alignment of the eight sequences tentatively assigned to *B. caballi* (T34 and T88) revealed two very similar alleles, represented in subsequent analyses by KGR-BC3 and KGR-BC4 (Figure 3:4).

In order to further clarify the likely identities of the piroplasms detected in the ticks, phylogenetic analyses were carried out based on alignments of the newly obtained sequences with relevant sequences drawn from GenBank. For each of the three putative taxa identified (*T. ovis, T. velifera* and *B. caballi*) all 18S rDNA sequences deposited in GenBank in their name were recovered and aligned (February 2018). These sequences, together with, where relevant, 18S rDNA sequences from other taxa and the representative sequences obtained in the current study, were aligned with one another and used a basis for phylogenetic analysis.

In the first analysis, the positions of KGR-TO4 and KGR-TV1were assessed in the context of the phylogenetic diversity of *T. ovis* and *T. velifera* (Figure 3:4). A total of 20 18S rDNA sequences purportedly from *T. ovis* were encountered in GenBank. Sixteen of these sequences clustered tightly together in the phylogenetic reconstruction (Figure 3:4). The remaining four sequences formed two distinct clusters, one of which lay intermediate to the main *T. ovis* and *T. velifera* clusters, whilst the other lay on a long, deeply divergent branch. KGR-TO4 lay within the large tight cluster that contained most of the *T. ovis* strains for which 18S rDNA data were available (Figure 3:4). Eight 18S rDNA sequences purportedly from *T. velifera* were encountered in GenBank. Seven of these clustered together in the phylogenetic reconstruction, whereas the eighth is loosely clustered with the intermediate *T. ovis* cluster described above (Figure 3:4). KGR-TV1 lay within the cluster containing 7/8 of the *T. velifera* strains and was tightly clustered with two strains from Ethiopian cattle and ticks (Figure 3:4).

In the second analysis, the phylogenetic positions of KGR-BC3 and KGR-BC4 were assessed in the context of *B. caballi* intraspecies diversity. *B. caballi* strains for which data were available in GenBank were inferred to lie in three well-supported divergent clusters (Figure 3.5). Two of these clusters were dominated by strains from Africa and, to a lesser extent Europe, whereas the third cluster the majority of strains were from the New World (although this cluster also contained strains from Europe and Asia). KGR-BC3 and KGR-BC4 both lay in one of the two “old World” clusters, which included strains recovered from donkeys, dogs, horses and ticks in Africa, Europe and Asia. KGR-BC3 was the outlier in this cluster, but its inclusion in the cluster was strongly supported by bootstrapping (Figure 3.5).

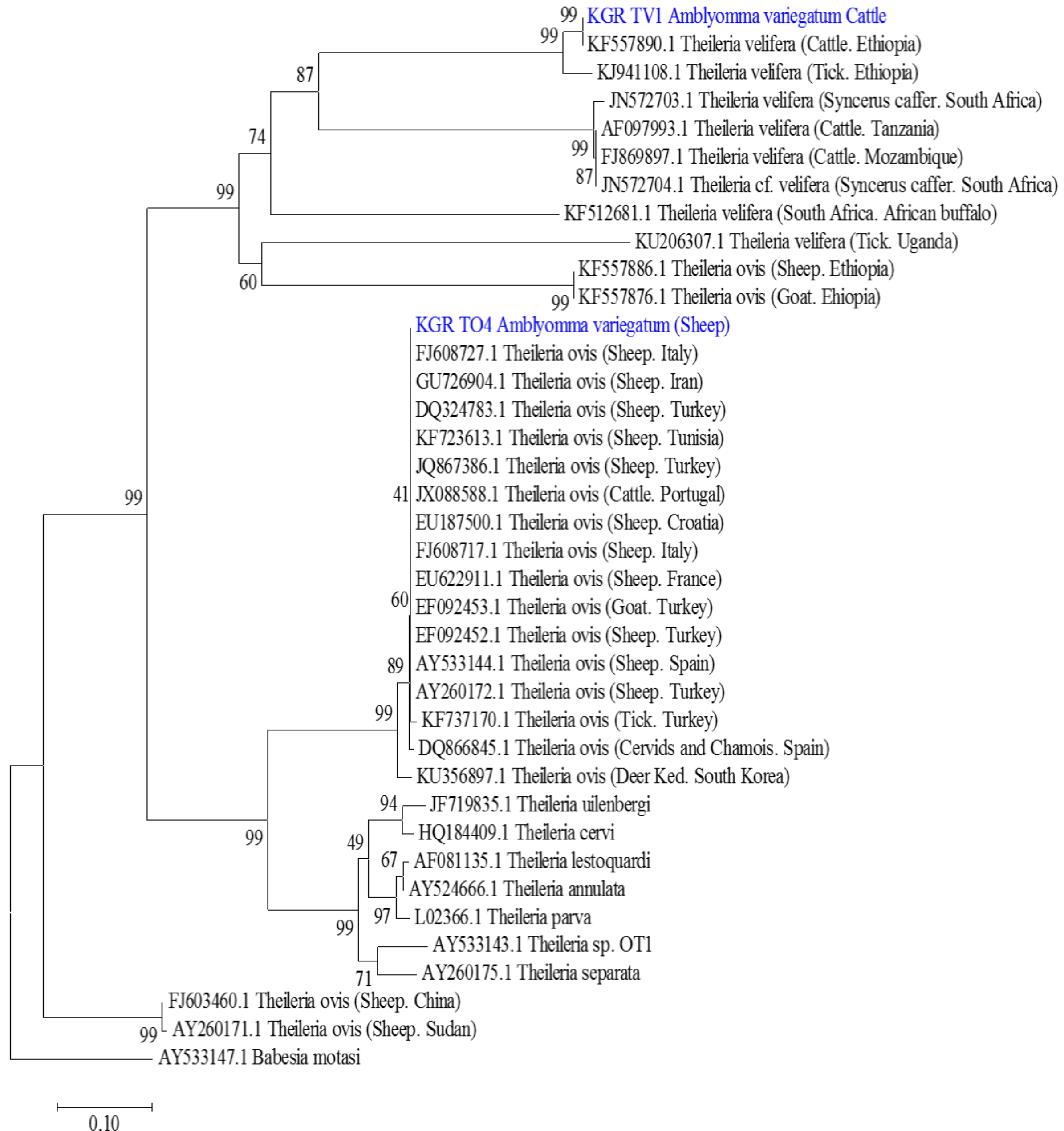


Figure 3:4: Phylogenetic tree of *T. ovis* and *T. velifera* 18S rRNA gene sequence obtained from ticks in this study

KGR TO4 sequence data obtained from these studies clustered tightly with isolates originating from different geographic regions however some *T. ovis* sequence data obtained from Genebank clustered with an out group of *Babesia* spp. these may properly be misidentified before it was deposited

The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987). The optimal tree with the sum of branch length = 2.38423084 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method (Nei & Kumar, 2000) and are in the units of the number of base differences per site. The analysis involved 45 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 42 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

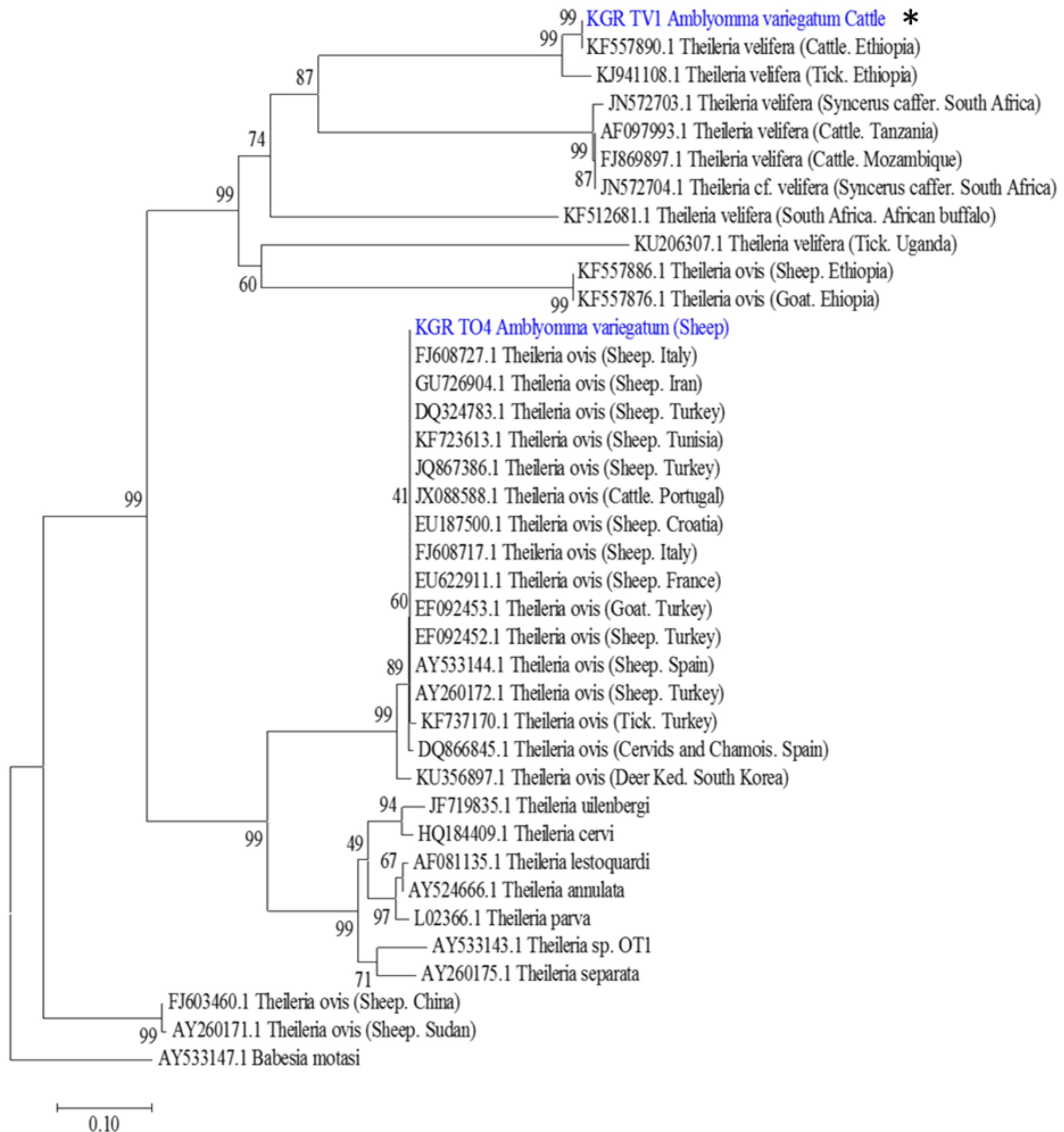


Figure 3:5: Phylogenetic tree of *B. caballi* strains demonstrating the inferred phylogenetic position of the two strains encountered in this study

* KGR TV1 sequence data obtained from these studies clustered with some *T. ovis* sequence data obtained from Genebank these may properly be misidentified before it was deposited.

The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987). The optimal tree with the sum of branch length = 2.18846055 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method (Nei & Kumar, 2000) and are in the units of the number of base differences per site. The analysis involved 37 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 122 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar, Stecher & Tamura, 2016).

3.4.3 Molecular detection of *Rickettsia* species in ticks

A total of 84/172 (48.8%) ticks produced a positive result when tested using the real-time PCR. All positive samples were further amplified with a conventional PCR as a means of generating PCR products for sequencing that would allow species identification through comparative sequence analysis. Out of 25 partial *gltA* amplicons sent for sequencing, 11 yielded no readable sequence data on either strand. Seven amplicons yielded readable sequence data on only one strand (reverse). The amount of readable data for these samples ranged from 188 to 592 base pairs. Three amplicons yielded readable sequence data on both strands and the combination of data from both strands provided between 568 and 643 base pairs of readable sequence data. For the remaining 4 amplicons, although readable data was obtained from one or both strands, these were ambiguous, probably derived from more than one amplicon (i.e. mixed traces). Thus, sequence data from 10 amplicons were submitted for BLAST analysis (Table 3:8).

Table 3:8: Results of BLAST analysis of *gltA* sequences obtained

Tick species from which amplicon was obtained	Data used for BLAST (bp)	BLAST result
A. variegatum	188*	100% similarity with numerous SFG species
A. variegatum	568	100% similarity with only *R. africae*
Rhipicephalus spp.	491*	100% similarity with only *R. massiliae*
A. variegatum	282*	100% similarity with only *R. africae*
A. variegatum	214*	100% similarity with numerous SFG species
Rhipicephalus spp.	643	100% similarity with only *R. massiliae*
A. variegatum	434*	Highest similarity (99.3%) with *R. africae*
A. variegatum	570	100% similarity with only *R. africae*

A. variegatum	592*	Highest similarity (99.8%) with *R. africae*
A. variegatum	330*	Highest similarity (99.7%) with *R. africae*

*data obtained some a single strand only

Sequence data from two of the amplicons were too short to allow the species from which they were derived to be identified. Six amplicons yielded sequence data that allowed them to be identified as *R. africae* based on identity or near-identity to *R. africae* sequences in GenBank (and marked dissimilarity with other SFG species). All these amplicons were derived from *A. variegatum* ticks. Two amplicons yielded sequence data that allowed them to be identified as *R. massiliae* based on identity with *R. massilae* sequences in Genbank and dissimilarity with sequences from other SFG species. Both these amplicons were derived from *Rhipicephalus* ticks.

3.4.4 Molecular detection of *Ehrlichia* and *Anaplasma* species in ticks

A total of 96/172 (55.8%) ticks yielded an amplicon when PCR tested for *Ehrlichia/Anaplasma* species. However, of these, only 5 amplicons with unambiguous sequence data were obtained. The amount of readable data for these samples ranged from 222 to 247 base pairs (see Table 3:9). One amplicon derived from *A. variegatum* yielded sequence data that was identified as a spotted fever group *Rickettsia* spp. Two amplicons derived from *Rhipicephalus* spp. yielded sequence data that was identified as *Ehrlichia canis* and *Anaplasma* spp. Two amplicons yielded sequence data was identified as *Anaplasma* spp. and *A. platys* based on identity with *R. massilae* sequences in Genbank

Table 3:9: Results of BLAST analysis of *Ehrlichia/Anaplasma* sequences obtained

Name	AC	Gene	Length of Sequence Query (bp)	Pct. (%)
A. variegatum	MG668812.1 MG744513.1 LS992663.1 MK007077.1	Spotted Fever Group *Rickettsia* species	247	100
Rhipicephalus spp.	KY594915.1	*Ehrlichia canis*	240	100
Rhipicephalus spp.	MH879781.1 CP023730.1 MG910980.1 MH588233.1 CP015994.2	*Anaplasma* spp.	240	100
A. variegatum	MK016525.1 MG668799.1 MG869594.1	*Anaplasma spp.*	247 296 222	100
A. variegatum	MH129061.1 MG050139.1 KY594914.1 KX792089.2	*Anaplasma spp.* *A. platys* *A. platys* *A. platys*	238 226 246 231	97

3.5 Discussion

3.5.1 Prevalence of tick infestation

In this study, the overall prevalence of tick infestation in cattle was 33.3%. The finding is less than that of Eyo et al. (2014) who surveyed tick infestation in cattle at four selected grazing reserves in South-South Nigeria between November - February and April- October and recorded a prevalence of 88.4%. Similarly, Abera et al., (2010) and Musa et al., (2014) found a high prevalence of 97.8% and 63.4% in Ethiopia and Maiduguri North Eastern Nigeria respectively. The prevalence of tick infestation was found to be 36.2% in sheep and 45.9% in goat which is higher than the work carried out by Ofukwu & Akwuobu, (2011) in Markurdi, North Central Nigeria. They reported a lower prevalence of 21.9% and 23.9% in sheep and goat respectively. Nonetheless, Abera et al. (2010) in a survey of ixodid ticks in domestic ruminants in South-Western Ethiopia report a high prevalence of 89.9% and 94.4% in sheep and goat respectively. The difference found in these studies may be associated with the difference in the climatic conditions such as relative humidity, environmental temperature and geographical location. This factor helps in the ecological structure for the reproduction and growth of tick populations (Pegram et al., 1981).

It is evident from the results that the tick prevalence significantly differed between animal hosts, which concurs with previous studies (Ghosh et al., 2007; Sajid et al., 2008). The observed lower tick prevalence in cattle as compared to sheep and goats might be linked with the manual removal of ticks i.e three times a week during the wet season (i.e., April to October) and twice a week during the dry season (i.e., November to March) by the Fulani pastoralist in KGR (Maina, 1986). Another possible explanation for low prevalence recorded in cattle could be that the Fulani pastoralist at KGR might have administered drugs such as ivermectin to the cattle before the sampling of ticks took place or they might have been consistent with the manual removal of ticks practice (Lorusso et al., 2013). Limited information is available about tick prevalence in small ruminants in Nigeria. In general, the tick prevalence observed in the present study was higher in goats (75.9%) as compared to sheep (33.5%), which is also in agreement with a local study in Pakistan (Sajid et al., 2008). Although a reason for lower tick prevalence in sheep is not evident, one could speculate that the hairy wool might be an important protective factor against tick infestation (Sajid et al., 2008).

3.5.2 Prevalence of tick infestation between sex

The prevalence of tick infestation was found to be less in female than male animals. This result is in line with the findings of Musa et al. (2014) who studied the prevalence of tick infestation in different breeds of cattle in Maiduguri North Eastern Nigeria. The authors proposed that the high prevalence of infestation observed among male cattle resulted from their use for farming activities and were taken out for grazing at a long distance. However, this observation is in contrast with other studies Rony et al., (2011), Asmaa et al., (2014), Fikru & Kasaye, (2015) in which females were observed to have higher tick infestations than males. These studies suggested that females were more prone to tick bite because they had great expanses of hairless skin (around the udders and vulva) that permitted easier attachment. Other explanations of sex bias in tick infestation rates include variation in hormones, with high expression of progesterone and prolactin making individuals more attractive to ticks (Sarkar et al., 2010).

Male to female ratio of identified tick species in the study indicated that males were found to be dominant. The finding agrees with that of Tessema & Gashaw, (2011) and Mohamed et al. (2014) having dominant males than females. One may speculate that the higher number male than females could be due to fully engorged female tick drop off to the ground to lay eggs while males tend to remain permanently attached to the host up to several months later to continue feeding and mating with other females on the host before dropping off and hence males normally remains on the host longer than female (Tadesse & Sultan, 2014).

3.5.3 Prevalence of tick infestation between age groups

A significantly low tick burden in young animals as compared to adults animals was recorded in this study which is in line with the finding of (Lorusso et al., 2013). The result of this survey also agrees with the finding of Islam et al., (2009) who also reported a high prevalence in older animals (61.54%). However, it is in contrast with the finding of Manan et al., (2007), Vatsya & Garg (2007), Kassa & Yalew (2012), Abraham & Kasaye, (2015), they reported that young animals were more susceptible than adults. The lower tick burdens recorded in young animals in this study could be due to a combination of factors, including the frequent grooming of calves, especially head, ears and neck regions, by their dams and the smaller surface area of younger animals as compared to adults (Mooring et al., 2000). Furthermore, young animals seem to be more capable of protecting themselves from ticks by innate and cell-mediated immunity (Okello-Onen et al., 1999), although it must be stressed that we did not evaluate the immune status of the animals in our study.

3.5.4 Prevalence of tick infestation between body condition score

Interestingly, a higher proportion of animals with a good or poor body condition were infested with ticks than animals judged to have medium body condition categories. Though, the finding is in contrast with the studies of Wasihun & Doda, (2013), who studied the prevalence and identification of tick in Humbo district in Ethiopia. They found high tick infestation in medium body condition (78%) followed by poor body condition (67%), and good body condition animal (57%). The high prevalence recorded in good body condition followed by poor scored animals in this study may be associated with a low resistance to tick infestation (Manan et al., 2007). This finding suggest that animals with medium body scored animals have reduced resistance and are exposed to any kind of disease when grazing on the field, and poor body conditioned animals were kept at home due to their inability to walk long distant areas, so they become less infested than medium sized animals but the well fed animals were very resistant to any kind of diseases when they grazed in the field or are kept at home One may speculate that the good body condition scored animal in this study might have been suffering from different gastrointestinal parasitic infections or any other diseases that weaken the immune system and makes them more susceptible to tick infestation when grazing.

3.5.5 Prevalence of tick infestation at the genus level

In this study, almost 80% of the ticks encountered were *A. variegatum*. This high prevalence is thought to reflect the adaptation of this tick species to the thick hides of the livestock breeds in the KGR (Igwe et al., 2017). The animals tend to be more resistant to *Rhipicephalus* species which are smaller ticks than *Amblyomma* spp (Bayer & Maina, 1984). According to Ellis and Hugh-Jones, 1976 cited in Bayer & Maina, (1984) who studied cattle resistance to tick infestation specifically on *Boophilus microplus* found zebu cattle were relatively more resistant to *Rhipicephalus* species than *Amblyomma*. The result they found may suggest the high prevalence of *Amblyomma variegatum* on the domestic animals studied. During the sampling period, the Fulani's were found to remove ticks by manual hand removal. However, it does not guarantee 100% success and does not prevent the animals from getting infected with pathogens. Due to hand removal practice at the grazing reserve, the animals surveyed were continuously checked for ticks by the Fulani and these control measures are likely to bias our sampling. This work agrees with that of Bayer & Maina (1984) who worked on the seasonal pattern of tick load in Bunaji cattle in the subhumid zone of Nigeria. Their findings are also in line with the results of other studies in SSA which also reported a high prevalence

of *A. variegatum* (Obadiah, 2012; Ejima & Obayumi, 2014; Ikpeze et al., 2015). This finding echoes the alarming need for intervention since *Amblyomma* spp. ticks are well-established pathogen vectors, most notably of *E. ruminantium*, with heartwater being one of the major constraints to livestock production in sub-Saharan Africa (Faburay et al., 2007).

Rhipicephalus spp. was the second most abundant tick genera encountered in this study. Similar to the current finding, Kamani et al., (2017) reported *Rhipicephalus* (including the subgenus *Boophilus*) species, as the second most abundant tick species in a survey carried out in the North Western part of Nigeria. On the contrary, the findings of Vincenzo et al., (2013) described *Rhipicephalus* (subgenus *Boophilus*) as the most abundant and most widely spread tick in North Central Nigeria. Their findings may not be surprising as their study was carried out in the late wet season, when relative humidity as well as the vegetation coverage, and therefore the abundance of adult ticks on cattle, are expected to be at their peak in Central Nigeria (Bayer & Maina, 1984; Iwuala & Okpala, 1978; Maina, 1986). The difference may also be associated with geographical location and altitude factors. The ticks of the genera *Rhipicephalus* spp. have been documented worldwide as vectors of many important disease agents in animal and human diseases such as Q fever, monocytic Ehrlichiosis, Babesiosis, Hepatozoonosis, East Coast fever, Borreliosis, and Anaplasmosis (Parola & Raoult, 2001).

Hyalomma spp. was the third most abundant tick genera encountered in this study which is compatible with the work of Kamani (2017). On the contrary, the findings of Vincenzo et al., (2013) described *Hyalomma* tick as the second tick in North Central Nigeria. The low prevalence recorded in this study may reflect the seasonality of this tick in Nigeria, where it is known to peak in the late wet season (Bayer & Maina, 1984; Iwuala & Okpala, 1978). In addition, adults *Hyalomma* preferentially localize in the inter-digital clefts and the tail switch (MacLeod, 1975; Ndhlovu et al., 2009), which may be overlooked. *Hyalomma* spp. ticks are the most important vectors responsible in transmitting the bacterium *Anaplasma marginale* to cattle causing Bovine Anaplasmosis (gall sickness), viral disease Crimean-Congo haemorrhagic fever in human and animals, a protozoan disease leading to Babesiosis and the bacterium *Rickettsia Conorii* causing tick typhus in humans. The adult ticks that feed on animal's cause large lesion at the attachment sites resulting in a severe abscess.

3.5.6 Tick distribution pattern and predilection site

The distribution of tick infestation in different body parts of animals examined reveals that udder and external genitalia, inner thigh and under tail/perineum were the most tick infested sites in the body of examined animals. This further confirms that ticks prefer to attach and feed on some parts of the body of animals. This finding is in agreement with the work by Opara & Ezeh (2011) in Borno State, North Eastern Nigeria who found that ticks infesting cattle in this area prefer to attach and feed on inner thighs, dewlap, abdomen, legs, udder, dorsum, ear and hump in this order. Asmaa et al., (2014) also reported that udders and external genitalia were the most tick infested sites (70.7% each) followed by neck & chest (63.0% each), inner thighs (61.1%), perineum (41.7%), ears (14.6%) and around eyes (11.7%). Atif et al., (2012) in the same vein reported that the perineum, udder and external genitalia (98%) were the most tick infested sites in cattle followed by dewlap (92%), inner thighs (90%), neck & back (54%), tail (26%), ears (13%), around eyes (10%), flanks (4%) and legs (2%) in this order of infestation. These findings could be attributed to the fact that external genitals, perineum and inguinal/groin region of the body are highly supplied with blood and ticks usually prefer thinner and short hair skin for infestation. This helps in easy penetration of mouthparts of ticks into the richly vascular area for feeding (Sajid et al., 2009).

3.5.7 Molecular detection of piroplasms in ticks

This study provides evidence of piroplasm infections in livestock in the KGR and explores their diversity. Such information is critical for controlling and preventing infections caused by these pathogens, which leads to the loss of livelihoods of many livestock owners (Moumouni et al., 2015). In the present study, *T. ovis* and *T. velifera* which are protozoan associated with benign theileriosis in cattle, sheep and goats) were previously reported in Nigeria at a prevalence of 32.7% in goats at study carried out in North Western Nigeria (Onoja et al., 2013) and 52.4% in cattle in North Central Nigeria respectively (Lorusso et al., 2016).The single *T. ovis* sequence type encountered in this study, in both *Rhipicephalus* spp. and *A. variegatum* ticks collected off sheep and goats, KGR-TO4, cluster tightly with isolates originating from different geographic regions see Figure 3:4. This suggests that *T. ovis* represent a single species apart from some divergent strains from China and Ethiopia, all *T. ovis* strains from across its range have almost indistinguishable 18s rDNA sequences, thus the species as a whole is indistinguishable, with little/no evidence of marked divergence across the species – no geographical signal. All *T. ovis* strains in Europe/Africa/Western Asia are pretty much the same. It's likely that the Chinese and Ethiopian outliers are not the same

species (in ecological/evolutionary terms). Thus, not much is known about the vectors of *T. ovis* (Zakkyeh et al., 2012). *Rhipicephalus spp.* including *R. bursa*, *R. evertsi*, and *R. sanguineus* have been reported as vectors of *T. ovis* (Zakkyeh et al., 2012). These species are widely distributed throughout the world (Zakkyeh et al., 2012). Therefore, the detection of *T. ovis* in *A. variegatum* ticks in this study points towards the existence of alternative vectors for these protozoa. Phylogenetic analysis of the single *T. velifera* sequence type encountered in this study in *A. variegatum* ticks collected off cattle, sheep and goats, KGR-TV1, demonstrated it clustered tightly with *T. velifera* strains associated with cattle and their ticks in Ethiopia. However, unlike *T. ovis, T. velifera* appears to be phylogenetically diverse, this suggests that *T.velifera* sequence encountered in this study is a divergent strain from South Africa, Tanzania, Mozambique and Uganda.

On the other hand, the single *B. caballi* sequence type encountered in this study, in both *Rhipicephalus* spp. and *A. variegatum* ticks collected off cattle, KGR-TO4, clusters tightly with isolates originating from different geographic regions (Kenya, Italy, Ethiopia, Croatia, and China) see Figure 3.5.This suggests that *B. caballi* represent a monophyletic species, from some divergent strains from South Africa, Jordan, Romania, Spain Malaysia and Iran. It's likely that the Caribbean's island, Mongolia, Brazil outliers are not the same species (in ecological/evolutionary terms). *Hyalomma* spp. ticks (*H. marginatum rufipes* and *H. truncatum*, in particular) are the main vectors of *B. caballi* in Sub-Saharan Africa, *Rhipicephalus evertsi evertsi* and *R. bursa* in South Africa, while *R. sanguineus*, *H. dromedarii* and *H. excavatum* are the most important vectors in North Africa (Kaufmann, 1996). As the transstadial transmission of this pathogen has only been reported in *Rhipicephalus* spp. ticks (De Waal & Potgieter, 1987), it is not known whether *A. variegatum* nymphs possibly fed on *B. caballi* infected hosts (e.g. equids) could maintain the infection and pass it to the adult stage. As an alternative hypothesis, *A. variegatum* positive ticks might have acquired the infection through co-feeding on the same anatomic region infested by other tick species, e.g. *Hyalomma* and *Rhipicephalus*, known vectors of *B. caballi*. The transmission of tick-borne pathogens through co-feeding has been demonstrated for viruses and spirochetes (Randolph & Nuttall, 1996). However, in the study area, equids are not present but might have interacted with some of the livestock when taken out for grazing. In

order to better clarify these findings, other tick species, likely to be involved in the transmission of *B. caballi* (*Hyalomma* and *Rhipicephalus spp*.) could be tested.

3.5.8 Molecular detection of *Rickettsia* microorganisms in ticks

This study further ascertained the presence of *Rickettsia africae* in *A. variegatum* ticks collected from domestic ruminants at KGR in Nigeria. *R. africae* was previously reported at a prevalence between 8% in three locations in Jos North Central Nigeria from *A. variegatum, R. decoloratus* and *R. sanguineus* (Ogo et al., 2012). Lorusso et al., (2013) also reported a prevalence of 62% of *A. variegatum* ticks were positive of SFG rickettsiae DNA of which 22 showed 100% similarity with published sequences of *R. africae*. The confirmation of the presence of *R. africae* provides background for further epidemiologic and clinical investigations of tick-borne diseases in the country. In sub-Saharan Africa, several rickettsial strains have been isolated and detected from ticks and vertebrate animals (Parola, 2006), among which *R. africae*, the etiological agent of African tick-bite fever (ATBF), is the most common (Parola et al., 1999; Mura et al., 2008). The main tick-vectors of *R. africae* are *A. hebraeum* in Southern Africa and *A. variegatum* in West, Central and Eastern Africa (Ndip et al., 2004; Eldin et al., 2011). The presence of *R. africae* infected ticks at KGR is consistent with the prevalence of *A. variegatum* and *Hyalomma* spp. reported in this thesis. Ticks of the genus *Amblyomma* spp. are considered to be the main vectors for *R. africae* although this bacterium has recently been found infecting other genera, including *Rhipicephalus* (Macaluso et al., 2003; Mediannikov et al., 2012) and *H. impeltatum* (Ogo et al., 2012). Thus, experimental evaluations of vector competence clearly need to be carried out in order to establish the vector competence of *Rhipicephalus* spp. ticks.

