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The distribution of *Babesia bigemina* and *Babesia bovis* transmitted by *Rhipicephalus* spp. on a farm in the Eastern Cape.

By

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Submitted in fulfilment of the requirements in respect of the

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Supervisor: Ms. EMSP van Dalen

DECLARATION

I, Abrè Marais declare that the Master's degree research dissertation that I herewith submit for the Master's degree qualification, MSc. Entomology at the University of the Free State is my independent work and that I have not previously submitted it for a qualification at another institution of higher education.

Signed:  Date: 24-01-2020

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ABBREVIATIONS

°C- degree Celsius

Bp- Base pairs

CTAB- Cetyltrimethylammonium-bromide

DNA- Deoxyribonucleic acid

EDTA- Ethylenediaminetetraacetic acid

ELISA- enzyme-linked immunosorbent assay

g- gram

IFA- Indirect fluorescent antibody

M- molar

ml- millilitres

mM- milli-molar

NaCl- Sodium chloride

PCR- Polymerase chain reaction

PVP- Polyvinylpyrrolidone

QBC- Quantitative buffy coat

RBC- Red blood cells

RNase- Ribonuclease

SOP- Standard Operating Procedure

TBE- Tris-borate-EDTA

TE- Tris-EDTA

Tris- Trisaminomethane

µg- microgram

µl- microliter

UV- ultraviolet

v/v- volume per volume

ETHICAL STATEMENT

The organisms that were tested in this study, the adult blue ticks, were removed from their natural host, cattle. Ticks are ectoparasites and thus their removal did not have a negative effect on the ecosystem and was of an advantage to the cattle and the producer. Small amounts of blood were extracted with clean needles from cattle, which did not have a lasting effect on the animals. The study collections were conducted during routine farming practices in a familiar environment. The producer and farm workers were present at the collections in order to create a familiar environment. Minimal contact was made with the cattle and collections occurred as quickly as possible. Any animal that exhibited excessive physical distress was released from the cattle crush and not used in the study.

Ethical clearance was obtained from the Animal Ethics Committee of the University of the Free State. **Student project number: AED2017/0034**

***See Appendix 1 for Ethical Clearance document and Appendix 2 for producers consent form.**

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Abstract

Rhipicephalus (Boophilus) microplus, the Asiatic blue tick, was introduced from Madagascar to South Africa during 1896, after a rinderpest epidemic. Displacement of *Rhipicephalus (Boophilus) decoloratus*, the African blue tick, by *R. (B.) microplus* has been reported in the Eastern Cape and Limpopo provinces. These two tick species are known vectors for protozoan bovine blood parasites. With the introduction of *R. (B.) microplus*, *Babesia bovis,* one of these parasites*,* was also introduced into South Africa*. Babesia bovis* is more virulent than *B. bigemina,* the native species. Control of the disease, babesiosis, caused by these blood parasites, is mainly accomplished by control of the tick vector with chemicals. This however is becoming less effective due to emerging resistance of both tick species to these chemicals. Methods for detecting *B. bigemina* and *B. bovis* are therefore becoming all the more important to be able to detect outbreaks early, as well to determine new areas where both the vectors and blood parasites are present.

The aim of this study was to confirm the presence of both vector species, *R. (B.) decoloratus* and *R. (B.) microplus*, on an Eastern Cape farm after reports of possible *B. bovis* infections. Larvae, collected by field drags, and adult ticks, collected from cattle, were identified morphologically by using a dissection microscopes. The presence of the protozoan parasites *B. bigemina* and *B. bovis* in the blood of cattle hosts was further investigated through Giemsa-stained blood smears from blood samples, collected from 10% of the cattle on the farm. DNA extractions and PCR were performed on the progeny of adult ticks and blood collected from cattle hosts, to scan for *Babesia* infections.

Morphological identification of larval and adult ticks indicated that both *R. (B.) decoloratus* and *R. (B.) microplus* were present on the test farm. Over the study period, *R. (B.) microplus* was found in all camps investigated but *R. (B.) decoloratus* was present in significantly higher numbers than *R. (B.) microplus* with 97% ($P = 0.0000402$) of the larvae and 98% ($P = 0.000041$) of the adults collected, identified as *R. (B.) decoloratus*. Displacement of *R. (B.) decoloratus* by *R. (B.) microplus* thus did not take place since the first discovery of *R. (B.) microplus* on the farm during 2014.

By means of Polymerase chain reactions the presence of *B. bigemina* was found in two adult ticks and *B. bovis* in one. The blood smears yielded one positive *B. bovis* identification in the blood of one host animal that was confirmed with PCR. DNA extracted and PCR performed on a second blood sample showed one animal host with a double infection of both *B. bigemina* and *B. bovis*. The presence of both parasite species, although at a low frequency of 1.85% for *B. bovis* and 1.08% for *B. bigemina*, was also confirmed for this farm. It was however only identified from three camps of the 11 camps tested.

This study confirmed the prevalence of both of the tick vector species well as the pathogens they transmit on this commercial farm. The presence of *R. (B.) microplus* and *B. bovis*, currently still present in low numbers, should be monitored for potential further distribution of this parasite to prevent unexpected outbreaks of babesiosis and the financial implication it can cause.

Key words: Polymerase Chain Reaction (PCR), blue ticks, cattle, blood smears, *Babesia*, Babesiosis, larvae, adults, lifecycle, DNA

Chapter 1: Introduction

Ticks are obligate blood-feeding ecto-parasites that feed on mammals, birds, and reptiles (Walker *et al.* 2003). They also have the ability to transfer blood-parasites to the host animals they are feeding on. African babesiosis disease, caused by *Babesia bigemina* and transmitted by *Rhipicephalus (Boophilus) decoloratus* as well as by *Rhipicephalus (Boophilus) microplus* and the Asiatic babesiosis, caused by *Babesia bovis* and transmitted by *R. (B.) microplus,* are two tick-transmitted diseases that play an important role in the cattle-farming industry in South Africa. Due to a lack of alternative options, ticks as vectors of these diseases, are controlled in order to prevent transmission thereof. With approximately 186 million cattle in Africa running the risk of infection, information pertaining to these diseases is of considerable economic importance (Madder *et al.* 2013, Schroder & Reilly 2013).

These diseases result in economic expenses due to animal deaths, milk and meat production losses, abortions, treatment and control costs of the diseases as well as international trade embargos (Bock *et al.* 2004). Total economic losses caused by babesiosis and anaplasmosis in the cattle industry amounted to 5.1 million US dollars in Kenya, 5.4 million in Zimbabwe, 68 million in Tanzania, 21.6 million in South Africa, 19.4 million in China, 57.2 million in India, 3.1 million in Indonesia and 0.6 million in Philippines, annually during the late 1990's (Bock *et al.* 2004). Although no recent studies indicated the full extent of the economic impact of these ticks and diseases, these figures still indicate the severity of the potential economic loss.

According to Tønnesen *et al.* (2004) *R. (B.) microplus* was introduced into South Africa during the late 1800`s during importation of cattle from Madagascar after a rinderpest epidemic struck Southern Africa. The introduction of *R. (B.) microplus* to Africa and South Africa also introduced *B. bovis*, which causes a much more virulent form of babesiosis. Furthermore *R. (B.) microplus* was shown to displace *R. (B.) decoloratus* once introduced into an area (Nyangiwe *et al.* 2013) and almost completely displaced *R. (B.) decoloratus* in some parts of South Africa not previously infested by this tick species. This displacement occurred in areas of the Eastern Cape and Limpopo Provinces as indicated by Waladde & Rice (1982), Tønnesen *et al.* (2004) and Nyangiwe *et al.* (2013). Distribution of this invasive species could have been caused

by trading of cattle by means of local and international trade of cattle between farmers. Suitable conditions for *R. (B.) microplus,* such as humid and warm conditions, can cause the establishment and further displacement of *R. (B.) decoloratus*. Once *R. (B.) microplus* is established in an area, the possibility of the appearance of Asiatic babesiosis also increases. It is therefore important to investigate new spatial distribution of *R. (B.) microplus*, in order to determine the presence of *B. bovis* in the cattle hosts. This will enable producers to pro-actively plan treatment necessary for the prevention and further distribution of, or early treatment of Asiatic babesiosis disease.

The reliable detection of blood parasites is important as they greatly reduce economic losses of farmers in the cattle industry through timeous interventions including curbing any further spread of the diseases. The conventional identification through blood smears may sometimes be misleading and inaccurate as it is often only useful during acute infections. An inexperienced person might also misidentify the *Babesia* species due to small differences between the species that need to be recognised in blood smears as well as low prevalence during routine testing. Molecular identification is becoming the more preferred method to determine the presence of tick transmitted pathogens (Morzaria *et al.* 1992). This study made use of both methods to determine the presence of *B. bovis* on a farm in the Eastern Cape Province where *R. (B.) microplus* had recently been detected.

1.1. Literature review

1.1.1. Vectors of *Babesia* species

The most important vectors for transmission of *Babesia* species to cattle in South Africa are *R. (B.) decoloratus* and *R. (B.) microplus*. Both species fall into the Kingdom, Animalia; Phylum, Arthropoda; Class: Arachnida; Subclass: Acari; Superorder Parasitiformes; Order, Ixodida; Family, Ixodidae; Subfamily, Rhipicephalinidae. They were previously classified under the genus *Boophilus* but in 2000 were moved to the genus *Rhipicephalus* after discovering that *Rhipicephalus* were paraphyletic. Murrell *et al.* (2000) found that 12S mitochondrial DNA showed a 93% bootstrap support that

R. (B.) decoloratus and *R. (B.) microplus* shared a clade with *R. evertsi* (Murrell & Barker 2003).

Rhipicephalus (Boophilus) decoloratus (Koch, 1844) also known as the African blue tick, is indigenous to Africa south of the Sahara (Figure 1.1). This tick is commonly found in temperate climates among wooded areas and grasslands where their hosts are found (Walker *et al.* 2003). It is also the main vector of *B. bigemina* causing African babesiosis in cattle which is transmitted transovarially (Smith & Kilborne 1893). In cattle it can also transstadially transmit *Anaplasma marginale*, causing anaplasmosis or gall sickness or transovarially transmit *Borrelia theileri*, causing spirochaetosis. The maintenance host of *R. (B.) decoloratus* is cattle but they can also feed on a wide variety of host species that includes wild ungulates, horses and donkeys, carnivores, rodents and birds, in the absence of cattle (Horak *et al.* 2018).

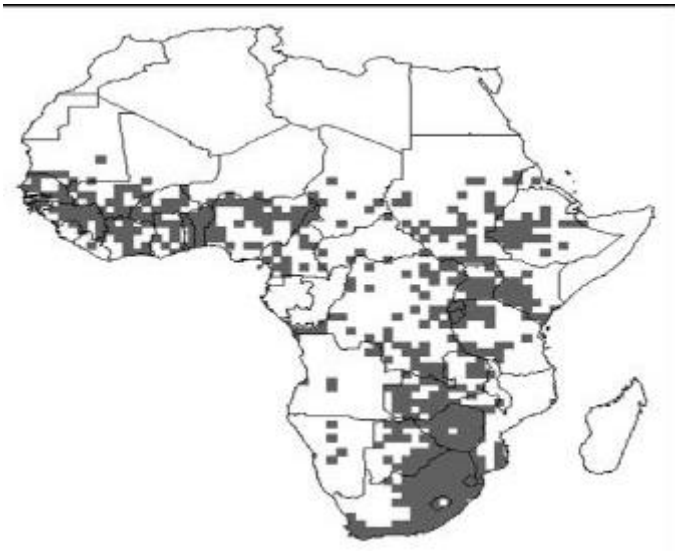

Figure 1.1: Distribution of *R. (B.) decoloratus* in Africa (Walker *et al.* 2003).

Rhipicephalus (*Boophilus*) *microplus* is also known as the pantropical blue tick or Asiatic blue tick (Horak *et al.* 2018). It is currently also widely spread through Africa, mainly in Eastern Africa, Western Africa and Southern Africa with Namibia being the most recent country (Figure 1.2) (Nyangiwe *et al.* 2018) These ticks can be found on cattle and goats, with cattle being the only preferred host of this species (Horak *et al.* 2018). *Rhipicephalus (Boophilus) microplus* is a vector of two economically important parasites namely *B. bigemina* and *B. bovis* in Southern Africa (Horak *et al.* 2018).

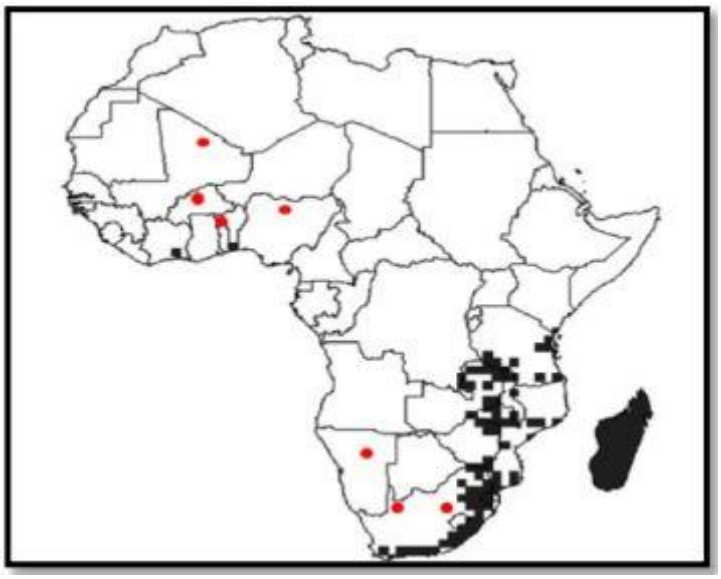

Figure 1.2: Distribution of *R. (B.) microplus* in Africa (Nyangiwe *et al.* 2018).

Both species are one-host ticks and are found in humid areas where rainfall exceeds 500 mm (Horak *et al.* 2018). Both species have previously been collected in Savanna, Grassland and Fynbos biomes in South Africa with *R. (B.) microplus* also found in Thicket and Forest biomes. A possible exclusion of the presence of *R. (B.) microplus* in a specific geographic area, can be made through temperature tolerance, as *R. (B.) decoloratus* can withstand lower mean temperature as the 15°C set for *R. (B.) microplus* (Tønnesen 2006). *Rhipicephalus (Boophilus) decoloratus* and *R. (B.) microplus* larvae can tolerate 0°C for a maximum of 72 hours according to Gothe (1967), thus limiting the spread of these ticks.

The two vector species can morphologically be distinguished from each other through the following characteristics. *Rhipicephalus (Boophilus) decoloratus* has a dental organisation consisting of 3+3 columns of teeth organised on the ventral aspect of the hypostome whereas *R.* (*B.*) *microplus* has a 4+4 column teeth arrangement (Walker *et al.* 2003). Males can further be distinguished by the spur lengths on coxae 1 with those of *R. (B.) decoloratus* being shorter than for *R. (B.) microplus*. The ventral plates of *R. (B.) decoloratus* are visible from a dorsal view, but usually not possible for *R. (B.) microplus* (Walker *et al.* 2003).

1.1.1.1. Feeding, life cycle and reproduction

Rhipicephalus (B.) decoloratus and *R. (B.) microplus* are both hematophagous one-host ticks. After eggs have hatched, larvae begin to wait on vegetation, waiting for a

host to pass to grab onto the host pelage. Once on a host, they seek for a suitable place where they could attach to establish a feeding site. Feeding sites were found to be mostly on the dewlap, stomach and between the hind legs of cattle (Fourie *et al.* 2013). A suitable feeding site results in the ticks cutting through the epidermis of the hosts with their chelicerae. The hypostome is then inserted into the lesion and a cement is excreted together with the saliva to attach the tick to the host. This is of importance for the transmission of babesiosis via the saliva that is subsequently regurgitated into the host (Walker *et al.* 2003).