The detection of *Rickettsia massiliae* further ascertained the presence of this microorganism in the country. *R. massiliae* was first isolated in 1992 from ticks in France near Marseille (Beati & Raoult, 1993). Subsequently, this rickettsia has been detected by molecular methods and isolated in several countries in Europe (Socolovschi et al., 2010), China (Wei et al., 2015) and recently in livestock (cattle) in Nigeria (Reye et al., 2012; Lorusso et al., 2016). *R. massiliae* was previously reported in a questing *R. evertsi* ticks at a prevalence of (3%) from Oyo State in South Western Nigeria (Reye et al., 2012), and was also detected at a prevalence of (3.5 %) in seven villages in Jos North Central Nigeria (Lorusso et al., 2016). The finding of 100 % similarity of *Rickettsia* spp. positive amplicons with *R. massiliae* 16S rDNA is suggestive of the occurrence of this SFG rickettsia in the study area. *R. massiliae* is one of the most widely distributed SFG rickettsiae, described so far in all five continents

(Parola et al., 2013). The presence of this microorganism's DNA was recently documented in questing *Rh. evertsi* ticks, collected from the vegetation in the South-Western part of Nigeria (Reye et al., 2012). In other SSA countries (i.e. Central African Republic, Guinea, Ivory Coast and Mali), *R. massiliae* was detected in several *Rhipicephalus* spp. ticks collected from cattle (e.g. *Rh. guilhoni*, *Rh. lunulatus*, *Rh. muhsamae*, *Rh. senegalensis* and *Rh. sulcatus*) (Cazorla et al., 2008; Parola et al., 2013); most of these tick species were previously reported in Nigeria (Lorusso al., 2013). The first human case was described in a Sicilian patient who was admitted at Palermo hospital in 1985. However, it was not until 2005 that the isolate discovered 20 years before was characterized and identified as *R. massiliae* (Vitale et al., 2006).

3.5.9 Molecular detection of *Ehrlichia/Anaplasma* species in ticks

This study confirms the presence of *Anaplasma* spp. based on their 16S rRNA sequences includes *A. platys*, the agent of infectious canine cyclic thrombocytopenia (Yang et al., 2015), which has been described in dogs in Asia (Unver et al, 2003; Kongklieng et al., 2014), in sheep from Senegal (Djiba et al., 2013), in sika deer, goats, and cattle in China (Zhang et al., 2015; Li et al., 2016) and in cattle from Nigeria (Lorusso et al., 2016). The other three *Anaplasma* spp. reported are poorly characterized it would seem appropriate that species are further studied as they could be important pathogens.

In this study, *Ehrlichia* ruminantium was not encountered in the ticks surveyed which are known to infect ruminants (Allsopp, 2015), however, evidence of infection with *E. canis* in ticks collected off domestic ruminants studied in KGR was recorded. Although *E. canis* is best known as a very common dog pathogen around the world, infections have also been described in people (Perez et al., 2006) and in cats (Braga et al., 2014), and there is thus growing evidence that *E. canis* has a wider host range than previously thought (Yu et al., 2007; Aguiar et al., 2014; Zhang et al., 2015). Our finding of *E. canis* in ticks collected off domestic ruminants in Nigeria further supports previous evidence of its occurrence and is consistent with the findings of a study showing that *E. canis* are present in domestic in cattle in the Caribbean (Zhang et al., 2015). The presence of *E. canis* in ticks is not surprising as the Fulani pastoralist in KGR, own dogs which they use for hunting while they take their animals for grazing. Further studies are required to determine the pathogenicity of *E. canis* in domestic ruminants.

3.5.10 Coinfection of ticks

Ehrlichia/Anaplasma species (*E. canis, A. platys*) were found to be likely associated with *Theileria/Babesia* (*T. ovis, T. velifera* and *B.* c*aballi*) and *Rickettsia* species (*R. africae* and *R. massiliae*). This may suggest a synergism between these infection patterns. The frequent association may be related to transmissions through the same tick vector (i.e. *A. variegatum*) and to the fact that infections are characterized by durable carrier statuses (Uilenberg et al., 1974; Young et al., 1978). The results of coinfection with *Ehrlichia/Anaplasma* and *Theileria/Babesia* is insufficiently understood and the possible pathological, immunological and epidemiological consequences of such coinfections should be further investigated, possibly by applying longitudinal sampling of individual e.g cattle, exposed to babesiosis. Similarly, the finding that *Theileria/Babesia* spp. co-infected with *Rickettsia* spp. in ticks suggest that *Theileria/Babesia* spp. might be more likely to co-exist with other pathogens in ticks (Chen et al., 2014). The spatial distribution of those pathogens suggested that animals in KGR are at a high risk of exposure to coinfections.

3.6 Conclusion

This study demonstrated that cattle, sheep and goats in KGR are infested with three species of hard ticks namely; *A. variegatum, Rhipicephalus* species*,* and *Hyalomma* species. Between age groups, young animals were significantly less infested than adults and male animals were found to be more susceptible to tick infestation than female. The finding of *B. caballi* in ticks collected off cattle warrant further investigation and in order to better clarify these findings, other tick species, likely to be involved in the transmission of *B. caballi* (*Hyalomma* and *Rhipicephalus* spp.) need be tested. In addition, future studies aiming to better understand the prevalence of *Rickettsia massiliae* in the study area and the risk of exposure for the local population handling livestock (e.g. pastoralists, veterinary and para-veterinary personnel) will be desirable.

Chapter 4: Molecular Epidemiology of Tick-Borne Haemoparasites in Cattle in KGR, Nigeria

4.1 Introduction

Tick-borne infections constitute a major constraint on cattle production and the expansion of the dairy industry in many countries across sub-Saharan Africa (Uilenberg, 1995). The diseases caused by tick-borne pathogens cause substantial economic loss and the improvement of strategies to control the diseases caused by these organisms requires more detailed knowledge of their prevalence and how they interact with each other (Njiiri et al., 2015). In order to prioritise future research on the development of improved control measures against TBIs, it is essential to define the prevalence of tick-borne pathogens in target cattle populations. The most serious TBIs in eastern and central Africa are ECF, caused by the intracellular protozoan parasite *Theileria parva* (Gachohi et al., 2012) and heartwater disease caused by *E. ruminantium* (Faburay et al., 2007). The diseases are associated with high levels of mortality, primarily in exotic and crossbred cattle, but also in indigenous calves and adult cattle in endemically unstable areas (Faburay et al., 2007). Several TBIs transmitted by *Ehrlichia/Anaplasma*, *Theileria/Babesia,* and *Rickettsia* have been reported in many studies conducted on cattle in Nigeria (Kamani et al., 2010; Ogo et al., 2012; Lorusso et al., 2013; Lorusso et al., 2016). *Bartonella* was studied on rodents in the country but to the best of knowledge; these studies first describe and report *Bartonella* in cattle in much of sub-Saharan Africa (Kamani et al., 2013). Nonetheless, there is limited information on the prevalence of these pathogens in grazing reserve in the country.

In Nigeria, the most commonly used technique for the diagnosis of TBIs in naturally infected cattle is clinical data in combination with the examination of Giemsa-stained blood smears, and occasionally lymph node biopsies are also examined for schizonts, but this is not routine (Leeflang & Ilemobade, 1977; Kamani et al., 2010). The major shortfall of these techniques is that the differentiation of haemoparasites from other less pathogenic infection in cattle is difficult, and such microscope-based techniques are relatively insensitive (Friedhoff & Bose, 1994). A specific and sensitive serological method (e.g. indirect fluorescent antibody test, (IFAT), rapid card agglutination test, capillary tube-agglutination test) for detection of antibodies against TBIs is also available (Obi, 1978; Akinboade & Dipeolu, 1984; Ajayi & Dipeolu, 1986) but this cannot be used for differentiating current from previous infections. Assays to differentiate cattle-infective TBIs on the basis of their rRNA sequences have been described by Allsopp et al., (1993), Bishop et al., (1995) but these did not include all TBIs. In the last decade, several molecular techniques have been developed to detect tick-borne haemoparasites, in both their vertebrate or arthropod hosts, with the majority of these assays

consisting of species-specific Polymerase chain reaction (PCR) assays, often with a downstream nested approach in order to obtain an enhanced sensitivity (reviewed in Sparagano et al., 1999; Collins et al., 2002; Bock et al., 2004; Aubry & Geale, 2011; Eremeeva, 2012). However, most of the time, these tools are designed to detect single infections and are unable to diagnose coinfections by several pathogens within the same host(Sparagano et al., 1999; Collins et al., 2002; Bock et al., 2004; Aubry & Geale, 2011; Eremeeva, 2012). Mixed infections of tick-borne haemoparasites frequently occur, hence a multiple detection test such as the reverse line blot hybridisation is a valuable tool in the simultaneous detection of such infections (Sparagano et al., 1999). The reverse line blot (RLB) hybridization assay has been developed to detect and differentiate between several parasite species simultaneously. This method, indeed, allows and enables the detection of up to eight genera of tick-borne microorganisms, including *Bartonella* spp. (Schouls et al., 1999) *Borrelia* spp. (Schouls et al., 1999), *Ehrlichia* spp. and *Anaplasma* spp. (Bekker et al., 2002), *Hepatozoon* spp. (Matjila et al., 2008) and *Rickettsia* spp. (Christova et al., 2003), *Babesia* and *Theileria* spp. (Georges et al., 2001). The RLB technique has not been applied in the field situation in North Western Nigeria but the assay has been used to identify haemoparasites in North Central Nigeria in cattle (Lorusso et al., 2016).

The present study aimed to investigate, by molecular means, the occurrence of tick-borne microorganisms, of both veterinary and zoonotic importance, infecting cattle in KGR North-Western Nigeria. This study relied on the application of a broad-spectrum reverse line blotting (RLB) combining four different polymerase chain reaction (PCR) approaches, enabling the detection of microorganisms belonging to the genera *Ehrlichia, Anaplasma*, *Theileria*, *Babesia*, *Rickettsia*, and *Bartonella*

4.2 Materials and Method

4.2.1 Sample collection

Blood samples and field data were collected as described in Chapter 2 section 2.5.

4.2.2 DNA extraction and elution

For each of the blood samples spotted on FTA® cards, five discs of 3 mm of diameter were punched using a Harris Micro-Punch™ (Whatman BioScience Ltd, Cambridge, UK) and placed into a 1.5 ml Eppendorf tube (see detailed description in Chapter 2 section 2.1).

4.2.3 Reverse line blotting (RLB)

4.2.3.1 PCR for haemoparasites

The RLB used was made up of 4 group-specific PCRs then probes to delineate species within each group. Following DNA extraction and elution, each sample was subjected to four simultaneous PCRs targeting, respectively:

(i) a 460–520 bp long fragment from the V1 hypervariable region of the 16S SSU rRNA gene for *Ehrlichia* and *Anaplasma* spp. (Schouls et al., 1999; Bekker et al., 2002),
(ii) A 460–540 bp long fragment from the V4 hypervariable region of the 18S ribosomal RNA (rRNA) gene for *Theileria* and *Babesia* spp. (Georges et al., 2001),
(iii) a 350–400 bp variable region in the 16S rRNA gene for *Rickettsia* spp. (Christova et al., 2003),
(iv) and a 296 bp long fragment of 16S rRNA gene for *Bartonella* (see a detailed description of primers in Chapter 2 section 2.2.1).

All DNA amplifications were carried out using the same cycling conditions, as described in (Table 2:3). The outcome of PCR was determined following the electrophoretic run of the positive and negative controls as described in Chapter 2 section 2.1.

4.2.3.2 RLB hybridization of PCR product

Hybridization of PCR products with catch-all and species-specific probes was performed as (described in Chapter 2 section 2.2.3).

4.2.3.3 X-ray film development

After the series of washing steps illustrated above, 5ml of solutions ECL1 and ECL2 (Amersham, UK) were spread sequentially over the membrane, which was then incubated at room temperature for a minimum of 1 minute (see detailed description in Chapter 2 section 2.2.4).

4.2.4 PCR product purification and sequencing

PCR products were purified using an Isolate II PCR and gel kit according to the manufacturer's instructions (Qiagen) (see detailed description in Chapter 2 section 2.3).

4.2.5 Haematology

Haematology work was carried out during field work while in Nigeria with others but not as part of the PhD thesis to determine the haematological reference values of apparently healthy cattle raised under free-range traditional husbandry practice as influenced by age, and sex to establish a data baseline for the animals.

4.3 Epidemiological statistical analysis

4.3.1 Univariant analysis

Field data and results generated in the laboratory were entered and managed in Microsoft-excel. SPSS version 20.0 software program was initially employed for the data analysis as described in (Chapter 2 section 2.5.1).

4.3.2 Multivariant analysis

To investigate those factors that influence an individual's probability of testing positive for infection with tick-borne haemoparasites, generalized linear mixed models (GLMMs) were used that assumed a binomial error term and a logit link as described in (Chapter 2 section 2.5.2).

4.4 Results in cattle

4.4.1 Overall infection rates in cattle

RLB was successfully completed on DNA extracts prepared from blood collected from all 268 cattle surveyed. Of these animals, only 43 (16%) were not found to be infected with a tick-borne parasite/pathogen. Thus, the vast majority (84%) of cattle tested were infected with at least one tick-borne parasite. The prevalence of infection varied between KGR blocks Chi-squared tests revealed that the only significant associations between haemoparasites prevalence and blocks ($p < 0.05$) were between the *A. marginale* ($\chi2 = 27.57$, P = 0.001), *B. bovis* ($\chi2 = 26.74$, P = 0.001), *B. caballi* ($\chi2 = 14.400$, P = 0.006), *T. equi*-like ($\chi2 = 29.85$, P = 0.001), *T. mutans* ($\chi2 = 33.39$, P = 0.001), *T. velifera* ($\chi2 = 21.76$, P = 0.001), and *Theileria* spp. MSD4 ($\chi2 = 17.90$, P = 0.001), with the parasites being more prevalent in block 1 and 5 (98.3%, and 95.8%, respectively) than elsewhere see Table 4:2. The prevalence of infection was greater in older animals than younger animals, but this difference was not statistically significant ($\chi2 = 3.39$, P = 0.065 (Table 4.2). On the whole, sex-related haemoparasitaemia did not vary significantly ($\chi2 = 0.139$, P = 0.708) in the study, however, parasitaemia was higher in males (53/62, 85.4%) than female (172/206, 83.4%).

Table 4:1: Proportion of infected animals within the sampled population

Blocks	Infected Animals/Animals sampled (%)		
	Young	Adults	Total
Block 1	14/14 (100)	46/47 (97.8)	60/61 (98.3)
Block 2	10/16 (62.5)	30/38 (78.9)	40/54 (74.0)
Block 3	6/6 (100)	17/18 (94.4)	23/24 (95.0)
Block 4	17/27 (62.9)	39/54 (72.2)	56/81 (69.1)
Block 5	11/12 (91.6)	35/36 (97.2)	46/48 (95.8)
Total	58/75 (77.3)	167/193 (86.5)	225/268 (84.0)

4.4.2 Prevalence of tick-borne haemoparasites in cattle

The prevalence's of single and mixed-species infections are summarised in (Table 4:2). In all, 14 different species of haemoparasites were detected (Table 4:2). In addition, (3) *Ehrlichia/Anaplasma,* (2) *Theileria*, (7) *Rickettsia* and (9) *Bartonella* samples produced a signal only with the group-specific probes, and not with the corresponding species-specific probes. These could not be assigned to a particular species. Table 4:2 shows the number of cattle which were positive for each of the haemoparasites. The most prevalent species were the *Theileria* (207/268, 77.2%), *Ehrlichia/Anaplasma* (191/268, 71.3%), and *Babesia* (84/268, 31.3%) respectively.

Table 4:2: Prevalence of each tick-borne haemoparasites in cattle

Microorganism	**Prevalence (%)**	**total No.=268**	**Confidence Interval Lower - Upper bound (%)**
		No. Infected	
Theileria mutans	69.4	186	63.5 – 74.9
Theileria velifera	69.4	186	63.5 – 74.9
Theileria equi-like	56	150	49.8 – 62.0
Anaplasma marginale	54.9	147	48.7 – 60.9
Ehrlichia spp. Omatjenne	50.4	135	44.2 – 56.5
Theileria spp. MSD4	36.2	97	30.4 – 42.3
Babesia bovis	22.8	61	17.9 – 28.3
Babesia bigemina	16	43	11.9 – 21.0
Anaplasma centrale	7.5	20	4.6 – 11.3
Bartonella spp.	3.4	9	1.5 – 6.3
Babesia caballi	3	8	1.3 – 5.8

Rickettsia spp.	2.6	7	1.1 – 5.3
Anaplasma phagocytophilum	1.9	5	0.6 – 4.3
Ehrlichia ruminantium	0.7	2	0.1 – 2.7

4.4.3 Confirmation of amplicon identity in cattle

Unambiguous sequence data (460 – 520 bp) were obtained from *Ehrlichia*/*Anaplasma* catch-all –only positive (n= 6) samples only (n=2) were found 99-100% similar with *Anaplasma platys* (Accession No. KU586124.1, KJ832067.1). Furthermore, (350–400 bp) sequence data were obtained for *Rickettsia* catch-all only positive (n= 7) samples only (n= 1) were found 99% similar with *Rickettsia felis* (Accession No. CP000053.1). In the same way, (296 bp) sequence data for *Bartonella* catch-all only positive (n=9) sample only (n=8) were found 98-100% similar to *Bartonella bovis* (Accession No. KF199899.1, KR733183.1)

4.4.4 Age-wise prevalence of tick-borne infection in cattle

The prevalence of infection of specific pathogens differed between young and older animals (Table 4.4). Infection prevalence was significantly higher in older animals than younger animals for with regards *Theileria mutans* (χ^2=5.3, p=0.020), *Theileria velifera* (χ^2=5.7, p=0.017), *Theileria equi*-like (χ^2=4.8, p=0.029), *Anaplasma marginale* (χ^2=10.9, p=0.001), and *Babesia bovis* (χ^2=5.3, p=0.022). No *Ehrlichia ruminantium* infection was detected in young animals (see Table 4:3).

Table 4:3: Age-wise prevalence of tick-borne infection in cattle

Micro-organism	Prevalence of infection (%)		Chi-Square Tests
	Young	Adults	
Theileria mutans	48 (64)	150 (78)	χ^2=5.3, p=0.020
Theileria velifera	44 (59)	142 (74)	χ^2=5.7, p=0.017
Theileria equi-like	34 (45)	116 (60)	χ^2=4.8, p=0.029
Anaplasma marginale	30 (40)	117 (61)	χ^2=10.9, p=0.001
Ehrlichia spp. Omatjenne	35 (47)	100 (52)	χ^2=0.6, p=0.449
Theileria spp. MSD4	30 (40)	67 (35)	χ^2=0.6, p=0.419
Babesia bovis	10 (13)	51 (26)	χ^2=5.3, p=0.022
Babesia bigemina	13 (170)	30 (16)	χ^2=0.1, p=0.720
Anaplasma centrale	3 (4)	17 (9)	χ^2=1.8, p=0.179
Bartonella species	1 (1)	8 (4)	χ^2=1.3, p=0.251
Babesia caballi	2 (3)	6 (3)	χ^2=0.0, p=0.849
Rickettsia species	2 (3)	5 (3)	χ^2=0.0, p=0.972
Ehrlichia ruminantium	0 (0)	2 (1)	n/a

4.4.5 Sex-wise prevalence of tick-borne infection in cattle

The prevalence of infection of specific pathogens also varied between male and female animals. Out of 268 animals studied for one or more heamoparasite infections, 206 (76.8%) were females while 62 (23.1%) were males. However, no significant (P>0.05) difference was observed in all the species studied (Table 4:4).

Table 4:4: Sex-wise prevalence of tick-borne infection in cattle

Microorganisms	Prevalence of infection (%)		Chi-Square Tests
	Male	**Female**	
Theileria mutans	44 (70.9)	154 (74.7)	χ^2=0.3, p=0.551
Theileria velifera	41 (66.1)	145(70.3)	χ^2=0.4, p=0.523
Theileria equi-like	34 (54.8)	116 (56.3)	χ^2=0.04, p=0.838
Anaplasma marginale	35 (56.4)	112 (54.3)	χ^2=0.0, p=0.773
Ehrlichia spp. Omatjenne	32 (51.6)	103 (50)	χ^2=0.0, p=0.824
Theileria spp. MSD4	30 (48.3)	67 (32.5)	χ^2=5.1, p=0.23
Babesia bovis	13 (4.8)	48 (23.3)	χ^2=0.1, p=0.701
Babesia bigemina	12 (19.3)	31 (15.0)	χ^2=0.6, p=0.418
Anaplasma centrale	5 (8.0)	15 (7.2)	χ^2=0.0, p=0.837
Bartonella species	1 (1.6)	8 (1.9)	χ^2=0.7, p=0.384
Babesia caballi	3 (4.8)	5 (2.4)	χ^2=0.9, p=0.328
Rickettsia species	3 (4.8)	4 (1.9)	χ^2=1.5, p=0.210
Ehrlichia ruminantium	0 (0)	2 (0.9)	n/a

4.4.6 Single infections

24 single infections were detected, amongst 225 positive microorganisms (10.6 %), of which 11 were in young cattle (45.8 % of positive animals) and 13 in adult cattle (54.1 % of positive animals). Cases of single infections were mostly represented by *T. mutans* (*n* = 7), *A. marginale* (*n* = 5), *T. velifera* (*n* = 4), *E.* spp. Omatjenne (*n* = 3), *T. equi*-like (*n* = 2), *A. phagocytophilum* (*n* = 1), *A. platys* (n = 1), and *Rickettsia* spp. (*n* = 1).

4.4.7 Coinfections

201/268 (75 %) cattle were positive for two or more microorganisms simultaneously. Overall 92 different combinations of microorganisms were found. The largest variety of coinfections was recorded in (*A. centrale* + *A. marginale* + *E.* spp. Omatjenne + *B. bigemina* + *B. caballi* + *T. equi*-like + *T. mutans* + *T. velifera* + *T.* spp. MSD4 and *A. marginale* + *E.* spp. Omatjenne + *B. bigemina* + *B. bovis* + *T. equi*-like + *T. mutans* + *T. velifera* + *T.* spp. MSD4 + *Bartonella* specie). On the whole, the most frequent combinations included (i.e. *A. marginale* + *E.* spp. Omatjenne + *T. equi*-like + *T. mutans* + *T. velifera* + *T.* spp. MSD4) to five co-infective species (*E.* spp. Omatjenne + *T. equi*-like + *T. mutans* + *T. velifera* + *T.* spp. MSD4). Table 4:5 reports only the 37 most frequent coinfections. For a more detailed overview, the reader is referred to the extensive format of the table in the Appendix Section (Table 5:11). Out of 201 cattle that were positive for two or more microorganism the largest variety of coinfection differed between young 47 (23.3%) and adult's animals 154 (76.6%). Coinfection prevalence was significantly higher in adults' animals than younger animals (χ^2=8.4, p=0.001). while sex recorded 44 (21.8%) in males and 157 (78.1%) in females. Age wise prevalence recorded a significance difference while no significant difference was observed in sex (χ^2=0.46 p=0.402).

Table 4:5: Thirty-seven most frequent multiple infections by tick-borne haemoparasites according to sex, age classes and overall number of cattle.

(*Ac* = *Anaplasma centrale*; *Am* = *Anaplasma marginale*; *E*spO = *Ehrlichia* spp. Omatjenne; *Er* = *Ehrlichia ruminantium*; *R* = *Rickettsia* spp.; *B* = *Bartonella*; *Bbov* = *Babesia bovis*; *Tel* = *Theileria equi*-like; *Tm* = *Theileria mutans*; *T*spMSD4 = *Theileria* sp MSD4; *Tv* =*Theileria velifera*).

	Tick-borne haemoparasites species combinations	Frequency				Totals
		Males	Females	Young	Adults	
1	*Am* + *E*spO + *Tel* + *Tm* + *Tv* + *T*spMSD4	5	12	4	13	17
2	*E*spO + *Tel* + *Tm* + *Tv* + *T*spMSD4	2	7	3	6	9
3	*Am* + *E*spO + *Bbov* + *Tel* + *Tm* + *Tv*	-	8	1	7	8
4	*Am* + *Tel* + *Tm* + *Tv* + *T*spMSD4	3	5	1	7	8
5	*Am* + *E*spO + *Bb* + *Tel* + *Tm* + *Tv* + *T*spMSD4	2	6	3	5	8
6	*Am* + *E*spO + *Tm* + *Tv*	3	4	1	6	7
7	*Am* + *E*spO	1	6	1	6	7
8	*Tel* + *Tm* + *Tv*	-	6	-	6	6
9	*Am* + *E*spO + *Tel* + *Tm* + *Tv*	-	5	-	5	5
10	*Am* + *Tel* + *Tm* + *Tv*	-	5	-	5	5
11	*E*spO + *Tel* + *Tm* + *Tv*	-	5	1	4	5
12	*Tm* + *Tv*	-	5	1	4	5
13	*Am* + *Tel* + *Tm* + *Tv* + *T*spMSD4	3	2	2	3	5

14	*Ac* + *Am* + EspO + *Tel* + *Tm* + *Tv* + *T*spMSD4	2	2	-	4	4
15	*Am* + *Bb* + *Tel* + *Tm* + *Tv* + *T*spMSD4	-	3	3	1	4
16	*Am* + *Bbov* + *Tel* + *Tm* + *Tv* + *T*spMSD4	1	2	1	2	3
17	*Am* + EspO + *Bb*+ *Tel* + *Tm* + *Tv* + *T*spMSD4	1	-	3	-	3
18	*Am* + EspO + *Bbov* + *Tel* + *Tm* + *Tv* + *T*spMSD4	1	2	1	2	3
19	*Am* + *Tm*	-	3	2	1	3
20	*Am* + *Tm* + *Tv*	-	3	-	3	3
21	EspO + *Bbov* + *Tel* + *Tm* + *Tv* + *T*spMSD4	2	1	2	1	3
22	*Am* + EspO + *Bb* + *Bbov* + *Tel* + *Tm* + *Tv* + *T*spMSD4	1	2	-	3	3
23	*Am* + EspO + *Bb*+ *Bc* + *Tel* + *Tm* + *Tv* + *T*spMSD4	2	1	1	2	3
24	*Ac* + *Am* + *Tm*	1	2	1	2	3
25	EspO + *Tm* + *Tv*	1	2	1	2	3
26	*Ac* + *Am* + *Bb* + *Bbov* + *Tel* + *Tm* + *Tv* + *T*spMSD4	-	2	-	2	2
27	*Ac* + *Am* + EspO + *Bbov* + *Tel* + *Tm* + *Tv* + *T*spMSD4	-	2	-	2	2

28	*Am* + *Bb* + *Bbov* + *Tel* + *Tm* + *Tv*	1	1	-	2	2
29	*Am* + *E*spO + *Ap* + *Tel* + *Tm* + *Tv* + *T*spMSD4	-	2	-	2	2
30	*Am* + *E*spO + *Bb* + *Tel* + *Tm* + *Tv* + *B*	1	1	-	2	2
31	*Am* + *E*spO + *Bb* + *Bbov* + *Tel* + *Tm* + *Tv*	-	2	-	2	2
32	*Am* + *E*spO + *Bb* + *Tel* + *Tm* + *Tv*	-	2	-	2	2
33	*Bb* + *Bbov* + *Tel* + *Tm* + *Tv* + *T*spMSD4	-	2	-	2	2
34	*Bbov* + *Tel* + *Tm* + *Tv*	-	2	-	2	2
35	*E*spO + *Bb* + *Tel* + *Tm* + *Tv*	-	2	-	2	2
36	*E*spO + *Tm*	1	1	2	-	2
37	*Tel* + *Tm* + *Tv* + *T*spMSD4	1	1	2	-	2

4.5 Results from General Linear Mixed-Model (GLMM) analyses

GLMM analyses were carried out to identify ecological determinants that were significantly correlated to haemoparasites infection prevalence in the KGR cattle sampled in this study. Two models were analysed for each – one GLM and one GLMM (with owner included as a random effect). The use of the owner as a random effect is to negate the effect of different practices that the different owners may employ.

4.5.1 *Anaplasma marginale* infection prevalence model

Cattle infected with *A. marginale* were significantly more likely to be co-infected with *E.* spp. Omatjenne (p= 0.003, odds ratio 2.51) and *T. mutans* (p=0.001, odds ratio = 2.24) and have a decrease (p=0.03, odds ratio 4.64) or increase in PCV (p=0.04, odds ratio 2.06). In addition, older animals were shown to be significantly more likely to be infected with *A. marginale* than younger animals (p= 0.015, odds ratio = 2.25) (see Table 4:6).

Table 4:6: Results of the GLMM analysis for *A. marginale* infection in cattle

Parameters	Estimate	(SE)	Z value	Pr(>|z|)	Odds ratio
Intercept	-2.4066	0.5082	-4.736	2.18e-06 ***	-
E. spp. Omatjenne	0.9207	0.3082	2.987	0.00282 **	2.51
PCV decrease	1.5348	0.7021	2.186	0.02882 *	4.64
PCV increase	0.7271	0.3643	1.996	0.04593 *	2.06
T. mutans	1.7991	0.3950	4.555	5.24e-06 ***	2.24
Adults	0.8090	0.3318	2.438	0.01477 *	2.25

Significance codes : 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 '' 1

4.5.2 *Ehrlichia* spp. Omatjenne prevalence model

Cattle infected with *E.* spp. Omatjenne were significantly more likely to be co-infected with *T. velifera* (P= 0.001, odds ratio 5.82) and, as aforementioned, *A. marginale* (p= 0.01, odds ratio 2.25) (see Table 4:7).

Table 4:7: Results of the GLMM analysis for *Ehrlichia* spp. Omatjenne infection in cattle

Parameters	Estimate	(SE)	Z value	Pr(>|z|)	Odds ratio
Intercept	-1.7120	0.3023	-5.662	1.49e-08 ***	-
T. velifera	1.7622	0.3431	5.136	2.80e-07 ***	5.82
A. marginale	0.8151	0.2902	2.808	0.00498 **	2.25

Significance codes : 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 '' 1

4.5.3 *Anaplasma centrale* prevalence model

Cattle infected with *A. centrale* were more likely to be co-infected with *A. marginale* (p= 0.01, odds ratio 5.36) and that reduced tick burden was associated with increased risk of *A. centrale* infection (p= 0.004, odds ratio 5.24) (see Table 4:8).

Table 4:8: Results of the GLMM analysis for *A. centrale* infection in cattle

Parameters	Estimate	(SE)	Z value	Pr(>|z|)	Odds ratio
Intercept	-3.1983	0.6413	-4.987	6.12e-07 ***	-
Tick burden	-1.6565	0.5815	-2.849	0.00439 **	5.36
A. marginale	1.6805	0.6548	2.566	0.01027 *	5.24

Significance codes : 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 '' 1

4.5.4 *Babesia bigemina* prevalence model

Cattle infected with *B. bigemina* were significantly associated with increased risk of coinfection with *A. marginale* (p= 0.03, odds ratio 2.52), *B. bovis* (p= 0.01, odds ratio 2.65) and *T. annulata* (p= 0.0001, odds ratio 5.76) (see Table 4:9).

Table 4:9: Results of the GLMM analysis for *B. bigemina* infection in cattle

Parameters	Estimate	(SE)	Z value	Pr(>|z|)	Odds ratio
Intercept	-3.7174	0.5230	-7.108	18e-12 ***	-
A. marginale	0.9262	0.4479	2.068	0.038645 *	2.25
B. bovis	0.9760	0.3866	2.524	0.011589 *	2.65
T. annulata	1.7520	0.4521	3.875	0.000107 ***	5.76

Significance codes : 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

4.5.5 *Babesia bovis* prevalence model

Cattle infected with *B. bovis* were significantly more likely to have an increased risk of coinfection with *T. mutans* (p= 0.001, odds ratio 27.2), *B. bigemina* (p= 0.003, odds ratio 3.43), with reduced risk of coinfection with *B. caballi* (p= 0.03, odds ratio 11.5). In addition, older animals were significantly more likely to be infected with *B. bovis* (p= 0.02, odds ratio 2.63) (see Table 4:10).

Table 4:10: Results of the GLMM analysis for *B. bovis* infection in cattle

Parameters	Estimate	(SE)	Z value	Pr(>|z|)	Odds ratio
(Intercept)	-5.3110	1.1509	-4.614	3.94e-06 ***	-
T. mutans	3.3046	1.0333	3.198	0.00138 **	27.2
B. bigemina	1.2339	0.4216	2.926	0.00343 **	3.43
B. caballi	-2.4459	1.1619	-2.105	0.03528 *	11.5
Adult	0.9697	0.4361	2.224	0.02618 *	2.63

Significance codes : 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

4.5.6 *Theileria equi*- like prevalence model

Cattle infected with *T. equi*-like were more likely to be co-infected with *T. velifera* (p= 0.001, odds ratio 36.3) (see Table 4:11).

Table 4:11: GLMM analysis for *T. equi*-like infection in cattle

Parameters	Estimate	(SE)	Z value	Pr(>|z|)	Odds ratio
Intercept	-3.6922	0.7222	-5.112	3.19e-07 ***	-
T. velifera	3.5929	0.6675	5.382	7.36e-08 ***	36.3

Significance codes : 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

4.5.7 *Theileria mutans* prevalence model

Cattle infected with *T. mutans* were also more likely to have coinfections with *A. marginale* (p= 0.13, odds ratio 2.46), *T. velifera* (p= 0.001, odds ratio 29.3) and *T. equi*-like (p= 0.01, odds ratio 8.87) (see Table 4:12).

Table 4:12: GLMM analysis for *T. mutans* infection in cattle

Parameters	Estimate	(SE)	Z value	Pr(>|z|)	Odds ratio
Intercept	-1.8279	0.6632	-2.756	0.00585 **	-
A. marginale	0.9005	0.5979	1.506	0.13203	2.46
T. velifera	3.3803	0.6596	5.124	2.98e-07 ***	29.3
T. equi-like	2.1835	0.8744	2.497	0.01251 *	8.87

Significance codes : 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

4.5.8 *Theileria velifera* prevalence model

Cattle infected with *T. velifera* were significantly more likely to be co-infected with *E.* spp. Omatjenne (p= 0.01, odds ratio 4.12), *T. equi*-like (p= 0.001, odds ratio 34.1) and *T. mutans* (p= 0.001, odds ratio 36.7) (see Table 4:13).

Table 4:13: GLMM analysis for *T. velifera* infection in cattle

Parameters	Estimate	(SE)	Z value	Pr(>|z|)	Odds ratio
Intercept	-3.3548	0.5640	-5.948	2.71e-09 ***	-
E spp. Omatjenne	1.4180	0.5435	2.609	0.00908 **	4.12
T. equi-like	3.5316	0.7147	4.941	7.76e-07 ***	34.1
T. mutans	3.6030	0.5904	6.102	1.05e-09 ***	36.7

Significance codes : 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

4.5.9 *Theileria* spp. MSD4 prevalence model

Cattle infected with *Theileria* spp. MSD4 were significantly more likely to have coinfections with *A. centrale* (p= 0.02, odds ratio 4.60), or *T. equi*-like (p= 0.001, odds ratio 4.81). Female animals were also significantly more likely to be infected with *Theileria* MSD4 than males. Moreover, *Theileria* MSD4 infection was found to be linked, although not significantly, to decrease (p= 0.05 odds ratio 3.49) or increase in PCV (p= 0.05, odds ratio 2.23) (see Table 4:14).

Table 4:14: GLMM analysis for *Theileria* spp. MSD4 infection in cattle

Parameters	Estimate	(SE)	Z value	Pr(>|z|)	Odds ratio
Intercept	-2.4954	0.5254	-4.750	2.04e-06 ***	-
Female	-1.2698	0.4437	-2.862	0.00421 **	2.33
PCV decrease	1.2522	0.6574	1.905	0.05682	3.49
PCV increase	0.8055	0.4233	1.903	0.05703	2.23
A. centrale	1.5281	0.7037	2.171	0.02990 *	4.60
T. equi-like	1.5719	0.4777	3.290	0.00100 **	4.81

Significance codes : 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

4.6 Discussion

4.6.1 Prevalence of tick-borne haemoparasites detected in cattle

In the present study, a comprehensive investigation of the distribution and prevalence of haemoparasites in all six Blocks of KGR in Kaduna State, North Western Nigeria, was undertaken. The findings of this study revealed an overall prevalence rate of 84.0% of any TBIs in cattle. Of these, *Theileria* species recorded the highest prevalence, followed by *Ehrlichia/Anaplasma* species, *Babesia* species, *Bartonella* species and *Rickettsia* species. The present study is comparable to previous studies on the prevalence of haemoparasites in cattle 25.7% (Kamani et al., 2010), 10.8% (Paul et al., 2016), and 82.6% (Lorusso, et al., 2016) in Nigeria. This suggests the occurrence of a continuous challenge of cattle by parasites and the existence of a carrier state in cattle in the country. Though the reason for the differences in the reported prevalence rates might be due to the difference in the diagnostic

technique employed, climatic/geographical variation of sampling areas, the period of sample collections, and sample size among others.

The effects of age on prevalence of haemoparasites has been previously reported (Kamani et al., 2010; Ademola & Onyiche, 2013; Okorafor, & Nzeako, 2014; Lorusso et al., 2016). The GLMM analysis suggested that age of cattle had a significant effect on the probability of animal to be infected with haemoparasites and the finding suggested that older animals were shown to be significantly more likely to be infected than younger animals. This finding is in line with previous studies (Lorusso et al., 2016; Weny et al., 2016; Bariso & Worku, 2018). However, it is in contrast with the findings of Paul et al., (2016), who reported a higher prevalence in *Anaplasma* and *Babesia* spp.in younger cattle than the adult. The difference in the findings is in line with the concept of 'inverse age immunity', with colostral first, and then innate immunity, enabling the low likelihood of the appearance of clinical disease in young animals at the time of their peak exposure to a pathogen. In addition, it may be due to the fact that the young animals are closely watched and cared for by the Fulani pastoralists during grazing at KGR than the adults this may probably reduce infestation rate with arthropod vectors such as ticks and the corresponding disease (Lorusso et al., 2016).

The effects of risk factors such as sex on the prevalence of haemoparasites have been previously reported (Kamani et al.,2010; Alim et al.,2011; Ademola & Onyiche, 2013; Okorafor & Nakao, 2014). The higher prevalence recorded in males than females is contradictory to previous finding Kamani et al., (2010), Hailemariam et al., (2017), and Debbarma et al., (2018) who attributed their finding to the fact that female animals were generally herded much longer for the purpose of breeding and milk production, thereby prolonging their exposure to challenges of disease. It has also been reported that female ruminants are generally more prone to infection by haemoparasites due to their extended breeding for economic purposes such as parturition and milk production (Ukwueze & Kalu, 2015; Anyanwu, et al., 2016) as well as the stress of breeding, milking and cyclical hormonal changes associated with gestation, parturition and calving processes.

4.6.2 Prevalence of *Theileria/Babesia* species in cattle

Theileria mutans and *T. velifera* infection were the two most prevalent microorganisms recorded in cattle, with the prevalence of 69.4% and 69.4%, respectively. These two *Theileria* species have long been recognised to be present in Nigeria (Saidu et al., 1984), and are known to be transmitted by *A. variegatum* (Uilenberg et al., 1974; Young et al., 1978),

endemically present in the whole of Nigeria (Bayer & Maina, 1984; Lorusso et al., 2013) including KGR (see Chapter 3). Reports of the consequences of infection vary; most infections appear to be asymptomatic (Saidu et al., 1984), but occasionally mild clinical signs can develop (Saidu et al., 1984) and one report details severe anaemia and sometimes death in cattle (Yusufmia et al., 2010). The high prevalence recorded in this study agrees with the findings reported (by RLB) in North Central Nigeria (Lorusso et al., 2016) and other SSA countries such as Uganda (Oura et al., 2014) and South Sudan (Salih et al., 2007). However, the finding in this thesis is higher than the 30.9% reported on the basis of blood smear microscopy, and serology by ELISA from southern and eastern Ethiopia (Solomon et al., 1998). This variation in infection rates reported in this study might be related to the differences in diagnostic techniques with serology indicating exposure but not active infection or not necessarily carrier status (Salih et al., 2015). Alternatively, variation in the detected prevalence of infection may reflect ecological differences such as vector abundance, host abundance and susceptibility (García-Sanmartín et al., 2006). The high haemoparasitemia detected in adults than young cattle suggest an early exposure of cattle in KGR with these piroplasms, due to early infestations with *A. variegatum*; this finding is also in agreement with a previous report (Asiimwe et al., 2013).

This study detected the presence of *T. equi*-like at a high prevalence of (56%) in cattle. In a study conducted by Kouam, (2010) to determine the Genetic diversity of equine piroplasms in Greece within *Theileria* genotypes (*T. equi* and *T. equi*-like). A partial sequence (509 bp) of the V4 region of the 18S rRNA gene of a *T. equi*-like isolate showed only 99% similarity with the reference *T. equi*-like isolates from Northern Spain from which the detecting probe used was designed but showed 100% similarity with the *T. equi*-like variants from Southern Spain. This indicated a noticeable degree of polymorphism within the population of *T. equi*-like. This microorganisms is well recognized as the cause of equine piroplasmosis (Wise et al., 2013), an important disease of horses which has been recognized and studied in Nigeria (Turaki et al., 2014; Mshelia et al., 2016). Our finding that *T. equi*-like occurs in cattle agrees with the previous report of a longitudinal survey between March 2012- February 2013 from North Central Nigeria Lorusso, (2015), this further expands the host range of this organism. The significance, extent, and consequences of infections with *T. equi*-like in domestic ruminants require further investigation. Numerous species of ticks such as *Amblyomma, Dermacentor*, *Hyalomma*, *Ixodes*, and *Rhipicephalus* are confirmed or suspected vectors of *T. equi*-like (Scoles & Ueti, 2015). Of these, only *Amblyomma*,

Hyalomma and *Rhipicephalus* spp. are known to occur in Nigeria (Bayer and Maina, 1984; Lorusso et al., 2013). Studies also suggested that *Rhipicephalus* (*Boophilus*) *microplus* may likely serve as an agent of transmission (Asgarali et al., 2007), which is found on cattle in sub-Saharan Africa including Nigeria (Kamani et al., 2017). The higher prevalence obtained in adults than young cattle suggests that age plays a role in the infection of cattle with *T. equi* as adult animals, especially under stress and immune suppression, are susceptible to infection of this microorganism (Salib et al, 2013)

This study provides the first record of the occurrence of *Theileria* spp. MSD4 in cattle in Nigeria. This species was first identified from a naturally infected bovine at the Merck, Sharp & Dome (MSD) experimental centre at Hartebeespoort, Pretoria, South Africa (Chae et al., 1999). It was initially suspected to be a variant of *T. velifera*, but sequence and phylogenetic analyses based on 18S rRNA gene sequences indicated that it is most closely related to *T. mutans* (Chae et al., 1999; Martins et al., 2010; Chaisi et al., 2011; Mans et al., 2011). Although no attempts have been made to clarify the identity of *Theileria* spp. (strain MSD) after its first description by Chae et al., (1999), the identification of similar sequences at a high prevalence in cattle indicates that this genotype is circulating in some ruminants populations in KGR. This species has also been described in a Sika deer (*Cervus nippon*) from Japan but there is little sequence data on the organism with only a 552 bp sequence of the 18S rRNA gene reported in GenBank (AB981984). GLMM analysis suggested that female animals had a significant effect on the probability of infection with *Theileria* spp. MSD4. The prevalence of parasitaemia was higher in females than male animals possibly due to the fact that females are kept much longer for breeding and milk production purposes and cyclical hormonal changes associated with gestation, parturition and calving processes (Ukwueze & Kalu, 2015; Anyanwu, et al., 2016).

In the present study, *B. bigemina* and *B. bovis* were recorded at a prevalence of (16.0 % and 22.8 % respectively). In Nigeria, these infections are recognized as an economically important in cattle. The tick vectors (*Rhipicephalus* (*Boophilus*) species) responsible for transmitting the infection were the second most abundant ticks identified from the study area (see Chapter 3). The finding of *B. bigemina* in cattle in this study is higher than the previous report of 7.9% from North Central Nigeria (Lorusso et al., 2016), and 9.5% reported in North Western Nigeria (Onoja et al., 2013). However, it is in line with the previous study by means of serological examination (IFAT) in Northern Nigeria with a prevalence of 29.4% (Ajayi &

Dipeolu, 1986). Similarly, several other studies have reported the presence of *B. bovis* in cattle in Nigeria relying on morphological (Leeflang, 1977; Leeflang & Ilemobade, 1977; Kamani et al., 2010) and serological (i.e. IFAT) characterization (Akinboade & Dipeolu, 1984). The difference in the prevalence might be due to different factors like management condition of the focus area, farming system and proper use of antiparasitic drugs, fluctuations of parasites during chronic course of the disease and in carriers animals, sensitivity of test used, distribution of infected vector and accessibility of animals to wildlife sanctuary and forest area harbouring the *Babesia* vectors (Gubbels et al., 1999). Other cause of variation may be due to different geographic conditions and or due to different breeds of cattle studied. GLMM suggested that age groups of cattle significantly affected the rate of infection with *B. bovis*. In particular, young cattle had a lower infection rate of *B. bovis* infections compared with older cattle. As aforementioned, this difference might be caused by the higher innate immune response of cattle at the young age, also proved for the *Babesia* species (Zintl et al., 2005; Jonsson et al., 2008; Terkawi et al., 2012).

This study reports the presence of *B. caballi* DNA in cattle with at a very low prevalence in Nigeria. Previously, DNA of *B. caballi* was reported in *Haemaphysalis longicornis* ticks collected from cattle in Korea (Kang et al., 2013) amplifying a 403 bp fragment of the V4 region of the 18S rRNA gene (Battsetseg et al., 2001). The finding in this study is not surprising as this pathogen has been detected in *A. variegatum* ticks that were collected off the same cattle as reported in Chapter 3. In sub-Saharan African, *B. caballi* is known to be transmitted by ticks of the genus *Hyalomma* spp. (e.g *H. marginatum rufipes* and *H. truncatum*, in particular) which are the main vectors, *Rhipicephalus evertsi evertsi* and *R. bursa* in South Africa, while *R. sanguineus*, *H. dromedarii* and *H. excavatum* are the most important vectors in North Africa (Kaufmann, 1996). However, this microorganism's DNA was also found in 10/504 (2%) and 1/43 (2.3%) *A. variegatum* ticks collected from cattle in the Republic of Guinea and North Central Nigeria respectively, screened by RLB targeting the same 18S rRNA gene fragment used in the present study (Tomassone et al., 2005; Lorusso, 2015). The pathogenicity of this organism is not known in cattle and during the fieldwork of this study donkeys where not encounter at the grazing reserve. Therefore, the presence of this protozoa in cattle in the Nigerian KGR may be likely attributed to the presence of its confirmed (i.e. *H. truncatum*) and potentially additional (i.e. *A. variegatum*) vector in this study area. This further expands the host range of this infection and a more

extended epidemiological survey will be required to ascertain the role of ruminants as a reservoir of this apparent infection.

4.6.3 Prevalence of *Ehrlichia/Anaplasma* species in cattle

In this study, the prevalence of *A. marginale* was 54.9%. This is, however, higher than (53.3%; 8/15) and (38.0%; 268/704) reported by PCR and RLB in southern (Reye et al., 2012) and North Central Nigeria (Lorusso et al., 2016). In contrast, it is lower than 75.9% reported by qPCR in North Central Nigeria (Elelu et al., 2016). The difference may be due to the diagnostic techniques employed in the different studies. qPCR, targeting the *msp*4 and *msp*2 genes, has been reported to be more sensitive for the genetic characterization of *A. marginale* strains than RLB (de la Fuente et al., 2007), the heat-shock gene *groEL* (Park et al., 2005), the 23S rRNA (Dahmani et al., 2015) and the 16S rRNA gene (Reinbold et al., 2010). The significant prevalence of *A. marginale* warrants further investigation to evaluate the impact of this bacterium on livestock production, since it is considered to be a pathogenic species in cattle across Africa, causing severe clinical symptoms (e.g. abortion) and very serious economic losses (Said et al., 2018). However, at the time of blood sampling between (May-August in 2015 and June-August in 2016), the cattle infected with *A. marginale* showed no clinical signs. These animals could be considered asymptomatic carriers. The age of cattle appears to influence the prevalence of anaplasmosis, with *A. marginale* infection rate being significantly higher in older animals. Similarly, in Morocco, Hamou et al. (2012) reported the difference in the prevalence of *A. marginale* infections in calves (26.1 %) and adults (52.4 %). Our results were consistent with those reported in Uganda (Magona & Mayende, 2001). This suggests the occurrence of a continuous challenge of cattle by parasites and the existence of a carrier state in cattle in the country. This difference might be explained by the more sustained exposure of adults to tick vectors (Bouattour et al., 1996). Moreover, it appears that calves are less susceptible to the disease. Indeed, anaplasmosis is rare in animals younger than six months, while those between six months and one year usually develop only a mild illness, and cattle between one and two years old develop multiple signs of the disease (weakness, fever, depression, constipation, decreased milk production, jaundice, and abortion) which is rarely fatal. However, the disease is often fatal after acute infection in adults over two years old, with a mortality risk ranging from 29 % to 49 % (Kocan et al., 2003). Calves are temporarily protected by the colostrum and a mother's immunity (maternal antibodies), providing short-term protection (M'Ghirbi et al., 2016). GLMM analysis showed a significant effect on the probability of animal to be

infected with *A. marginale*, with a decrease or increase in PCV. These results are similar to those reported by Nazifi et al., (2012) in cattle naturally infected by *A. marginale*, which showed a significant decrease in PCV in positive animals. These findings are supportive of decreased erythrocyte life span and extravascular erythrophagocytosis, as suggested for bovine anaplasmosis (Ristic, 1981). It is noteworthy that in *A. marginale* acute infection, the anaemia is expected to be normocytic evolving to macrocytic, with bone marrow hyperplasia, reticulocytosis, and increased MCV and osmotic fragility of red blood cells (Thrall et al., 2012). On the other hand, the probability of increased PCV in *A. marginale* reported in this study is in line with the finding of Gotze et al., (2008) in a study of the effects of oxytetracycline in the increase of PCV. Their results reveal the activity of the oxytetracycline in the recuperation of clinical cases with anaemia by bovine anaplasmosis increases the PCV. This finding is not surprising in the present situation where both trained and untrained persons are involved in offering animal health services at KGR coupled with an increased uncontrolled variety of antibiotics particularly oxytetracycline (L.A) veterinary drugs marketed freely to herdsmen.

In this study, *Ehrlichia* spp. 'Omatjenne' was recorded in a high prevalence in cattle. The pathological role of this infection is still not well known (Allsopp et al., 1997). But the detection in blood collected from cattle suggests the presence of this rickettsia in the resident ruminant population. This poorly known *Ehrlichia* species was initially isolated in *Hyalomma truncatum* ticks in Southern Africa (du Plessis, 1990). The tick survey elucidated in Chapter 3 of this thesis confirmed indeed the presence of this tick genus in the study area. The proportion reported in this study in cattle (50.4%) is higher than that of Lorusso et al., (2016) who reported a prevalence of (34.7%). The previous detection of infection with this pathogen in cattle in Nigeria (Lorusso et al., 2016) confirms the occurrence of this natural infection. The role of this taxon in causing disease in livestock needs to be investigated. Furthermore, the high prevalence of infection recorded in adult than young cattle, suggests the occurrence of carrier status in older animals, as already proven for *E. ruminantium* (Andrew & Norval, 1989).

This study confirms the presence of *A. centrale* in cattle. Little is known about the epidemiology of this infection in Nigeria, which has rarely been examined on its own due to the overlapping occurrence of the seemingly more pathogenic *A. marginale* infection (Leeflang & Ilemobade, 1977). The low prevalence recorded in cattle (i.e. 7.5%) agrees with the finding of Lorusso et al., (2016) in North Central Nigeria and the conventional methods

(e.g ELISA, blood smears and cytological analysis) often used for the detection of *Anaplasma* spp. infection in Nigerian cattle, do not enable the discrimination between *A. marginale* and *A. centrale* (Dreher et al., 2005; Bello et al., 2017). The presence of competent vectors *Rhipicephalus* spp. (e.g *Rh. microplus*) (Reye et al., 2012) and *Amblyomma* ticks (e.g *A. variegatum*) in the study area as shown in Chapter 3, suggests the establishment of this species in KGR and its potential to spread within the grazing reserve. Furthermore, as an endemically stable setting for *Anaplasma* spp. infections are usually characterised by higher infection rates in adult cattle, due to their carrier status (Lorusso et al., 2016), the rather high prevalence detected in adults than in young cattle suggests an extent of epidemiological 'stability' for this microorganism. This situation may favour the onset of sporadic episodes of acute anaplasmosis in the indigenous cattle population. GLMM analysis suggested that reduced ectoparasites burden is associated with increased risk of *A. centrale* infection. Interestingly, during the fieldwork, the Fulani's were found to remove ticks by manual hand removal. However, it does not guarantee 100% success and does not prevent the animals from getting infected with pathogens. Infestation by ectoparasites could lead to considerable economic losses to farmers due to loss of productivity, mortality, and skin diseases (Minjauw & Mcleod, 2003). Further epidemiological investigations are required to fully understand the output of the GLMM on reduced ectoparasites burden is associated with increased risk of *A. centrale* infection.

In the present study, only 2 (0.7%) cattle tested positive for *E. ruminantium* even though *A. variegatum* ticks were identified as the most abundant tick (79.6%) in the study area see Chapter 3. *E. ruminantium* is the causative agent of heartwater in both cattle and small ruminants in SSA and the Caribbean, as well as several islands of the Indian Ocean including Madagascar (Provost & Bezuidenhout, 1987), and it is known to be widespread in Nigeria (Ilemobade & Leeflang, 1977). *E. ruminantium* is believed to be responsible for numerous deaths occurring throughout the year, especially during the rainy season from March to September in much of sub-Saharan African countries (Bekker et al., 2001). In Southern Africa, the disease is mainly seen in areas where regularly dipped taurine breed animals are reared in close proximity to indigenous livestock, kept with no acaricidal treatment and also where wild ruminants are frequently seen in the grazing areas (Allsopp, 2010). The animals sampled in this study are not dipped in any acaricidal bath and no wild ruminants have been spotted in the grazing areas. The low apparent prevalence detected here might be attributed to the biology of *E. ruminantium*, as it mainly resides in endothelial cells and is only

periodically found in the bloodstream (Andrew & Norval, 1989; Lorusso et al., 2016). One may also speculate that the RLB hybridization assay may not have been sensitive enough to detect *E. ruminantium* infections if the rickettsiaemia was very low. It is advised that the samples also would be subjected to the *E. ruminantium*-specific pCS20 real-time PCR assay (Steyn et al., 2008) to determine more accurately the *E. ruminantium* prevalence in cattle in Nigeria.

This study further confirms the presence of *A. platys* in cattle in Nigeria. Previous studies have reported the presence of this organism in cattle in North Central Nigeria (Lorusso et al., 2016). The sequence data in this study were 99-100% similar to the *A. platys* pathogen previously reported in China and Nigeria GenBank accession no. KU586124.1 and KJ832067, respectively. At present no information is available on the pathogenicity of *A. platys* in cattle, causing a syndrome in dog known as canine cyclic thrombocytopenia (Woody & Hoskins, 1991). *A. platys* has regularly been found in ticks, usually, those of the *Rhipicephalus* genus in Africa (Marié et al., 2009). The present results suggest that cattle likely represent an alternative host for *A. platys* and further studies are required to ascertain the pathogenicity of this organism in cattle and which tick species are involved in its transmission to vertebrate hosts in Nigeria.

4.6.4 Prevalence of *Bartonella* species in cattle

This is the first study investigating the prevalence of *Bartonella* infection in cattle in Nigeria and provides the first molecular evidence of *Bartonella bovis* in the country. Thus far, the presence of *Bartonella* infection in the country has been reported in commensal rodents and ectoparasites (*Xenopsylla cheopis*, *Haemolaelaps* spp., *Ctenophthalmus* spp., *Hemimerus talpoides*, and *Rhipicephalus sanguineus*) (Kamani et al., 2013). Our finding suggests that cattle are a possible reservoir of *Bartonella* infection. The first report of infection of cattle with a *Bartonella* organism was made in 1934 by (Donatien and Lestoquard), who proposed the name '*B. bovis*' or '*Haemobartonella bovis*' to the detected microorganism (Piémont et al., 2002). This organism was then reported in Poland where the proportion of infected domestic cattle was 6.8% (Welc-Faleciak & Grono, 2013). In France, a higher prevalence was reported at an estimated prevalence of 59% (Maillard et al., 2006). In Spain, *Bartonella chomelii* is considered the most frequent species infecting cattle, as compared to *B. bovis* (Antequera-Gomez et al., 2015), whereas another investigation of *B. bovis* in cattle from five countries around the world reported a prevalence of 57.2% in Georgia, 20.6% in Guatemala, 10% in Thailand, and 0% in Japan and Kenya (Bai et al., 2013). The difference in infection

rates reported from different regions of the world may be related to the occurrence and abundance of the vectors implicated in the transmission of these pathogens (probably *Hippobscidae* flies), and the impact of intensive livestock production in the prevalence/intensity of infection in cattle (Antequera-Gomez et al., 2015). This study confirms the presence of *Ba. bovis* in cattle in Nigeria; following characterization, isolates were found 99-100% similar to sequence data deposited in Genbank. Further studies are needed to clarify the pathogenic and clinical importance of *Bartonella* species in cattle and to identify the vectors as well as the potential susceptibility of humans and other animals and livestock species.

4.6.5 The association with coinfection pattern in cattle

Coinfections have rather significant implications; it is assumed that they can be a cause of polymorphism of symptoms, more severe course of the disease or difficulties in the treatment and resistance to treatment. The results of the present study illustrate that coinfection by several tick-borne pathogens is very common occurrence in cattle in Nigeria. The findings highlight that infection with *A. marginale, E.* spp. Omatjenne, *A. centrale, B. bigemina, B. bovis, T. equi, T. mutans, T. velifera, T.* spp. MSD4 appear to be the most probable infections in cattle. Potential vectors of these TBIs have been reported in this thesis (Chapter 2) and elsewhere for different parts of Nigeria (Lorusso et al., 2013). Coinfection of *A. centrale* with *A. marginale* is not surprising as both species may share the same tick vectors (*Boophilus* spp.), which is distinct from the ticks that transmit *E.* spp. Omatjenne. (*Hyalomma* spp.).

The *Theileria* species found in Nigeria (*T. mutans* and *T. velifera*) are both vectored by *A. variegatum* (Horak et al., 2011). *Theileria* parasites primarily utilize white blood cells, transferring to red blood cells, after the merogony, only for the uptake by the tick vector. Resource-based interactions between *Theileria* and *Anaplasma* species are thus expected to be absent or weakly negative. Immunological interactions between *Anaplasma* species should be also strongly negative, due to possible cross-immunity between these potentially antigenically similar congeneric parasites. On the other hand, *Theileria* is not closely related to *Anaplasma*, and cross-immunity in the sense of the host immune system recognizing similar surface markers on both taxa is unlikely. Weak cross-immunity, if any, is thus expected only as resulting from non-specific activation of inflammatory responses required to curb infection by any intracellular parasite (Th1 responses), including both *Theileria* and *Anaplasma* (McKeever 2009; Aubry & Geale 2011). However, because *Theileria* (ECF)

utilizes and destroy the host's immune effector cells (lymphocytes), they can have significant immunosuppressive effects (McKeever 2009) and might thus facilitate colonization and population growth of *Anaplasma*.

The outcome of coinfection of *B. bigemina* with *A. marginale* and *B. bovis* can be more severe than a single infection alone, in light of the pathogenicity of each of these pathogens (Bilgiç et al., 2013). Similarly, coinfection with *A. marginale and T. velifera* may be associated with negative prognosis and economic losses (Hailemariam et al., 2017).

4.7 Conclusion

This study shows a very high prevalence of tick-borne pathogens close to 85% in the study area, with coinfections being more common than single infections. This might have implications for potential interactions of pathogens and the patterns of clinical symptoms. Future studies aiming to better understand the pathogenicity of *Ehrlichia* spp. Omatjenne, *A. platys, A. centrale, B. caballi, Theileria* spp. MSD4, *T. equi*-like and *Bartonella* species are also recommended. With special regards to the *Bartonella* spp. detected, the significance to animal health, zoonotic potential and vector capacity need to be further investigated.
On the whole, the epidemiological data from this study provided significant information on tick-borne diseases in the study area and will serve as a scientific basis for planning future control strategies.