Tick larvae feed for approximately seven days on the host before moulting into the nymphal stage (Walker *et al.* 2003). During this period *R. (B.) microplus* larvae can successfully transmit babesiosis, where the babesiosis remains infective during the larval stages (Bock *et al.* 2004). The nymphal stage feeds on the same host for a further approximately seven days to fully engorge (Walker *et al.* 2003). In the case of *R. (B.) decoloratus*, *B. bigemina* is transmitted during the nymphal and adult stages due to infective sporozoites taking nine days to develop and the tick larvae taking approximately seven days to engorge (Bock *et al.* 2004). Nymphs moulting into females will take in a small blood meal before mating takes place and get fully engorge after they have mated. Males do not engorge fully, but only take in a sufficient blood meal to mature their sexual organs. After mating and engorgement the females detach from the host and oviposition takes place in the soil in a sheltered environment (Figure 1.3) (Walker *et al.* 2003). The entire life cycle on a single host takes around three weeks with approximately seven days to complete each stage. Oviposition starts more or less one week after females have dropped from the host and each female can produce 1000 to 2500 eggs over a period of approximately 21 days. Egg hatching may take three to six weeks depending on environmental conditions (Walker *et al.* 2003).

Rhipicephalus (B.) decoloratus go into diapause during winter when temperatures decrease and no oviposition occurs when temperatures drop below 10°C and temperatures do not increase 13 – 15 days after female detachment. Spickett & Heyne (1990) found that this period of diapause during the egg stage, may be caused by the inverse relationship that the eggs hatch faster during warmer temperatures and slower during low temperatures due to temperature accumulation. This helps to synchronize the hatching dates of the pre-winter eggs with eggs that were laid during early spring. Larvae that hatched before the onset of colder temperatures as described by Spickett

& Heyne (1990), survived likely due to the immobility that the cold provided, causing them to stay in the microhabitat in an immobile state rather than climbing onto grasses in search for hosts. This allow these larvae to synchronize with larvae that hatched during warmer temperatures later on.

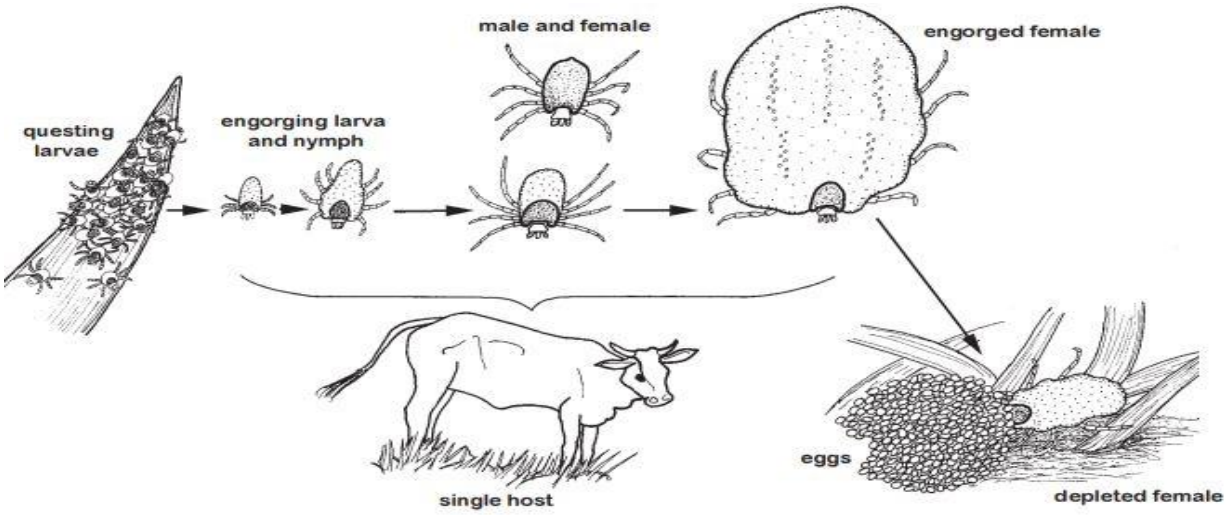

Figure 1.3: The life cycle of a one-host tick as described by Walker *et al. (*2003).

1.1.1.2. Inter-species competition

A number of factors modulate the capacity to support populations, including climate, vegetation and cattle biotypes. The fact that *R. (B.) decoloratus* is a more generalist host feeder makes it more widely distributed than *R. (B.) microplus* which tends to be restricted to areas with cattle as available hosts. However, collections of *R. (B.) microplus* feeding successfully on goats (Horak *et al.* 2018), as well as collections made on horses and eland, may indicate that *R. (B.) microplus* are gradually adapting to feeding on wild bovid species other than cattle. A buffer zone can however be found in the fact that *R. (B.) decoloratus* can survive in less humid and colder areas (Guglielmone 1995, Bock *et al.* 2004).

The displacement of *R. (B.) decoloratus* by *R. (B.) microplus* seems to be due to several factors. In areas suitable for both tick species, the shorter life cycle of *R. (B.) microplus,* is used to its advantage. *Rhipicephalus (B.) microplus* males reach maturity well before the *R. (B.) decoloratus* males*,* making it possible to mate with *R. (B.) decoloratus* females thereby preventing *R. (B.) decoloratus* males to mate with their females. The ability of males to move between cattle in close contact with each other further makes it possible to mate with more females before *R. (B.) decoloratus* males

are ready to do so. The resultant offspring of mating between *R. (B.) decoloratus* and *R. (B.) microplus* has been previously reported to be infertile. Hence, the interspecific mating results in reduced numbers of *R. (B.) decoloratus* (Waladde & Rice 1982, Tønnesen *et al.* 2004).

The number of eggs produced by *R. (B.) microplus* females was estimated by De Vos *et al.* (2001) to be about 500 more than those produced by *R. (B.) decoloratus,* causing an increase in the numbers game in favour of *R. (B.) microplus*. Almost complete displacement of *R. (B.) decoloratus* in parts of South Africa, not previously infested by *R. (B.) microplus*, has been reported in areas in the Eastern Cape (Waladde *et al*. 1982)(Nyangiwe *et al.* 2013) and Limpopo (Tønnesen *et al.* 2004). In both areas, it was found that *R. (B.) microplus* males even copulate with *R. (B.) decoloratus* females or nymphs before *R. (B.) decoloratus* males are sexually mature, causing interbreeding and sterile progeny that contribute to the decrease in *R. (B.) decoloratus* numbers (Horak *et al.* 2009). This in turn makes it difficult for *R. (B.) microplus* to spread more successfully due to *R. (B.) microplus* being unable to breed with the hybrid ticks (Bock *et al.* 2004).

1.1.2. Bovine babesiosis

The two most economically important species causing bovine babesiosis in South Africa are *B. bigemina*, first described by Babes (1888) in Rumania and *B. bovis,* first described by Canestrini in the same year (Uilenberg 2006). They belong to the Phylum: Apicomplexa, Class: Aconoidasida, Order: Piroplasmida, Family: Babesiidae, Genus: *Babesia* and can be transmitted to their animal hosts by either *R. (B.) decoloratus (B. bigemina)* or *R. (B.) microplus* (both *B. bigemina* and *B. bovis)*. Two other *Babesia* species found in South Africa, *B. occultans*, transmitted by *Hyalomma truncatum* and an unnamed *Babesia* species, transmitted by *Hyalomma truncatum* seems to be of no significant economic importance (Penzhorn 2015).

1.1.2.1. *Babesia bigemina*

Babesia bigemina is wide spread throughout South Africa and the rest of Africa due to its vectors *R. (B.) decoloratus* and *R. evertsi* having a larger distribution than *R. (B.) microplus,* the other vector. *Babesia bigemina* is not found in drier parts of South Africa as its vectors have a limited distribution under lower humidity.

Babesia bigemina is large and its merozoites is paired at an acute angle within red blood cells (RBC) of the hosts. The pathogenic effects, of *B. bigemina*, are associated with the destruction of the host RBCs causing haemoglobinuria seen earlier and more consistently in the urine of cattle infected with *B. bigemina* than with *B. bovis*. Fever is also less severe with no cerebral involvement. Animals that were infected and survived make a complete and rapid recovery. In severe cases, animals may develop severe anaemia, jaundice and sudden death with little to no symptoms (Smeenk *et al.* 2000, Bock *et al.* 2004).

1.1.2.2. *Babesia bovis*

Babesia bovis can only be transmitted by *R. (B.) microplus* and had expanded its distribution throughout South Africa due to the increased invasion of its vector to different regions of South Africa (Waladde & Rice 1982, Tønnesen *et al.* 2004). The wider distribution of this pathogen, synchronised with the increased occurrence of its vector in areas with suitable environmental conditions for the vector to survive and reproduce, caused the disease to slowly spread to these areas. (Tønnesen *et al.* 2004). Of note is the high prevalence of *B. bovis* DNA that may be found in *R. (B.) decoloratus*, which indicates that it can become infected with *B. bovis*, but is not able to transmit the parasite (Smeenk *et al.* 2000).

Babesia bovis is smaller than *B. bigemina* and its merozoites is paired at an obtuse angle within the RBC. The acute phase of the disease caused by *B. bovis* infections may last for three to seven days. The more severe signs of the disease may be masked by the presence of only fever for a few days before inappetence, depression, increased respiratory rate, weakness and reluctance to move occurs. Symptoms of muscle mass loss, tremors and the constant tendency to lie down present in advanced cases in infected animals, followed by a coma. After non-fatal infections a stable condition and a complete recovery usually takes several weeks. Sub-acute infections are difficult to detect, due to clinical symptoms being less noticeable. Cattle that recover may stay infective for up to four years, depending on the breed of cattle (Bock *et al.* 2004).

1.1.2.3. Transmission, lifecycle and reproduction

In a review of babesiosis in cattle, Bock *et al* (2004) described the biology of this protozoan in the host and in the vector (Figure 1.4). The vector becomes infected with the *Babesia* parasite when feeding on an infected vertebrate host. The parasite goes through several stages, within the tick vector, until it infects the next vertebrate host during feeding. Therefore, for a *Babesia* infection to become established in the cattle host, a minimum of two ticks needs to feed on the same host, not necessarily at the same time. One needs to infect the host and this infection needs to be established in the host before another tick feeds again to become infected (Pfäffle *et al.* 2013).

According to Friedhoff & Ristic (1988) *Babesia* species can only parasitize RBCs within vertebrate hosts. In the vertebrate RBCs infested with *B. bigemina*, a gamont precursor in the form of an ovoid type of merozoite with diploid DNA levels (Mackenstedt *et al.* 1995) is passed from the blood of the host to the tick vector through the mid gut. This passage stimulates an initial development of two ray body populations that further multiply within the RBC (Golgh *et al.* 1998). After completion of division, single-nucleated haploid ray bodies, assumed to be gametes (Mackenstedt *et al.* 1995), fuse in pairs (Golgh *et al.* 1998) and form a spherical cell, the zygote (Friedhoff & Ristic 1988).

Selective infection of the digestive cells of the tick gut and then of the basophilic cells, is followed by further multiplication and development into polypoid kinetes due to multiple fission or schizogony (Mackenstedt *et al.* 1995). Once released into the tick haemolymph (Agbede *et al.* 1986), kinetes reach the ovaries and infect the developing oocytes. Repeated cycles of secondary schizogony occur in the oocytes. This causes transovarial transmission to take place at transfer rates of between 20% and 40% for *B. bigemina* and less than 14.5% for *B. bovis* (Oliveira *et al.* 2005). Further development of both *B. bovis* and *B. bigemina* takes place within the larvae of *R. (B.) decoloratus* and *R. (B.) microplus*, respectively, after being dormant in the eggs. This further helps the parasite to survive and be transmitted to new hosts during the next generation (Bock *et al.* 2004). Transmission takes place to the larval stage through kinetes that enter the salivary glands of the larvae.

The attachment of the infected tick to the vertebrate host seems to stimulate sporozoite development (Mackenstedt *et al.* 1995) in the feeding larvae. Full

development of *B. bigemina* sporozoites however takes around nine days, causing transmission of the *Babesia* parasite to the vertebrate host during feeding of the nymphal and adult stages (Hoyte 1961, Potgieter & Els 1977b). Development of infective sporozoites of *B. bovis* occurs within two to three days after attachment of the larvae, causing the parasite to be transmitted to the vertebrate host during the larval feeding period (Riek 1966). The infective stage of *B. bovis* thus does not continue past the larval stage.

The host is infected with *Babesia* sporozoites during injection of saliva into the host skin when feeding. The sporozoites make use of a specialised apical complex to penetrate the membrane of the host RBC, where they form a ring-shaped trophozoite once inside the red blood cell (Potgieter & Els 1977a, Potgieter & Els 1979, Friedhoff & Ristic 1988). Binary fission causes the development of two merozoites, observed as pairs of attached pear-shaped parasites, considered to be the gamont precursor, which will then be ready to be transmitted to a blue tick vector upon feeding for continued development (Mackenstedt *et al.* 1995). Further division in the same red blood cell can also eventually destroy the cell allowing new cells to be invaded (Cruthers 2019).

Despite the status as a one-host tick, *R. (B.) microplus*, particularly in the case of males, are able to be transferred between cattle in close proximity with each other which can lead to a shortened infection period of only 6–12 days for *B. bigemina* infections that usually take 12–18 days after tick attachment (Bock *et al.* 2004).

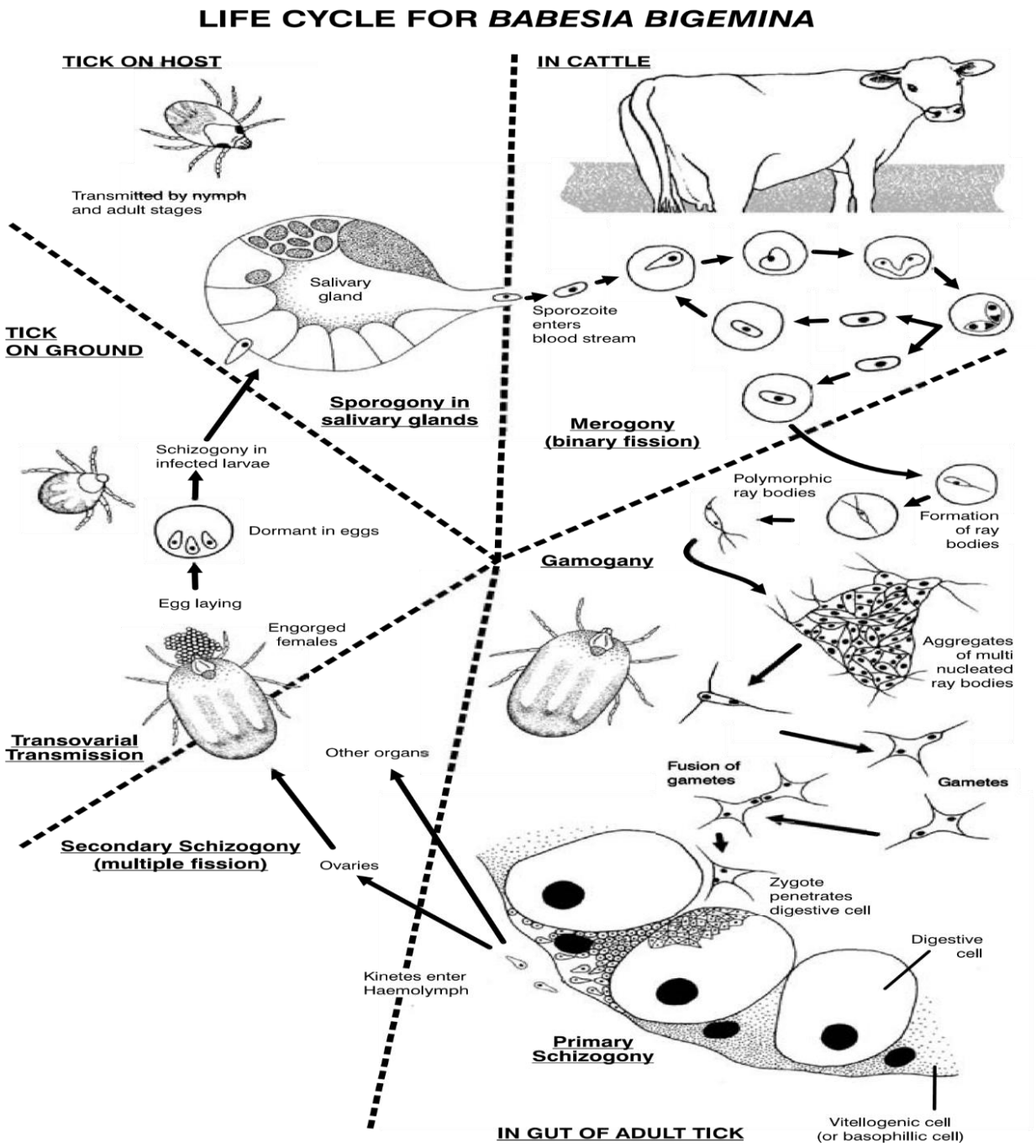

Figure 1.4: Life cycle of *Babesia bigemina* within the tick vector and cattle host (Bock *et al.* 2004).