Chapter 5: Molecular Epidemiology of Tick-Borne Haemoparasites in Sheep and Goats in KGR, Nigeria

5.1 Introduction

Small ruminants represent an important source of good quality animal protein in many developing countries of Africa including Nigeria (Unigwe et al., 2016). The small ruminants which include sheep and goats represent an important segment of the livestock system in Nigeria (Ademola & Onyiche, 2013). They form an important source of income, meat and dairy products in urban and rural areas in Nigeria (Nwosu et al., 2007). Apart from being the source of animal protein, their wastes are also very important organic manure in agriculture (Nwosu et al., 2007). These animals are also used for sacrifices during religious worships, traditional ceremonies and cultural festivals in most parts of Nigeria (Elele et al.,2013). Nigeria has a population of about 34.5 million goats and 8 to 13.2 million sheep out of which about 3.4 million are found in the southern/humid region and between the larger proportion of the animal in the Northern region of the country (Bourn et al., 1994; Lawal-Adebowale, 2012). Generally, three breeds of goats (Sahel, Sokoto red and West African dwarf) and four breeds of sheep (Balami, Ouda, Yankasa and West African dwarf) are recognized in the country (Blench, 1999). Out of these four major indigenous breeds of sheep in the country, the WAD breed are most commonly found in the southern region while the Balami, Uda and Yakansa breed are in the Northern region of the country (Lawal-Adebowale, 2012). Presently, there is either little or no attention given to these animals in the rural areas of the country where most of the sheep and goats are reared in large flocks (Omoike, 2014). In general, sheep husbandry is inadequately managed in Nigeria's agricultural sector owing to the fact that large flocks of these animals are mostly owned and managed by the Fulani pastoralists either on free-range nomadic extensive or semi-intensive system. Under the free-range nomadic system, sheep and goats move about freely even with other ruminants to feed on forages, pastures, hay, grasses and farm produce left over, which are usually available on grazing fields during the rainy and dry seasons. Though, this management system is basically influenced by cheap means of feeding all year round (Omoike, 2014). Unfortunately, this type of rearing system, on the other hand, predisposed the animals to vectors of infectious diseases and parasites (Lawal-Adebowale, 2012). High prevalence of diseases and parasites has been reported as a major constraint, causing high mortalities and preventing these animals to express their full genetic potential which is generally considered low (Akande et al., 2010; Mirkena et al., 2010). Haemoparasitic diseases in sheep and goats caused by the various haemoparasites is widespread in some parts of Nigeria and they occur in the Northern, Southern and Western regions of the country (Thornton, 2010; Jatau et al., 2011; Adamu & Balarabe 2012; Ukwueze & Kalu, 2015; Opara et al., 2016). Haemoparasitic disease

especially Babesiosis, Anaplasmosis, Theileriosis and Trypanosomosis are considered as major impediments to ruminant production including sheep and goats (Lako et al., 2007; Useh et al., 2011). They have generally been shown to cause destruction of red blood cells resulting in anaemia, anorexia, high morbidity and mortality, infertility, jaundice and weight loss (Akande et al., 2010; Ademola & Onyiche, 2013; Opara et al., 2016; Sharifi et al., 2016). Anaemia has been reported as a reliable indicator for the severity of haemoparasitic infections (Adejinmi et al., 2004; Opara et al., 2016). Despite several reported incidence rates of haemoparasitic infections in Nigeria, infection with haemoparasites persists as the major challenge to livestock production (Okaiyeto et al., 2008; Jatau et al., 2011; Onoja et al., 2013; Gebrekidan et al., 2014; Ukwueze & Kalu, 2015; Anyanwu et al., 2016; Opara et al., 2016). A proper understanding of the epidemiology of haemoparasitic diseases is a prerequisite to having a rational design for the effective control and preventive strategies against these dreadful diseases (Opara et al., 2016). Several in-depth studies on the prevalence of these infections have been conducted in various parts of Sub-Saharan Africa, including Nigeria. Despite these economic significant consequences of haemoparasites to ruminants, the prevalence and magnitude of haemoparasites infection in sheep and goats have not been assessed in grazing reserves in Nigeria. Therefore, information on prevalence, distribution, and potential risk factors of haemoparasites of sheep and goats is significant because the outcome could be used to make objective decisions on control strategies. The finding would also be useful in formulating strategies to meet the current shortage of protein from animal products created by the rapidly increasing human population. The present study was designed to use RLB assay for simultaneous detection and identification of multiple pathogens *Ehrlichia/Anaplasma*, *Theileria/Babesia*, *Rickettsia* and *Bartonella* spp. in sheep and goat blood samples from KGR in order to develop baselines about the presence of haemoparasites in the local population.

5.2 Materials and Method

5.2.1 Sample collection

Blood samples and field data were collected as described in Chapter 2 section 2.5.

5.2.2 DNA extraction and elution

For each of the blood samples spotted on FTA® cards, five discs of 3 mm of diameter were punched using a Harris Micro-Punch™ (Whatman BioScience Ltd, Cambridge, UK) and placed into a 1.5 ml Eppendorf tube (see detailed description in Chapter 2 section 2.1).

5.2.3 Reverse line blotting (RLB)

5.2.3.1 PCR for haemoparasites

The RLB used was made up of 4 group-specific PCRs then probes to delineate species within each group. Following DNA extraction and elution, each sample was subjected to four simultaneous PCRs targeting, respectively:

(i) a 460–520 bp long fragment from the V1 hypervariable region of the 16S SSU rRNA gene for *Ehrlichia* and *Anaplasma* spp. (Schouls et al., 1999; Bekker et al., 2002),
(ii) A 460–540 bp long fragment from the V4 hypervariable region of the 18S ribosomal RNA (rRNA) gene for *Theileria* and *Babesia* spp. (Georges et al., 2001),
(iii) a 350–400 bp variable region in the 16S rRNA gene for *Rickettsia* spp. (Christova et al., 2003),
(iv) and a 296 bp long fragment of 16S rRNA gene for *Bartonella* (see a detailed description of primers in (Chapter 2 section 2.2.1).

All DNA amplifications were carried out using the same cycling conditions, as described in (Table 2:3). The outcome of PCR was determined following the electrophoretic run of the positive and negative controls as described in (Chapter 2 section 2.1).

5.2.3.2 RLB hybridization of PCR products

Hybridization of PCR products to catch-all and species-specific probes was performed as (see detailed description in (Chapter 2 section 2.2.3)

5.2.3.3 X-ray film development

After the series of washing steps illustrated above, 5ml of solutions ECL1 and ECL2 (Amersham, UK) were spread sequentially over the membrane, which was then incubated at room temperature for a minimum of 1 minute (see detailed description in Chapter 2 section 2.2.4).

5.2.4 PCR product purification and sequencing

PCR products were purified using an Isolate II PCR and gel kit according to the manufacturer's instructions (Qiagen) (see detailed description in Chapter 2 section 2.3).

5.3 Epidemiological statistical analysis

5.3.1 Univariant analysis

Field data and results generated in the laboratory were entered and managed in Microsoft-excel. SPSS version 20.0 software program was initially employed for the data analysis as described in (Chapter 2 section 2.5.1).

5.4 Results in sheep

5.4.1 Overall infection rates in sheep

RLB was successfully completed on DNA extracts prepared from blood collected from all 257 sheep surveyed. Of these animals, only 79 (30.7%) were not found to be infected with a tick-borne parasite/pathogen. Thus, the vast majority (69.3%) of sheep tested were infected with at least one tick-borne parasite. The prevalence of infection varied between KGR blocks Chi-squared tests revealed that the only significant associations between haemoparasites prevalence and blocks ($p < 0.05$) were between the *A. centrale* ($\chi2 = 69.38$, P = 0.000), *E.* spp. Omatjenne ($\chi2 = 22.70$, P = 0.000), *B. bovis* ($\chi2 = 25.71$, P = 0.000), *T. equi*-like ($\chi2 = 22.80$, P = 0.000), *T. mutans* ($\chi2 = 24.76$, P = 0.000), *T. velifera* ($\chi2 = 23.24$, P = 0.000), *T.* spp. MSD4 ($\chi2 = 15.63$, P = 0.008), *Rickettsia* species ($\chi2 = 41.65$, P = 0.000) with the parasites being more prevalent in block 2 and 3 (73.8%, and 91.1%, respectively) than elsewhere see Table 5:1. The prevalence of infection was greater in older animals than younger animals, but this difference was not statistically significant ($\chi2 = 2.00$, P = 0.156) (Table 5:1). On the whole, sex-related haemoparasitaemia did not vary significantly ($\chi2 = 2.444$, P = 0.117) in the study, however, parasitaemia was higher in female (142/198, 71.7%) than males (36/59, 61.0%).

Table 5:1: Proportion of infected animals within the sampled population

Blocks	Infected Animals/Animals sampled (%)		
	Young	Adults	Total
Block 1	9/18 (50)	24/39 (61.5)	33/57 (57.8)
Block 2	10/16 (62.5)	21/26 (80.7)	31/42 (73.8)
Block 3	21/22 (95.4)	41/46 (89.1)	62/68 (91.1)
Block 4	0/0 (0.0)	3/6 (50.0)	3/6 (50.0)
Block 5	30/51 (58.8)	17/28 (60.7)	47/79 (59.4)
Block 6	1/3 (33.3)	1/2 (50.0)	2/5 (40.0)
Total	71/110 (64.5)	107/147 (72.7)	178/257 (69.3)

5.4.2 Prevalence of tick-borne haemoparasites prevalence in sheep

The prevalence of single and mixed-species infections is summarised in (Table 5:2). In all, 12 different species of haemoparasites were detected (Table 5:2). In addition, (11) *Ehrlichia/Anaplasma,* (18) *Theileria*, (51) *Rickettsia* and (2) *Bartonella* samples produced a signal only with the group-specific probes, and not with the corresponding species-specific probes. These could not be assigned to a particular species. Table 5:2 shows the number of sheep which were positive for each of the haemoparasites. The most prevalent species were *Theileria equi*-like (109/257, 42.4%) and *Rickettsia* species (251/257, 19.8%), respectively.

Table 5:2: Prevalence of tick-borne haemoparasites in sheep

Microorganism	**Prevalence (%)**	**Total No.=257**	**Confidence Interval Lower - Upper bound (%)**
		No. infected	
Theileria equi-like	42.4	109	36.3 – 48.7
Rickettsia species	19.8	51	15.1 – 25.3
Anaplasma centrale	17.5	45	13.1 – 22.7
Theileria velifera	12.1	31	8.3 – 16.7
Ehrlichia spp. Omatjenne	10.1	26	6.7 – 14.5
Theileria mutans	8.9	23	5.8 – 13.1
Theileria spp. MSD4	7.0	18	4.2 – 10.8
Babesia bovis	2.7	7	1.1 – 5.5
Bartonella species	0.8	2	0.1 – 2.8
Babesia caballi	0.4	1	0 – 2.1
Ehrlichia ruminantium	0.4	1	0 – 2.1
Typhus group	0.4	1	0 – 2.1

5.4.3 Confirmation of amplicon identity in sheep

Unambiguous sequence data (460 – 520 bp) were obtained from *Ehrlichia/Anaplasma* catch-all only positive (n =11) samples all (n=11) were found 98-100% similar with *Anaplasma ovis* (Accession No. KU569700.1, KF293705.1, KX579073.1). Furthermore, (460–540 bp) sequence data for *Theileria* catch-all only positive (n= 18) all (n =18) were found 98-100% similar with *Theileria ovis* with (Accession No. JN412660.1, KX671114.1, KX273858.1, JN412664.1, KJ832065.1, KJ832064.1). In the same way, (296 bp) sequence data for *Bartonella* catch-all only positive (n= 2) all (n= 2) were found 98-100% similar *Bartonella* spp. (Accession No. CP019781.1).

5.4.4 Age-wise prevalence of tick-borne infections in sheep

The prevalence of infection of specific pathogens differed between young and older animals (Table 5:3). Out of 257 animals studied for one or more heamoparasite infections, 110 (42.8%) were young while 147 (57.1%) were adults. Infection prevalence was significantly higher in older animals than younger animals with regards *T. velifera* (χ^2=5.8, p=0.015), *E.* spp. Omatjenne (χ^2=6.5, p=0.010). No infection was observed in young *Bartonella species* and *E. ruminantium* likewise in adult *B. caballi* and Typhus group (Table 5:3).

Table 5:3: Age-wise prevalence of tick-borne infection in sheep

Microorganism	Prevalence of infection (%)		Chi-Square Tests
	Young	Adults	
Theileria equi-like	41 (37.2)	68 (46.2)	χ^2=2.0, p=0.149
Rickettsia species	20 (18.1)	31 (21.0)	χ^2=0.3, p=0.563
Anaplasma centrale	17 (15.4)	28 (19.0)	χ^2=5.6, p=0.453
Theileria velifera	7 (6.3)	24 (16.3)	χ^2=5.8, p=0.015
Ehrlichia spp. Omatjenne	5 (4.5)	21 (14.2)	χ^2=6.5, p=0.010
Theileria mutans	7 (6.3)	16 (10.8)	χ^2=1.5, p=0.209
Theileria spp. MSD4	6 (5.4)	12 (8.1)	χ^2=0.7, p=0.400
Babesia bovis	3 (2.7)	4 (2.7)	χ^2=0.0, p=0.998

Bartonella species	0 (0)	2 (1.3)	n/a
Babesia caballi	1 (0.9)	0 (0)	n/a
Ehrlichia ruminantium	0 (0)	1 (0.6)	n/a
Typhus group	1 (0.9)	0 (0)	n/a

n/a = not applicable

5.4.5 Sex-wise prevalence of tick-borne infections in sheep

The prevalence of infection of specific pathogens also varied between male and female animals. Out of 257 animals studied for one or more heamoparasite infections, 198 (77.0%) were females while 59 (22.9%) were males. In particular, no significant (P>0.05) difference was observed in all the species studied. Furthermore, no infection is observed in male *E. ruminantium* and *Typhus* group likewise *B. caballi* in female (Table 5:4).

Table 5:4: Sex-wise prevalence of tick-borne infection in sheep

Microorganism	Prevalence of infection (%)		Chi-Square Tests
	Males	Females	
Theileria equi-like	21(35.5)	88 (44.4)	χ^2=1.4, p=0.227
Rickettsia species	10 (16.9)	41 (20.7)	χ^2=0.4, p=0.525
Anaplasma centrale	9 (15.2)	36 (18.1)	χ^2=0.2, p=0.604
Theileria velifera	7 (11.8)	24 (12.1)	χ^2=0.0, p=0.958
Ehrlichia spp. Omatjenne	2 (3.3)	24 (12.1)	χ^2=3.8, p=0.051
Theileria mutans	3 (5.0)	20 (10.1)	χ^2=1.4, p=0.236
Theileria spp. MSD4	4 (6.7)	14 (7.0)	χ^2=0.0, p=0.939
Bartonella species	0 (0)	2 (1.0)	n/a
Babesia bovis	1 (1.6)	6 (3.0)	χ^2=0.3, p=0.580
Babesia caballi	1 (1.6)	0 (0)	n/a

Ehrlichia ruminantium	0 (0)	1 (0.50)	χ^2=0.2, p=0.584
Typhus group	0 (0)	1 (0.50)	n/a

n/a = not applicable

5.4.6 Single infections

78 single infections were detected, amongst 178 positive microorganisms (78/178, 43.8 %), of which 34 were in young sheep (34/78, 43.5 % of positive animals) and 44 in adult sheep (44/78, 56.4 % of positive animals). Cases of single infections were mostly represented by *T. equi*-like (*n* = 23), *Theileria* spp. (*n* = 18), *Rickettsia* spp. (*n* = 11), *Ehrlichia/Anaplasma* spp. (*n* = 9), *A. centrale* (*n* = 7), *T. mutans* (*n* = 4), *B. bovis* (*n* = 3), *E.* spp. Omatjenne (*n* = 2) and *T. velifera* (*n* = 1).

5.4.7 Coinfections

101/257 (39.2 %) sheep were positive for two or more microorganisms simultaneously. Overall 36 different combinations of microorganisms were found. The largest variety of coinfections was recorded in (*A. centrale* + *B. caballi* + *T. equi*-like + *T. mutans* + *T. velifera* + *T.* sp MSD4 + *Rickettsia* spp). On the whole, the most frequent combinations included two (i.e. *T. equi*-like + *Rickettsia*) to three co-infective species (i.e. *A. centrale* + *T. equi*-like + *Rickettsia* species). Table 5:5 reports only the 12 most frequent coinfections. For a more detailed overview, the reader is referred to the extensive format of the table in the Appendix Section (Table 5:12). Out of 101 sheep that were positive for two or more microorganisms the largest variety of coinfection differed with age and adult's animals were morelikely to be infected than (63; 63%) than young (37; 37%) thus, no significant difference recorded ($\chi2$=2.2, p=0.133). While sex recorded (19; 18.8%) in males and (80; 79.2%) in females with no significant difference was observed ($\chi2$=1.23 p=0.267).

Table 5:5: Twelve most frequent multiple infections by tick-borne haemoparasites according to sex, age classes and overall number of sheep.

(*Ac* = *Anaplasma centrale*; *Ao* = *Anaplasma ovis*; *E*spO = *Ehrlichia* spp. Omatjenne; *Er* = *Ehrlichia ruminantium*; *R* = *Rickettsia* spp.; *B* = *Bartonella*; *Bbov* = *Babesia bovis*; *Tel* = *Theileria equi*-like; *Tg* = Typhus group *Tm* = *Theileria mutans*; *To* = *Theileria ovis;* *T*spMSD4 = *Theileria* sp MSD4; *Tv* =*Theileria velifera*).

	Tick-borne haemoparasites species combinations	Frequency				Totals
		Males	Females	Young	Adults	
1	*Tel* + *R*	3	13	8	8	16
2	*Ac* + *Tel*	5	6	4	7	11
3	*Ac* + *Tel* + *R*	2	9	6	5	11
4	*Tel* + *Tv* + *T*spMSD4	3	6	2	6	9
5	*E*spO + *Tel* + *Tm* + *Tv*	3	5	1	7	8
6	*Ac* + *E*spO + *Tel*	-	5	2	3	5
7	*E*spO + *Tel*	-	4	1	3	4
8	*Tel* + *T*spMSD4	-	4	1	3	4
9	*Ac* + *Tel* + *Tm*	-	2	1	1	2
10	*Ac* + *To*	-	2	1	1	2
11	*Ao* + *To*	1	1	1	1	2
12	*E*spO + *To*	-	2	-	2	2

5.5 Results in goats

5.5.1 Overall infection rates in goats

RLB was successfully completed on DNA extracts prepared from blood collected from all 196 goats surveyed. Of these animals, 146 (74.4%) were not found to be infected with a tick-borne parasite/pathogen. Thus, only (25.5%) of goats tested were infected with at least one tick-borne parasite. The prevalence of infection varied between KGR blocks Chi-squared tests revealed that the only significant associations between haemoparasites prevalence and blocks ($p < 0.05$) were between the *A. centrale* ($\chi2 = 12.75$, P = 0.001), *A. marginale* ($\chi2 = 65.20$, P = 0.000), *B. bigemina* ($\chi2 = 42.93$, P = 0.000), *B. bovis* ($\chi2 = 55.55$, P = 0.000), *B. caballi* ($\chi2 = 14.48$, P = 0.006), *T. equi*-like ($\chi2 = 153.7$, P = 0.000), *T. mutans* ($\chi2 = 161.7$, P = 0.000), *T. velifera* ($\chi2 = 161.7$, P = 0.000), *T.* spp. MSD4 ($\chi2 = 161.7$, P = 0.000), *Rickettsia* species ($\chi2 = 30.87$, P = 0.000) with the parasites being more prevalent in block 1 and 3 (96%, and 50%, respectively) than elsewhere. The prevalence of infection was greater in older animals than younger animals, a significant difference was observed ($\chi2 = 3.89$, P = 0.048) (Table 5:6). On the whole, sex-related haemoparasitameia did not vary significantly ($\chi2 = 1.91$, P = 0.1668) in the study, however, parasitaemia was higher in female (48/170, 28.2%) than males (4/26, 15.3%).

Table 5:6: Proportion of infected animals within the sampled population

Blocks	Infected Animals/Animals sampled (%)		
	Young	Adults	Total
Block 1	3/3 (100)	21/22 (95.4)	24/25 (96)
Block 2	1/5 (20)	1/10 (10)	2/15 (13.3)
Block 3	5/10 (50)	0/0 (0)	5/10 (50)
Block 4	1/3 (33.3)	1/11 (9.0)	2/14 (14.2)
Block 5	0/0 (0)	0/0 (0)	0/0 (0)
Block 6	4/57 (7.0)	13/75 (17.3)	17/132 (12.8)
Total	14/78 (17.9)	36/118 (30.5)	50/196 (25.5)

5.5.2 Prevalence of tick-borne haemoparasites in goats

The prevalence of single and mixed-species infections is summarised in (Table 5:7). In all, 12 different species of haemoparasites were detected (Table 5:7). In addition, (1) *Ehrlichia/Anaplasma,* (1) *Theileria*, (6) *Rickettsia* and (2) *Bartonella* samples produced a signal only with the group-specific probes, and not with the corresponding species-specific probes. These could not be assigned to a particular species. Table 5:7 shows the number of cattle which were positive for each of the haemoparasites. The most prevalent species were *Ehrlichia/Anaplasma* (31/196, 15.8%), *Theileria* (26/196, 13.2%), and *Babesia* (11/196, 5.6%) respectively.

Table 5:7: Prevalence of each tick-borne haemoparasites in goats

Microorganism	**Prevalence (%)**	**Total No.=196**	**Confidence Interval Lower – Upper bound (%)**
		No. infected	
Theileria equi-like	14.3	28	9.7 – 20.0
Anaplasma centrale	13.3	26	8.9 – 18.8
Theileria mutans	13.3	26	8.9 – 18.8
Theileria velifera	13.3	26	8.9 – 18.8
Theileria spp. MSD4	13.3	26	8.9 – 18.8
Anaplasma marginale	6.6	13	3.6 – 11.1
Babesia bovis	5.6	11	2.8 – 9.8
Rickettsia species	3.1	6	1.1 – 6.5
Babesia bigemina	2	4	0.6 – 5.1
Ehrlichia spp. Omatjenne	1.5	3	0.3 – 4.4
Bartonella species	1	2	0.1 – 3.6
Babesia caballi	1	2	0.1 – 3.6

5.5.3 Confirmation of amplicon identity in goats

Unambiguous sequence data (460 – 520 bp) were obtained from *Ehrlichia*/*Anaplasma* catch-all only positive (n =1) samples were found 99-100% similar to *Anaplasma ovis* (Accession No. KU569700.1). In the same way, (296 bp) sequence data for *Bartonella* catch-all only positive (n=2) sample all (n =2) was found 98-100% similar with *Bartonella* spp. (Accession No. CP014012.1).

5.5.4 Age-wise prevalence of tick-borne infections in goats

The prevalence of infection of specific pathogens differed between young and older animals (Table 5:8). Out of 196 animals studied for one or more haemoparasite infections, 78 (39.8%) were young while 118 (60.2%) were adults. Young animals are no more significantly likely to have TBIs than older animals (Table 5:8).

Table 5:8: Age-wise prevalence of tick-borne in goats

Microorganism	**Prevalence of infection (%)**		**Chi-Square Tests**
	Young	**Adults**	
Theileria equi-like	8 (10.2)	20 (16.9)	χ^2=1.71, p=0.190
Anaplasma centrale	7 (8.9)	19 (16.1)	χ^2=2.07, p=0.150
Theileria mutans	6 (7.6)	20 (16.9)	χ^2=3.49, p=0.061
Theileria velifera	6 (7.6)	20 (16.9)	χ^2=3.49, p=0.061
Theileria spp. MSD4	6 (7.6)	20 (16.9)	χ^2=3.49, p=0.061
Anaplasma marginale	4 (5.1)	9 (7.6)	χ^2=0.47, p=0.491
Babesia bovis	4 (5.1)	7 (5.9)	χ^2=0.05, p=0.811
Rickettsia species	1 (1.2)	5 (4.2)	χ^2=1.38, p=0.240
Babesia bigemina	3 (3.8)	1 (0.8)	χ^2=2.11, p=0.146
Ehrlichia spp. Omatjenne	1 (1.2)	2 (1.6)	χ^2=0.05, p=0.818
Bartonella species	1 (1.2)	1 (0.8)	χ^2=0.08, p=0.767
Babesia caballi	1 (1.2)	1 (0.8)	χ^2=0.08, p=0.767

5.5.5 Sex-wise prevalence of tick-borne infections in goats

The prevalence of infection of specific pathogens also varied between male and female animals. Out of 196 animals studied for one or more haemoparasite infections, 170 (86.7%) were females while 26 (13.2%) were males. No significant (P>0.05) difference was observed in all the pathogens detected. Furthermore, no infection is observed in male *A. centrale* and *Bartonella* species (Table 5:9).

Table 5:9: Sex-wise prevalence of tick-borne infection in goats

Microorganism	Prevalence of infection (%)		Chi-Square Tests
	Male	Female	
Theileria equi-like	4 (15.3)	24 (14.1)	χ^2=0.03, p=0.863
Anaplasma centrale	0 (0)	26 (15.2)	n/a
Theileria mutans	2 (7.6)	11 (6.4)	χ^2=0.809, p=0.368
Theileria velifera	2 (7.6)	24 (14.1)	χ^2=0.809, p=0.368
Theileria spp. MSD4	2 (7.6)	24 (14.1)	χ^2=0.809, p=0.368
Anaplasma marginale	2 (7.6)	11 (6.4)	χ^2=0.05, p=0.816
Babesia bovis	1 (3.8)	10 (5.8)	χ^2=0.17, p=0.674
Rickettsia species	1 (3.8)	5 (2.9)	χ^2=0.06, p=0.803
Babesia bigemina	1 (3.8)	3 (1.7)	χ^2=0.48, p=0.485
Ehrlichia spp. Omatjenne	1 (3.8)	2 (1.1)	χ^2=1.06, p=0.302
Bartonella species	0 (0)	2 (1.1)	n/a
Babesia caballi	1 (3.8)	1 (3.8)	χ^2=2.3, p=0.124

n/a = not applicable

5.5.6 Single infections

23 single infections were detected, amongst 50 positive cases (23/50, 46 %), of which 7 were in young goats (7/23, 30.4 % of positive animals) and 16 in adult goats (16/23, 69.5 % of positive animals). Cases of single infections were mostly represented by *A. centrale* (*n* = 15), *Bartonella* species (*n*= 2), *E*. spp. Omatjenne (*n* = 2), *T. equi-like* (*n* = 2), *A. ovis* (*n* = 1).

5.5.7 Coinfections

28/196 (14.2 %) goats were positive for two or more microorganisms simultaneously. Overall 14 different combinations of microorganisms were found. The largest variety of coinfections was recorded in (*A. marginale* + *E.* spp. Omatjenne + *B. bigemina* + *B. bovis* + *B. caballi* + *T. equi*-like + *T. mutans* + *T. velifera* + *T.* spp. MSD4 + *Rickettsia* species). On the whole, the most frequent combinations included (i.e *T. equi*-like + *T. mutans* + *T. velifera* + *T.* spp. MSD4). Table 5:10 reports only the 8 most frequent coinfections. For a more detailed overview, the reader is referred to the extensive format of the table in the Appendix Section (Table 5.13). Out of 28 goats that were positive for two or more microorganisms the largest variety of coinfection differed between young 8 (28.5%) and adult's animals 20 (71.4%). Coinfection prevalence was significantly higher in adults' animals than younger animals thus no significant difference recorded (χ^2=1.7, p=0.189). while sex recorded 3 (10.7%) in males and 25 (89.2%) in females. Though, no significant difference was observed between sex (χ^2=0.18 p=0.667).

Table 5:10: Seven most frequent multiple infections by tick-borne haemoparasites according to sex, age classes and overall number of goats

(*Ac = Anaplasma centrale*; *Am = Anaplasma marginale; E*spO = *Ehrlichia* spp. Omatjenne; *R = Rickettsia* spp.; *B = Bartonella*; *Bb = Babesia bigemina; Bc = Babesia caballi; Bbov = Babesia bovis*; *Tel = Theileria equi*-like; *Tm = Theileria mutans*; *T*spMSD4 = *Theileria* spp. MSD4; *Tv =Theileria velifera*).

	Tick-borne haemoparasites species combinations	Frequency				Totals
		Males	Females	Young	Adults	
1	*Tel* + *Tm* + *Tv* + *T*spMSD4	-	7	2	5	7
2	*Am* + *Bbo* + *Tel* + *Tm* + *Tv* + *T*spMSD4	-	3	-	3	3
3	*Ac* + *Am* + *Bb* + *Bbo* + *Tel* + *Tm* + *Tv* + *T*spMSD4	-	2	-	2	2
4	*Ac* + *Am* + *Tel* + *Tm* + *Tv* + *T*spMSD4	-	2	-	2	2
5	*Ac* + *Am* + *Tel* + *Tm* + *Tv* + *T*spMSD4 + *R*	-	2	-	2	2
6	*Am* + *Tel* + *Tm* + *Tv* + *T*spMSD4	1	-	1	1	2
7	*Tel* + *Tm* + *Tv* + *T*spMSD4 + *R*	-	2	-	2	2

5.6 Discussion

5.6.1 Prevalence of tick-borne haemoparasites detected in sheep and goats

Haemoparasitic infections are considered as major constraints to farm animal production in Nigeria which significantly affect their productivity (Opara et al., 2016). Therefore, a survey on the prevalence of haemoparasites and their economic significance is important to the livestock industry especially the small ruminants in developing countries including Nigeria (Ademola & Onyiche, 2013; Opara et al., 2016). In the present study, a comprehensive investigation of the distribution and prevalence of haemoparasites in six Blocks of KGR in Kaduna State, North Western Nigeria, was undertaken. The finding of this present study revealed an overall prevalence rate, for any tick-borne infection, of 69.3% and 25.5% in sheep and goats, respectively. The present study confirms the reports of previous studies on the prevalence of tick-borne haemoparasites in sheep of 33.0% (Okaiyeto et al., 2008), 95.51% (Jatau et al., 2011), 21.0% (Adamu and Balarabe, 2012), 12.50% (Opara et al., 2016), 56.32% (Anyanwu et al., 2016) and in goats 57.6% (Ukwueze & Kalu, 2015), and 23.61% (Opara et al., 2016). This suggests a continuous challenge of the parasites and the existence of a carrier state in the country. Though, the reason for the differences in the reported prevalence rates might be due to the difference in the technique employed, climatic/geographical variation of sampling areas, the period of sample collections, sample size and breed variation among others.

The finding of the present study revealed that young sheep and goats were less infected when compared with adults. This finding agrees with those of Opara et al. (2016), and Bello et al., (2017) who also reported a significantly high level of haemoparasites infection rate in adult sheep compared to the adults. Previous studies have confirmed a higher level of haemoparasitamia in adult sheep reared under nomadism when compared to young sheep also reared under pastoralist transhumance (Ademola & Onyiche, 2013; Nwoha et al., 2014; Anyanwu et al., 2016). Similarly, in goats the infection rate was higher in adults when compared to young animals; this finding is also supported by the work of Anyanwu et al., (2016) and Opara et al., (2016). In KGR, young sheep and goats are closely more watched and cared for by the Fulani pastoralists (especially women and children) (Lorusso, personal communication) rather than the adults; this may probably reduce infestation rate with arthropod vectors such as ticks and the corresponding infections. In addition, this is in line with the concept of ‘inverse age immunity’, with colostral first, and then innate immunity, enabling the low likelihood of the appearance of clinical disease in young animals at the time

of their peak exposure to a pathogen (Jonsson et al., 2012; Lorusso et al., 2016). Importantly, the lower infections rates in sheep and goats could also be attributable to the significantly lower tick burden they bear, compared to adult as explained in Chapter 3.

The finding of the present study also revealed high haemoparasitaemia in female sheep and goats when compared to the male individuals, although this difference was not statistically significant (P>0.05). This result supported the previous finding of Opara et al. (2016), who have reported a high prevalence rate of haemoparasites in female sheep (21.40%) compared to male ones (5.60%). Our finding also corroborates the reports of Samdi et al. (2008), Ademola & Onyiche (2013) and Nwoha et al. (2014) who have also reported the more frequent occurrence of haemoparasitaemia in female sheep compared to their males. Similarly, in goats, the general infection rate was higher in the female when compared to male individuals. This result is also supported by the work of Samdi et al. (2008), who recorded a prevalence of 2.27% rate in females as against 1.60% males. Out of 257 sheep sampled 198 were females while 59 males while in goats 170 were females and 26 were males. Indeed, most of the Fulani pastoralists keep a larger number of female sheep and goats than males especially for the purposes of breeding while the males are usually sold out for cash, which may affect the proportion of the sex infection. It has also been reported that female ruminants are generally more prone to infection by haemoparasites due to their extended breeding for economic purposes such as parturition and milk production as well as the stress linked to breeding and milking, and cyclical hormonal changes associated with gestation, parturition and calving processes (Ukwueze & Kalu, 2015; Anyanwu et al., 2016). Accordingly, the susceptibility of females might be attributed to reduced immunity as a result of stress due to pregnancy and lactation.

5.6.1 Prevalence of *Theileria/Babesia* species in sheep and goats

Both infections by *T. mutans* and *T. velifera* were recorded in sheep (8.9% and 12.1%) and goats (13.3% and 13.3% respectively) and this finding expands the host range of this organism and warrants further investigation. These two non-pathogenic *Theileria* species have been long recognised to be present in Nigeria cattle (Saidu et al., 1984), and are known to be transmitted by *A. variegatum* (Uilenberg et al., 1974; Young et al., 1978), endemically present in the whole of Nigeria (Bayer & Maina, 1984) including KGR (see Chapter 3) and can result in mild clinical signs, but some pathogenic strains for example in Eastern Africa cause severe anaemia and sometimes death in cattle (Yusufmia et al., 2010).

In this present study, *T. equi*-like was recorded at a high prevalence in sheep, while goats had a lower prevalence as discussed in (Chapter 4 section 4.5.6) this microorganism is well recognized as the cause of equine piroplasmosis. Our finding that *T. equi*-like occurs in sheep and goats further expands the host range of this organism. The significance, extent, and consequences of infections with *T. equi*-like in domestic ruminants require further investigation.

In this study, *Theileria* spp. (strain MSD) was detected in both sheep and goats at a prevalence of (7.0% and 13.3% respectively). As discussed in (Chapter 4 section 4.6.2) no attempts have been made to clarify the identity of specie after its first description by Chae et al. (1999), the identification of similar result suggest that this genotype is circulating in some ruminants populations in KGR. Further studies are needed to clarify the identity and extent of the geographical distribution of this distinct *Theileria* spp. 'strain MSD'

In Nigeria, *Babesia bigemina* and *Babesia bovis* infections are recognized as an economic important pathogen in cattle. The tick vectors (*Rhipicephalus* species of the sub-genus *Boophilus*) responsible for transmitting the infection was the second most abundant tick identified from the study area (see Chapter 3). In the present study, *B. bovis* was recorded at a low prevalence in sheep and goats (2.7% and 5.6% respectively), while *B. bigemina* was not detected in sheep. However, in goats, it was recorded at very low prevalence of (2.0%). Interestingly, this study reports the presence of *B. bovis* DNA in sheep and goats although small ruminants are not a known host for these parasites. The significance, extent, and consequences of infections with *B. bovis* in small ruminants require further investigation. Similarly, the detection of *B. bigemina* in goats in this study is in line with the previous report recorded at a prevalence of (0.8%; 1/127) in goats from Vietnam (Sivakumar et al., 2013). Although the pathobiological significance of this host shifting is not known, it might be one of the survival strategies used by haemoprotozoan parasites.

This study reports the presence of *B. caballi* in Nigeria at a very low prevalence in sheep and goats (0.4% and 1%) respectively. The finding in this study is consistent with the detection of this pathogen in *A. variegatum* ticks that were collected off cattle in the same study area as reported in (Chapter 3). The pathogenicity of this organism is not known in domestic ruminants and during fieldwork donkeys where not encounter at the grazing reserve. This finding further expands the known host range of this infection. A more extended

epidemiological survey would be required to ascertain the role of ruminants as a reservoir of this apparent infection.

5.6.2 Prevalence of *Ehrlichia/Anaplasma* species in sheep and goats

This study provides the first molecular report of *A. marginale* in goats in Nigeria, while no infection with this pathogen was recorded in sheep. *Anaplasma* spp. antibodies have previously been identified in goats from North Eastern Brazil (Ramos et al., 2008) and Pakistan (Hussain et al., 2017). However, direct molecular detection of *A. marginale* in small ruminants has been only reported in sheep from Iran (Yousefi et al., 2017). *Rh.* (*Bo.*) *annulatus, Rh.* (*Bo.*) *decoloratus, Rh. evertsi evertsi, Rh. simus* ticks have been described as the main vector of *A. marginale* (Gueye et al., 1994; Katherine et al., 2010). Previous studies have suggested that *Amblyomma* ticks may be involved in the transmission of *A. marginale* (Silva et al., 2014; da Silva et al., 2015).

In this study, *Ehrlichia* spp. 'Omatjenne' recorded a very lower prevalence in sheep and goats (10.1% and 1.5% respectively). To the best of our knowledge, this is the first reported case in the country and the pathological role of this infection is still not well known (Allsopp et al., 1997). This organism has been reported to be genetically close to *E. ruminantium* and was initially isolated in *Hyalomma truncatum* ticks collected from apparently healthy cattle in Namibia (du Plessis, 1990). This tick species is likely prevalent in the study area as reported in Chapter 3 of this thesis. Initially thought to be apathogenic in cattle, studies have also shown the association of *Ehrlichia* spp. (Omatjenne) with 'heartwater' (cowdriosis)-like syndrome in sheep under experimental conditions (du Plessis, 1990). The detection of this organism in blood collected from sheep and goats suggests the presence of this rickettsia in the resident ruminant population. The previous detection of infection with this pathogen in dogs (Adamu et al., 2014) and cattle from Nigeria (Lorusso et al., 2016) confirms the occurrence of natural infection in Nigerian ruminants, as reported also in Chapter 4 of this thesis. The role of this taxon in causing disease in livestock needs to be further investigated.

This study confirms the presence of *A. centrale* in sheep and goats with a very high prevalence (17.5% and 13.3% respectively). Little is known about the epidemiology of this infection in small ruminants and cattle in Nigeria, as the disease has rarely been detected and examined on its own, due to the greater interest attracted by the more pathogenic and widely distributed *A. marginale*. Nonetheless, the presence of competent vectors *Rhipicephalus* spp.

in the study area as shown in Chapter 3, confirms the likelihood of this species to spread within the grazing reserve.

In the present study, only one (0.4%) sheep tested positive for *E. ruminantium* even though *A. variegatum* ticks were identified as the most abundant tick species from cattle in the study area (see Chapter 3). To the best of our knowledge, this is the first report on the molecular detection of *E. ruminantium* in sheep from North Western Nigeria. As discussed in (Chapter 4 Section 4.6.3) *E. ruminantium* is believed to be responsible for numerous deaths occurring throughout the year, especially during the rainy season from March to September in many sub-Saharan African countries (Bekker et al., 2001). The presence of this pathogen should be taken into account when attempting livestock improvement through the introduction of exotic breeds, and other native breeds in the area.

The result of the present study confirms the presence of *Anaplasma ovis* infection in sheep with no infection recorded in goats in Nigeria. The agent of ovine and goat anaplasmosis, *A. ovis* is associated with haemolytic anaemia; however, the course of the disease is usually subclinical (Alessandra & Santo, 2012). The severity of the infection is associated with cross infection with other parasitic diseases (Alessandra & Santo, 2012). The acute phase of the disease is characterized by fever, progressive anaemia, icterus, weight loss, milk yield decrease, and sometimes death (Ben Said et al., 2017). Sheep develop persistent infection which allows them to be reservoirs of infection and the transmission of the pathogen occurs to feeding ticks (Kocan et al., 2010). The persistent infection is one of the reasons for the high rate of infection by *A. ovis* in small ruminants in the study area. The sequence data in this study were found 98-100% similar to *A. ovis* reported with GenBank accession no. from Kenya KU569700.1, Italy KF293705.1, and China KX579073.1. Thus, the previous study reported a prevalence of (16.8%) in Red Sokoto goats slaughtered in Umuahia, Abia state Nigeria (Ukwueze and Kalu 2014). The authors attributed the low prevalence recorded to the regular use of chemoprophylaxis and acaricides by small scale farmers or the diagnostic method used, which was based on the identification of *A. ovis* on Giemsa stained blood smears. This method is less sensitive in the detection of carriers with low levels or no evidence of parasitaemia (Jalali et al., 2016; Nasreen et al., 2016). They suggested the prevalence of *A. ovis* in goats in the study area is likely much higher than that detected in this study (Atif et al., 2012; Jalali et al., 2016).

5.6.3 Prevalence of *Bartonella* species in sheep and goats

This is the first study investigating the prevalence of *Bartonella* infection in domestic ruminants in Nigeria and provides the first molecular evidence of such occurrence in sheep and goats. Thus far, the presence of *Bartonella* infection has been reported in commensal rodents and ectoparasites (*Xenopsyllacheopis, Haemolaelaps* spp., *Ctenophthalmus* spp., *Hemimerus talpoides, and Rhipicephalus sanguineus*) in Nigeria (Kamani et al., 2013). The low prevalence of *Bartonella* bacteremia recorded in this study (0.8% and 1% in sheep and goats, respectively) suggests that domestic ruminants are possible reservoirs of *Bartonella* spp. In sheep and goats, to the best of our knowledge, *Bartonella* strains have never been isolated in Africa, despite the fact that *B. melophagi* has been reported in *M. ovinus* (the sheep ked) collected from sheep in Ethiopia (Kumsa et al., 2014). Recently, *B. melophagi* was detected in sheep and sheep keds *M. ovinus* in New Mexico, USA (Kosoy et al., 2016). Further studies are needed to clarify the importance of *Bartonella* species in sheep and goats and to identify their competent vectors as well as the potential susceptibility of humans and other animals. Based on the sequence data obtained in this study the diversity of *Bartonella* spp. associated with ruminants in Nigeria seems relatively low and efforts should be made to characterise this pathogen to species level targeting different genes such as the *gltA*.

5.6.4 Coinfections

The results of the present study illustrate that mixed infections by several tick-borne pathogens are very common in small ruminants in Nigeria. In this study, sheep and goats were found infected with more than two pathogens, a fact which increases the health problems of the animals, and thus, the loss of products as described by Lawal-Adebowale, (2012). This finding indicates that superinfection by several haemoparasitic species occurs and the absence of cross-protection between these species (Renneker et al., 2013). The results highlight that infections with *Theileria* and *Rickettsia* species appear to be the main risk in sheep, and *Theileria* species in goats. Moreover, there is also a clear risk of coinfection with *Anaplasma, Babesia, Theileria* and *Rickettsia* species. The presence of multiple pathogens within an individual host may affect the outcome of infection. Coinfection with either *Theileria ovis* and *A. ovis*, that are known to be non-pathogenic and mild pathogenic, respectively, can dramatically change the prognosis and increase the risk of severe disease and mortality. Accordingly, it has been suggested that the outcome of concurrent infection with *A. ovis* and *Babesia* species infection can be more severe than a single infection alone (Renneker et al., 2013). Thus, coinfections with *Anaplasma* and *Babesia* species may be

associated with a poor prognosis and economic loss. Testing for only the major pathogen species may, therefore, underestimate the overall risks for Nigerian livestock with regards to TBIs. Further studies should include a more detailed picture of the effect of multiple infections, including assessment of clinical parameters besides infection status. In conclusion, *Theileria, Anaplasma, Babesia* and *Rickettsia* species are present in sheep and goats in the KGR, and the demonstrated high infection rate suggests that ticks and tick-borne diseases have a considerable impact on these animals' productivity (Perry & Randolph, 1999).

5.7 Conclusion

The result of this present study reveals the presence of haemoparasitic infection in small ruminants reared by the Fulani pastoralists in KGR. The detection of *T. mutans, T. velifera, B. caballi* and *T. equi*-like infections in sheep and goats further expand the known host range of these species, warranting further investigation. The local Fulani herdsmen may not have noticed the effects of the parasites on their animals due to the subclinical or chronic nature of the infection, which in most cases do not result in severe clinical disease manifestation or mortality. However, their pathophysiological effects might usually manifest in significant losses in the animal productivity which may be in the form of stunted or decreased growth rate in young sheep and goats, progressive emaciation, late maturity, weight loss, and increased susceptibility to other infectious diseases (Leeflagn & Ilemobade, 1977). Therefore, there is a need for prevention and control programs against these parasites, which calls for routine screening to monitor and possibly reduce the pathophysiological effects of the parasites. Moreover, strategic measures should be taken to control the vectors involved in their transmission.

Chapter 6: General conclusion and recommendations

This thesis describes the first systematic epidemiological investigation of TBIs in North Western Nigeria. Despite the widespread distribution of tick-borne haemoparasitic infections in tropical and subtropical regions of the world and their constraint to livestock production (Minjauw & Mcleod, 2003; Jongejan & Uilenberg, 2004), relatively limited information on their abundance and distribution is currently available in free-range animals in grazing reserves in Nigeria.

Prior to this study, information on the presence and distribution of ruminants' TBIs in the country was fragmentary and based on clinical signs, microscopic examination of blood smears and/or lymph node biopsies and serological methods for the detection of antibodies in affected animals (Leeflang, 1977; Leeflang & Ilemobade, 1977; Ajayi & Dipeolu, 1986; Kamani et al., 2010). The absence of robust molecular epidemiological studies and lack of diagnostic capacities in local laboratories in the country resulted in a lack of records on the impact of the disease in livestock, particularly in small ruminants. The only available reports on the occurrence of TBIs in the country that were based on molecular studies mostly focussed on cattle (Kamani et al., 2010; Ogo et al., 2012; Reye et al., 2012; Lorusso et al., 2016). Accordingly, prior to this thesis, very limited information was available on the prevalence of these pathogens in small ruminants in the country. The present study reports and confirms the endemicity of TBIs in ruminants in Nigeria, indicating that small ruminants are highly susceptible to the infections. In order to control them effectively in susceptible livestock in Nigeria, especially in the traditional livestock husbandry systems, it is essential to understand the epidemiology of TBIs, particularly on the prevalence and distribution of infection in the target population. Similarly, to understand the risk of exposure to TBIs for livestock in the country, it was necessary to determine the prevalence of tick infestation and infection rates in the vector tick population.

This PhD thesis applied a research approach starting with identification of ticks using tick identification guide (Walker et al., 2003), followed by molecular PCR-based diagnostic tools, conventional 16S rRNA and nested 18S rRNA to determine infection rates of *Ehrlichia/Anaplasma*, and *Theileria/Babesia* species respectively. For *Rickettsia* species, two methods were initially compared in the identified ticks (Chapter 3), namely the Real-time PCR and the Conventional PCR both targeting a fragment of citrate synthase gene *gltA*. Furthermore, an RLB assay allowing the simultaneous detection, of multiple pathogens (i.e *Ehrlichia/Anaplasma*, *Theileria/Babesia*, *Rickettsia* and *Bartonella*) was then applied to cattle (Chapter 4) and in sheep and goat blood samples (Chapter 5).

Recommendations for future research

The epidemiological data and prevalence of tick-borne haemoparasites and their analysis presented in this study have raised major areas of focus for further studies to improve the knowledge and ultimately control TBIs in livestock and humans in Nigeria:

i. The epidemiological significance, extent, and consequences of infections with *T. equi*-like and *B. caballi* in domestic ruminants require further investigation. In order to clarify the vectorial competence in this respect, tick species likely to be involved in the transmission of *B. caballi* (e.g. *Hyalomma* and *Rhipicephalus* spp.) need to be tested. In addition, extensive testing of ticks molecularly is recommended to acquire more information on the circulation/prevalence of infection of tick-borne pathogens as these may provide a benchmark to design appropriate prevention and control strategies.

ii. Furthermore, this study confirms the presence of *R. africae* and *R. massiliae* infections in ticks in the study area thereby suggesting the presence of these parasites in the local livestock population. The Real-Time PCR showed high sensitivity and specificity in detecting *Rickettsia* species in ticks when compared to the conventional PCR. The sequence data obtained provide new information on the epidemiology of *R. africae* and *R. massiliae* in Nigeria. The detection of these zoonotic pathogens in ticks collected from cattle, sheep and goats represents a risk to local farms. This finding suggests the need for the assessment of the burden of ATBF in the human populations in contact with livestock in Nigeria. In addition, *R. africae* experimental evaluations of vector competence clearly need to be carried out in order to establish the vector competence of *Rhipicephalus* spp. ticks.

iii. It is recommended to carry out large-scale epidemiological surveillance of *A. phagocytophilum* to ascertain the true prevalence of the infection, the possible vectors/reservoirs of the pathogen amongst livestock populations in Nigeria and to assess the potential risk factors for infection in humans.

iv. Further epidemiological investigations on *Ehrlichia* spp. 'Omatjenne' are needed to fully understand the impact of the infection it causes in ruminants. In addition, detailed studies on role of *A. ovis* infection are also recommended. Livestock improvement plans such as smallholder dairy development schemes through the

introduction of high yielding breeds should take into account the occurrence of these infections and adopt the necessary precautions to avoid losses.

v. Further research should aim to clarify the identity of *Theileria* spp. (strain MSD) in Nigeria.

vi. Further studies are needed to clarify the importance of *Bartonella* species in sheep and goats and to identify the vectors as well as the potential susceptibility of humans and other animals. The diversity of *Bartonella* spp. associated with ruminants based on sequence data obtained in this study in Nigeria seems relatively low and an effort should be made to characterise this pathogen to species level targeting different genes such as the *gltA* in sheep and goats

vii. It is recommended that the blood and tick samples be subjected to the *E. ruminantium*-specific pCS20 real-time PCR assay (Steyn et al., 2008) to determine more accurately the *E. ruminantium* prevalence in cattle and small ruminants in Nigeria

viii. This study suggests that ruminants are likely an alternative host for *A. platys* and further studies are required to ascertain the pathogenicity of this organism in cattle.

Potential future avenues for RLB technique

This study demonstrated the applicability of the RLB technique in detecting up to six genera of tick-borne haemoparasites (i.e. *Ehrlichia, Anaplasma, Theileria, Babesia, Rickettsia* and *Bartonella* spp.) in cattle, sheep and goats in Nigeria. The RLB method is potentially suitable for use with large numbers of sample/specimens, with one blotter enabling the screening of 43 samples simultaneously; it does not require expensive instrumentation and provides rapid results when compared with conventional PCR and alternative molecular methods (e.g. sequencing and restriction fragment length polymorphism analysis). Therefore, this method could become a powerful and reliable tool for the identification of tick-borne pathogens (Wang et al., 2008). During laboratory analysis, there were minor differences in intensities of signal dots observed, in which clear signals similar to or stronger than positive controls on the membrane were regarded as positive. This approach was validated by the use of conventional PCR using genus-specific primers. As suggested by Wang et al. (2008) a weak signal reflects minor sequence variation (1–2 bp) and an absent signal represents at least a 2 bp sequence change in the corresponding probe region. However, discordant signals between (one positive

– weak or strong – and one negative) can occur but are rare. Some cross-reactions were observed between the genus *Theileria* and the spotted fever group of rickettsiae. This requires further attention with critical evaluation during the preparation of the membrane

Closing Statement

In conclusion, this PhD thesis has established baseline data on the epidemiology and prevalence of TBIs in North Western Nigeria using molecular diagnostic tools with high sensitivity and specificity. The outcome of this research will help create awareness in the Nigerian authorities on the true burden of TBIs of medical and veterinary importance in the country. Such work is timely as there is an increasingly acute demand on Nigerian farmers to improve their productivity in the quality of hide and skin of their livestock and to meet the growing demand for milk and meat by the country's rapidly increasing urban population (Lawal-Adebowale, 2012). Therefore, improving livestock production and productivity is a national priority to enhance the livelihood of poor small-scale farmers and to ensure food and nutritional security for rural farming communities and the country at large

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6 – Appendix

Appendix I Coinfections of tick-borne haemoparasites in cattle, sheep and goats

Table 5:11: Extensive format of Table 4:6. Patterns of multiple infections by tick-borne haemoparasites according to sex, age classes and overall number of animals in cattle.

(*Ac* = *Anaplasma centrale*; *Am* = *Anaplasma* marginale; *E*spO = *Ehrlichia* spp. Omatjenne; *Er* = *Ehrlichia ruminantium*; *R* = *Rickettsia* spp.; *B* = *Bartonella*; *Bbov* = *Babesia bovis*; *Tel* = *Theileria equi*-like; *Tm* = *Theileria mutans*; *T*spMSD4 = *Theileria* sp MSD4; *Tv* =*Theileria velifera*)

	Tick-borne haemoparasites species combinations	Frequency				Totals
		Males	Females	Young	Adults	
1	*Am* + *E*spO + *Tel* + *Tm* + *Tv* + *T*spMSD4	5	12	4	13	17
2	*E*spO + *Tel* + *Tm* + *Tv* + *T*spMSD4	2	7	3	6	9
3	*Am* + *E*spO + *Bbov* + *Tel* + *Tm* + *Tv*	-	8	1	7	8
4	*Am* + *Tel* + *Tm* + *Tv* + *T*spMSD4	3	5	1	7	8
5	*Am* + *E*spO + *Bb* + *Tel* + *Tm* + *Tv* + *T*spMSD4	2	6	3	5	8
6	*Am* + *E*spO + *Tm* + *Tv*	3	4	1	6	7
7	*Am* + *E*spO	1	6	1	6	7
8	*Tel* + *Tm* + *Tv*	-	6	-	6	6
9	*Am* + *E*spO + *Tel* + *Tm* + *Tv*	-	5	-	5	5
10	*Am* + *Tel* + *Tm* + *Tv*	-	5	-	5	5
11	*E*spO + *Tel* + *Tm* + *Tv*	-	5	1	4	5
12	*Tm* + *Tv*	-	5	1	4	5
13	*Am* + *Tel* + *Tm* + *Tv* + *T*spMSD4	3	2	2	3	5

14	*Ac* + *Am* + EspO + *Tel* + *Tm* + *Tv* + *T*spMSD4	2	2	-	4	4
15	*Am* + *Bb* + *Tel* + *Tm* + *Tv* + *T*spMSD4	-	3	3	1	4
16	*Am* + *Bbov* + *Tel* + *Tm* + *Tv* + *T*spMSD4	1	2	1	2	3
17	*Am* + *E*spO + *Bb*+ *Tel* + *Tm* + *Tv* + *T*spMSD4	1	-	3	-	3
18	*Am* + *E*spO + *Bbov* + *Tel* + *Tm* + *Tv* + *T*spMSD4	1	2	1	2	3
19	*Am* + *Tm*	-	3	2	1	3
20	*Am* + *Tm* + *Tv*	-	3	-	3	3
21	*E*spO + *Bbov* + *Tel* + *Tm* + *Tv* + *T*spMSD4	2	1	2	1	3
22	*Am* + *E*spO + *Bb* + *Bbov* + *Tel* + *Tm* + *Tv* + *T*spMSD4	1	2	-	3	3
23	*Am* + *E*spO + *Bb*+ *Bc* + *Tel* + *Tm* + *Tv* + *T*spMSD4	2	1	1	2	3
24	*Ac* + *Am* + *Tm*	1	2	1	2	3
25	*E*spO + *Tm* + *Tv*	1	2	1	2	3
26	*Ac* + *Am* + *Bb* + *Bbov* + *Tel* + *Tm* + *Tv* + *T*spMSD4	-	2	-	2	2
27	*Ac* + *Am* + *E*spO + *Bbov* + *Tel* + *Tm* + *Tv* + *T*spMSD4	-	2	-	2	2

28	*Am* + *Bb* + *Bbov* + *Tel* + *Tm* + *Tv*	1	1	-	2	2
29	*Am* + EspO + *Ap* + *Tel* + *Tm* + *Tv* + *T*spMSD4	-	2	-	2	2
30	*Am* + EspO + *Bb* + *Tel* + *Tm* + *Tv* + *B*	1	1	-	2	2
31	*Am* + EspO + *Bb* + *Bbov* + *Tel* + *Tm* + *Tv*	-	2	-	2	2
32	*Am* + EspO + *Bb* + *Tel* + *Tm* + *Tv*	-	2	-	2	2
33	*Bb* + *Bbov* + *Tel* + *Tm* + *Tv* + *T*spMSD4	-	2	-	2	2
34	*Bbov* + *Tel* + *Tm* + *Tv*	-	2	-	2	2
35	EspO + *Bb* + *Tel* + *Tm* + *Tv*	-	2	-	2	2
36	EspO + *Tm*	1	1	2	-	2
37	*Tel* + *Tm* + *Tv* + *T*spMSD4	1	1	2	-	2
38	EspO + *Bb* + *Bbov* + *Tel* + *Tm* + *Tv*	-	1	-	-	1
39	EspO + *Tel* + *Tm* + *Tv*	-	-	1	-	1
40	EspO + *Tm* + *Tv*	-	-	1	-	1
41	*Tm* + *Tv*	-	1	1	-	1
42	*Ac* + *Am* + *Er* + *Tel* + *Tm* + *Tv* + *T*spMSD4	-	1	-	1	1
43	*Ac* + *Am* + EspO + *Bb* + *Bc* + *Tel* + *Tm* + *Tv* + *T*spMSD4	-	1	-	1	1

44	*Ac* + *Am* + *E*spO + *Bb* + *Tel* + *Tm* + *Tv* + *B*	-	1	-	1	1
45	*Ac* + *Am* + *E*spO + *Bb*+ *Tel* + *Tm* + *Tv* + *T*spMSD4	-	1	-	1	1
46	*Ac* + *Am* + *E*spO + *Tm* + *Tv*	-	1	1	-	1
47	*Ac* + *Am* + *E*spO + *Tm* + *Tv* + *T*spMSD4	1	-	1	-	1
48	*Ac* + *Am* + *Tel* + *Tm* + *Tv* + *T*spMSD4	-	1	-	1	1
49	*Ac* + *E*spO + *Bb* + *Bc* + *Tel* + *Tm* + *Tv* + *T*spMSD4	-	1	-	1	1
50	*Am* + *Tel* + *Tm* + *Tv*	-	1	-	1	1
51	*Am* + *Bb* + *Bbov* + *Tel* + *Tm* + *Tv* + *B*	-	1	-	1	1
52	*Am* + *Bb* + *Bbov* + *Bc* + *Tel* + *Tm* + *Tv* + *T*spMSD4	1	-	-	1	1
53	*Am* + *Bb* + *Bbov* + *Tel* + *Tm* + *Tv* + *T*spMSD4	1	-	-	1	1
54	*Am* + *Bb* + *Bbov* + *Tel* + *Tm* +*Tv* + *T*spMSD4	1	-	-	1	1
55	*Am* + *Bb* + *Bc* + *Tel* + *Tm* + *Tv* + *TspMD4*	-	1	-	1	1
56	*Am* + *Bb* + *Tel* + *Tm* + *Tv* + *T*spMSD4 + *R*	1	-	-	1	1

57	*Am* + *Bb* + *Tm* + *Tv*	-	1	-	1	1
58	*Am* + *Bbov* + *Tel* + *Tm* + *Tv*	-	1	-	1	1
59	*Am* + *Bbov* + *Tel* + *Tm* + *Tv* + *T*spMSD4 + *B*	-	1	-	1	1
60	*Am* + *Bbov* + *Tm* + *Tv*	-	1	-	1	1
61	*Am* + *Er* + *Tel* + *Tm* + *Tv* + *R*	-	1	-	1	1
62	*Am* + *E*spO + *Tm* + *Tv* + *T*spMSD4	-	1	-	1	1
63	*Am* + *E*spO + *Tm* + *Tv*	-	1	-	1	1
64	*Am* + *E*spO + *Bbov* + *Tm* + *Tv*	-	1	-	1	1
65	*Am* + *E*spO + *Ap* + *Bbov* + *Tel* + *Tm* + *Tv* + *T*spMSD4	1	-	-	1	1
66	*Am* + *E*spO + *Bb* + *Bbov* + *Tel* + *Tm* + *Tv* + *T*spMSD4	-	1	-	1	1
67	*Am* + *E*spO + *Bb* + *Bbov* + *Tel* + *Tm* + *Tv* + *T*spMSD4 + *B*	-	1	-	-	1
68	*Am* + *E*spO + *Bb* + *Bbov* + *Tm* + *Tv* + *T*spMSD4 + *B*	-	1	-	1	1
69	*Am* + *E*spO + *Bb* + *Bc* + *Tel* + *Tm* + *Tv*	-	1	-	1	1
70	*Am* + *E*spO + *Bb* + *Bc* + *Tel* + *Tm* + *Tv* + *T*spMSD4	-	1	-	1	1
71	*Am* + *E*spO + *Bb* + *Tel* + *Tm* + *Tv* + *T*spMSD4 + *R*	1	-	1	-	1

72	*Am* + *Esp*O + *Bbov* + *Tel* + *Tm* + *Tv* + *B*	1	-	1	-	1
73	*Am* + *Esp*O + *Bbov* + *Tel* + *Tm* + *Tv* + *B*	-	1	-	1	1
74	*Am* + *Esp*O + *Bbov* + *Tm*	-	1	-	1	1
75	*Am* + *Esp*O + *R*	-	1	-	1	1
76	*Am* + EspO + *Tel* + *Tm* + *Tv* + *B*	-	1	-	1	1
77	*Am* + *Esp*O + *Tel* + *Tm* + *Tv* + *R*	-	1	-	1	1
78	*Am* + *Esp*O + *Tm* + *Tv* + *T*spMSD4	-	1	1	-	1
79	*Am* + *Esp*O +*Bbov* + *Tm* + *Tv* + *T*spMSD4	1	-	-	1	1
80	*Am* + *Esp*O +*Tel* + *Tm* + *Tv*	-	1	-	1	1
81	*Ap* + *Bbov* + *Tel* + *Tm* + *Tv* + *T*spMSD4 + *B*	-	1	-	1	1
82	*Ap* + *Tm*	-	1	-	1	1
83	*Ap* + *Tm* + *Tv* + *T*spMSD4 + *B*	-	1	-	1	1
84	*Bbov* + *Tm* + *Tv*	1	-	-	1	1
85	*Esp*O + *Bb* + *Bbov* + *Tv* + *T*spMSD4	-	1	-	1	1
86	*Esp*O + *Bb* + *Bbov* + *Tel* + *Tm* + *Tv* + *T*spMSD4	1	-	1	-	1
87	*Esp*O + *Bb* + *Tm* + *Tv*	-	1	1	-	1
88	*Esp*O + *Bb* + *Tm* + *Tv* + *T*spMSD4	-	1	1	-	1

89	*E*spO + *Bbov* + *Tm*	-	1	-	1	1
90	*E*spO + *Tm* + *Tv* + *T*spMSD4	-	1	-	1	1
91	*Tel* + *R*	1	-	1	-	1
92	*Tm* + *Tv* + *T*spMSD4	1	-	1	-	1

Table 5:12: Extensive format of Table 5:5. Patterns of multiple infections by tick-borne haemoparasites according to age classes and overall number of animals in sheep

(*Ac* = *Anaplasma centrale*; *Ao* = *Anaplasma ovis*; *E*spO = *Ehrlichia* spp. Omatjenne; *Er* = *Ehrlichia ruminantium*; *R* = *Rickettsia* spp.; *B* = *Bartonella*; *Bbov* = *Babesia bovis*; *Tel* = *Theileria equi*-like; *Tg* = Thyphus group *Tm* = *Theileria mutans*; *To* = *Theileria ovis;* *T*spMSD4 = *Theileria* sp MSD4; *Tv* =*Theileria velifera*).