1.1.3. Control

Endemic stability, as defined by Norval *et al.* (1992), is mostly seen as the ideal situation where the absence of clinical disease is strived for. This requires a stable relationship between the host, vector and environment and therefore requires the presence of *Babesia*-infected ticks to maintain a constant antigenic stimulus. This will also help to maintain the immunity of calves acquired from their mothers which

protects them against infection for the first two months after birth followed by innate immunity for up to nine months (Bock *et al.* 2004). When little to no infections take place, animals will not be infected for a long period of time after birth and will develop severe infections, that can be life-threatening, if they are exposed to *Babesia*-infected ticks later in life, due to little immunity. *Babesia bovis* does not induce immunity against *B. bigemina* infections, although *B. bigemina* immunity does offer some protection against *B. bovis* (Bock *et al.* 2004). Control of babesiosis is challenging and both natural and chemical options can be employed with varying degrees of success.

1.1.3.1. Natural

In areas where cattle were not previously exposed, introduction of babesiosis will have a catastrophic effect due to no host immunity. Clinical signs will be severe and high mortality rates may follow (Guglielmone 1995). Cattle that were previously infected, but had recovered from babesiosis, become carriers, the parasite remaining in the blood of these recovered animals (Saad *et al.* 2015). *Babesia bovis* infection may last for up to four years, but in the case of *B. bigemina,* animals can stay infective for up to 6 months (Bock *et al.* 2004).

In environments with high tick burdens, the incorporation of *Bos indicus* genetics into a herd would depress the inoculation rate of *Babesia* due to the breed being highly resistant to ticks. This will lessen the tick burden and can reduce the occurrence of babesiosis (Guglielmone 1995). In studies conducted on Nguni cattle by Marufu *et al.* (2011) and Nyangiwe *et al.* (2011), it was found that the shorter hair length and the secretion of more sebum, a natural repellent for ticks, helped to protect cattle from ticks attaching and thus lowering the risk of an infection. On the other hand, cattle like Bonsmara breeds, have longer hair and secrete less sebum, allowing ticks to easily attach and have a protected environment from predators and weather conditions, thus allowing a higher chance of infection with *Babesia* spp.

Other means of natural control can be executed in the form of controlled pasture burning in camps or areas where cattle graze to eliminate ticks that may disperse *Babesia* spp. Infection rate will decline for a short time until the vegetation has recovered to support new generations of ticks (Horak *et al.* 2011, Abbas *et al.* 2014). Pasture resting, where camps are not in use for prolonged periods, will help to disrupt the life cycle of ticks, killing of ticks due to a lack of hosts and dehydration (Abbas *et*

al. 2014). Use of fertilization may also help to reduce tick loads on pastures as found by Da Cunha *et al.* (2010) and Leal *et al.* (2017) when they tested urea on pastures.

Natural enemies of ticks may also be used to lessen tick burdens. This may include the use of microorganisms such as fungi and bacteria, other animals such as insects, birds and genetically resistant cattle. Fungi of the class Deuteromycets (Samish *et al.* 2004) have the ability to penetrate the cuticle of tick vectors thus killing the host irrespective of life stages. Bacteria like *Rickettsia* and *Francisella,* interrupt the natural endosymbionts of ticks, thus resulting in death of the ticks (Samish *et al.* 2004). *Ixodiphagus* wasp species have been found to lay their eggs in the nymphal stage of the ticks, as observed from laboratory studies performed by Manjunathachar *et al.* (2014). These wasps' eggs hatch within the nymph and start eating the nymph from the inside. Oxpeckers *Buphagus africanus* and *Buphagus erythrorhynchus,* indigenous to Africa, feed on ectoparasites that consists largely of ticks. The limitation of relying on this control animal is that they are visual predators and feed mainly on engorged ticks and not immature stages (Samish *et al.* 2004).

1.1.3.2. Chemical

The use of chemical products to control babesiosis can be executed on different levels.

Anti-*Babesia* chemotherapeutic agents can be used to treat if infected cattle are diagnosed early. Currently the only effective chemical treatments are the anti-protozoal agents, Diminazene aceturate and Imidocarb dipropionate with the Diamadine derivative, currently being the most effective, due to the rapid activity against bovine babesiosis (Penzhorn 2015). These chemicals only provide a short-term protection against babesiosis (Gohil *et al.* 2013) and can be costly due to continued treatment being needed. In recent years these products were also deemed unsafe for use due to residues in the meat of treated animals (Mosqueda *et al.* 2012). Quinuronium Sulphate, a Quinoline derivative, is greatly effective against *B. bigemina* but has a slow effect on *B. bovis*. Acridine derivatives, with Euflavine, showed to be effective against both *B. bigemina* and *B. bovis* but Trypan blue is only effective against *B. bigemina*.

Vaccines, developed against ticks during the 1990's, showed to be effective in reducing tick burdens on cattle. These vaccines can be useful when used in

combination with acaricides to more effectively control high tick burdens. Another option could be vaccination against babesiosis but it might be difficult to produce vaccines due to little knowledge of the immune response that accompany infections. Nonetheless a live *B. bovis* vaccine was developed in splenectomised calves, which drastically decreases the parasite virulence. A number of practical limitations also accompany the use of vaccines. It is important to keep the vaccine cold and prevent contamination with other diseases. Vaccines also have a short shelf life (Gohil *et al.* 2013). Despite all these limitations it is possible for these vaccines to give lifelong protection as seen with the current Australian chilled tick fever vaccine (Gohil *et al.* 2013).

Indirect control by means of vector control is probably the most frequently used control method to prevent the transmission of babesiosis. Acaricides such as organophosphates, amidines, synthetic pyrethroids, macrocyclic lactones and fluazuron are the most common chemical control agents used to decrease or control tick infestations. These chemicals are also applied in different ways, such as running the animals through a spray race or dip tank, injecting the animals, or chemicals used as a pour-on. Development of tick resistance to these acaricides can however cause a breakdown in tick control with an increased potential to transmit babesiosis (Abbas *et al.* 2014).

Within the organophosphates, chemicals such as Chlorfenvinpos and Chlorpyrifos inhibit acetylcholine release from sodium channels in the synaptic cleft of the central nervous system of the ticks. This is done by competitive inhibition of the acaricide and the acetylcholine to the same target site to acetylcholinesterase, the enzyme responsible for breaking down of acetylcholine. The result is that the neurotransmitters repeatedly send increased electrical charges, resulting in neuro overstimulation and eventually death of the tick (Abbas *et al.* 2014). Resistance to this chemical was found to be mainly linked to mutations on the target site, creating an insensitivity to these chemicals (Abbas *et al.* 2014).

Amidine, a triazapentadien compound, marketed as in Amatraz, has a toxic effect on the octopamine receptors. Resistance is thought to be an alteration of two nucleotide base pairs that alter the target site but the exact mechanism is still unknown (Abbas *et al.* 2014).

Synthetic pyrethroids are based on pyrethrins that can be found in the Chrysanthemum family. This compound is a neurotoxin that acts on the sodium cannels effecting the permeability of the nerve membranes. Resistance is linked to a mutation that make sodium channels less sensitive to the chemical (Abbas *et al.* 2014).

Macrocyclic Lactones include avermectins and milbemycins, natural fermentation products of *Streptomyces avermitilis* and *S. hygroscopicus*. These chemicals increase the flow of chloride ions into cells resulting in paralysis of the neuromuscular systems. The mechanism for resistance is still unknown according to Abbas *et al.* (2014).

Insect Growth Regulators comes in two main forms, a juvenile hormone inhibitor and a chitin synthesis inhibitors. The juvenile hormone inhibitor acts like a juvenile hormone which is responsible for instar moulting, and prevents moulting into adult stages. Chitin synthesis inhibitors inhibits chitin formation, the major component in arthropod cuticles, thus preventing the formation of chitin (Mcnair 2015) and normal growth.

1.1.4. Identification techniques

There are four different stages of importance when it comes to detecting *Babesia* spp. infections as described by Morzaria *et al.* (1992). The first is the early infection that is known as the low parasitaemic phase. During this stage of the infection, parasite levels are still low with the parasitaemia being less than one RBC infected per every 1000. During this phase, the parasite is often undetectable on Giemsa-stained smears and under field conditions (Morzaria *et al.* 1992).

The second phase presents as the acute infection phase. During this stage, the parasite is easily observed by light microscopy when looking at Giemsa-stained blood smears. The detection during this phase is important as to select the correct treatment in the case of multi-infected animals (Morzaria *et al.* 1992).

The third phase is the recovery period. Detection during this phase may be important to establish if the correct parasite treatment was chosen. Lastly, the fourth phase is when the animal becomes a carrier and develops antibodies. It is further difficult for detection of parasites as the parasitic load is less than during the acute phase. The

antibodies are important for certain epidemiological studies like complement fixation and indirect fluorescent antibody (IFA) test as described by Morzaria *et al.* (1992).

Several techniques may be implemented to identify these protozoans in ticks and in hosts, including blood smears, indirect fluorescent antibody (IFA) tests and the Polymerase chain reaction (PCR) with the use of applicable primers (Lempereur *et al.* 2010, Abu Kwaik *et al*. 2011).

1.1.4.1. Blood smears

The most common means of identification is through Giemsa-stained blood-smears as it is fast and inexpensive in comparison to other means of detection. Blood smears however can miss infections and trained personnel may be needed to differentiate between the two main *Babesi*a spp. (Küttel *et al.* 2007). In this method the presence of *Babesia,* the trophozoites and merozoites, is observed with a light microscope under x1000 magnification. This method can detect parasitic loads as low as one RBC infected out of 10 000 non-infected RBC if 100-200 microscopic fields are observed as described by Morzaria *et al.* (1992).

Fluorescence microscopy as done by Winter (1967) and described in Morzaria *et al.* (1992) was found to be more sensitive than Giemsa-stained blood smear analyses. In this method, blood is stained with acridine orange and studied under a fluorescence microscope. This quantitative buffy coat (QBC) method was later refined to be more sensitive by concentrating 50-60 µl of stained blood in a capillary tube causing it to be up to 100-fold more sensitive than Giemsa-stained blood smears during field conditions. Although this method is more sensitive, Levine *et al.* (1989) concluded that it does not concentrate parasites within infected RBC.

1.1.4.2. Indirect fluorescent antibody test

This test is more sensitive and specific than other tests like blood smears. Indirect Fluorescent antibody test has limitations outside experimental situations as it binds irreversibly with serum containing *B. bovis* antibodies. It can however be useful in detecting *B. bigemina* antibodies within serum. The antigens that can be used in this test can be found within the blood of infected animals or alternatively be grown within culture and is derived from *Babesia* merozoites (Goodger 1971, Morzaria *et al.* 1992).

1.1.4.3. Polymerase chain reaction

Polymerase Chain Reaction is more suited for identification of *Babesia* spp. as it can detect *Babesia* infections at lower concentrations than blood smears.

The primers for detecting *Babesia* spp. are highly species-specific. No cross reactions are found with DNA of *Anaplasma* spp., *Theileria* spp. or *Rhipicephalus (Boophilus*) spp. if infection with *Babesia* is not present (Smeenk *et al.* 2000). It is possible to detect *Babesia* infections in animals as young as one month with the use of PCR techniques (Oliveira-Sequeira *et al.* 2005).

Among the drawbacks of PCR may be the occurrence of cross-reactions between *B. bovis* and *B. bigemina* and the fact that it may be unable to discriminate between previous exposures and currently affected animals (Oliveira-Sequeira *et al.* 2005).

DNA cloning may also be used. This may be done by injecting the sequenced DNA into a plasmid or bacteriophage and then growing this DNA within bacteria to amplify the DNA. The DNA is then purified by using parasite-specific probes labeled with radioisotopes as described by Morzaria *et al.* (1992).

1.2. Justification

In South Africa alone, ticks and tick-transmitted diseases account for millions of rand's in financial losses, among cattle producers due to cattle. The distribution of the vector tick species and protozoan parasites therefore needs to be identified in affected areas so that necessary steps can be followed to treat babesiosis appropriately and to lessen the chance of wrongful treatment and development of resistance (Tønnesen *et al.* 2004).

Cattle are the main host of the babesiosis protozoans, mainly found in the blood of the animals (Tønnesen *et al.* 2004). Detection of the presence of these diseases, especially Asiatic babesiosis in areas where it previously were not found, is of utmost importance as it results in a timeous implementation of control strategies. The aim of this study was therefore to establish the occurrence of both *B. bigemina* and *B. bovis*, and their vectors *R. (B.) decoloratus* and *R. (B.) microplus*, on a farm in the Eastern

Cape Province where *B. bovis* and its vector *R. (B.) microplus* were not previously present.

1.3. Objectives

The objectives of this study were:

1. To establish the presence of the two babesiosis vectors, *R. (B.) microplus* and *R. (B.) decoloratus* on a farm in the Eastern Cape Province where *B. bovis* and its vector *R. (B.) microplus* were not previously found.
2. To determine the presence of *B. bigemina* and *B. bovis* in larvae collected from the pasture of the different camps on the farm.
3. To investigate the presence of *B. bovis* and *B. bigemina* in larvae of fully engorged females collected from cattle grazing in different camps on the farm.
4. To determine the presence of *B. bovis* and *B. bigemina* in blood smears obtained from cattle, from which *Rhipicephalus (Boophilus)* tick species were collected.
5. To establish the extent of *B. bovis* infestation on this farm.

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Chapter 2: Materials and methods

During a preliminary study done in 2013, a single collection of *Rhipicephalus (Boophilus) microplus* was made on a farm in the Eastern Cape province. This tick species, a vector of the pathogen *Babesia bovis,* had not previously been found on commercial farms in this area. After an outbreak of Asiatic babesiosis caused by *B. bovis*, presented on the neighbouring farm, a further investigation of the presence of *B. bovis* on the study farm was needed. The diagnosis of Asiatic babesiosis on the neighbouring farm was made on grounds of the sudden death of more than one animal with a very short period of visible illness. No other diagnostic tools were used to confirm this diagnosis.

The aim of this study was to confirm the presence and extent of the distribution of *B. bovis* on the study farm where the presence of *R. (B.) microplus* had already previously been confirmed. For this purpose, two identification tools were employed to look for the presence of *B. bovis* in blood, collected from cattle grazing the fields of this farm. The more conventional blood smears were compared to PCR to establish the sensitivity of each method. Both methods were also used to determine the prevalence of *Babesia* infections on this farm. The PCR were also employed to determine the presence of *B. bovis* and *B. bigemina* in larvae obtained from field drags and adult ticks collected from cattle.

2.1. Study area

All collections were made from cattle and the fields that they were grazing on, from a farm located in the Coombs district, coordinates 33°19'25.5"S; 26°51'17.7"E, near Makhanda in the Eastern Cape province. Thickets in the Eastern Cape comprise of dense vegetation dominated by spiny, often succulent trees and shrubs. Mean midday temperatures range from 18°C during July to 26°C during February and rainfall occurs mainly during summer, making this area the perfect breeding ground for both vectors of babesiosis, *R. (B.) microplus* and *R. (B.) decoloratus* (Palmer 2004).