	Tick-borne haemoparasites species combinations	Frequency				Totals
		Males	Females	Young	Adults	
1	*Tel* + *R*	3	13	8	8	16
2	*Ac* + *Tel*	5	6	4	7	11
3	*Ac* + *Tel* + *R*	2	9	6	5	11
4	*Tel* + *Tv* + *T*spMSD4	3	6	2	6	9
5	*E*spO + *Tel* + *Tm* + *Tv*	3	5	1	7	8
6	*Ac* + *E*spO + *Tel*	-	5	2	3	5
7	*E*spO + *Tel*	-	4	1	3	4
8	*Tel* + *T*spMSD4	-	4	1	3	4
9	*Ac* + *Tel* + *Tm*	-	2	1	1	2
10	*Ac* + *To*	-	2	1	1	2
11	*Ao* + *To*	1	1	1	1	2
12	*E*spO + *To*	-	2	-	2	2
13	*Ac* + *Bc* + *Tel* + *Tm* + *Tv* + *TspMSD4* + *R*	1	-	1	-	1
14	*Ac* + *Er*	-	1	-	1	1

15	*Ac* + *E*spO + *Tel* + *R*	-	1	-	1	1
16	*Ac* + *E*spO + *Tel* + *Tm* + *Tv*	-	1	-	1	1
17	*Ac* + *E*spO + *Tm* + *Tv* + *B*	-	1	-	1	1
18	*Ac* + *E*spO + *Tm* +*Tv*	-	1	-	1	1
19	*Ac* + *Tel* + *Tm* + *Tv* + *T*spMSD4 + *R*	-	1	-	1	1
20	*Ao* + *Tel* + *Tv*	-	1	-	1	1
21	*Ao* + *Tel* + *Tv* + *R*	-	1	1	-	1
22	*Bbov* + *Tel* + *R*	-	1	1	-	1
23	*Bbov* + *Tel* + *Tm*	-	1	1	-	1
24	*Bbov* + *Tel* + *Tm* + *R*	-	1	-	1	1
25	*Bbov* + *To*	1	-	1	-	1
26	*E*spO + *Tel* + *R*	-	1	1	-	1
27	*Tel* + *Tm*	-	1	1	-	1
28	*Tel* + *Tm* + *Tv* + *B*	-	1	-	1	1
29	*Tel* + *T*spMSD4 + *R*	-	1	-	1	1
30	*Tel* + *Tv* + *R*	-	1	-	1	1
31	*Tel* + *Tv* + *T*spMSD4 + *R*	-	1	-	1	1
32	*Tel* + *Tv* + *T*spMSD4 + *Tg*	-	1	1	-	1
33	*Tm* + *R*	-	1	1	-	1
34	*Tm* + *Tv*	1	-	1	-	1

35	*To + R*	-	1	1	-	1
36	*Tv + R*	-	1	-	1	1

Table 5:13: Extensive format of Table 5:10. Patterns of multiple infections by tick-borne haemoparasites according to sex, age classes and overall number of animals in goats

(*Ac* = *Anaplasma centrale*; *Am* = *Anaplasma marginale; E*spO = *Ehrlichia* spp. Omatjenne; *R* = *Rickettsia* spp.; *B* = *Bartonella*; *Bb* = *Babesia bigemina; Bc* = *Babesia caballi; Bbov* = *Babesia bovis*; *Tel* = *Theileria equi*-like; *Tm* = *Theileria mutans*; *T*spMSD4 = *Theileria* spp. MSD4; *Tv* =*Theileria velifera*).

	Tick-borne haemoparasites species combinations	Frequency				Totals
		Males	Females	Young	Adults	
1	*Tel* + *Tm* + *Tv* + *T*spMSD4	-	7	2	5	7
2	*Am* + *Bbo* + *Tel* + *Tm* + *Tv* + *T*spMSD4	-	3	-	3	3
3	*Ac* + *Am* + *Bb* + *Bbo* + *Tel* + *Tm* + *Tv* + *T*spMSD4	-	2	-	2	2
4	*Ac* + *Am* + *Tel* + *Tm* + *Tv* + *T*spMSD4	-	2	-	2	2
5	*Ac* + *Am* + *Tel* + *Tm* + *Tv* + *T*spMSD4 + *R*	-	2	-	2	2
6	*Am* + *Tel* + *Tm* + *Tv* + *T*spMSD4	1	-	1	1	2
7	*Tel* + *Tm* + *Tv* + *T*spMSD4 + *R*	-	2	-	2	2
8	*Ac* + *Am* + *Bbo* + *Tel* + *Tm* + *Tv* + *T*spMSD4	-	1	-	1	1
9	*Ac* + *Bb* + *Bbo* + *Tel* + *Tm* + *Tv* + *T*spMSD4 + *R*	-	1	-	1	1
10	*Ac* + *Bbo*	-	1	-	1	1

11	*Ac* + *Bbo* + *Tel* + *Tm* + *Tv* + *T*spMSD4	-	1	-	1	1
12	*Ac* + *Tel* + *Tm* + *Tv* + *T*spMSD4	-	1	-	1	1
13	*Am* + *E*spO + *Bb* + *Bbo* + *Bc* + *Tel* + *Tm* + *Tv* + *TspMSD4* + *R*	1	-	-	1	1
14	*Bbo*+ *Tel* + *Tm* + *Tv* + *T*spMSD4	-	1	-	1	1

Appendix II Sequence data obtained from Apicomplexa

Apicomplexa 18S rDNA sequence data obtained from ticks in Chapter 3

>T24

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>T34

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>T108

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>T112

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>T118

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>T125

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>T127

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>T131

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>T133

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Appendix III Sequence data obtained from Apicomplexa

Rickettsia gltA **sequence data obtained from ticks in Chapter 3**

>14

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>47

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>59

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>19R

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>20R

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>57R

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>63R

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>67R

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Appendix IV Heamtology results of cattle, sheep and goats

Cattle Hematology Results

Serial Coding No	Herd No.	Sex	Age	BCS	Mucous membranes	Ectoparasites	Haematological findings										
							PCV	Hgb	WBC	RBC	Total Proteins	Neutrophil	Lymphocyte	Monocyte	Eosinophil	Basophil	Band
C1	1	M	1Y	M-	Pale	NO	35	11.6	2.8	5.7	6.4	33	67	0	0	0	0
C2	1	F	1Y	M	Normal	NO	44	14.6	4.5	7.4	6	36	60	0	4	0	0
C3	1	M	1Y	M-	Pale	NO	43	14.3	9	7.3	8.6	36	64	0	0	0	0
C4	1	F	1Y	M	Normal	NO	33	11	8.4	6	4.5	24	76	0	0	0	0
C5	1	M	1Y	M	Normal	NO	38	12.6	4.4	6.2	4.6	18	82	0	0	0	0
C6	1	M	1Y	L	Pale	NO	43	14.3	8.6	7.1	8.6	26	74	0	0	0	0
C7	1	M	3Y	M	Pale	Tick	37	12.3	7	6.1	4.5	33	67	0	0	0	0
C8	1	F	1.5Y	M	Normal	NO	49	13	8	8.1	6.8	35	65	0	0	0	0
C9	1	F	1Y	M	Normal	NO	43	14.3	9	7.2	8.2	30	64	1	0	0	0
C10	1	M	1Y	M	Normal	NO	36	12	5.4	6	6.8	27	69	1	3	0	0
C11	1	F	3Y	M	Normal	NO	44	14.6	5.6	7.5	8.4	36	56	1	0	0	7
C12	1	F	3Y	M	Normal	NO	41	13.6	8.1	6.8	6.4	21	78	1	0	0	0
C13	1	F	3Y	M-	Pale	NO	40	13.3	4.8	6.8	8.2	23	67	6	0	0	4
C14	1	F	7Y	M	Normal	NO	37	12.3	3.6	6.2	10.4	24	86	0	0	0	0
C15	1	F	15Y	M-	Pale	NO	53	17.6	14.8	8.8	6.8	29	71	2	0	0	0
C16	1	F	7Y	M	Normal	NO	40	13	9	6.6	8	20	78	2	0	0	0
C17	1	F	10Y	L	Pale	NO	41	13.6	8	6.8	8.6	16	84	0	0	0	0
C18	1	F	3Y	M-	Pale	NO	40	13	4.8	7	8.6	23	65	2	6	0	4
C19	1	F	7Y	M	Normal	NO	56	18.6	5.8	9.3	8.8	28	72	0	0	0	0
C20	1	F	8Y	M	Normal	NO	34	11.3	6	6.1	6.8	18	80	0	2	0	0
C21	1	F	7Y	M	Normal	NO	32	10.6	3.6	5.5	8.8	37	63	0	0	0	0

C22	1	F	7Y	M	Normal	NO	34	11.3	3.8	5.9	10	17	83	0	0	0	0
C23	1	M	4Y	M	Normal	NO	47	15.6	8.1	7.8	8.2	23	77	0	0	0	0
C24	1	M	3Y	M	Normal	Tick	54	18	18	9	4	18	82	0	0	0	0
C25	1	M	5Y	M	Pale	Tick	36	12	8.1	6.4	4.8	30	70	0	0	0	0
C26	1	F	6M	M	Normal	NO	49	13	6	8.2	5.4	15	83	2	0	0	0
C27	1	F	6Y	M	Normal	NO	46	15.3	7.6	7.6	6.2	30	70	0	0	0	0
C28	1	F	6Y	M	Normal	NO	44	14.6	8	7.6	8.2	24	70	6	0	0	0
C29	1	F	6Y	M	Normal	NO	57	19	7.4	9.6	7.4	14	86	0	0	0	0
C30	1	F	6Y	M	Normal	NO	31	10.3	10	5.2	7.2	28	80	0	0	0	0
C31	1	F	5Y	M	Normal	NO	72	24	6	12.1	3	39	61	0	0	0	2
C32	1	F	5Y	M	Normal	NO	42	14	8	7	7	26	74	0	0	0	0
C33	1	F	5Y	M-	Pale	NO	69	23	13.6	11.6	2	31	69	0	0	0	0
C34	1	F	6Y	M-	Pale	NO	47	15.6	4.4	8	6	30	69	0	0	0	0
C35	1	F	5Y	M	Normal	NO	14	4.6	5.1	2.5	6.4	35	65	0	0	0	1
C36	1	F	3Y	M	Normal	NO	75	25	6.2	12.8	7.2	41	59	0	0	0	0
C37	1	F	5Y	M	Normal	NO	59	19.3	8.6	10	8.2	32	68	0	0	0	0
C38	1	F	5Y	M	Normal	NO	38	12.3	8.4	6.4	5.8	30	69	1	0	0	0
C39	1	F	2Y	M	Normal	NO	34	11.3	4.9	6	2.4	29	71	0	0	0	0
C40	1	F	3Y	M	Normal	NO	28	9.3	8.2	5	6.2	45	44	6	5	0	0
C41	1	F	4 Y	M	Normal	NO	35	11.6	6.8	5.9	6	24	76	0	0	0	0
C42	1	M	2.5 Y	M-	Pale	NO	30	10	6	5.1	6.6	34	66	0	0	0	0
C43	1	F	4Y	M-	Pale	NO	35	11.6	8.8	6.7	6	26	74	0	0	0	0
C44	1	F	6Y	M	Normal	NO	39	13	9	6.6	8.4	22	78	0	0	0	0
C45	1	F	6Y	M	Normal	NO	56	18.6	16	9.4	6	18	74	2	0	0	6
C46	1	F	7Y	M	Normal	NO	41	13.6	15.3	6.9	5	15	84	1	0	0	0
C47	1	F	6Y	M-	Pale	NO	51	17	8.1	8.5	7	36	64	0	0	0	0
C48	1	F	6Y	M	Normal	NO	52	17.3	14	8.6	5.8	33	60	2	5	0	0
C49	1	F	6Y	M	Normal	NO	44	14.6	12	7.2	7.8	13	86	1	0	0	0
C50	1	F	6Y	L	Pale	NO	35	11.6	6.2	5.7	8	60	34	6	0	0	0

C51	1	F	7Y	M-	Pale	NO	46	15.3	5.3	7.7	8	24	76	0	0	0	0
C52	1	F	4Y	M-	Pale	NO	48	16	7	8	8.2	23	77	0	0	0	0
C53	1	F	3Y	M-	Pale	NO	39	13	18	7	7	36	64	0	0	0	0
C54	1	F	1Y	M	Normal	NO	38	12.3	6.8	6.3	5	26	74	0	0	0	0
C55	1	F	1Y	M	Normal	NO	66	23	6.1	11.2	8.6	40	60	0	0	0	0
C56	1	F	1.5Y	M	Normal	NO	39	13	11.2	6.6	6.4	31	69	0	0	0	0
C57	1	F	4Y	M	Normal	NO	17	5.6	2.8	2.8	6.2	16	82	2	0	0	0
C58	1	M	4Y	M	Normal	NO	46	15.3	8	7.6	8	18	82	0	0	0	0
C59	1	F	3Y	M-	Pale	NO	34	11.3	6.2	5.6	6.8	14	86	0	0	0	0
C60	1	F	4Y	L	Pale	NO	40	13.3	8.1	6.6	7.6	26	74	0	0	0	0
C61	3	M	1Y	M-	Pale	NO	49	16.3	8	8.4	6.2	30	70	0	0	0	0
C62	3	F	5Y	M	Normal	NO	64	21.3	7.2	10.5	7	31	63	0	6	0	0
C63	3	M	3Y	L	Pale	NO	47	15.6	8.2	7.3	7	36	64	0	0	0	0
C64	3	M	2Y	L	Pale	NO	36	12	8.6	6.4	7.8	15	85	0	0	0	0
C65	3	F	2Y	M	Normal	NO	64	21.3	8.1	4	7	22	78	0	0	0	0
C66	3	F	2Y	M	Normal	NO	46	15.3	9.2	7.7	8	26	74	0	0	0	0
C67	3	F	4Y	F	Pale	NO	54	18	8.8	9	11	30	65	1	4	0	0
C68	3	F	6Y	F	Normal	NO	47	15.6	8.1	7.9	11	24	76	0	0	0	0
C69	3	F	6Y	M	Normal	NO	66	22	6.8	11	7	27	73	0	0	0	0
C70	3	F	5Y	M	Normal	NO	19	6.3	12	3.4	6.8	12	88	0	0	0	0
C71	3	F	6Y	M	Normal	NO	17	5.6	7.6	3.3	7	32	68	0	0	0	0
C72	3	F	6Y	L	Pale	NO	44	14.6	8.6	7.3	8	30	70	0	0	0	0
C73	3	F	6Y	M	Normal	NO	41	13.6	9.2	7	9.3	33	67	0	0	0	0
C74	1	F	4Y	M	Normal	NO	45	15	4.9	7.6	8.2	44	56	0	0	0	0
C75	3	F	10Y	M	Normal	NO	49	16.3	5.3	8.1	7	18	82	0	0	0	0
C76	3	F	10Y	L	Pale	NO	52	17.3	6.6	8.6	7	24	76	0	0	0	0
C77	3	F	6Y	M-	Pale	NO	59	18.6	8.3	10	6.2	30	70	0	0	0	0
C78	3	M	4Y	M-	Pale	NO	50	16.6	8	8.4	7.4	25	74	1	0	0	0
C79	3	F	3Y	M	Normal	NO	61	20.3	16	10.2	8.4	15	80	1	4	0	0

C80	3	F	8Y	L	Pale	NO	58	19.3	8.4	9.4	8.4	24	76	0	0	0	0
C81	3	M	2Y	M	Normal	NO	40	13	8	6.8	7.2	31	69	0	0	0	0
C82	3	M	6Y	M	Normal	NO	45	15	3.6	8	8.4	37	60	3	0	0	0
C83	3	F	4Y	M-	Pale	NO	41	13.6	8	6.8	9	27	71	2	0	0	0
C84	3	F	5Y	M	Normal	NO	68	22.6	14.6	11.4	7.2	30	68	2	0	0	0
C85	3	M	1Y	L	Pale	NO	24	8	8	4	6.2	40	56	4	0	0	0
C86	2	F	7Y	L+	Pale	Tick	44	14.6	8.1	7.6	7.2	34	66	0	0	0	0
C87	2	M	3Y	M	Normal	NO	35	11.6	8.8	6	8.6	18	82	0	0	0	0
C88	2	F	4Y	M-	Normal	NO	41	13.6	14	7	8	18	75	0	7	0	0
C89	2	F	5Y	L	Pale	Tick	51	17	8.1	9	8.4	45	55	0	0	0	0
C90	2	M	1Y	M	Normal	NO	45	15	6.8	8	8.4	36	60	0	0	0	4
C91	2	M	1Y	M-	Pale	Tick	34	11.3	2.8	5.6	8.6	24	76	0	0	0	0
C92	2	F	1Y	M	Pale	Tick	29	9.6	15	4.8	7.8	26	70	4	0	0	0
C93	2	M	3Y	L+	Cyanotic	Tick	33	11	6	6	6.8	33	60	0	6	0	1
C94	2	F	5Y	L+	Pale	Tick	64	21.3	7.4	10.8	7.2	24	68	8	0	0	0
C95	2	F	9Y	L	Pale	Tick	30	10	6	5.4	6.6	33	66	1	0	0	0
C96	2	F	6Y	L+	Normal	NO	28	9.3	8	4.8	6.4	31	60	1	8	0	0
C97	2	F	5Y	M	Normal	NO	43	14.3	18.6	7.1	8.2	12	80	8	0	0	0
C98	2	F	5Y	M	Normal	NO	27	9	9	4.5	6.4	14	68	0	12	0	6
C99	2	F	3Y	M	Normal	NO	28	9.3	6.8	4.7	7.8	30	66	0	0	0	4
C100	2	F	3Y	M	Normal	NO	47	15.6	6	8	8.6	23	67	0	0	0	0
C101	2	F	5Y	L+	Normal	NO	36	12	5	6.4	7.2	24	76	0	0	0	0
C102	2	F	4Y	L	Pale	Tick	48	16	6	8	10	33	64	0	0	0	3
C103	2	F	2Y	L+	Pale	NO	52	17.3	8.1	8.6	8	26	74	0	0	0	0
C104	2	F	2Y	L+	Pale	NO	54	18	8	9.1	7.8	36	67	0	0	0	0
C105	2	M	2Y	M	Normal	NO	50	16.6	5.6	8.3	8.2	30	66	4	0	0	0
C106	2	M	4Y	L	Pale	Tick	0	0	0	0	0	0	0	0	0	0	0
C107	2	M	5Y	M	Normal	NO	59	19.6	8.2	9.9	5.4	39	51	6	0	0	4
C108	2	F	4Y	M	Normal	NO	41	13.6	18	6.8	8.6	36	64	0	0	0	0

C109	2	F	5Y	M-	Cyanotic	Tick	42	14	14	7	7	46	54	0	0	0	0
C110	2	F	4Y	L+	Pale	Tick	54	18	10.3	9	7	36	60	2	0	0	2
C111	2	F	3Y	L+	Pale	NO	50	16.6	5	5.2	9.2	24	76	0	0	0	0
C112	2	F	9Y	L	Pale	Tick	33	11	8.6	5.8	5.2	30	70	0	0	0	0
C113	2	F	8Y	L	Pale	NO	35	11.6	8.1	5.8	5.2	15	84	1	0	0	0
C114	2	M	3Y	M	Normal	NO	36	12	6	8.3	8	28	70	2	0	0	0
C115	2	F	4Y	L+	Pale	NO	29	9.6	7.4	5	6	36	64	0	0	0	0
C116	2	F	3Y	M-	Normal	NO	38	12.6	12	6.3	7.8	30	70	0	0	0	0
C117	2	F	4Y	L+	Pale	Tick	45	15	9	7.8	9.2	34	60	3	1	0	2
C118	2	F	5Y	M-	Normal	NO	56	18.6	10	9.3	6.8	48	52	0	0	0	0
C119	2	F	4Y	M	Normal	NO	47	15.6	6.3	8	7.8	30	69	1	0	0	0
C120	2	M	1.5Y	M	Normal	NO	51	17	5.2	8.8	6.6	36	60	0	2	0	2
C121	2	F	1Y	L+	Normal	NO	21	7	17	4	7.8	50	4	1	0	0	0
C122	2	F	1Y	M	Normal	NO	47	15.6	14	7.9	8.2	42	58	0	0	0	0
C123	2	M	1Y	M	Normal	NO	46	15.3	12	7.6	8.2	40	57	1	0	0	2
C124	4	F	7Y	M-	Pale	Tick	34	11.3	11	5.6	6.2	35	60	1	0	0	4
C125	4	F	6Y	M	Normal	NO	0	0	0	0	0	0	0	0	0	0	0
C126	4	F	8Y	M	Pale	NO	38	12.3	4.8	6.5	6.2	30	66	4	0	0	0
C127	4	F	6Y	M-	Pale	NO	47	15.6	8	7.9	8.2	36	64	0	0	0	0
C128	4	M	3Y	M	Pale	Tick	31	10.3	6	5.1	6.4	46	54	0	0	0	0
C129	4	F	6Y	L+	Pale	Tick	44	14.6	10.8	7	8.2	28	70	0	2	0	0
C130	4	F	5Y	M	Normal	NO	44	14.6	10	7.3	5.4	24	76	0	0	0	0
C131	4	M	4Y	M	Normal	NO	31	10.3	8.6	5.3	6.4	40	54	0	6	0	0
C132	4	F	3Y	L+	Pale	NO	37	12.3	8	6.1	6.8	18	70	2	8	0	2
C133	4	F	9Y	L	Pale	NO	25	8.6	4.8	4.2	5.4	18	80	2	0	0	0
C134	4	F	7Y	L+	Pale	NO	41	13.6	7	6.8	4.6	45	50	0	0	0	5
C135	4	F	6Y	M-	Normal	NO	40	13.3	4	6.6	4.8	24	76	0	0	0	0
C136	4	F	3Y	M-	Pale	NO	30	10	8.8	5.3	5.2	14	86	0	0	0	0
C137	4	F	4Y	M	Normal	NO	39	13	8.6	7	6.4	48	52	0	0	0	0

C138	4	F	6Y	M-	Normal	NO	38	12.6	9.2	6.4	7.2	46	54	0	0	0	0
C139	4	F	7Y	M-	Normal	NO	40	13.3	5.6	6.9	6.6	15	85	0	0	0	0
C140	4	M	4Y	M-	Normal	NO	43	14.3	7.2	7.1	6.8	30	65	0	5	0	0
C141	4	F	5Y	M	Normal	NO	43	14.3	10.6	7.1	8.2	12	88	0	0	0	0
C142	4	F	6	M	Normal	NO	0	0	0	0	0	0	0	0	0	0	0
C143	4	F	4Y	M	Normal	NO	40	13.3	4.9	6.6	4.8	28	70	0	0	0	2
C144	4	F	8Y	L+	Pale	Tick	43	14.3	6	7.1	7.2	33	67	0	0	0	0
C145	4	F	6Y	M-	Pale	NO	0	0	0	0	0	0	0	0	0	0	0
C146	4	M	5Y	M-	Normal	NO	34	11.3	15.6	5.6	6.6	23	77	0	0	0	0
C147	4	M	1.5Y	M	Normal	NO	33	11	16	5.9	7.4	17	83	0	0	0	0
C148	4	F	4Y	M	Normal	NO	30	10	8.2	5	6	30	70	0	0	0	0
C149	4	M	1.5Y	M	Normal	NO	49	16.3	10	8	7.4	72	18	0	6	0	0
C150	4	M	1.5Y	M-	Normal	NO	38	12.6	6.6	6.2	9	34	66	0	0	0	0
C151	4	M	1Y	M+	Normal	NO	61	20.3	9	10.1	9	22	78	0	0	0	0
C152	4	F	2Y	M-	Normal	NO	62	20.6	6	10.3	8	24	76	0	0	0	0
C153	4	F	3Y	M-	Pale	NO	17	5.6	2.8	3	6.4	28	72	0	0	0	0
C154	4	F	6Y	M-	Normal	NO	48	16	17.6	8.1	6.8	38	62	0	0	0	0
C155	4	F	5Y	M	Normal	NO	45	15	6.8	7.5	8.6	35	65	0	0	0	0
C156	4	F	7Y	M-	Normal	NO	14	4.6	22.1	2.3	7.6	12	88	0	0	0	0
C157	4	F	1Y	M	Normal	NO	55	18.3	8.1	9.3	9.6	31	68	1	0	0	0
C158	4	F	4Y	M	Normal	NO	28	8.6	14	4.8	6.4	20	64	0	1	0	0
C159	4	F	7Y	M-	Pale	NO	15	5	3.2	3.5	7	34	66	0	0	0	0
C160	4	M	2Y	M	Normal	NO	22	7.3	6	3.6	6	26	74	0	0	0	0
C161	4	F	3Y	M	Pale	NO	38	12.3	12.1	6.4	8.4	36	36	6	0	0	0
C162	4	F	7Y	M	Pale	NO	54	18	0.3	9	8.4	27	67	3	0	0	3
C163	4	F	4Y	M-	Normal	NO	24	8	11	4.1	8.2	24	73	3	0	0	0
C164	4	F	1.5Y	M	Normal	NO	38	12.6	5	6.3	7.8	33	66	1	0	0	0
C165	4	F	3Y	M	Normal	NO	30	10	6.2	5	8.2	20	76	0	2	0	2
C166	4	F	3Y	M	Normal	NO	24	8	6.1	4	7	18	80	2	0	0	0

C167	4	F	4Y	M	Normal	NO	32	10.6	8.1	5.3	10	26	74	0	0	0	0
C168	4	F	2Y	M-	Pale	NO	48	16	6.6	8	9.8	38	62	0	0	0	0
C169	4	F	3Y	M-	Normal	NO	38	12.6	10.2	6.9	9	17	83	0	0	0	0
C170	4	F	6Y	M	Normal	NO	58	19.3	6	9.8	8	40	60	0	0	0	0
C171	4	F	5Y	M-	Pale	NO	24	8	5	4	8.4	30	70	0	0	0	0
C172	4	F	1.5Y	M+	Normal	NO	32	10.6	6.2	5.3	6.4	40	60	0	0	0	0
C173	4	F	3Y	M	Normal	NO	43	14.3	6.2	7.4	6	32	68	0	0	0	0
C174	4	F	1Y	L+	Pale	Tick	63	21	11	10.5	10	51	49	0	0	0	0
C175	4	M	2Y	M	Normal	NO	35	11.6	14	6	8	28	72	0	0	0	0
C176	4	F	4Y	L+	Pale	Tick	35	11.6	7	6	8.2	18	79	0	3	0	0
C177	4	F	9M	M+	Normal	NO	31	10.3	10	5.2	7.4	40	60	0	0	0	0
C178	4	F	1Y	M+	Normal	NO	36	12	14	6	6.4	16	84	0	0	0	0
C179	4	F	1Y	M	Normal	NO	56	18.6	9	9.1	8	25	70	0	5	0	0
C180	4	M	2Y	M	Normal	Tick	52	17.3	11	8.6	7.4	45	50	0	0	0	5
C181	4	M	3Y	M+	Normal	NO	38	12.3	5.2	6.2	6.2	39	61	0	0	0	0
C182	4	F	2Y	L+	Pale	Tick	25	8.3	4.5	4.2	8.2	30	66	0	0	0	4
C183	4	F	2Y	M	Normal	NO	29	9.6	14	5	8.6	46	54	0	0	0	0
C184	4	F	3Y	M-	Pale	NO	31	10.3	6	5.4	6	18	82	0	0	0	0
C185	4	M	8M	M	Normal	NO	36	12	4	6.1	6.4	27	70	0	0	0	3
C186	4	M	3Y	M+	Normal	NO	25	8.3	8.9	4.2	6.8	38	62	0	0	0	0
C187	4	M	2Y	M	Normal	NO	31	10.3	5.5	5.1	6.8	25	74	1	0	0	0
C188	2	F	2Y	M	Normal	NO	39	13	9.4	6.5	6	15	85	0	0	0	0
C189	4	F	3Y	M	Normal	NO	39	13	10	7	6.8	38	60	0	0	0	2
C190	4	F	10M	M	Normal	NO	32	10.6	8.8	5.3	6.8	42	58	0	0	0	0
C191	2	M	4Y	M	Normal	NO	28	8.6	4	4.7	6.8	26	74	0	0	0	0
C192	2	F	2Y	M	Normal	NO	64	21.3	10.8	10.6	10	20	77	0	0	0	0
C193	2	F	2Y	M-	Pale	NO	44	14.6	10	7.4	7	18	82	0	0	0	0
C194	4	F	2Y	L+	Pale	NO	0	0	0	0	0	0	0	0	0	0	0
C195	2	F	5Y	M	Pale	Tick	39	13	14	6.6	8.2	50	49	0	1	0	0