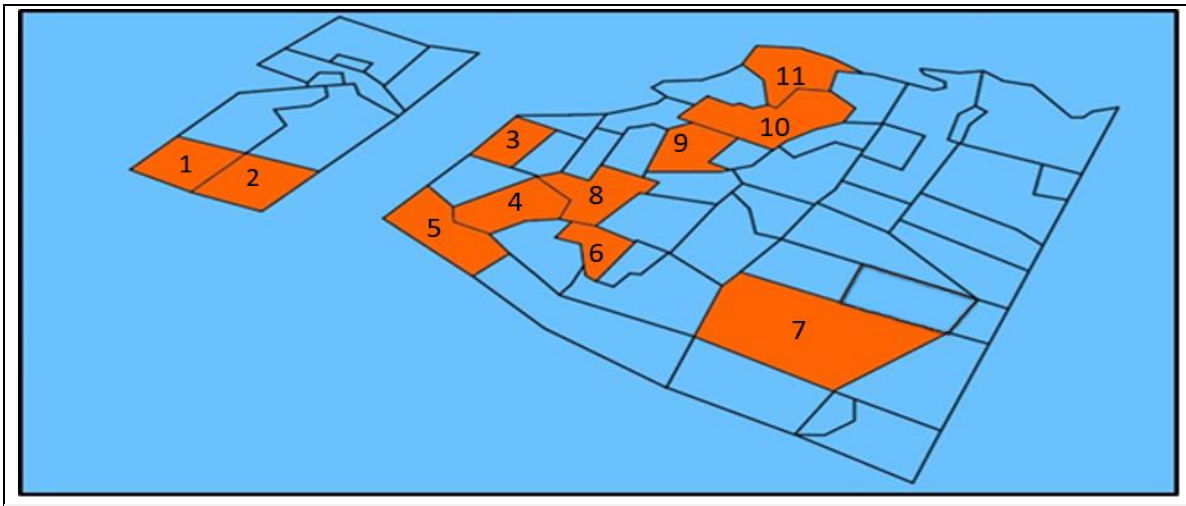
The farm consists of three different properties of approximately 2500 ha in total but only two properties were used in this study, although the cattle could have been moved among all three. The grazing areas were made up of a mixture of thickets, or bushy clumps, and grassland biomes containing either sweet or sour veld. The sweet veld areas were at the lower elevations of the farm and varied from camp to camp, with dense grass and bushes, to camps with fewer bushes. Some of the sweet veld camps were also used for cultivation. In contrast, the sour veld was found at higher elevations which were more open with few to no bushes.

2.2. Field collections

Collections were performed during April and November 2017 and again during April 2018. In total, 11 camps were used during this study (Figure 2.1). The camps selected depended on the movement of cattle according to the management strategy of the producer. This was determined by food availability in camps and the rotation program followed by the producer.

2.2.1. Tick larvae

Tick larvae were collected in the camps where the cattle had grazed approximately 18-23 days prior to adult tick and blood collections (Figure 2.1). Tick larvae were collected by means of a drag stick that consisted of 10 strips of flannel cloth, 1 m long and 10 cm wide. Each drag stick had a final area of 1 m^2. Four drags of 150 m each were performed near the walking pathways, near shrubs and trees, possible sleeping areas and water points in the camps (Figure 2.2A). After each drag, tick larvae were collected from each flannel strip using forceps and placed in a plastic tube, containing 70% ethanol, for storage until identification (Spickett *et al.* 2006)(Figure 2.2B). Tick larvae from each camp were pooled during storage and the container was then marked with the date of collection as well as the name of the camp where the collection was performed. Temperature, humidity, landscape coordinates, vegetation in the form of photos, cloud cover and the starting and ending times of field drags were recorded separately for each camp.



1.	**ARTHURS RESERVOIR**	**6.**	**MILKCOW**
2.	GAVIN HILL	7.	GUAVA
3.	LOLWENI	8.	SHEDS
4.	QUARRY	9.	BARBERS DAM
5.	BUSHALT	10.	LANDS AND OLD ORANGE
		11.	GAALBOOM

Figure 2.1: Map indicating sampled cattle grazing locations/camps.

A: Drags being performed for tick larvae collection.

B: Tick larvae being removed from flannel strips.

Figure 2.2: Tick larvae collected from vegetation near water point (A) and tick larvae being removed from flannel strips and stored in 70% ethanol (B). (Photo Credit: M. Pottinger)

2.2.2. Adult tick collection from cattle

During each collection period approximately 50 Bonsmara-cross cattle, not treated with any chemicals within the previous 14 days, were randomly selected from a herd of free grazing cattle from at least 5 different camps. Fully engorged female

Rhipicephalus spp. ticks, found on the posterior end of the cattle especially on the inner parts of the hind legs as well as the body and dewlap, were randomly collected from the selected animals. Ticks were collected by pulling them from the animals with forceps or gloved hands and placing the ticks into pre-marked, bottles, indicating the date and herd group /camp from which it was collected (Figure 2.3). Fully engorged females were collected to ensure higher egg production to maximise the degree of accuracy and possibilities of identifying *Babesia* species when present. Collections were only done from cattle older than nine months due to possible innate immunity against ticks and babesiosis (Guglielmone 1995).

Figure 2.3: Adult ticks being removed from an animal's dewlap with forceps and placed in storage container. (Photo Credit: M. Pottinger)

2.2.3. Blood collection from cattle

Blood was collected from at least 50 animals from five different camps, not treated for diseases or ticks burdens during the previous 14 days. Fifty animals made up approximately 10% of all the cattle on the farm. Blood was collected from cattle older than nine months, to ensure that innate immunity to the parasites, could not interfere with the test results (Guglielmone 1995).

A small amount of blood (2-4 ml) was collected from the coccygeal vein, under the tail of the animal by means of a bullnose into an Ethylenediaminetetraacetic acid (EDTA)

vacutainer suction tube, pre-marked with the cattle number. (Figure 2.4). This location was chosen for blood withdrawal as it most likely contain a sufficient number of parasites to be detected during blood DNA extractions and for blood smears. It is also easier to obtain blood from this vein than as from the jugular vein as that will necessitate the animal to be put in a neck clamp. Needles were replaced with new sterile needles between each collection. This was done by carefully removing the used needle and placing it into a field biological waste bag until it could be disposed of at the laboratory. In the event that the bullnose was contaminated with blood, it was immediately put in a container with a 10% Sodium Hypochlorite (bleach) and 70% Ethanol solution to prevent cross contamination of DNA or animal diseases.

Blood-filled EDTA tubes were stored on ice until further use. The time and location were documented on the blood collection forms (Appendix 3).

Figure 2.4: Blood being extracted with an EDTA vacutainer tube from the coccygeal vein, under the tail. (Photo Credit: M. Pottinger)

2.3. Handling of field samples

2.3.1. Field laboratory

At the temporary field laboratory, drag collected larvae were placed in plastic screw top tubes containing 70% ethanol and stored ambient temperature, ranging from 15-32°C, in test tube racks.

Engorged adult female ticks collected from the cattle were identified, at the field laboratory with a Nikon dissection microscope under 100X magnification, as either *R*. (*B*.) *decoloratus* or *R*. (*B*.) *microplus* by making use of the differences in dentition organisation between the two species and other factors described in section 4 (Walker *et al.* 2003). They were then stored individually in tubes with cotton wool stoppers to allow air exchange and prevent injury during short term storage. The tubes were then placed in an incubation box, lined with wet tissue paper to create a higher humidity and temperature to prevent dehydration and promote metabolism.

Collected blood specimens were stored at 4°C, for the duration of the collection period to help preserve the blood and blood parasite DNA within the blood samples. At the field laboratory three smears were made of each blood sample. The method entailed a total volume of 10 µl of blood to be placed on a pre-marked microscope slide. The blood drop was then smeared with a separate slide by placing the spreader slide before the drop, drawing it at a 45 degree angle to make contact with the blood and then drawing the spreader to the opposite end of the slide as to get an even thin smear. The blood smears were then air-dried for approximately two minutes and if not completely dry observed every 30 second until dry. The dried blood smears were then fixed using 100% methanol for 5 minutes. The methanol solution was changed every 100 slides, to minimise contamination. Blood smear samples were marked on the microscope slide with a reference index consisting of the collection date and animal number and stored in microscope plate holders. The blood smears were stored at the same conditions as the blood for simplicity of storage.

2.3.2. Field laboratory cleaning procedures

Contaminated bullnoses were scrubbed within a mild solvent, then soaked in 10% Sodium Hypochlorite (bleach) for 5 minutes followed by an additional 5 minutes soak in 70% Ethanol before the next day's collection. All blood spillages were cleaned by wiping the surfaces with a paper towel, followed by cleaning with a 10% Sodium Hypochlorite (bleach) solution and then with a 70% Ethanol solution. All the paper towels used for cleaning were discarded in the biological waste container.

2.3.3. Transportation of collections

At the end of the collection period all collected samples were transported to the University of the Free State in Bloemfontein for further evaluation. Ethanol tubes, containing larvae were placed in test tube racks and secured with masking tape to prevent tubes falling from the racks during transportation and placed within the incubation boxes for more convenient transport.

Live tick samples within the tubes had a cotton wool stopper securely placed in the tube. Double-sided tape was placed around the top of the tube. The tubes were then stabilized, within the incubation box, to prevent falling over during transportation. The incubation box was closed securely with the lid hinges.

Blood samples were tightly packed, to prevent breakage and movement, placed in a polystyrene ice box with dry ice cubes and the lids of the cooler boxes were taped down to prevent opening during transportation.

Laboratory and fieldwork waste was transported within appropriately marked plastic bags for disposal in biological waste containers at the laboratory situated at the University of the Free State.

2.3.4. Laboratory procedures

Upon return to the main laboratory, all samples were logged onto accountability forms (Appendix 4). All samples were stored in secure rooms with key-card access at the appropriate storage or incubation conditions. Larvae were stored in 70% Ethanol at room temperature until identification. Engorged females were placed in incubation containers at a relative humidity of higher than 70% in a room kept at 27 ± 2°C. The temperature and humidity was monitored weekly and documented to assure optimal incubation conditions for oviposition and hatching of larvae. Egg production was

monitored and hatch dates of larvae were documented. The hatch date was considered to be the date when approximately 75% of the eggs had hatched. This was also indicated by larvae moving to the top of the tube. Sixteen days after the hatch date, larvae were transferred to a tube containing 70% ethanol. They were then stored together with larvae obtained from field drags at 4°C for preservation of the parasites until DNA extraction and PCR could be performed on the tick larvae (Kolobov & Vainberg 1976).

Blood samples were stored in a fridge in a molecular laboratory at 4°C to preserve the parasite DNA within the blood samples until DNA extraction and PCR were performed (Kolobov & Vainberg 1976). Blood smears were stored at 4°C within the microscope slide holders until further use.

2.4. Identification methodology

2.4.1. Tick identification

The tick species under investigation, *Rhipicephalus* (*B.*) *decoloratus* and *Rhipicephalus* (*B.*) *microplus* look very similar, not only in colour but also in shape and size. In both cases, there are no discernible differences between the two species, when viewed with the naked eye, especially during the larval and nymphal stages. Identification was made, making use of a Nikon dissection microscope with 100x magnification was used.

2.4.1.1. Females

The main visible distinction between the two species can be observed from the ventral view. Hypostomal teeth are arranged in rows of 3+3 within two columns for *R. (B.) decoloratus* and in rows of 4+4 within two columns for *R. (B.) microplus*. There is also a protuberance with pectinate setae on the internal margin of the palp article one for *R. (B.) decoloratus* which is lacking in *R. (B.) microplus.* This lack of protuberance makes the palp article inner margin distinctly concave in the latter. Lastly, the genital aperture posterior lips of *R. (B.) decoloratus* has a narrow “U” shape, in contrast to *R. (B.) microplus* which has a wide “U” shape (Walker *et al.* 2003).

2.4.1.2. Males

Although males were not specifically collected, if found in the collection they were also grouped into their respective species. Male mouthparts are similar to those of the females in both of these species. The spur length on coxae 1 can be used to distinguish between the two species as it is short for *R. (B.) decoloratus* and long for *R. (B.) microplus*. Lastly, the ventral plate spurs are dorsally visible in *R. (B.) decoloratus* and are not visible from the dorsal side for *R. (B.) microplus* (Walker *et al.* 2003).

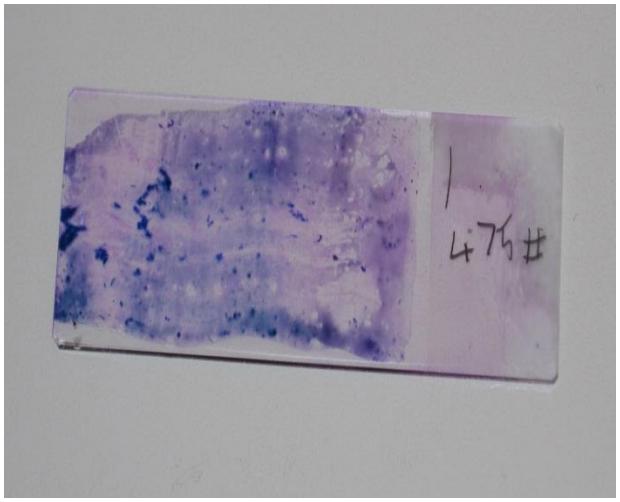
2.4.1.3. Larvae

Identification keys were used to identify the larvae to species level. Both species can be described as having a broad and oval shape. The hypostome also has a tooth configuration of 2+2 for both of these species. The main difference between these species is that *R. (B.) microplus* has a scutum that extends over two thirds of the dorsal surface. The scutum of this species is also smooth. In the case of *R. (B.) decoloratus* larvae, the scutum can be seen as broader than long, has small setae and is punctuated. The last notable difference is that *R. (B.) microplus* has a short internal spur on the first coxa, while *R. (B.) decoloratus* has no spur (Gothe 1967, Berry 2017).

2.4.2. Identification of *Babesia* species

2.4.2.1. Blood smears

Blood smears (Figure 2.5A) were made at the field laboratory as described in section 3.1, from the blood obtained from cattle. At the main laboratory the smears were stained with Giemsa to be able to identify the *Babesia* parasites in the blood. Giemsa staining was performed by placing the slides in a microscope stain rack in pairs of 10 and submerging the slides in 10% Giemsa stain solution for 15 minutes (Figure 2.5B). The slides were then carefully rinsed with running tap water for approximately 10 seconds, lightly tapped on a paper towel to remove most of the water and air dried for a further 10 minutes. The slides were then placed back into the microscope slide holder for later use (Mtshali *et al.* 2004).



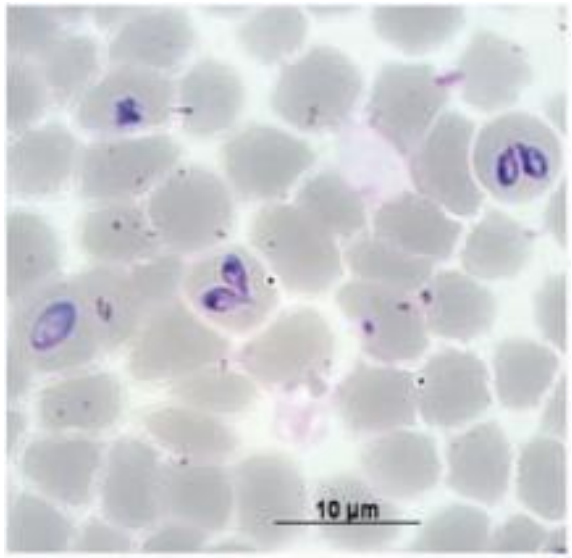
A: Giemsa-stained blood smear.

B: Microscope slides being stained

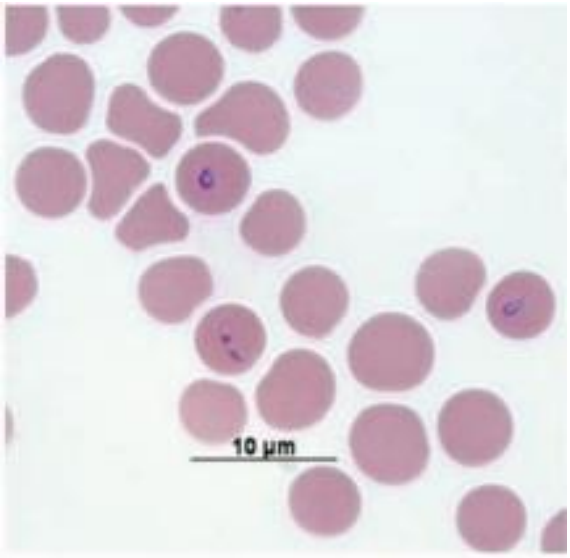
Figure 2.5: A single Giemsa-stained blood smear (A) and blood smears being stained in 10% Giemsa stain (B).

The stained microscope slides were observed under a Nikon light microscope at 1000x magnification, using immersion oil. One hundred observation fields per microscope slide were performed to search for the presence of *B. bovis* or *B. bigemina*. When found, measurements were made using a camera mounted on the microscope and camera measuring equipment for accuracy to get the correct length of the parasites. The slides were lightly marked with a permanent marker on the bottom of the slide in the approximate location of an identified specimen for later identification in the case of data corruption. Two images per identified parasite were taken, one containing measurements and one containing only the identified parasite (Mtshali *et al.* 2004). These images were labelled with the slide number and indication of measurements.

Parasites were identified making use of the following criteria: *Babesia bigemina* merozoites are is large in size with dimensions 3.24±0.22 x 1.29±0.17 and are paired at an acute angle within the RBC. *Babesia bovis* merozoites are smaller with a size of 1.29±0.21 x 0.71±0.17 and are paired at an obtuse angle within the RBC according to Chaudhry *et al.* (2010) (Figure 2.6).



A: Giemsa stain of *Babesia bigemina*



B: Giemsa stain of *Babesia bovis*

Figure 2.6: Giemsa-stained images of *Babesia bigemina* and *Babesia bovis*. (Source: Mosqueda *et al.* 2012).

2.4.2.2. DNA extraction

A modified Cetyltrimethylammonium-bromide (CTAB) method was used to extract DNA from cattle blood and tick larvae to be able to perform PCR.

The CTAB DNA extraction method, usually used for freeze-dried plant material, was implemented for DNA extraction in this study. The process was started by adding 500 µl of CTAB buffer, consisting of a mixture containing 1 M Tris-Cl, 0.5 M EDTA, 5 ml NaCl, 10% CTAB, β-mercapto-ethanol, polyvinylpyrrolidone (PVP) and water (Table 2.1). The contents of the CTAB mixture was kept the same as the original CTAB extraction mixture used to extract DNA from freeze-dried plant matter. The volume of the extraction buffer mixture was however decreased to a volume of 500 µl instead of 750 µl buffer. Previous results showed that reducing the volume of CTAB buffer did not influence the end results for DNA extraction (Palumbi & Ferraris 1996, Piertney & Webster 2010).

The method was further adapted by incubation for a minimum of five hours at 50-55°C as indicated by the salting out method for DNA extraction versus one hour at 65°C recommended by the original CTAB method. Higher concentrations of DNA were extracted due to this adaptation as the lower temperature implemented prevented

denaturing of the DNA due to the high temperature. It also had an added benefit of being able to be run overnight when necessary (Palumbi & Ferraris 1996).

DNA was separated from the extraction buffer by adding a 500 µl mixture of chloroform (96% v/v) and isoamylalcohol (4% v/v) and centrifugation at 1200 g for 10 minutes (Palumbi & Ferraris 1996).

Table 2.1: Preparation of chemical dilutions used during the CTAB DNA extraction method.

1M Tris pH 8.0	
Tris	60.5 g
Water	500 ml
0.5M EDTA pH8.0	
EDTA-NA.2H2O	93 g
Water	500 ml
5M NaCl	
NaCl	146.1 g
Water	500 ml
10% CTAB	
CTAB	10 g
Water	100 ml
CTAB extraction buffer (50ml)	
1M Tris-Cl (100mM)	5 ml
0.5 M EDTA (20 mM)	2 ml
5M NaCl (1.4 M)	14 ml
10% CTAB (2%)	10 ml
β-mercapto-ethanol (2%)	2.5 ml
PVP (1%)	0.5 g
Water	18.9 ml
Chloroform/Isoamylalcohol	
Chloroform (24 parts)	96 ml
Isoamylalcohol (1 Part)	4 ml
70% Ethanol	
100% Ethanol	10 ml
Water	100 ml
TE	
1 M Tris-Cl (10nm)	1 ml
0.5 M EDTA (1 mM)	0.2 ml
Water	100 ml
7M Ammonium acetate	
Ammonium acetate	57.8 g
Water	100 ml

DNA was precipitated from the aqueous phase by adding 500 µl isopropanol, incubating at room temperature for 20 minutes and centrifugation at 1200 g for 10 minutes. It was also found that incubation for 30 minutes yielded more DNA precipitate and time can be extended if insufficient precipitate has formed. All liquid was then drawn off using a vacuum aspirator (Palumbi & Ferraris 1996).

The precipitate was washed with ice-cold 70% ethanol and centrifuged at 1200 g for 10 minutes. This step was performed two or more times where needed, to obtain a better DNA yield (Palumbi & Ferraris 1996).

The DNA was then re-suspended overnight in 100 µl Tris- EDTA (TE) buffer. If a pallet was still visible after re-suspension, the process was repeated with only 1 hour incubation with the CTAB buffer. If the concentration as determent by a Nano Drop spectrophotometer was too high to use for PCR, additional TE buffer was added (Palumbi & Ferraris 1996).

2.4.2.3. RNase elimination.

Two microliter RNase was added to the mixture and incubated at 37°C for two hours. Twenty microliters of a 7.5 M ammonium acetate and 200 µl mixture of chloroform (96% v/v) and isoamylalcohol (4% v/v) were added. DNA was suspended in ice-cold 100% ethanol and centrifuged at 1200 g for 15 minutes. The DNA was then re-suspended in 50 µl TE buffer for one hour at 37°C. The DNA concentration was calculated by means of a Nano drop spectrophotometer and diluted to a 10 µg DNA concentration (Palumbi & Ferraris 1996). After initially adding the RNase elimination step it was found that the RNA did not interfere with the primers used and this part was discarded from the methodology.

2.4.2.4. DNA extraction from blood

Approximately 100 µl cattle blood was added to 500 µl CTAB extraction buffer and thoroughly mixed after which the extraction procedure as described was followed.

2.4.2.5. DNA extraction from ticks

The engorged female ticks were allowed to oviposit and the produced eggs, to hatch over a period of approximately 42 days. Larvae from each female were separated from egg debris and transferred to a plastic screw-top tube for storage in 70% ethanol at

4°C. Approximately 100 larvae from each female were manually crushed with a tool that fits tightly into the bottom of an Eppendorf tube. The resulted homogenate was used to extract DNA from the larvae by means of the modified CTAB method as described previously. Extracted DNA was used to detect the presence of *Babesia* DNA in the larvae of the female ticks, transovarially transmitted by the females to the larvae (Bock *et al.* 2004, Oliveira *et al.* 2005).

To determine the presence of *Babesia* spp. in larvae collected by drag sampling, at least 30 tick larvae, where possible, were crushed individually and used for DNA extraction, making use of the described modified CTAB method. All volumes more than 100 µl were decreased to 100 µl.

2.4.3. DNA amplification

2.4.3.1. Polymerase chain reaction

The DNA amplification was started by mixing the extracted DNA, obtained from blood and tick larval extractions, with the different components of the Kappa2g Robust HotStart PCR kit according to the kit protocol. The different components were as follows:

- 5.0 µl of 5X KAPA enhancer 1,
- 0.5 µl of 10 mM KAPPA dTNP mix,
- 1.25 µl of 10 µM forward and reverse primers each,
- 0.1 µl of 5 U/ul KAPPA2G Robust HotStart DNA Polymerase
- 5.0 µl of 5X KAPPA2G Buffer A.

Extracted DNA template was added at a concentration of 10 ng-100 ng, prescribed by the kit protocol; and this mixture was made up to 25 µl with PCR-grade water. Primers for both *B. bigemina* and *B. bovis* (Figure 2.7) were added simultaneously as these were found not to interfere with each other (Lempereur *et al.* 2010).

Figure 2.7: Forward and reverse primers for *Babesia bigemina* and *Babesia bovis*.

The primers used for PCR, described by Lempereur *et al*. (2010), were as follows:

Babesia bigemina:

- GAU5 (Forward) 5'- TGGCGGCGTTTATTAGTTCG- 3'
- GAU6 (Reverse) 5'- CCACGCTTGAAGCACAGGA- 3’

Babesia bovis:

- GAU9 (Forward) 5'- CTGTCGTACCGTTGGTTGAC-3'
- GAU10 (Reverse) 5'- CGCACGGACGGAGACCGA-3'

Polymerase Chain Reaction conditions consisted of an initial denaturing of the DNA at 95°C for three minutes during the first cycle. This was followed by 30 cycles of denaturation of DNA at 95°C for 15 seconds, annealing of the primers at 60°C for 15 seconds and then extending the DNA at 72°C for 15 seconds/kb. The final step was done at 72°C for 1 minute/kb for the final extension.

2.4.3.2. Gel electrophoresis

DNA fragments were identified by gel electrophoresis by running the DNA through a 1.5% agarose gel and comparing the fragments with a 50 bp ladder run simultaneously. The agarose gel was prepared by mixing 1.57 g of agarose powder with a 1X Tris-borate-EDTA (TBE) buffer, made by dissolving 10.8 g of Trisaminomethane (Tris) and 5.5 g Boric acid in 900 ml distilled water before adding 4 ml of 0.5 M Na_2EDTA and adjusting the volume to 1 litter.

The agarose was heated, in a Media Bottle, for three minutes in a microwave oven and mixed occasionally until the agarose was completely dissolved. The glass bottle was closed with the lid and cooled to approximately 55°C; this helped to create a vacuum inside the bottle pulling out any air bubbles within the agarose dilution, before adding 4 µl Ethidium bromide (0.5 ug/ml) and gently mixing by twirling the bottle. The mixture was then cast into a casting tray, gently adding a desired sized comb to create the wells.

After the agarose gel was set, a 15 µl mixture consisting of 10 µl amplified DNA, and 5 µl purple loading gel dye, that came with the ladder, were loaded into each well with an appropriate micropipette. The loading dye was used at a 1X concentration made up from a 6X stock solution. Amplified DNA was run through the 1.5% agarose gel at 80 mA until the dye moved three quarters of the gel length (Figure 2.8).

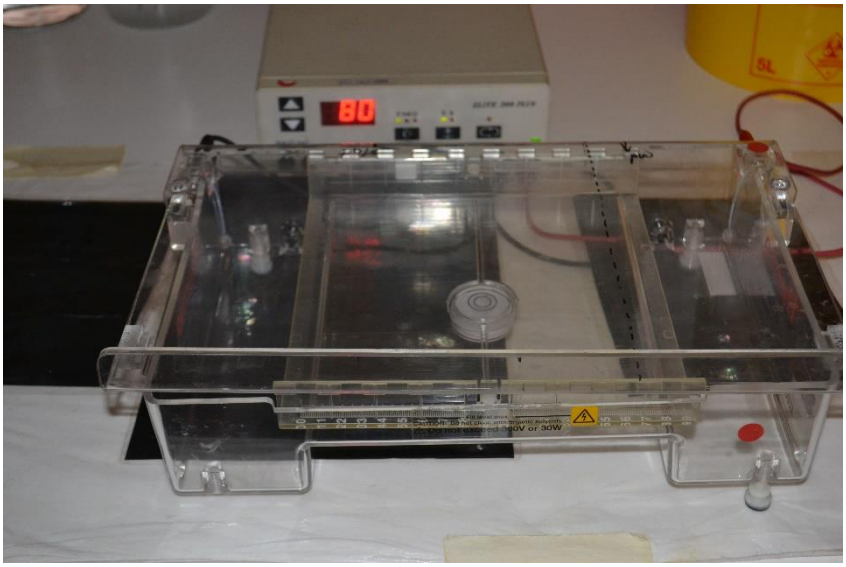


Figure 2.8: Gel electrophoresis setup running at 80 mA.

The extracted DNA fragment in the gel was observed with a UV-Trans illuminator. Photos taken were processed using an E-Capt program that came with the E-Box UV-Trans illuminator to identify the bands that cannot be directly seen due to impurities. This was done by comparing the E-Capt processed images with those of the 50 bp ladder (Figure 2.9) programmed into the E-Capt program.

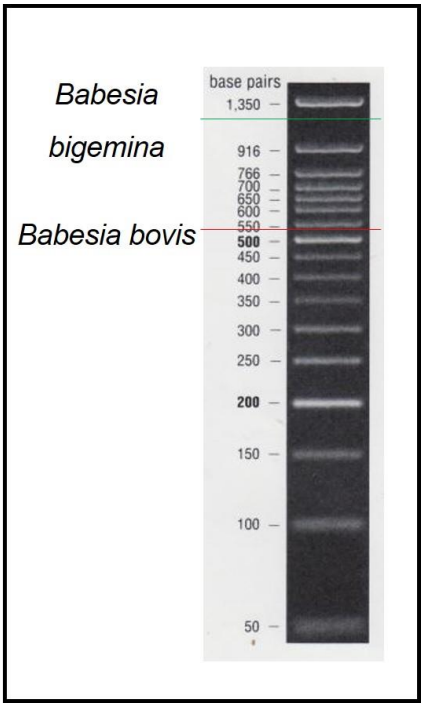


Figure 2.9: Fifty (50) bp ladder indicating the positions of DNA-PCR fragment from *Babesia bigemina* and *Babesia bovis.*

The DNA fragments on the gel were then used to identify *B. bigemina* and *B. bovis*. The amplified *B. bigemina* DNA produced a fragment of 1124 bp and *B. bovis* one of 541 bp as was also found by Chaudhry *et al.* (2010) and Lempereur *et al.* (2010) (Table 2.2).

Table 2.2: Base pair lengths of the different PCR bands of the protozoans and tick DNA according to Chaudhry *et al.* (2010) and Lempereur *et al.* (2010).

Protozoan species	Position of BP
Babesia bigemina	1124 bp
Babesia bovis	541 bp

2.5. Statistical analysis

Microsoft Excel 2013 was used to calculate the descriptive statistics and the significance of differences between the numbers of *R. (B.) decoloratus* and *R. (B.) microplus* collected during each collection (April 2017 - November 2017, April 2017 - April 2018 and November 2017 – April 2018). A 95% confidence interval was assumed. Significance test was also performed between the numbers of each species collected compared to the other.

2.6. Problems that occurred and how they were handled

2.6.1. Incubation

Larvae from ticks collected during the last field trip during April 2018 escaped from the individual vials. The larvae were still contained in terms of camps from which they were collected as well as separation of the tick species but larvae of different females from one camp and each species were pooled for DNA extractions. This enabled the detection of the presence of *Babesia* spp. for each camp for each of the two tick species collected. The methodology was thus altered by extracting DNA from tick larvae pooled for each camp and species instead of pooling larvae from individual females collected from each camp. This lead to significantly fewer samples being tested during April 2018. This alteration was made possible due to the way the samples were stored during incubation that separated the samples per camp and species.

Although this occurred, the samples could still be used to indicate if *Babesia* spp. were present in the test population and thus did not significantly alter the results.

2.7. Disposal

2.7.1. Blood

Blood vacutainer tubes were stored until the end of the study at 4°C in the fridge. At the end of the study, the blood tubes were discarded into a biological waste container into a clear plastic bag and disposal box provided for biological waste by the disposal company. Disposal boxes were periodically collected the accredited disposal company, Compass, for incineration.

2.7.2. Ticks

Ticks were disposed of according to the internal laboratory SOP for tick disposal by treating cotton stopper and conical flask with acetone for a day and then pouring boiling water over flask and stopper. Ticks were then discarded into a biological waste container for removal by Compass biological waste removal.

2.7.3. DNA

Extracted DNA was stored during the study period. After the study was concluded, the DNA was placed within an autoclave for 20 minutes at 121°C. The remaining chemicals in the DNA extraction were then disposed of as described below.