C196	4	F	2Y	L+	Pale	Tick	37	12.3	16	6.1	7.2	35	65	0	0	0	0
C197	4	F	3Y	M	Normal	NO	25	8.3	6	4.2	7.6	32	64	0	0	0	4
C198	2	F	5Y	L+	Pale	NO	30	10	8	5.1	8.4	16	80	0	0	0	4
C199	4	F	7Y	M-	Pale	Tick	30	10	8	5.1	6.4	25	75	0	0	0	0
C200	4	F	6Y	M	Normal	Tick	38	12.3	10	6.3	8.2	33	65	2	0	0	0
C201	2	F	6Y	M	Normal	NO	30	10	9	5.3	8.4	45	50	0	3	0	2
C202	2	F	2Y	M	Normal	NO	30	10	6	5	6.9	45	50	0	0	0	5
C203	2	F	2Y	M-	Normal	NO	59	19.6	12	10	5.4	20	80	0	0	0	0
C204	2	M	3Y	M	Normal	NO	55	18.3	18	9.1	7	23	77	0	0	0	0
C205	2	F	5Y	M-	Normal	NO	0	0	0	0	0	0	0	0	0	0	0
C206	2	F	7Y	L+	Pale	NO	30	10	10	6	7.6	30	69	1	0	0	0
C207	4	M	10M	M	Normal	NO	30	10	10	6	7.6	30	69	1	0	0	0
C208	4	M	9M	M	Normal	NO	37	12.3	11.8	6.2	7.2	24	76	0	0	0	0
C209	4	F	8Y	F	Pale	Tick	27	9	8.4	4.6	7.6	36	64	0	0	0	0
C210	4	F	4Y	M	Normal	NO	26	8.6	14	4.3	6.2	24	76	0	0	0	0
C211	2	F	8M	M	Normal	NO	36	12	8	6	9	40	60	0	0	0	0
C212	4	M	3Y	M	Normal	NO	30	10	12	5	7.4	32	68	0	0	0	0
C213	2	F	7Y	M-	Pale	NO	31	10.3	8	5.2	10	55	40	3	0	0	2
C214	2	F	8Y	M-	Pale	NO	47	15.6	7	7.6	7.6	40	60	0	0	0	0
C215	2	F	7Y	M-	Pale	NO	50	16.6	4.8	8.2	7.4	30	70	0	0	0	0
C216	4	M	7Y	M	Normal	NO	51	17	15.2	8.6	7.4	49	51	0	0	0	0
C217	4	F	9M	M	Normal	NO	47	15.6	10	8	6	44	56	0	0	0	0
C218	4	F	6Y	M-	Pale	Tick	26	8.6	8.1	4.4	7.2	35	63	0	0	0	2
C219	4	F	11M	L+	Pale	NO	31	10.3	12	5.2	7.2	66	30	4	0	0	0
C220	4	M	4Y	M	Normal	Tick	31	10.3	5.1	5.3	6	33	67	0	0	0	0
C221	5	F	8Y	M	Pale	NO	28	9.3	8.1	4.5	8.8	34	65	1	0	0	0
C222	5	M	5Y	M	Normal	NO	31	10.3	7.4	5.3	8.2	32	68	0	0	0	0
C223	5	F	2Y	M	Normal	Tick	30	10	5.4	5	7	45	53	2	0	0	0

C224	5	M	5Y	M	Normal	Tick	30	10	6	5.2	8.4	55	44	1	0	0	0
C225	5	F	4Y	M	Normal	Tick	34	11.3	6.8	5.6	6	46	54	0	0	0	0
C226	5	F	2.5Y	L+	Normal	NO	38	12.6	6	6.2	7.6	26	74	0	0	0	0
C227	5	F	6Y	M	Normal	NO	39	13	6.8	7	6	28	70	2	0	0	0
C228	5	F	7Y	M	Normal	NO	36	12	7	6	8.4	38	52	4	4	0	2
C229	5	M	2Y	M+	Normal	NO	45	15	9.8	7.5	7.4	33	50	1	0	0	0
C230	5	F	4Y	M	Normal	NO	35	11.6	10.2	6	4.6	40	50	0	5	0	0
C231	5	F	4Y	M	Pale	Tick	24	8	7.4	4	6.8	45	45	0	6	1	3
C232	5	F	5Y	M	Normal	Tick	28	9.3	6.4	4.8	6.6	38	52	2	0	0	6
C233	5	F	2Y	M	Pale	NO	25	8.3	6.2	4.1	4.6	45	50	0	5	0	0
C234	5	M	4Y	M	Normal	Tick	32	10.6	8.2	5.2	7.8	40	60	0	0	0	0
C235	5	F	5Y	M+	Normal	Tick	34	11.3	8.2	5.9	7.4	30	65	5	0	0	0
C236	5	M	2Y	M	Normal	NO	37	12.3	11.4	6	5.4	45	45	2	6	0	2
C237	5	F	2Y	M	Normal	NO	30	10	10.2	5	7	30	60	3	5	0	5
C238	5	F	2Y	M	Normal	NO	34	11.3	9.9	6	6.4	35	55	3	5	0	2
C239	5	F	10Y	M	Pale	Tick	30	10	6.7	5	7.6	25	58	1	2	0	2
C240	5	M	2Y	M	Normal	Tick	29	9.6	8.9	5	7	30	60	2	4	1	3
C241	5	F	3Y	M	Normal	NO	31	10.3	9	5.1	7.4	25	64	6	5	0	0
C242	5	F	2Y	M	Normal	NO	30	10	15	5	6.6	15	85	0	0	0	0
C243	5	F	3Y	M+	Normal	Tick	50	16.6	7.1	8.3	7.8	22	78	0	0	0	0
C244	5	F	6Y	M	Pale	NO	18	6	11.2	3	7.4	10	85	2	0	0	3
C245	5	F	6Y	M	Normal	NO	49	16.3	9.6	8.1	9	23	75	2	0	0	0
C246	5	F	8Y	M	Normal	NO	54	18	13	9	8	18	82	0	0	0	0
C247	5	F	8Y	M	Normal	NO	41	13.6	4	7	3	25	65	4	6	0	0
C248	5	F	9Y	L+	Pale	NO	26	8.6	10	4.2	8.2	16	82	2	0	0	0
C249	5	F	10Y	M	Normal	NO	31	10.3	3.2	5.2	8.2	12	88	0	0	0	0
C250	5	M	2Y	M	Normal	NO	38	12.6	4.8	6.3	6.6	16	84	0	0	0	0

C251	5	M	3Y	M	Normal	NO	28	9.3	9	5.1	7.6	33	66	0	0	0	1
C252	5	F	7Y	M	Normal	NO	34	11.3	7	6	8.6	40	60	0	0	0	0
C253	5	F	5Y	M	Normal	NO	26	8.6	8.1	4.2	7.8	40	58	2	0	0	0
C254	5	F	4Y	M	Normal	NO	30	10	5.8	5.1	8.6	39	61	0	0	0	0
C255	5	F	1.5Y	M	Normal	NO	35	11.3	6.8	5.8	6.8	44	56	0	0	0	0
C256	5	F	6Y	M	Normal	NO	36	12	8.1	6	8.4	28	70	0	0	0	2
C257	5	M	1Y	M	Normal	NO	23	7.6	9.6	4	6.2	20	78	2	0	0	0
C258	5	F	7Y	M	Normal	NO	24	8	6.4	4.1	8.4	27	72	1	0	0	0
C259	5	M	3Y	L	Normal	NO	22	7.3	7.6	4	7.4	35	65	0	0	0	0
C260	5	F	5Y	M	Normal	NO	25	8.3	8	4.1	7.4	60	20	1	0	0	5
C261	5	F	5Y	M	Normal	NO	24	8	5.4	4	8.2	41	59	0	0	0	0
C262	5	M	3Y	M	Normal	NO	34	11.3	4	5.6	7	26	74	0	0	0	0
C263	5	F	6Y	M	Normal	NO	25	8.3	6.6	4.2	8	26	74	0	0	0	0
C264	5	F	5Y	M	Normal	NO	29	9.6	8	5	8.8	28	65	2	2	0	3
C265	5	F	3Y	M	Normal	NO	23	7.6	13	3.8	6.8	29	71	0	0	0	0
C266	5	F	4Y	M	Normal	NO	37	12.3	6.2	6.2	7.4	28	70	0	0	0	2
C267	5	M	3Y	L	Pale	NO	16	5.3	9.9	6	6	30	65	4	0	0	1
C268	5	F	1Y	M	Normal	NO	28	9.3	2.8	5	6	28	70	2	0	0	0

Sheep Hematology Results

Serial Coding No	Herd No.	Sex	Age	BCS	Mucous membrane	Ectoparasites	Haematological findings										
							PCV	Hb	WBC	RBC	Total Proteins	Neutrophil	Lymphocyte	Monocyte	Eosinophil	Basophil	Band
S1	1	M	2Y	M	Normal	Tick	42	14	4	7	8	24	76	0	0	0	0
S2	1	M	1Y	M	Pale	Tick	19	6.3	5.6	3.3	8.6	32	68	0	0	0	0
S3	1	F	4Y	M	Normal	NO	34	11.3	14	5.8	8.6	34	66	0	0	0	0
S4	1	F	5Y	L	Pale	NO	31	10.3	8	5.1	8	42	58	0	0	0	0
S5	1	F	5Y	M	Normal	NO	64	21.3	7	10.6	8	14	86	0	0	0	0
S6	1	F	5Y	M	Normal	NO	28	9.3	6	4.5	6.2	42	50	0	8	0	0
S7	1	F	3Y	M-	Pale	NO	27	9	6.2	4.5	7	35	60	0	5	0	0
S8	1	F	3Y	F	Pale	NO	35	11.6	8.1	5.9	6	40	60	0	0	0	0
S9	1	F	2Y	M	Normal	NO	23	7.6	8.4	4	6.2	36	60	0	0	0	4
S10	1	F	2Y	M	Normal	NO	28	9.3	8.1	4.6	6	28	72	0	0	0	0
S11	1	F	3Y	M	Normal	NO	36	12	6	6.2	5	16	84	0	0	0	0
S12	1	F	2Y	L	Pale	NO	43	14.3	5	7.1	4.6	20	80	0	0	0	0
S113	1	F	6Y	L	Pale	Tick	15	5	16	2.5	8	27	73	0	0	0	0
S114	1	F	2Y	L+	Normal	Tick	14	4.6	5.8	2.3	9	26	74	0	0	0	0
S115	1	M	1.5Y	M-	Normal	Tick	29	9.6	9	5	8.4	30	70	0	0	0	0
S116	1	F	1Y	L	Normal	Tick	12	4	9.1	2.1	2.4	20	78	0	0	0	2
S117	1	F	3Y	M	Normal	Tick	51	17	8	9	9	24	76	0	0	0	0
S118	1	F	2Y	M	Pale	Tick	16	5.3	14.2	2.8	8	16	84	0	0	0	0
S119	1	M	3M	M	Normal	NO	19	6.3	6.2	3.1	8.2	21	79	0	0	0	0
S120	1	F	2M	M	Normal	NO	11	3.6	2.4	2.2	3	31	78	1	0	0	0
S121	1	F	3Y	M	Normal	NO	31	10.3	6	5.2	6	45	53	2	0	0	0
S130	1	F	12m	M	Normal	Tick	26	8.6	8	4.2	6	15	80	0	0	0	5
S131	1	F	24m	M	Pale	Tick	16	5.3	16	3	6.6	30	70	0	0	0	0

S132	1	F	12m	M	Normal	Tick	18	6	8.1	3.1	6.4	40	60	0	0	0	0
S133	1	F	24	M	Normal	Tick	15	5	15	2.8	2.2	43	53	3	0	0	0
S134	1	F	24	M	Pale	Tick	11	3.6	2.6	1.9	3.8	18	75	4	2	0	1
S135	1	F	12	M	Normal	Tick	22	7.3	6	6	7	28	72	0	0	0	0
S136	1	F	24	M	Normal	Tick	10	3.3	4	2.1	3.8	18	80	0	0	0	2
S137	1	F	36	M	Normal	Tick	30	10	6	5.2	9	36	64	0	0	0	0
S151	1	F	3Y	M	Normal	Tick	20	6.6	17	3	6.2	25	74	1	0	0	0
S152	1	F	5Y	M	Normal	Tick	26	8.6	2.6	4.4	6	28	70	0	0	0	2
S153	1	F	1Y	M	Normal	NO	14	4.6	5.6	3	4.8	34	64	2	0	0	0
S154	1	F	5Y	M	Normal	Tick	23	7.6	10	4.1	6.2	20	80	0	0	0	0
S155	1	F	6M	M	Normal	Tick	29	9.6	8.1	5	9	27	69	1	3	0	0
S156	1	F	2Y	M	Normal	NO	16	6	9	3.1	4	60	37	3	0	0	0
S157	1	F	2Y	M	Normal	NO	22	7.3	9.2	4	6	18	80	0	0	0	2
S158	1	F	5Y	M	Normal	NO	11	3.6	5	2.2	4	16	84	0	0	0	0
S159	1	F	2Y	M	Normal	Tick	28	9.3	9	4.6	6.2	36	60	0	0	0	4
S160	1	F	3Y	M	Normal	Tick	24	8	7	3.8	6	48	52	0	0	0	0
S161	1	F	4Y	M	Normal	Tick	16	5.3	9	3.2	8	34	66	0	0	0	0
S162	1	F	3Y	M	Normal	Tick	13	4.3	13	2.2	6.8	70	28	0	0	0	2
S163	1	F	4Y	M	Normal	Tick	17	5.6	6	2.9	6.6	32	68	0	0	0	0
S164	1	F	3Y	M	Normal	NO	14	4.6	6	2.8	2.4	28	70	0	0	0	2
S165	1	F	4Y	M	Normal	NO	36	12	6	6.2	8	55	44	1	0	0	0
S166	1	F	4Y	M	Normal	NO	26	8.6	7.8	4.2	5.2	5.2	44	5	0	0	0
S167	1	F	4Y	M	Normal	NO	12	4	6	2	3.8	19	81	0	0	0	0
S168	1	F	6M	M	Normal	NO	22	7.3	6.7	3.9	6	10	90	0	0	0	0
S169	1	F	3M	M	Normal	NO	32	10.6	6.4	5.4	4	25	74	0	1	0	0
S170	1	F	3M	M	Normal	NO	26	8.6	6	4	8	65	30	5	0	0	0
S171	1	F	4M	M	Normal	NO	32	10.6	6	5.1	5	20	74	2	2	0	4

S172	1	M	3M	M	Normal	NO	21	7	7.2	3.6	3.6	13	79	2	5	0	1
S173	1	M	4M	M	Normal	NO	22	7.3	8	4	8	35	64	0	0	0	1
S174	1	M	5M	M	Normal	NO	25	8.3	13	4.1	9	15	84	0	1	0	0
S175	1	M	5M	M	Normal	NO	33	11	6	6.1	6.4	45	54	0	0	1	0
S176	1	F	5Y	M	Normal	NO	9	3	20.1	2	4	15	85	0	0	0	0
S177	1	F	4Y	M	Pale	NO	18	6	3	3.1	11	12	88	0	0	0	0
S178	1	F	1Y	M	Normal	NO	18	6	6.8	3	8	22	78	0	0	0	0
S81	2	F	1Y	M	Normal	NO	54	18	8.8	8.9	7.6	44	56	0	0	0	0
S82	2	F	2Y	L+	Pale	NO	38	12.3	6.8	6.3	8	30	66	2	0	0	2
S83	2	F	1Y	L+	Pale	Tick	35	11.6	8.2	6	8.2	15	80	0	0	0	5
S84	2	F	1Y	M	Normal	NO	51	16.6	7.4	8.6	7.8	31	69	0	0	0	0
S85	2	F	2Y	M	Normal	NO	42	14	8	7	7.8	27	70	0	3	0	0
S86	2	M	2Y	L	Pale	Tick	49	16.3	7.4	8.1	6.8	27	70	3	0	0	0
S87	2	F	2Y	L+	Pale	NO	31	10.3	4.9	5.6	6.8	19	81	0	0	0	0
S88	2	F	1Y	M-	Normal	NO	54	18	9	9.4	9	21	78	1	0	0	0
S89	2	F	2Y	M-	Normal	NO	30	10	8	4.8	6.8	30	67	0	2	0	1
S90	2	F	2Y	M	Normal	NO	38	12.6	6	6.2	5.6	33	67	0	0	0	0
S91	2	F	2Y	L+	Normal	Tick	46	15.3	20	7.6	7	40	60	0	0	0	0
S92	2	M	2Y	M-	Normal	NO	0	0	0	0	0	0	0	0	0	0	0
S93	2	M	1.5Y	M-	Normal	NO	28	9.3	12	4.6	9.2	27	68	0	5	0	0
S94	2	F	10M	L	Pale	Lice	13	4.3	14	2.1	6.6	12	73	0	1	0	0
S95	2	F	8M	L+	Normal	NO	30	10	6.8	5	6.4	30	70	0	0	0	0
S96	2	F	1Y	M-	Pale	Tick	28	9.3	8	6.8	7.4	28	72	0	0	0	0
S97	2	F	1Y	L+	Normal	NO	11	3	13.2	2	6.2	38	62	0	0	0	0
S98	2	F	1.5Y	M-	Pale	NO	33	11	12.3	5.8	7.2	46	54	0	0	0	0
S99	2	F	9M	M	Normal	NO	38	12.6	6	6	7.2	18	72	6	0	0	4
S100	2	F	2Y	M	Normal	Tick	27	9	12	4.6	7.2	28	70	2	0	0	0
S103	2	F	2Y	M-	Normal	NO	42	14	8.1	7	6.4	24	76	0	0	0	0

S105	2	F	3Y	M	Normal	NO	33	11	6.8	6	7.8	30	62	0	8	0	0
S106	2	M	10M	M	Normal	NO	36	12	6.8	6	68	16	84	0	0	0	0
S107	2	F	4Y	M	Normal	NO	31	10.3	5.8	5	6.8	23	68	0	9	0	0
S108	2	M	3Y	M	Normal	NO	33	11	6	5.6	6.8	60	37	1	0	0	2
S110	2	F	3Y	M	Normal	NO	51	17	14	9	7.2	20	76	4	0	0	0
S122	2	M	24	M	Normal	NO	39	13	6.8	6.6	8.2	34	66	0	0	0	0
S123	2	F	36	M	Normal	NO	44	14.6	8	7.3	6.7	28	72	0	0	0	0
S124	2	F	24	M	Normal	NO	34	11.3	7	5	8.2	45	53	2	0	0	0
S125	2	F	24	M	Normal	Tick	36	12	8	6	7.4	32	68	0	0	0	0
S126	2	F	24	M	Normal	NO	37	12.3	7.2	6.1	7	40	60	0	0	0	0
S127	2	F	A	M	Normal	NO	31	10.3	7	5.2	5.6	25	75	0	0	0	0
S128	2	F	Y	M	Normal	NO	25	8.3	4	4.1	7	40	60	0	0	0	0
S129	2	F	Y	M	Normal	NO	27	9	9	5	7.8	28	72	0	0	0	0
S138	2	F	A	M	Normal	Tick	27	9	6	5	8.1	31	65	0	3	0	1
S139	2	F	A	M	Normal	NO	26	8.6	7	4.2	6	30	70	0	0	0	0
S140	2	M	Y	M	Normal	NO	22	7.3	5.2	4	7	48	52	0	0	0	0
S141	2	F	A	M	Normal	Tick	23	7.6	6.24	33	7.6	65	2	0	0	0	0
S142	2	F	4Y	M	Normal	NO	20	6.6	6.8	3.4	6.4	18	82	0	0	0	0
S143	2	F	5Y	M	Pale	Tick	12	4	6	2.4	3.4	12	88	0	0	0	0
S144	2	F	4Y	M	Normal	NO	35	11.6	8.6	5.8	8.8	28	70	2	0	0	0
S145	2	M	1Y	M	Normal	Tick	31	10.3	8	5.1	6.4	33	66	1	0	0	0
S13	3	F	11M	M	Normal	NO	45	15	6	7	6.4	20	78	2	0	0	0
S14	3	F	3Y	M	Normal	NO	36	12	2	6	8.2	21	79	0	0	0	0
S15	3	F	1Y	M-	Pale	NO	32	10.6	8	5.6	5	30	70	0	0	0	0
S16	3	F	11M	M	Normal	NO	39	13	6	6.5	8	50	50	0	0	0	0
S17	3	M	1Y	L	Pale	NO	33	11	2.5	6	6.2	42	55	3	0	0	0
S18	3	F	1Y	M	Normal	NO	30	10	6.8	5.2	4.2	25	74	1	0	0	0
S19	3	F	1Y	M	Normal	NO	41	13.6	5.2	6.8	6.4	42	58	0	0	0	0

S20	3	M	2Y	M	Normal	NO	88	19.3	6	9.8	5.4	36	64	0	0	0	0
S21	3	F	2Y	M	Normal	NO	62	20.6	18	10.3	7.4	38	52	6	0	0	4
S22	3	F	2Y	M	Normal	NO	40	13.3	18.1	6.6	7.2	22	78	0	0	0	0
S23	3	F	2Y	M-	Pale	NO	60	20	4.6	10.1	7.2	31	69	0	0	0	0
S24	3	F	3Y	M	Normal	NO	64	21.3	5.2	11	6.4	23	77	0	0	0	0
S25	3	F	2Y	M-	Pale	NO	29	9.6	16	5	5.4	43	57	0	0	0	0
S26	3	F	2Y	L	Pale	NO	61	20.3	11	10.1	7	60	40	0	0	0	0
S27	3	F	2Y	L	Pale	NO	49	16.3	4.8	8.2	6.4	32	60	0	8	0	0
S28	3	M	3Y	M	Normal	Tick	44	14.6	5	7.4	7.2	41	56	2	0	0	1
S29	3	F	2Y	M	Normal	NO	33	11	9	3.5	6.2	19	80	1	0	0	0
S30	3	F	2Y	M-	Pale	NO	18	6	6	3	2	22	78	0	0	0	0
S31	3	F	2Y	M	Normal	NO	40	13.3	4.4	6.4	5	24	76	0	0	0	0
S32	3	F	11M	L	Pale	NO	39	13	6	6.6	7.2	45	55	0	0	0	0
S33	3	F	3Y	M	Normal	NO	34	11.3	8	5.8	6.4	26	66	1	7	0	0
S34	3	F	3Y	M	Normal	NO	31	10.3	8.2	5.3	6.6	30	64	6	0	0	0
S35	3	F	2Y	M	Normal	NO	35	11.6	8	6	7	24	70	6	0	0	0
S36	3	F	2Y	L	Pale	NO	25	8.3	6.6	4.2	7.8	40	60	0	0	0	0
S37	3	F	2Y	M-	Pale	NO	34	11.3	5.6	5.6	4.8	60	40	0	0	0	0
S38	3	F	2Y	M	Normal	NO	36	12	9.4	6	5.4	45	54	1	0	0	0
S39	3	M	10M	M	Normal	NO	45	15	8	7.6	8	44	55	1	0	0	0
S40	3	F	2Y	M	Normal	NO	27	9	5.8	4.6	7	40	60	0	0	0	0
S41	3	F	3Y	M-	Pale	NO	64	21.3	8	11	5	33	67	0	0	0	0
S42	3	F	2Y	M	Normal	NO	31	10.3	6	5.1	6	30	68	0	0	0	2
S43	3	F	3Y	L	Pale	NO	30	10	8.4	5.1	7	28	80	1	0	0	1
S44	3	F	3Y	M	Normal	NO	19	6.3	5	3.2	6.2	20	74	0	6	0	0
S45	3	M	6M	M	Normal	NO	51	17	4.8	8.5	5.8	23	75	1	0	0	1
S46	3	F	2Y	M	Normal	NO	57	19	7	9.4	6.4	22	78	0	0	0	0
S47	3	F	2Y	M-	Pale	NO	46	15.3	4.8	7.8	5.8	40	60	0	0	0	0
S48	3	F	2Y	M-	Pale	NO	40	13.3	4.8	5.9	6	34	66	0	0	0	0

S49	3	F	3Y	M	Normal	NO	30	10	8.2	5	8	23	77	0	0	0	0
S50	3	F	9M	M	Normal	NO	57	19	8	10	7.4	22	66	2	7	0	3
S51	3	F	1Y	M-	Pale	NO	52	17.3	8	8.6	6	34	60	0	6	0	0
S52	3	F	1Y	L	Pale	NO	42	14	6.7	7	6	23	77	0	0	0	0
S53	3	F	2Y	M	Normal	NO	40	13.3	8.2	6.7	5.6	34	66	0	0	0	0
S54	3	F	1Y	M	Normal	NO	31	10.3	7.8	5.3	5	13	86	1	0	0	0
S55	3	M	2Y	L	Pale	NO	33	11	6.1	6	7.2	30	70	0	0	0	0
S56	3	F	1Y	M	Normal	NO	26	8.6	8	4.2	6	33	66	1	0	0	0
S57	3	F	2Y	M	Normal	NO	26	8.6	8	4.3	5.8	24	76	0	0	0	0
S58	3	F	2Y	M	Normal	NO	27	9	6.7	5	8	18	82	0	0	0	0
S59	3	M	4Y	L	Pale	NO	22	7.3	8.4	5.3	6.4	36	61	3	0	0	0
S60	3	F	3Y	M-	Pale	NO	40	13.3	6	6.6	6.8	31	69	0	0	0	0
S61	3	F	2Y	M	Normal	NO	27	9	6	5	6	25	73	0	0	0	2
S62	3	F	2Y	L	Pale	NO	25	8.3	4.4	4.2	6.4	16	84	0	0	0	0
S63	3	F	1Y	M-	Pale	NO	23	7.6	6	3.9	7	35	64	1	0	0	0
S64	3	F	2Y	M	Normal	NO	29	9.6	7	3.6	7	42	58	0	0	0	0
S65	3	M	2Y	M	Normal	NO	30	10	6.4	5	5.6	25	75	0	0	0	0
S66	3	F	1Y	M	Normal	NO	35	11.6	9.2	5.9	5.4	44	56	0	0	0	0
S67	3	F	1Y	M	Normal	NO	44	14.6	6	7.3	6.5	33	67	0	0	0	0
S68	3	F	1Y	M	Normal	NO	30	10	6.8	5	8	13	87	0	0	0	0
S69	3	F	2Y	M	Normal	NO	45	15	8	8	8.2	69	31	0	0	0	0
S70	3	M	4Y	M	Normal	NO	24	8	8.1	4.1	6.2	13	87	0	0	0	0
S71	3	F	2Y	M-	Pale	NO	31	10.3	8	5.4	4.8	26	74	0	0	0	0
S72	3	F	3Y	M	Normal	NO	19	6.3	4.4	3.3	5.6	14	86	0	0	0	0
S73	3	F	2Y	M	Normal	NO	25	8.3	13	4.2	6	20	74	0	6	0	0
S74	3	F	3Y	M	Normal	NO	43	14.3	9	7	7	15	75	5	0	0	5
S75	3	M	3Y	M-	Pale	NO	22	7.3	8.6	3.6	7	42	58	0	0	0	0
S76	3	M	2Y	M	Pale	Tick	26	8.6	5	4.2	6.8	20	80	0	0	0	0
S77	3	M	3Y	M	Normal	NO	40	13.3	5	6.6	8	30	67	2	0	0	1

S78	3	M	11M	M	Normal	NO	54	18	14	9	6.2	38	62	0	0	0	0
S79	3	M	2Y	M-	Pale	NO	43	14.3	5	7.2	6.8	18	78	0	0	0	0
S80	3	M	1Y	M	Pale	NO	39	13	6.2	7	6.4	29	71	0	0	0	0
S101	4	F	7Y	M-	Normal	NO	30	10	6.2	5	6.4	24	76	0	0	0	0
S102	4	M	4Y	M-	Pale	NO	41	13.6	6.4	5.6	6.6	48	50	2	0	0	0
S104	4	F	5Y	M-	Pale	NO	35	11.6	6.4	6	7.2	18	80	0	0	0	2
S109	4	M	4Y	M	Normal	NO	25	8.3	6.8	4.1	7.4	45	53	2	0	0	0
S111	4	F	3Y	M	Normal	NO	30	10	6.8	5.4	6.8	34	66	0	0	0	0
S112	4	F	7Y	M-	Pale	Tick	34	11.3	8	5.1	8.2	50	50	0	0	0	0
S179	5	F	5Y	M	Normal	NO	41	13.6	11.7	6.7	6.8	37	60	0	3	0	0
S180	5	M	1Y	M	Normal	NO	24	8	5.6	4.1	6.4	25	70	3	2	0	0
S181	5	F	1Y	M	Normal	NO	24	8	5.6	4	6	28	52	4	1	0	2
S182	5	F	1Y	M	Normal	NO	15	5	3.1	3	4.8	25	67	2	1	1	0
S183	5	F	1Y	M	Normal	NO	20	6.6	4.1	3.4	6.4	50	46	0	4	0	0
S184	5	F	1Y	M	Normal	NO	39	13	10.3	7	6.4	30	70	0	0	0	0
S185	5	F	1.5Y	M	Normal	NO	32	10.6	9.2	5.2	6	30	64	2	2	0	2
S186	5	F	5M	M	Normal	NO	24	8	8.2	4	5	50	45	2	3	0	0
S187	5	F	1Y	M	Normal	NO	21	7	3.4	4	6.4	16	80	3	1	0	0
S188	5	F	1Y	M	Normal	NO	23	7.6	3.9	4	7	30	70	0	0	0	0
S189	5	F	5Y	M	Normal	NO	36	12	9.1	6	8.4	20	75	2	3	0	0
S190	5	M	1Y	M	Normal	NO	25	8.3	6.8	4.1	7	33	65	0	2	3	0
S191	5	F	4Y	M	Normal	NO	23	7.6	8.6	4	5.2	22	73	0	2	1	2
S192	5	F	1Y	M	Normal	NO	53	17.6	5	8.8	7	26	68	6	0	0	0
S193	5	F	5Y	M	Normal	NO	25	8.3	6	4.2	7.2	30	64	2	3	0	1
S194	5	F	1Y	M	Normal	NO	36	12	16	6	6	24	76	0	0	0	0
S195	5	F	1Y	M	Normal	NO	13	4.3	18	3	3	22	78	0	0	0	0
S196	5	F	7Y	M	Normal	NO	36	12	10	6	8.6	26	74	0	0	0	0
S197	5	F	1.5Y	M	Normal	NO	27	9	4	4.5	6	35	60	5	0	0	0