2.7.4. Chemicals

Chemicals were placed in internal chemical waste disposal containers in the laboratory and was disposed of periodically by an appointed internal department of the University of the Free State according to the appropriate disposal laws for chemical waste.

2.7.5. Waste

Normal laboratory waste was disposed of according to the internal laboratory procedure by placing non-biological, non-sharp and non-chemical waste in the bin marked normal laboratory waste. This was discarded once a week through Municipal waste removal.

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Chapter 3: Results

Several techniques can be implemented to identify *Babesia bovis* and *Babesia bigemina* in tick and bovine hosts. These techniques include blood smears, Polymerase chain reactions (PCR), with the use of applicable primers, as well as the enzyme-linked immunosorbent assay (ELISA). Only the first two techniques were used in this study (Lempereur *et al.* 2010, Abu Kwaik *et al*. 2011).

The most common means of identification of these protozoan parasites is through blood smears, as it is fast and inexpensive compared to the other means of detection. Blood smears however can miss acute infections and trained personal may be needed to differentiate between the two main *Babesia* spp. that are found in South Africa (Küttel *et al.* 2007).

Polymerase Chain Reaction (PCR) is more suited for identification of *Babesia* spp. as it can detect these protozoan parasites even at lower parasitaemia or DNA concentrations. *Babesia* spp. in blood smears can mainly be seen during acute infections when high concentrations of the parasite in the merogony stage can be found within the RBC of the host. The PCR is useful to identify samples where uncertainty of correct identification from blood smears exists. It is however more expensive and requires molecular expertise and equipment, causing blood smears to still be the preferred method of identification by many veterinarians, as first level of investigation.

In this chapter, results obtained from blood and DNA extractions of *Rhipicephalus (Boophilus)* spp. collected from cattle, kept in different camps on a farm near Makhanda, are presented.

3.1. Larval field collections

Eleven from a total of 53 camps were dragged for larval collections. These camps were chosen as they presented the highest probability for the presence of larvae that could have infected the cattle groups under investigation. These cattle groups were

allocated to these camps for grazing, during the preceding four weeks before collection.

3.1.1. Morphological identification

Rhipicephalus (Boophilus) decoloratus larvae were found in all six camps where drags were performed during the April 2017 collection, with a mean number of 8 ± 5 larvae and a total of 50 larvae. The highest number of larvae were collected in Milk Cow camp (30 ± 12) with only one larva each found from drags in the camps Quarry, Barbers Dam and Gaalboom. Drags from Bushalt and Guava camps had 15 and 2 larvae, respectively. *Rhipicephalus (Boophilus) microplus* was only found on drags from four of the six camps with a mean of 2 ± 1 and a total of 11 larvae. The highest number of larvae were collected in Milk Cow camp (6 ± 2) with one in Guava and two in each of Quarry and Bushalt camps (Table 3.1).

Rhipicephalus (Boophilus) decoloratus larvae were found in all (9) camps where drags were performed during the November 2017 collection with a mean number of 18 ± 4 and a total of 169 larvae. Drags from Bushalt, Milk Cow, Guava, Sheds and Gaalboom camp each had the highest (30 ± 13) larvae with only two larvae found from drags done at Gavin Hill. Drags from Lolweni, Quarry and Barbers Dam had six, seven and four tick larvae, respectively. *Rhipicephalus (Boophilus) microplus* was only collected in one camp, Bushalt, where two (2 ± 1) larvae were found on one drag (Table 3.1).

Rhipicephalus (Boophilus) decoloratus larvae were found on drags from all ten camps during the April 2018 collection with a mean number of 17 ± 3 larvae and a total of 170 larvae. The highest number of larvae were collected in Arthurs Reservoir, Milk Cow and Guava camp, with 30 ± 11 larvae each; only three larvae were found from drags in Sheds camp. *Rhipicephalus (Boophilus) decoloratus* larvae were also collected from Gavin Hill (20), Lolweni (15), Quarry (5), Barbers Dam (15), Lands and Old Orange (18) and Gaalboom (4). No *R. (B.) microplus* were collected from any drags during this period (Table 3.1).

During the total study period, *R. (B.) decoloratus* was the most dominant species found on all the drags in all the camps. In the camps Arthurs Reservoir, Gavin Hill, Lolweni, Sheds, Barbers Dam, Lands and Old Orange and Gaalboom, all the larvae collected from drags were *R. (B.) decoloratus. Rhipicephalus (Boophilus) microplus* larvae were only collected from the camps Quarry, Bushalt, Milk Cow and Guava at a percentage

of 13%, 8%, 6% and 2%, of the total larval count for each camp, respectively (Table 3.1).

Overall the 389 *R. (B.) decoloratus* larvae found during this study represented 97% of all blue tick larvae found on grasses from the farm. The minimum percentage *R. (B.) decoloratus* found in a camp was 87% and several camps had only *R. (B.) decoloratus* larvae. *Rhipicephalus (Boophilus) microplus* consisted of a total of 13 larvae representing 3% of the total blue tick larvae found. Most of the test camps had no *R. (B.) microplus* while Quarry had the most at 13%. A significantly higher number of *R. (B.) decoloratus* compared to *R. (B.) microplus* was therefore present on this farm with a strong P-value (95%) of 0.0000402(Table 3.1).

Table 3.1: The total number of tick larvae and descriptive statistics for, *R. (B.) decoloratus* and *R. (B.) microplus*, collected during April 2017, November 2017 and April 2018 and the percentage of total ticks collected from each camp over the collection period.

	Apr-17		Nov-17		Apr-18		Totals		% of total	
	R dec	**R mic**	**R dec**	**R mic**	**R dec**	**R mic**	**R dec**	**R mic**	**R dec**	**R mic**
ARTHURS RESERVOIR	-	-	-	-	30	0	**30**	**0**	100	0
GAVIN HILL	-	-	2	0	20	0	**22**	**0**	100	0
LOLWENI	-	-	6	0	15	0	**21**	**0**	100	0
QUARRY	1	2	7	0	5	0	**13**	**2**	87	13
BUSHALT	15	2	30	2	-	-	**45**	**4**	92	8
MILKCOW	30	6	30	0	30	0	**90**	**6**	94	6
GUAVA	2	1	30	0	30	0	**62**	**1**	98	2
SHEDS	-	-	30	0	3	0	**33**	**0**	100	0
BARBERS DAM	1	0	4	0	15	0	**20**	**0**	100	0
LANDS AND OLD ORANGE	-	-	-	-	18	0	**18**	**0**	100	0
GAALBOOM	1	0	30	0	4	0	**35**	**0**	100	0
Sum	**50**	**11**	**169**	**2**	**170**	**0**	**389**	**13**		
Mean	**8**	**2**	**18**	**2**	**17**	**0**	**35**	**1**	**97**	**3**
Standard Error	**5**	**1**	**4**	**0**	**3**	**0**	**7**	**1**	**1**	**1**
Minimum	**1**	**1**	**2**	**2**	**3**	**0**	**13**	**1**	**87**	**0**
Maximum	**30**	**6**	**30**	**2**	**30**	**0**	**90**	**6**	**100**	**13**
Standard Deviation	**12**	**2**	**13**	**1**	**11**	**0**	**23**	**2**	**4**	**4**

P-value (95%) Total *R. (B.) decoloratus* versus total *R. (B.) microplus*= 0.0000402

- : No collections were performed R. dec: *R. (B) decoloratus* R. mic: *R. (B.) microplus*

3.1.2. Polymerase chain reaction identification

3.1.2.1. DNA extraction from larvae collected from drags

DNA was extracted from 402 individual larvae collected from field drags during all three collection periods. Sufficient DNA concentrations for obtaining successful PCR products is considered to be 10 ng/ µl and a 260/280 DNA purity of 1.5 - 2.5. This is the minimum DNA concentration required for the Kappa2G Robust HotStart PCR kit. Only 60 individual larval DNA extractions indicated sufficient DNA to perform PCR (Table 3.2).

DNA extractions of larvae collected from drags done during April 2017, had two *R. (B.) decoloratus* (Table 3.2A; Lane 1-2) (Figure 3.1A) and two *R. (B.) microplus* (Table 3.2A; Lane 3-4) (Figure 3.1A) larval DNA extractions from 50 and 11 individual larvae collected, respectively, with sufficient DNA to perform a PCR. Collections from November 2017 had 28 *R. (B.) decoloratus* (Table 3.2B; Lane 16-20, Table 3.2C; Lane 2-22) (Figure 3.1B & Figure 3.1C) of 169 individual larvae collected and one *R. (B.) microplus* (Table 3.2A; Lane 5) (Figure 3.1A) from two larval DNA extraction samples with sufficient DNA for PCR. The collection during April 2018 had 26 *R. (B.) decoloratus* (Table 3.2A; Lane 6-20, Table 3.2B; Lane 2-15) (Figure 3.1A & Figure 3.1B) from 170 larvae collected, that provided sufficient DNA for PCR. No *R. (B.) microplus* larvae were collected from drags during this period. Insufficient DNA concentrations and purity requirements were obtained from DNA extractions from the remaining 342 samples and PCR was not performed on these samples (Table 3.3).

None of the larval DNA extractions however showed any presence of *Babesia* spp. after they were subjected to the PCR. No DNA fragments could be visualised on agarose electrophoresis at 1124 bp fragment size as indication of the presence of *B. bigemina* and 541 bp for *B. bovis.* Fragments seen on the agarose gels in Figure 3.1 were much smaller than those expected for the two protozoan species and were most probably due to primer dimers (Figure 3.1A-3.1C).

Table 3.2A: Extracted larval DNA concentrations and PCR results from different camps and species collected during April 2017 and November 2017, with gel lane numbers corresponding to those indicated in Figure 3.1A.

Collection date	Lane		DNA purity		Camp	DNA extracted from
-	Lane number	Identified	ng/ µl	260/280	-	-
April 2017	1	None	138.7	2.37	QUARRY	*R. (B.) decoloratus*
	2	None	49.2	2.36	BUSHALT	
	3	None	259.2	2.25	MILK COW	*R. (B.) microplus*
	4	None	336.6	2.13		
November 2017	5	None	11.9	2.10	BUSHALT	*R. (B.) microplus*
	6	None	153.5	1.60	LOLWENI	*R. (B.) decoloratus*
	7	None	142.2	1.67		
	8	None	417.0	2.05	QUARRY	
	9	None	452.4	1.60		
	10	None	344.8	2.10		
	11	None	36.5	1.90	BUSHALT	
	12	None	314.4	1.77		
	13	None	268.2	1.89		
	14	None	322.5	2.30		
	15	None	326.7	1.92		
	16	None	68.7	1.70		
	17	None	359.8	1.94		
	18	None	274.4	1.93	MILKCOW	
	19	None	16.7	1.69		
	20	None	315.2	1.60		
-	Ladder	-	-	-	-	-

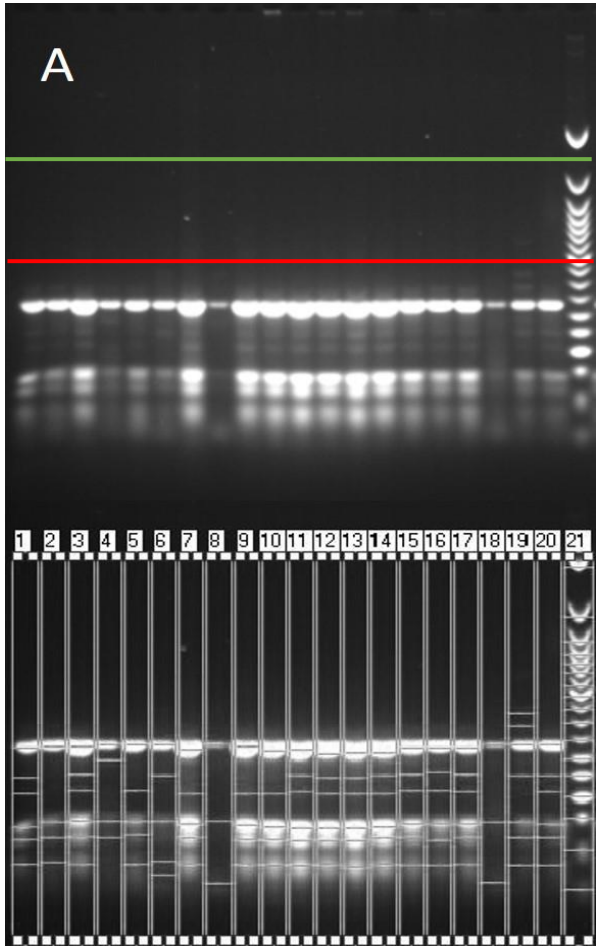

Figure 3.1A: Agarose gel of *Babesia* within larvae from the field. Green *B. bigemina* (1124 bp fragment)*;* Red *B. bovis* (541 bp fragment).

Table 3.2B: Extracted larval DNA concentrations and PCR results from different camps and species collected during November 2017 and April 2018, with gel lane numbers corresponding to those indicated in Figure 3.1B.

Collection date	Lane		DNA purity		Camp	DNA extracted from
-	Lane number	Identified	ng/ µl	260/280	-	-
-	Ladder	-	-	-	-	-
November 2017	2	None	303.8	2.09	MILKCOW	*R. (B.) decoloratus*
	3	None	329.2	1.92		
	4	None	120.6	1.63		
	5	None	202.0	2.21		
	6	None	392.6	1.65		
	7	None	117.2	1.73		
	8	None	234.3	2.13	SHEDS	
	9	None	226.1	2.30		
	10	None	390.1	2.40	GAALBOOM	
	11	None	385.6	2.00		
	12	None	151.5	2.10		
	13	None	152.5	2.06		
	14	None	159.3	2.23		
	15	None	59.6	2.02		
April 2018	16	None	462.5	2.30	ARTHURS RESERVOIR	
	17	None	417.5	1.80		
	18	None	178.9	2.39		
	19	None	257.9	2.09		
	20	None	75.9	1.60		

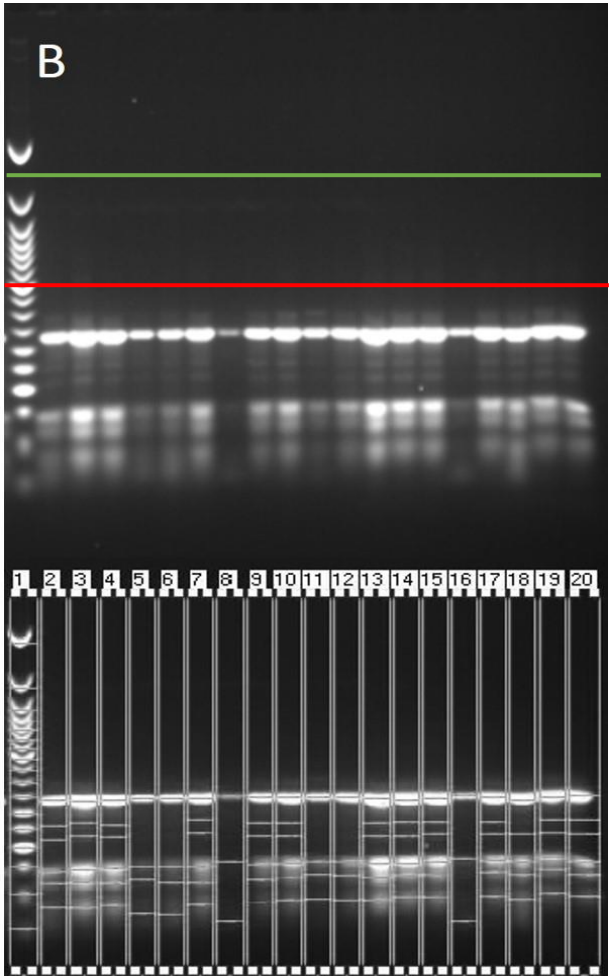

Figure 3.1B: Agarose gel of *Babesia* within larvae from the field. Green *B. bigemina* (1124 bp fragment)*;* Red *B. bovis* (541 bp fragment).

Table 3.2C: Extracted larval DNA concentrations and PCR results from different camps and species collected during April 2018, with gel lane numbers corresponding to those indicated in Figure 3.1C.

Collection date	Lane		DNA purity		Camp	DNA extracted from
-	Lane number	Identified	ng/ µl	260/280	-	-
-	Ladder	-	-	-	-	-
April 2018	2	None	481.7	2.12	GAVIN HILL	*R. (B.) decoloratus*
	3	None	202.5	2.30		
	4	None	244.6	1.52	QUARR	
	5	None	488.3	2.34		
	6	None	197.4	1.65		
	7	None	82.2	1.96	MILKCOW	
	8	None	91.3	2.28		
	9	None	372.6	2.04		
	10	None	327.7	1.94		
	11	None	416.7	2.24		
	12	None	122.6	2.41		
	13	None	300.4	2.15		
	14	None	248.9	1.96	GUAVA	
	15	None	424.0	1.90		
	16	None	53.7	1.78		
	17	None	294.3	2.18		
	18	None	260.6	1.99	BARBERS DAM	
	19	None	397.9	1.60	LANDS AND OLD ORANGE	
	20	None	471.1	2.10		
	21	None	289.8	2.28		
	22	None	99.3	1.59	ARTHURS RESERVOIR	

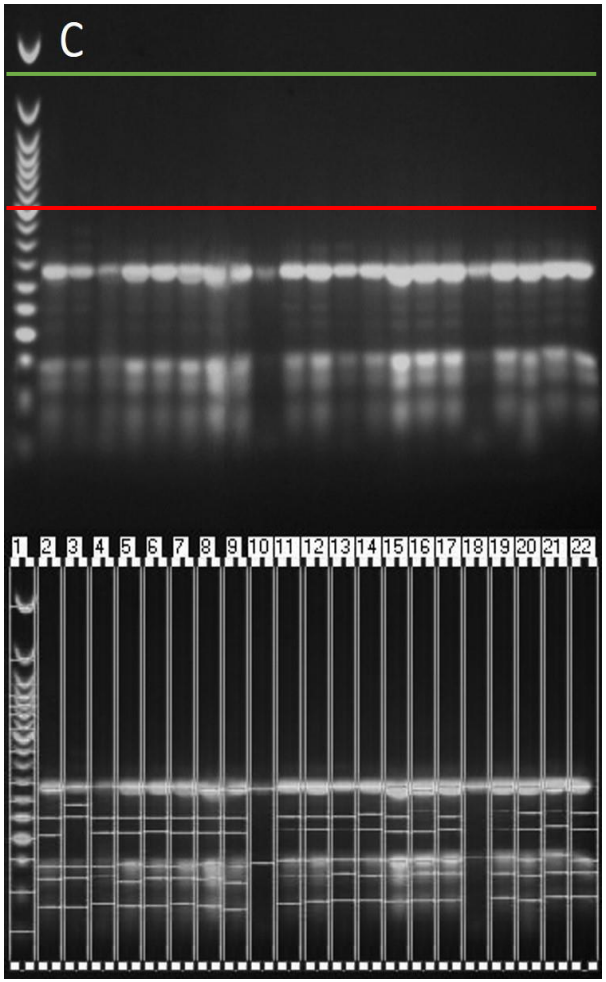

Figure 3.1C: Agarose gel of *Babesia* within larvae from the field. Green *B. bigemina* (1124 bp fragment)*;* Red *B. bovis* (541 bp fragment).

Table 3.3: Extracted larval DNA with insufficient DNA concentrations and PCR results from different camps and species collected during April 201, November 2017 and April 2018.

Collection date		DNA purity		Camp	**DNA extracted from**
		ng/ µl	260/280		
April 2017	1	0.5	1.84	BUSHALT	*R. (B.) decoloratus*
	2	2.8	2.29		
	3	7.8	3.64		
	4	363.1	3.3		
	5	0.8	3.57		
	6	8	1		
	7	6.3	1.72		
	8	5.7	0.66		
	9	0.3	1.58		
	10	0.3	1.8		
	11	221.7	0.69		
	12	3.8	2.12		
	13	1.1	2.52		
	14	2.1	3.49		
	15	3.3	3.43	MILK COW	
	16	6.7	0.29		
	17	1.1	0.25		
	18	6.3	2		
	19	7.2	2.57		
	20	3.8	0.15		
	21	4.5	2.16		
	22	7	1.91		
	23	5.1	0.4		
	24	7.4	2.92		
	25	2.9	1.17		
	26	477.9	1.14		
	27	2.4	3.06		
	28	3.4	2.76		
	29	9.6	2.15		
	30	223.4	1.43		
	31	0.1	1.99		
	32	0.1	0.96		
	33	9	1.01		
	34	0.9	1.1		
	35	6.3	1.43		
	36	4.7	1.72		
	37	1.2	3.2		
	38	6.7	1.61		
	39	9.3	0.09		
	40	5.4	1.75		
	41	8.8	3.29		
	42	7.7	2.1		

Table 3.3 continued: Extracted larval DNA with insufficient DNA concentrations and PCR results from different camps and species collected during April 201, November 2017 and April 2018.

Collection date		DNA purity		Camp	**DNA extracted from**
		ng/ µl	260/280		
April 2017	43	2.4	0.27	MILK COW	*R. (B.) decoloratus*
	44	6.4	2.46		
	45	2.9	2.07	GUAVA	
	46	6.8	0.55		
	47	0.4	2.04	BARBERS DAM	
	48	9.1	0.48	GAALBOOM	
	49	2	0.76	QUARRY	*R. (B.) microplus*
	50	8	3.15		
	51	454.3	3	BUSHALT	
	52	3.6	3.65		
	53	8.2	2.43	MILK COW	
	54	89.4	0.69		
	55	264.3	0.35		
	56	208	0.49		
	57	2.6	3.49	GUAVA	
November 2017	58	346.9	0.93	BUSHALT	*R. (B.) microplus*
	59	0.3	1.56	GAVIN HILL	*R. (B.) decoloratus*
	60	9.8	1.79		
	61	9.7	0.44	LOLWENI	
	62	5.7	2.74		
	63	324	3.04		
	64	2.7	0.22		
	65	9.7	2.65	QUARRY	
	66	388	2.6		
	67	110.9	0.01		
	68	6.7	1.02		
	69	340.8	2.54	BUSHALT	
	70	205.5	1.1		
	71	428	2.85		
	72	99.3	3.35		
	73	6.7	3.33		
	74	3.3	0.18		
	75	6.2	3.78		
	76	8.3	3.32		
	77	4.9	0.43		
	78	4	2.88		
	79	4.6	3.55		
	80	3.5	2.11		
	81	196.1	3.1		
	82	5	3.24		
	83	25.9	3.74		
	84	8.9	0.39		

Table 3.3 continued: Extracted larval DNA with insufficient DNA concentrations and PCR results from different camps and species collected during April 201, November 2017 and April 2018.

Collection date			DNA purity		Camp	**DNA extracted from**
			ng/ µl	260/280		
November 2017		85	160.8	3.42	BUSHALT	*R. (B.) decoloratus*
		86	9	2.74		
		87	380.5	1.32		
		88	6	3.69		
		89	4.5	0.93		
		90	396.9	2.5		
		91	335.8	2.85		
		92	6.9	1.88	MILKCOW	
		93	5.8	3.59		
		94	248	3.44		
		95	6.9	3.22		
		96	7.4	3.36		
		97	5.6	2.86		
		98	1	2.05		
		99	1.1	2.9		
		100	3.9	2.46		
		101	419.4	3.07		
		102	422.2	0.88		
		103	7.2	0.66		
		104	9.4	0.76		
		105	7.8	3.56		
		106	430.3	3.92		
		107	61.1	0.92		
		108	79.6	2.59		
		109	2.4	3.08		
		110	9	0.48		
		111	7	2.1		
		112	368.3	0.97		
		113	161	1.43	GUAVA	
		114	9.1	3.62		
		115	358.8	1.38		
		116	1.7	3.11		
		117	0.8	1.4		
		118	104.9	3.04		
		119	320.2	2.73		
		120	8.6	3.27		
		121	1.3	2.46		
		122	2	1.2		
		123	4.1	1.73		
		124	0.7	0.15		
		125	3.8	3.83		
		126	278.9	2.79		

Table 3.3 continued: Extracted larval DNA with insufficient DNA concentrations and PCR results from different camps and species collected during April 201, November 2017 and April 2018.

Collection date		DNA purity		Camp	**DNA extracted from**
		ng/ µl	260/280		
November 2017	106	430.3	3.92	MILKCOW	*R. (B.) decoloratus*
	107	61.1	0.92		
	108	79.6	2.59		
	109	2.4	3.08		
	110	9	0.48		
	111	7	2.1		
	112	368.3	0.97		
	113	161	1.43	GUAVA	
	114	9.1	3.62		
	115	358.8	1.38		
	116	1.7	3.11		
	117	0.8	1.4		
	118	104.9	3.04		
	119	320.2	2.73		
	120	8.6	3.27		
	121	1.3	2.46		
	122	2	1.2		
	123	4.1	1.73		
	124	0.7	0.15		
	125	3.8	3.83		
	126	278.9	2.79		
	148	8.6	1.83		
	149	393	1.42		
	150	349.5	0.53		
	151	306.1	0.01		
	152	0.1	2.32		
	153	163.9	0.74		
	154	1.4	3.91		
	155	7	1.66		
	156	2	0.68		
	157	399.4	3.14		
	158	8.1	1.58		
	159	161.8	3.87		
	160	8.4	1.63		
	161	9.8	3.05		
	162	2.1	0.84		
	163	1.5	3.06		
	164	104.6	3.77		
	165	1.5	2.59		
	166	2	0.78		
	167	7.5	1.34		
	168	9.2	3.74		

Table 3.3 continued: Extracted larval DNA with insufficient DNA concentrations and PCR results from different camps and species collected during April 201, November 2017 and April 2018.

Collection date		DNA purity		Camp	**DNA extracted from**
		ng/ µl	260/280		
November 2017	169	3.2	2.49	SHEDS	*R. (B.) decoloratus*
	170	4.8	1.58		
	171	194.5	2.63	BARBERS DAM	
	172	8.2	2.78		
	173	127.1	3.53		
	174	1.4	0.01		
	175	1.2	0.99	GAALBOOM	
	176	2.3	2.22		
	177	4.2	0.27		
	178	197.6	1.44		
	179	4.7	1.23		
	180	147	2.73		
	181	134	2.76		
	182	0	3.88		
	183	2.1	1.9		
	184	369.6	3.6		
	185	474.7	3.79		
	186	12.4	2.91		
	187	3.9	2.06		
	188	1.2	2.82		
	189	5.4	3.94		
	190	6.6	3.75		
	191	1	0.71		
	192	249.1	0.33		
	193	2.7	1.57		
	194	7.4	2.4		
	195	9.8	3.63		
	196	8.8	3.63		
	197	461.6	3.95		
	198	7.7	3.94		
April 2018	199	1	3.99	ARTHURS RESERVOIR	
	200	8.5	3.92		
	201	3.9	1.04		
	202	9.5	0.23		
	203	43	0.62		
	204	6.8	0.4		
	205	437.7	0.46		
	206	216.8	1.14		
	207	1.9	0.08		
	208	8.7	2.41		
	209	2.3	1.48		
	210	3.2	3.89		

Table 3.3 continued: Extracted larval DNA with insufficient DNA concentrations and PCR results from different camps and species collected during April 201, November 2017 and April 2018.

Collection date		DNA purity		Camp	**DNA extracted from**
		ng/ µl	260/280		
April 2018	211	3	1.77	ARTHURS RESERVOIR	*R. (B.) decoloratus*
	212	138.3	3.18		
	213	3	2.79		
	214	497.9	0.04		
	215	259.7	0.4		
	216	6.6	2.12		
	217	385.3	3.93		
	218	7.2	2.95		
	219	4.5	2.47		
	220	5.7	0.89		
	221	8.8	0.78		
	222	178.4	3.38		
	223	3.5	0.27	GAVIN HILL	
	224	5.8	0.44		
	225	1.4	3.97		
	226	9	0.98		
	227	8.1	1.75		
	228	352.9	3.59		
	229	6.2	0.96		
	230	0.4	1.11		
	231	254.3	0.18		
	232	7.3	2.92		
	233	7.6	2.4		
	234	1.3	0.75		
	235	4	0.53		
	236	483.2	3.59		
	237	8.4	1.95		
	238	6	1.69		
	239	1.6	1.93		
	240	227.6	0.52		
	241	2.9	1.21	LOLWENI	
	242	7.8	2.73		
	243	6.6	1.18		
	244	6.3	2.67		
	245	6.2	1.05		
	246	0.3	0.69		
	247	6.5	2.09		
	248	3.4	1.99		
	249	2.6	0.39		
	250	5.1	3.12		
	251	352.1	0.91		
	252	162.6	3.32		

Table 3.3 continued: Extracted larval DNA with insufficient DNA concentrations and PCR results from different camps and species collected during April 201, November 2017 and April 2018.

Collection date		DNA purity		Camp	**DNA extracted from**
		ng/ µl	260/280		
April 2018	253	266.6	0.07	LOLWENI	*R. (B.) decoloratus*
	254	0.9	2.63		
	255	109.9	2.7		
	256	2.8	1.95	QUARRY	
	257	1.9	2.46		
	258	202.5	3.86	MILKCOW	
	259	59.6	0.65		
	260	6.4	2.87		
	261	66.9	2.94		
	262	219.6	3.99		
	263	261.2	3.72		
	264	4.4	0.98		
	265	2.8	3.28		
	266	9.5	2.91		
	267	242	3.04		
	268	5.2	3.28		
	269	326.1	0.42		
	270	25.3	3.36		
	271	1.3	3.11		
	272	1.9	0.59		
	273	6.3	1.22		
	274	211.5	0.92		
	275	265.8	3.38		
	276	169.6	1.24		
	277	6.2	1.17		
	278	4.3	3.78		
	279	4.4	3.74		
	280	3.5	1.93		
	281	0.8	3.12	GUAVA	
	282	83.2	1.44		
	283	16.1	0.87		
	284	34.8	2.98		
	285	9.6	2.38		
	286	5	3.39		
	287	2.8	2.62		
	288	1	1.63		
	289	207.9	2.72		
	290	7	2.04		
	291	53.8	2.6		
	292	1.2	0.62		
	293	6.5	3.12		
	294	492.4	0.84		
	295	0	1.3		
	296	2.9	3		

Table 3.3 continued: Extracted larval DNA with insufficient DNA concentrations and PCR results from different camps and species collected during April 201, November 2017 and April 2018.

Collection date		DNA purity		Camp	**DNA extracted from**
		ng/ µl	260/280		
April 2018	297	215.2	3.89	GUAVA	*R. (B.) decoloratus*
	298	286.9	0.73		
	299	3.8	1.84		
	300	6.8	1.21		
	301	9.7	3.56		
	302	9.3	1.47		
	303	1.7	1.65		
	304	6	2.67		
	305	241.4	0.77		
	306	90.7	1.24		
	307	2	2.41	SHEDS	
	308	334.4	2.7		
	309	6.2	0.96		
	310	5.4	1.32	BARBERS DAM	
	311	87.8	3.11		
	312	0.9	1		
	313	9.4	3.19		
	314	300.8	1.04		
	315	2.4	2.55		
	316	100.9	0.65		
	317	5.8	1.88		
	318	8.7	0.02		
	319	6	3.61		
	320	0.8	0.57		
	321	8.3	1.32		
	322	8.8	2.5		
	323	0.7	2.52		
	324	5.2	2.12	LANDS AND OLD ORANGE	
	325	241.3	0.45		
	326	9.1	1.01		
	327	3.3	0.33		
	328	9.6	0.68		
	329	8.3	2.69		
	330	437.7	0.99		
	331	3.1	1.77		
	332	1.9	2.73		
	333	8.6	0.98		
	334	8.8	2.1		
	335	6.9	1.17		
	336	9.5	3.24		
	337	2.4	3.11		
	338	7.5	3.07		
	339	2.3	2.74	GAALBOOM	
	340	103.4	2.99		
	341	4.9	3.69		
	342	490.4	3.66		

3.2. Adult ticks collected from cattle

Cattle that grazed on grass in the camps dragged for larvae were investigated for the presence of adult ticks during each collection period. Fully engorged female blue ticks were collected from at least ten animals, where possible, from each camp.