S198	5	F	3Y	M	Normal	NO	31	10.3	2.4	5.1	6	86	14	0	0	0	0
S199	5	F	1Y	M	Normal	NO	25	8.3	6	4.1	6.4	20	68	1	0	0	0
S200	5	M	1Y	M	Normal	NO	33	11	15	5.5	6	32	66	0	0	0	2
S201	5	M	1.5Y	M	Normal	NO	51	17	4	8.6	6.8	30	66	3	0	0	1
S202	5	M	1Y	M	Normal	NO	50	16.6	6.2	8.2	7	48	52	0	0	0	0
S203	5	F	7Y	M	Normal	NO	58	19.3	3.6	9.7	6	42	60	4	4	0	2
S204	5	F	7Y	M	Pale	NO	13	4.3	8.6	2.2	5.4	23	77	0	0	0	0
S205	5	M	6M	M	Normal	NO	24	8	3	4	4	27	70	3	0	0	0
S206	5	M	1Y	M	Normal	NO	23	7.6	5.8	4.1	6.4	45	55	0	0	0	0
S207	5	M	1Y	M	Normal	NO	29	9.6	8.6	5	6	38	60	0	0	0	2
S208	5	F	1Y	M	Normal	NO	30	10	10	5	6	42	58	0	0	0	0
S209	5	F	3Y	M	Normal	NO	33	11	8.2	6	6.6	25	70	5	0	0	0
S210	5	F	2Y	M	Normal	NO	28	9.3	12	4.6	7.2	40	60	0	0	0	0
S211	5	F	2Y	M	Normal	NO	27	9	9	5	7	35	65	0	0	0	0
S212	5	F	1Y	M	Normal	NO	18	6	7	3.1	5.8	33	67	0	0	0	0
S213	5	F	2Y	M	Normal	NO	20	6.6	6	3.3	6.6	28	72	0	0	0	0
S214	5	F	3Y	M	Normal	NO	22	7.3	9	3.8	7.6	25	75	0	0	0	0
S215	5	F	1Y	M	Normal	NO	23	7.6	8	3.9	7	20	70	3	6	0	0
S216	5	M	1Y	M	Normal	NO	19	6.3	7.8	3.2	5.6	48	50	0	0	0	2
S217	5	M	1Y	M	Normal	NO	29	9.6	10	4.8	7	30	70	0	0	0	0
S218	5	M	3M	M	Normal	NO	32	10.6	7.4	5.2	4	35	65	0	0	0	0
S219	5	M	2M	M	Normal	NO	24	8	6	4	5	42	58	0	0	0	0
S220	5	M	6M	M	Normal	NO	20	6.6	5.3	3.6	6	13	76	0	0	0	11
S221	5	M	7M	M	Normal	NO	28	9.3	8	5	6.6	36	64	0	0	0	0
S222	5	F	3Y	M	Normal	NO	30	10	6.8	5	7	36	62	2	0	0	0
S223	5	F	3Y	M	Normal	NO	21	7	9	4	9	45	55	0	0	0	0
S224	5	F	2Y	M	Normal	NO	30	10	8.6	5.1	7.4	25	70	2	0	0	3

S225	5	F	5Y	M	Normal	NO	28	9.3	6.4	4.7	6	38	62	0	0	0	0
S226	5	M	4M	M	Normal	NO	19	6.3	4	4	6.8	18	80	2	0	0	0
S227	5	F	1Y	M	Normal	NO	23	7.6	4	4	6	36	64	0	0	0	0
S228	5	M	5M	M	Normal	NO	26	8.6	6.2	4.2	7.6	40	60	0	0	0	0
S229	5	F	1Y	M	Normal	NO	24	8	6.8	4.3	6.8	36	63	1	0	0	0
S230	5	F	1Y	M	Pale	NO	18	6	8.1	3.2	8	15	80	1	0	0	4
S231	5	F	1Y	M	Normal	NO	20	6.6	13.2	3.3	7.6	37	63	0	0	0	0
S232	5	F	2Y	M	Normal	NO	26	8.6	11	4.1	6	30	70	0	0	0	0
S233	5	M	9M	M	Normal	NO	26	8.6	8	4.3	7	24	76	0	0	0	0
S234	5	F	6M	M	Normal	NO	20	6.6	16	3	6.4	40	60	0	0	0	0
S235	5	F	2Y	M	Normal	NO	27	9	7	5	8.4	16	84	0	0	0	0
S236	5	M	7M	M	Normal	NO	22	7.3	9.6	4	8.2	40	60	0	0	0	0
S237	5	F	1Y	M	Normal	NO	24	8	16	4	8.8	19	81	0	0	0	0
S238	5	F	5Y	M	Normal	NO	21	7	4.4	3.5	6.4	45	55	0	0	0	0
S239	5	F	7M	M	Normal	NO	11	3.6	4	1.6	3	25	74	1	0	0	0
S240	5	M	2M	M	Normal	NO	22	7.3	6	3.6	5.8	36	60	4	0	0	0
S241	5	F	7M	M	Normal	NO	29	9.6	10.2	4.8	7.2	48	52	0	0	0	0
S242	5	F	1Y	M	Normal	NO	25	8.3	8.9	4.2	6	46	54	0	0	0	0
S243	5	F	2Y	M	Normal	NO	24	8	8.1	4.1	6.4	60	40	0	0	0	0
S244	5	F	2Y	M	Normal	NO	20	6.6	16	3.3	6.6	43	57	0	0	0	0
S245	5	F	2Y	M	Normal	NO	22	7.3	6	3.9	7	38	60	2	0	0	0
S246	5	M	1Y	M	Normal	NO	30	10	8	5.1	6	45	45	5	3	0	2
S247	5	M	1Y	M	Normal	NO	32	10.6	4	5.3	7	21	70	6	3	0	0
S248	5	F	2Y	M	Normal	NO	27	9	4.4	5	6.6	45	55	0	0	0	0
S249	5	F	2Y	M	Normal	NO	21	7	9	4	7.8	46	50	4	0	0	0
S250	5	F	6M	M	Normal	NO	24	8	8	4	5.6	45	55	0	0	0	0
S251	5	F	5M	M	Pale	NO	18	6	6	3	4.4	40	60	0	0	0	0

S252	5	F	5M	M	Normal	NO	22	7.3	8	3.7	4.2	34	66	0	0	0	0
S253	5	F	1Y	M	Normal	NO	10	3.3	16.2	2.2	3.2	32	66	1	0	0	1
S254	5	M	1	M	Normal	NO	9	3	10	1.4	4	11	89	0	0	0	0
S255	5	F	2Y	M	Normal	NO	12	4	6	3	3.2	28	72	0	0	0	0
S256	5	M	3M	M	Normal	NO	28	9.3	9.1	4.8	6.6	35	53	4	6	0	2
S257	5	M	8M	M	Normal	NO	26	8.6	4.9	4.2	5.4	23	75	2	0	0	0
S146	6	M	6M	M	Normal	NO	12	4	5.6	2.1	4.6	45	55	0	0	0	0
S147	6	F	4Y	M	Normal	NO	62	20.6	8	10	5.4	45	55	0	0	0	0
S148	6	F	2M	M	Normal	NO	24	8	8	4	7.8	23	66	1	0	0	0
S149	6	F	2Y	M	Normal	NO	26	8.6	8	4.2	6.8	30	70	0	0	0	0
S150	6	M	1Y	M	Normal	NO	19	6.3	12.6	3.3	6	42	58	0	0	0	0

Goats Hematology Results

Serial Coding No	Herd No.	Sex	Age	BCS	Mucous membrane	Ectoparasites	Haematological findings										
							PCV	Hb	WBC	RBC	Total Proteins	Neutrophil	Lymphocyte	Monocyte	Eosinophil	Basophil	Band
G1	1	F	9M	M	Normal	NO	18	6	9	3	4.2	25	74	0	0	0	0
G2	1	F	2Y	M	Normal	NO	25	8.3	3.4	4.1	8	29	61	1	9	0	0
G3	1	F	3Y	M	Normal	NO	14	4.6	6.8	2.6	4.8	38	60	2	0	0	0
G4	1	F	3Y	M-	Pale	NO	51	17	6.8	9	7	30	70	0	0	0	0
G5	1	F	4Y	M	Normal	NO	37	12.3	2.8	6.3	9.2	30	65	5	0	0	0
G6	1	F	4Y	M	Normal	Tick	34	11.3	10	5.6	6.8	32	68	0	0	0	0
G7	1	F	4Y	M-	Pale	Tick	26	8.6	2.8	4.3	6	28	72	0	0	0	0
G8	1	F	3Y	M	Normal	NO	29	9.6	9	5	6	24	76	0	0	0	0
G9	1	F	1Y	M	Normal	NO	23	7.6	8.4	3.9	5	28	82	0	0	0	0
G10	1	F	3Y	M	Normal	NO	26	8.6	6.2	4.4	6	26	67	0	0	0	0
G11	1	M	2Y	M-	Pale	NO	45	15	6.6	8.1	6.2	20	65	1	0	0	0
G12	1	F	3Y	M-	Pale	NO	60	20	9	9.9	7	30	69	1	0	0	0
G13	1	F	2Y	M	Normal	NO	40	13.3	6.8	6.8	7	27	73	0	0	0	0
G14	1	F	3Y	M	Normal	Tick	50	16.6	9.6	8.3	5	36	63	0	0	0	1
G15	1	F	2Y	L	Pale	NO	21	7	6.8	3.6	5	45	55	0	0	0	0
G16	1	F	2Y	M	Normal	NO	35	11.6	5	5.8	5.8	28	72	0	0	0	0
G17	1	F	2Y	M	Normal	Tick	38	12.6	8	6.4	6.2	20	76	0	4	0	0
G18	1	F	2Y	M	Normal	NO	48	16	6	8	7	12	88	0	0	0	0
G19	1	F	2Y	M-	Pale	NO	29	9.6	16	4.9	6.8	23	67	0	0	0	0
G20	1	M	2Y	M	Normal	NO	31	10.3	8.6	5.2	5.8	68	27	0	4	0	1
G21	1	F	2Y	M	Normal	NO	31	10.3	8.9	5.2	7	34	66	0	0	0	0
G22	1	F	2Y	M	Normal	NO	21	7	8.4	3.8	6	27	73	0	0	0	0
G23	1	F	3Y	M	Normal	NO	40	13	8	6.8	6.2	28	70	0	2	0	0

G24	1	F	3Y	M	Normal	NO	34	11.3	8.4	6	6.4	17	83	0	0	0	0
G25	1	F	2Y	M	Normal	NO	29	9.6	2.9	4.8	7.8	18	82	0	0	0	0
G26	3	M	1Y	M	Normal	NO	32	10.6	10.2	5.2	7.4	33	67	0	0	0	0
G27	3	M	1Y	L	Pale	NO	21	7	9.2	3.8	6	24	69	0	7	0	0
G28	3	F	1Y	M	Normal	NO	36	12	9	6.6	6	36	66	0	0	0	1
G29	3	F	1Y	M	Normal	NO	58	19.3	12.6	9.5	5.4	24	76	0	0	0	0
G30	3	F	1Y	M	Normal	NO	55	18.3	4	9.1	5.2	28	68	2	0	0	2
G31	3	F	1Y	M	Normal	NO	40	13.3	4.5	6.5	6	30	56	0	4	0	0
G32	3	F	9M	L	Pale	NO	54	18	6.6	9	5	26	74	0	0	0	0
G33	3	M	1Y	M-	Pale	NO	57	19	4.8	10	6.2	24	76	0	0	0	0
G34	3	F	1y	L	Pale	NO	64	21.3	9.2	10	6.4	20	76	4	0	0	0
G35	3	F	1Y	M	Normal	NO	41	13.6	6.8	7	5.4	13	86	1	0	0	0
G36	2	F	3Y	M	Normal	NO	45	15	6.2	8	6	25	74	1	0	0	0
G37	4	F	6Y	M	Pale	Tick	44	14.6	5	7.3	6	32	67	1	0	0	0
G38	4	F	6Y	M-	Pale	NO	49	16.3	10.3	8.1	9.8	35	65	0	0	0	0
G39	4	F	3Y	M	Normal	NO	24	8	10	4	5.8	24	73	0	0	0	3
G40	4	F	3Y	M-	Pale	NO	15	5	11	3	4.8	14	76	0	10	0	0
G41	2	F	1Y	M-	Normal	NO	50	16.6	8.8	8.4	8.8	15	80	0	5	0	0
G42	2	M	1Y	M-	Normal	NO	14	4.6	15	2.3	7	13	87	0	0	0	0
G43	4	M	2Y	L+	Pale	Tick	37	12.3	12	6.1	8.2	15	80	4	0	0	1
G44	4	F	2Y	M	Normal	NO	17	5.6	18	3.1	6.4	24	73	2	0	0	1
G45	4	F	7M	M-	Normal	NO	34	11.3	8	5.6	7.2	24	76	0	0	0	0
G46	2	F	1Y	M-	Pale	NO	41	13.6	6.8	6.9	8	18	82	0	0	0	0
G47	2	F	2Y	M-	Normal	NO	32	10.6	8	5.8	6	30	65	2	0	0	3
G48	2	F	2Y	M	Normal	NO	56	18.6	6.8	9	8.4	45	52	0	3	0	0
G49	4	M	2Y	M-	Normal	NO	61	20.3	16.2	10.1	8.2	14	80	0	6	0	0
G50	4	F	3Y	M-	Pale	NO	31	10.3	10.8	5.1	6.4	25	72	3	0	0	0
G51	2	F	2Y	M-	Normal	NO	30	10	9.6	5.5	6.4	14	71	0	14	0	1
G52	2	F	2Y	M	Normal	Tick	38	12.6	9	6.3	7.4	32	68	0	0	0	0

G53	2	M	3Y	M	Normal	Tick	38	12.6	16	6.2	5.8	40	60	0	0	0	0
G54	2	F	1.5Y	M	Normal	NO	32	10.6	10	5.3	8	23	77	0	0	0	0
G55	4	F	2Y	M-	Normal	NO	43	14.3	13	7.1	6	24	83	3	0	0	0
G56	4	M	1Y	M-	Normal	NO	26	8.6	14	4.3	7	46	54	0	0	0	0
G57	4	F	3Y	M	Normal	NO	42	14	14	7	6.8	35	65	0	0	0	0
G58	4	F	1.5Y	M-	Normal	NO	27	9	10.2	4.6	7.8	40	55	5	0	0	0
G59	4	M	1Y	M-	Pale	NO	38	12.6	5.1	6.5	6.6	28	72	0	0	0	0
G60	2	F	1.5Y	M	Normal	NO	13	4.3	12	2.2	6.4	50	45	4	0	0	1
G61	6	F	5Y	L	Pale	Tick	11	3.6	3	2.1	4.4	35	65	0	0	0	0
G62	6	F	3Y	M	Normal	NO	30	10	11	5.1	5.6	40	60	0	0	0	0
G63	6	F	3Y	M	Normal	NO	24	8	6.2	3.9	6.2	40	60	0	0	0	0
G64	6	F	5M	L+	Pale	NO	9	3	17	2	6	15	85	0	0	0	0
G65	6	M	9M	M	Normal	NO	21	7	3.8	3.9	6	50	45	0	5	0	0
G66	6	F	6Y	M	Normal	NO	22	7.3	6	6	7	28	72	0	0	0	0
G67	6	F	1Y	M	Normal	NO	25	8.3	10.9	4	6.2	42	58	0	0	0	0
G68	6	F	9M	M	Normal	NO	31	10.3	8	4	7.8	30	70	0	0	0	0
G69	6	F	1Y	M	Normal	NO	18	6	15	3.6	6.4	30	70	0	0	0	0
G70	6	F	9M	M	Normal	NO	22	7.3	13	3.6	6.4	38	60	2	0	0	0
G71	6	F	1.5Y	M	Normal	NO	19	6.3	6.2	3.1	4.2	16	82	2	0	0	0
G72	6	F	2Y	M	Normal	NO	32	10.6	9	5.4	7	40	60	0	0	0	0
G73	6	F	6M	M-	Pale	NO	16	5.3	16	3	7.4	33	67	0	0	0	0
G74	6	F	2Y	M	Normal	NO	21	7	6	4	5.8	33	65	2	0	0	0
G75	6	F	6M	M	Normal	NO	23	7.6	12	3.5	6	53	47	0	0	0	0
G76	6	F	3M	M	Normal	NO	29	9.6	10	4.8	6.2	43	57	0	0	0	0
G77	6	M	6M	M	Normal	NO	27	9	9	4.5	7.2	38	62	0	0	0	0
G78	6	M	6M	L	Pale	NO	15	5	14.1	3	6.2	25	74	1	0	0	0
G79	6	F	6M	M	Normal	NO	15	5	4	2.6	6.2	22	78	0	0	0	0
G80	6	F	4Y	M	Normal	NO	28	9.3	6	4.6	6.2	36	62	2	0	0	0

G81	6	F	4Y	M	Normal	NO	16	5.3	12.6	2.9	4.4	60	34	0	0	0	5
G82	6	F	1.5Y	M	Pale	NO	14	4.6	3	2.4	6.4	28	72	0	0	0	0
G83	6	F	1Y	M	Normal	NO	15	5	9	2.6	9	31	69	0	0	0	0
G84	6	F	4Y	M	Pale	Tick	12	4	4	2	6	42	58	0	0	0	0
G85	6	F	5M	M	Normal	NO	19	6.3	6	3.1	4.8	38	62	0	0	0	0
G86	6	F	5M	M	Normal	NO	24	8	3	4.2	7.6	30	70	0	0	0	0
G87	6	F	5M	M	Normal	NO	18	6	14	3.1	6	45	55	0	0	0	0
G88	6	M	6M	M	Normal	Tick	16	5.3	12.6	2.9	4.4	60	34	0	0	0	5
G89	6	F	1Y	M	Normal	NO	18	7	17	4	8.2	28	72	0	0	0	0
G90	6	F	3Y	M	Normal	NO	23	7.6	4	4	6.4	40	60	0	0	0	0
G91	6	F	4Y	M	Normal	NO	26	8.6	12	4	6.4	40	60	0	0	0	0
G92	6	F	2Y	M	Normal	NO	14	4.6	18	2.4	7	33	65	2	0	0	0
G93	6	F	4Y	M	Normal	NO	15	5	9.2	3	5.4	19	81	0	0	0	0
G94	6	F	1Y	M	Normal	NO	22	7.3	9.2	4	6	18	80	0	0	0	2
G95	6	F	3Y	M	Normal	Tick	14	4.6	10.2	2.4	3	28	72	0	0	0	0
G96	6	F	4M	L	Pale	NO	10	3.3	3	1.8	7.2	46	54	0	0	0	0
G97	6	F	3M	M	Normal	NO	25	8.3	4.8	4.1	4.8	33	67	0	0	0	0
G98	6	F	3Y	M	Normal	NO	21	7	8.1	4	7.4	39	61	0	0	0	0
G99	6	F	6Y	M	Normal	NO	22	7.3	9	4	8	30	68	2	0	0	0
G100	6	F	1Y	M	Normal	NO	28	9.3	16	5	7	65	35	0	0	0	0
G101	6	F	6M	M	Normal	NO	16	5.3	8	2.7	4	26	74	0	0	0	0
G102	6	M	5M	M	Normal	Tick	16	5.3	8	2.7	4	26	74	0	0	0	0
G103	6	F	4Y	M	Normal	Tick	11	3.6	5	2	6.4	48	50	2	0	0	0
G104	6	F	2Y	M	Pale	NO	10	3.3	5	2	5.6	38	60	2	0	0	0
G105	6	F	2Y	M	Normal	NO	22	7.3	4.8	4	6	25	75	0	0	0	0
G106	6	F	3Y	M	Normal	NO	21	7	4	4	5.2	33	65	2	0	0	3
G107	6	F	3Y	M	Normal	NO	18	6	4.8	3.3	6	25	75	0	0	0	0

G108	6	F	4Y	M	Normal	NO	18	6	9	3	4.6	20	80	0	0	0	0
G109	6	F	6M	M	Normal	NO	25	8.3	6	4.2	6	39	61	0	0	0	0
G110	6	F	8Y	M	Normal	NO	38	12.6	6	6.2	6	33	67	0	0	0	0
G111	6	F	2Y	M	Normal	NO	27	9	2.6	5.1	4.6	26	74	0	0	0	0
G112	6	F	1Y	M	Pale	NO	13	4.3	4	2.3	5.8	16	84	0	0	0	0
G113	6	M	6M	M	Normal	NO	20	6.6	17	3	6.2	25	74	1	0	0	0
G114	6	F	4Y	M	Normal	NO	25	8.3	6	4.1	7	13	72	5	2	0	8
G115	6	F	3Y	M	Normal	NO	17	5.6	6.1	3	4.8	12	88	0	0	0	0
G116	6	F	7M	M	Normal	NO	15	5	9	2.4	4	12	88	0	0	0	0
G117	6	F	2Y	M	Normal	NO	24	8	10	4	8.2	27	74	0	0	0	2
G118	6	M	4M	M	Normal	NO	21	7	17	4	6.4	28	72	0	0	0	0
G119	6	F	3Y	M	Normal	NO	22	7.3	8	4	6.2	24	76	0	0	0	0
G120	6	F	3Y	M	Normal	NO	17	5.6	12	2.9	6.2	20	80	0	0	0	0
G121	6	F	3Y	M	Normal	NO	18	6	15	3.6	6.4	30	70	0	0	0	0
G122	6	F	2Y	M	Normal	NO	10	3.3	8	2	6	60	33	2	0	0	5
G123	6	F	3Y	M	Normal	Tick	16	5.3	6	2.6	2.2	22	78	0	0	0	0
G124	6	M	5M	M	Normal	NO	55	18.3	6.2	9.2	5.2	48	52	0	0	0	0
G125	6	F	4M	M	Normal	NO	18	6	22	5	5	30	70	0	0	0	0
G126	6	F	2Y	M	Normal	NO	39	13	8.1	7	5.2	30	65	2	0	0	3
G127	6	F	2Y	M	Pale	NO	16	5.3	14	3.6	3.8	51	49	0	0	0	0
G128	6	F	4Y	M	Normal	NO	16	5.3	4.5	2.8	4.8	24	76	0	0	0	0
G129	6	M	1Y	M+	Normal	Tick	20	6.6	20.1	3.4	5	6	74	0	0	0	0
G130	6	F	3Y	M	Normal	NO	15	5	4.6	2.5	6.2	15	85	0	0	0	0
G131	6	F	2Y	M	Normal	NO	15	5	5	3.1	6	34	66	0	0	0	0
G132	6	F	6M	M	Normal	NO	34	11.3	10	5.6	5.8	40	60	0	0	0	0
G133	6	F	4Y	M	Normal	NO	25	8.3	4.2	4.1	4	16	84	0	0	0	0
G134	6	F	2Y	M	Normal	NO	16	5.3	2.6	2.6	4	14	86	0	0	0	0

G135	6	M	3M	M+	Normal	NO	40	13.3	6.9	6.7	6	44	66	0	0	0	0
G136	6	F	2Y	M	Normal	NO	17	5.6	3.6	3	6.4	26	74	0	0	0	0
G137	6	F	2M	M	Normal	NO	21	7	3	4	3.8	20	80	0	0	0	0
G138	6	F	2Y	M	Normal	NO	25	8.3	4	4.1	6.8	48	52	0	0	0	0
G139	6	F	2M	L+	Normal	NO	25	8.3	2	4.2	6	18	82	0	0	0	0
G140	6	F	2M	M	Pale	NO	14	4.6	6	2.6	4	14	86	0	0	0	0
G141	6	F	6M	M	Normal	NO	21	7	4	4	5.4	36	64	0	0	0	0
G142	6	F	6M	M	Normal	NO	20	6.6	14	3.4	6.4	30	74	3	1	0	2
G143	6	F	2Y	M-	Normal	Tick	19	6.3	19	3.4	6.2	18	82	0	0	0	0
G144	6	F	3Y	M	Normal	NO	17	5.6	14	3	5.2	18	80	2	0	0	0
G145	6	F	5Y	M	Normal	NO	16	5.3	6	2.6	2.2	22	78	0	0	0	0
G146	6	F	3Y	M	Normal	NO	13	4.3	5	2.4	5.8	14	81	0	0	0	0
G147	6	F	4M	M	Pale	NO	15	5	12	3	4.4	27	73	0	0	0	0
G148	6	M	4M	M	Normal	NO	28	9.3	9	5	6.4	40	58	0	0	0	2
G149	6	F	4Y	M	Normal	NO	28	9.6	6	5	6.2	46	54	0	0	0	0
G150	6	F	2M	M	Normal	NO	51	17	9	9	6.4	55	40	2	3	0	0
G151	6	M	2M	M	Normal	NO	28	9.3	8.2	5	5	28	72	0	0	0	0
G152	6	F	4Y	M	Normal	NO	22	7.3	8.6	4	6.4	28	70	2	0	0	0
G153	6	F	4Y	M	Normal	Tick	16	5.3	6	3.1	4.2	38	62	0	0	0	0
G154	6	F	2Y	M	Normal	NO	15	5	3	2.8	2.2	60	39	1	0	0	0
G155	6	M	1Y	M-	Normal	NO	11	3.6	18	2.2	4.2	33	67	0	0	0	0
G156	6	F	2Y	M	Normal	NO	18	6	18.2	3.3	6.4	40	60	0	0	0	0
G157	6	F	2Y	M	Normal	NO	22	7.3	5	5	6.2	34	66	0	0	0	0
G158	6	F	2Y	M	Normal	NO	28	9.3	6	3.2	7	24	76	0	0	0	0
G159	6	F	1.5Y	L+	Normal	NO	32	10.6	5.4	5.1	7.4	45	55	0	0	0	0
G160	6	F	3Y	M	Normal	NO	10	3.3	14	3	7.2	45	53	0	0	0	2
G161	6	F	1Y	M	Normal	NO	34	11.3	8	5.7	6.2	45	54	1	0	0	0

G162	6	F	5Y	M	Pale	NO	18	6	15	3.6	6.4	30	70	0	0	0	0
G163	6	F	2Y	M	Normal	NO	24	8	8	4.6	5	31	69	0	0	0	0
G164	6	F	2Y	M+	Normal	NO	45	15	12	8	6.2	26	74	0	0	0	0
G165	6	F	1Y	M	Normal	NO	22	7.3	8.6	4	6.4	28	70	2	0	0	0
G166	6	F	6M	M	Normal	NO	13	4.3	17	2.1	7.4	40	60	0	0	0	0
G167	6	F	3Y	M	Normal	NO	21	7	5.1	3.6	5.8	40	55	3	0	0	2
G168	6	F	2Y	M	Normal	NO	24	8	5.1	4	4.8	34	66	0	0	0	0
G169	6	F	6M	M	Normal	NO	20	6.6	20.1	3.4	5	6	74	0	0	0	0
G170	6	F	2Y	M	Normal	NO	23	7.6	6	4	8	33	66	1	0	0	0
G171	6	F	4M	M+	Normal	NO	36	12	10.2	6.1	7	37	57	3	3	0	0
G172	6	F	2Y	M	Normal	NO	25	8.3	9	4.6	6	26	74	0	0	0	0
G173	6	F	2Y	M	Normal	NO	11	3.6	11	3	6.2	40	60	0	0	0	0
G174	6	F	8M	M	Normal	NO	25	8.3	2	4.2	6	18	82	0	0	0	0
G175	6	F	3Y	M	Normal	NO	21	7	4.8	3.8	7	32	68	0	0	0	0
G176	6	F	4M	M	Normal	NO	44	14.6	6	7.3	7	30	60	7	0	0	3
G177	6	F	4Y	M	Normal	NO	31	10.3	8.1	5.1	7	20	75	0	0	0	5
G178	6	M	1.5Y	M	Normal	NO	22	7.3	6.6	3.8	5.6	15	74	2	3	0	1
G179	6	F	2Y	M	Normal	NO	35	11.6	6	6	5	37	63	0	0	0	0
G180	6	F	9M	M	Normal	NO	32	10.6	9	5.4	7	40	60	0	0	0	0
G181	6	F	9M	M	Normal	NO	48	16	6.4	5.1	6	45	54	1	0	0	0
G182	6	F	2Y	M	Normal	NO	26	8.3	10	4.2	6.8	28	72	0	0	0	0
G183	6	F	2Y	M	Normal	NO	24	8	6.2	4.1	7.2	22	78	0	0	0	0
G184	6	F	1Y	M	Pale	NO	8	2.6	10	2.1	6	12	86	0	0	0	4
G185	6	F	2Y	M	Normal	NO	26	8.3	6	4.3	7.8	30	67	3	0	0	0
G186	6	F	2Y	M	Normal	NO	33	11	10.2	6	5.2	40	60	0	0	0	0
G187	6	F	1Y	M	Normal	NO	26	8.6	7.4	4	4.8	30	70	0	0	0	0
G188	6	F	6M	M	Normal	NO	29	9.6	11	4.6	8	16	84	0	0	0	0

G189	6	F	2Y	M	Normal	NO	29	9.6	11	4.6	8	16	84	0	0	0	0
G190	6	F	1.5Y	L	Normal	NO	14	4.6	14.4	2.8	6.8	62	30	2	0	0	2
G191	6	F	9M	M	Pale	NO	15	5	8	2.6	5.4	25	70	0	0	0	5
G192	6	F	6M	M	Normal	NO	14	4.6	18	2.4	7	33	65	2	0	0	0
G193	2	M	8M	M	Normal	NO	20	6.6	17	3	6.2	25	74	1	0	0	0
G194	2	F	5Y	M	Normal	NO	16	5.3	16	3	7.4	33	67	0	0	0	0
G195	2	F	2Y	M	Normal	NO	23	7.6	10	4.1	6.2	20	80	0	0	0	0
G196	2	F	1Y	L	Normal	NO	25	8.3	9	4.6	6	26	74	0	0	0	0