3.2.1. Morphological identification

A total of 1144 engorged female *R. (B.) decoloratus* ticks with a mean of 229 ± 90 per camp were obtained from the cattle grazing in the five camps investigated during the April 2017 collection period (Table 3.4). Of these, four ticks from Quarry camp, nine from Bushalt, three from Milk Cow and ten from Guava camp were allocated to this project for evaluation of the presence of *Babesia* species (Table 3.4). The other ticks were allocated to two other projects running simultaneously with the *Babesia* study (Lesenyeho 2019, Pottinger 2019). During the same period a total of 32 engorged female *R. (B.) microplus* with a mean of 6 ± 3 per camp, were obtained from the same cattle group (Table 3.4). Again one adult *R. (B.) microplus* each from Quarry and Milk Cow and three and five adults from Lolweni and Guava camps were allocated to this project to investigate the presence of *Babesia* species from cattle grazing in these camps.

The most adult females were collected from cattle grazing in Guava camp with 528 ± 202 *R. (B.) decoloratus* and 15 ± 6 *R. (B.) microplus*. This was followed by Bushalt with 328 *R. (B.) decoloratus* and 11 *R. (B.) microplus* and Quarry camp with 163 *R. (B.) decoloratus* and 2 *R. (B.) microplus* adult ticks. The lowest number of ticks were found from cattle in Milk Cow camp where only three animals were grazing (Table 3.4).

Eight hundred and twenty-two engorged female *R. (B.) decoloratus* and 26 *R. (B.) microplus* females were obtained from the five camps investigated during the November 2017 collection period. Gavin Hill camp had the most ticks collected during this period with 422 ± 149 *R. (B.) decoloratus* and 19 ± 8 *R. (B.) microplus* and with 12 and five adult ticks allocated from each species, respectively, to investigate *Babesia* presence in the progeny of these ticks (Table 3.4). Lolweni camp had the lowest number of *R. (B.) decoloratus* (55 ± 149) and only 1 ± 8 *R. (B.) microplus* tick collected. From Lolweni camp three *R. (B.) decoloratus* and one *R. (B.) microplus* were used to test for *Babesia* presence. *Rhipicephalus (Boophilus) decoloratus* were also

collected from Bushalt (90), Milk Cow (91) and Sheds (164) with nine, nine and two *R. (B.) decoloratus,* respectively, used for DNA extractions. *Rhipicephalus (Boophilus) microplus* was also collected in camps Bushalt (1), Sheds (5) with one and two *R. (B.) microplus,* respectively being used. No *R. (B.) microplus* was collected from animals that grazed in Milk Cow camp (Table 3.4).

During the April 2018 collection a total of 1057 *R. (B.) decoloratus* and 12 *R. (B.) microplus* were collected from animals that grazed in four camps. (Table 3.4).

The most ticks were obtained from cattle that grazed in Lands and Old Orange camp during April 2018 with 427 ± 121 *R. (B.) decoloratus* and 6 ± 2 *R. (B.) microplus* adult females found. Twenty *R. (B.) decoloratus* and six *R. (B.) microplus* were used for further investigation for the presence of *Babesia* spp. Barbers Dam camp had the lowest number of *R. (B.) decoloratus* (140) and only three *R. (B.) microplus*. From Barbers Dam camp, six *R. (B.) decoloratus* and three *R. (B.) microplus* adults were used. During this period *R. (B.) decoloratus* were also collected from Arthurs Reservoir (217) and Milk Cow (273) with 15 and 10 *R. (B.) decoloratus* respectively being used for each camp. *Rhipicephalus (Boophilus) microplus* was only found in the camp Arthurs Reservoir (3) and the progeny of all three were used for DNA extraction. No *R. (B.) microplus* was collected from cattle in Milk Cow camp (Table 3.4).

Overall, engorged female *R. (B.) microplus* ticks were found on cattle from all camps investigated throughout the total study period, but were present at a low percentage of between 1% and 5% of the total blue tick count.

A total of 3023 *R. (B.) decoloratus* adults, were representing of 98% of all blue ticks found on the farm. The minimum percentage *R. (B.) decoloratus* found in a camp was 95%. *Rhipicephalus (Boophilus) microplus* consisted of a total of 70 adults representing only 2% of the total blue ticks found. A significantly higher number of *R. (B.) decoloratus* than *R. (B.) microplus* adults were collected from the cattle, with a strong P-value (95%) 0.000041 (Table 3.4).

Table 3.4: Total number of adult *R. (B.) decoloratus* and *R. (B.) microplus*, collected from cattle during April 2017, November 2017 and April 2018 for two participating studies. The number of ticks allocated to this study for investigation for the presence of *Babesia* spp. are indicated in parentheses over the rest were allocated to the project of Pottinger (2019).

	Apr-17		Nov-17		Apr-18		Total		% of Total	
	R dec	**R mic**	**R dec**	**R mic**	**R dec**	**R mic**	**R dec**	**R mic**	**R dec**	**R mic**
ARTHURS RESERVOIR	-	-	-	-	217(15)	3(3)	**217**	**3**	**99**	**1**
GAVIN HILL	-	-	422(12)**	19(5)	-	-	**422**	**19**	**96**	**4**
LOLWENI	-	-	55(3)	1(1)	-	-	**55**	**1**	**98**	**2**
QUARRY	163(4)	2(1)	-	-	-	-	**163**	**2**	**99**	**1**
BUSHALT	328(9)	11(3)	90(9)	1(1)	-	-	**418**	**12**	**97**	**3**
MILKCOW	105(3)	3(1)	91(9)	0	273(10)	0	**469**	**3**	**99**	**1**
GUAVA	528(10)	15(5)	-	-	-	-	**528**	**15**	**97**	**3**
SHEDS	-	-	164(2)	5(2)*	-	-	**164**	**5**	**97**	**3**
BARBERS DAM	-	-	-	-	140(6)	3(3)	**140**	**3**	**98**	**2**
LANDS AND OLD ORANGE	-	-	-	-	427(20)	6(6)	**427**	**6**	**99**	**1**
GAALBOOM	20(2)	1(1)	-	-	-	-	**20**	**1**	**95**	**5**
Sum	**1144**	**32**	**822**	**26**	**1057**	**12**	**3023**	**70**		
Mean	**229**	**6**	**164**	**7**	**264**	**4**	**275**	**6**	**98**	**2**
Standard Error	**90**	**3**	**67**	**4**	**61**	**1**	**54**	**2**	**0**	**0**
Minimum	**20**	**1**	**55**	**1**	**140**	**3**	**20**	**1**	**95**	**1**
Maximum	**528**	**15**	**422**	**19**	**427**	**6**	**528**	**19**	**99**	**5**
Standard Deviation	**202**	**6**	**149**	**8**	**121**	**2**	**181**	**6**	**1**	**1**

P-value (95%) Total *R. (B.) decoloratus* versus total *R. (B.) microplus*= 0.000041

- : No collections were performed *: *B. bovis* infection **: *B. bigemina* infection R. dec: *R. (B) decoloratus* R. mic: *R. (B.) microplus*

3.2.2. Polymerase chain reaction identification

Larval progeny of all 146 adult ticks collected were used in DNA extractions with a total of 76 samples that had sufficient DNA concentrations for PCR.

Thirty-two DNA extractions, from the larval progeny of each individual adult female tick collected during April 2017, had sufficient DNA for PCR. No *Babesia* spp. DNA fragments were found after PCR were done for this sample group from a total of 24 *R. (B.) decoloratus* (Table 3.5A; Lane 1-20 & Table 3.5B; Lane 2-5) (Figure 3.2A-3.2B) and eight *R. (B.) microplus* (Table 3.5B; Lane 5-1) (Figure 3.3A) larval DNA preparations.

The November 2017 blue tick collections had 37 DNA preparations from the progeny of individual adult ticks collected with sufficient concentrations of extracted DNA to perform PCR. Twenty-seven DNA preparations were extracted from *R. (B.) decoloratus* and 10 from *R. (B.) microplus* progeny. The PCR fragments visualised on the agarose gel indicated two *R. (B.) decoloratus* from Gavin Hill that were positive for *B. bigemina* (Table 3.5C; Lane 15, Table 3.5D; Lane 2) (Figure 3.2C-3.2D). One *R. (B.) microplus,* collected from Sheds was positive for *B. bovis* (Table 3.5C; Lane 6) (Figure 3.2C). No other DNA preparations showed *Babesia* DNA fragments after PCR for this collection period (Table 3.5B; Lane 14-20, Table 3.5C; Lane1-20 & Table 3.5D; Lane 2-11) (Figure 3.2B-3.2D).

DNA extractions for the 63 blue tick collections made during April 2018, were grouped by camp and tick species as the tick larvae from individual ticks escaped form the vials in which individual ticks were kept. The larvae were still contained in terms of camps from which they were collected as well as separation of the tick species but larvae of different females were pooled for DNA extractions. All seven of the pooled samples, had sufficient DNA concentrations to perform PCR. Four *R. (B.) decoloratus* and three *R. (B.) microplus* larval DNA preparations representing four camps. No *Babesia* DNA fragments were found for this collection period (Table 3.5D; Lane 12-18) (Figure 3.2D).

Fourteen samples did not have sufficient DNA concentrations to perform PCR, consisting of seven for the April 2017 collection and seven for the November 2017 collection (Table 3.6).

Table 3.5A: Extracted adult tick DNA concentrations and PCR results from different camps and species collected during April 2017, with gel lane numbers corresponding to those indicated in Figure 3.2A.

Collection date	lane		DNA purity		Camp	Species extracted from
-	Lane number	Identified	ng/ µl	260/280	-	-
April 2017	1	None	838.7	2.07	BUSHALT	*R. (B.) decoloratus*
	2	None	196.1	2.03		
	3	None	320.5	2.05		
	4	None	903.8	2.02		
	5	None	1027.4	2.05		
	6	None	235.7	2.07		
	7	None	2329.7	2.1		
	8	None	819.9	2.44		
	9	None	696.7	1.9		
	10	None	256.7	2.01	GAALBOOM	
	11	None	309.5	2.02	GUAVA	
	12	None	253.3	1.73		
	13	None	655.1	2.11		
	14	None	518.4	2.1		
	15	None	233.7	1.97		
	16	None	2094.7	2		
	17	None	361.8	1.99		
	18	None	3889.2	1.98		
	19	None	2256	2	MILKCOW	
	20	None	2003.1	2.03		
-	Ladder	-	-	-	-	-

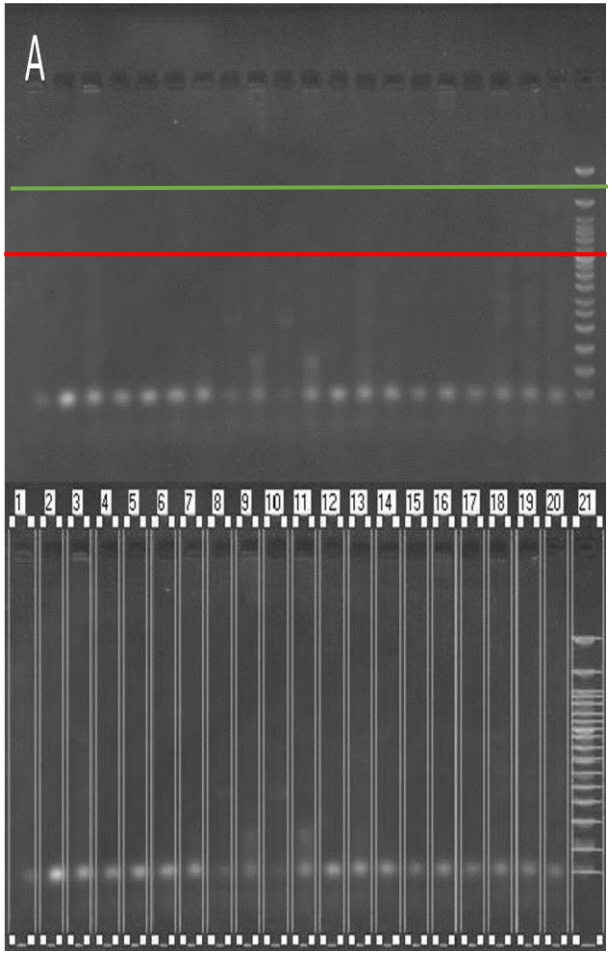

Figure 3.2A: Agarose gel of *Babesia* within adult ticks collected from cattle. Green *B. bigemina* (1124 bp fragment)*;* Red *B. bovis* (541 bp fragment).

Table 3.5B: Extracted adult tick DNA concentrations and PCR results from different camps and species collected during April 2017and November 2017, with gel lane numbers corresponding to those indicated in Figure 3.2B.

Collection date	Lane		DNA purity		Camp	Species extracted from
-	Lane number	Identified	ng/ µl	260/280	-	-
-	Ladder	-	-	-	-	-
April 2017	2	None	789.5	2.02	MILKCOW	*R. (B.) decoloratus*
	3	None	1496	2.12	QUARRY	
	4	None	1213.6	2.12		
	5	None	4039.4	2.08		
	6	None	914.3	2.05	BUSHALT	*R. (B.) microplus*
	7	None	629.3	2.04		
	8	None	1021.2	2.01		
	9	None	3479.9	2	GUAVA	
	10	None	3231.5	1.94		
	11	None	1042.6	1.96		
	12	None	1408	2.12		
	13	None	339.1	2.06	MILKCOW	
November 2017	14	None	20.1	2.47	BUSHALT	*R. (B.) decoloratus*
	15	None	58	2.05		
	16	None	77.2	2.06		
	17	None	50.6	2.06		
	18	None	45.2	2.14		
	19	None	18.8	2.44		
	20	None	23.4	2.45		

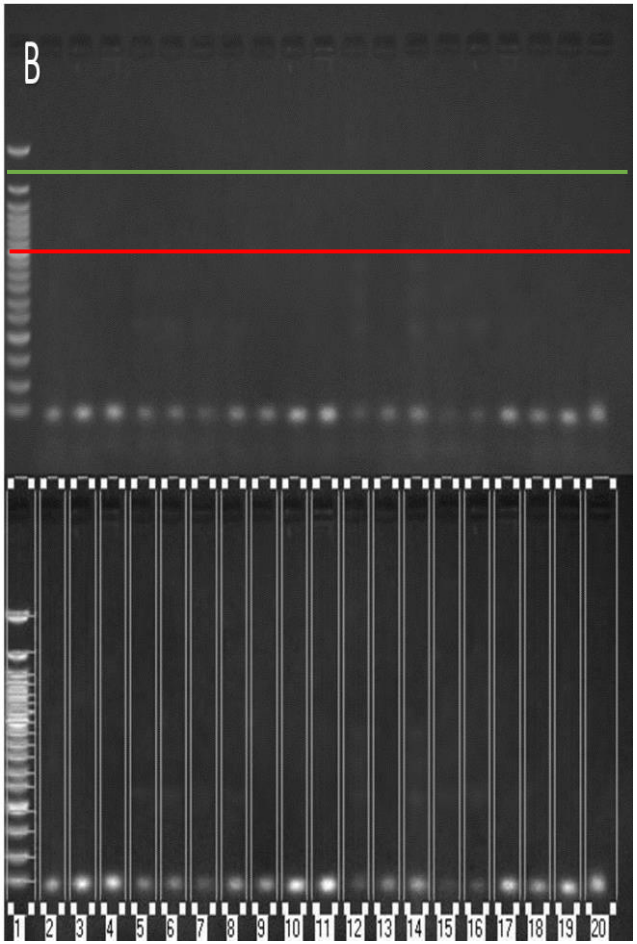

Figure 3.2B: Agarose gel of *Babesia* within adult ticks collected from cattle. Green *B. bigemina* (1124 bp fragment)*;* Red *B. bovis* (541 bp fragment).

Table 3.5C: Extracted adult tick DNA concentrations and PCR results from different camps and species collected during November 2017, with gel lane numbers corresponding to those indicated in Figure 3.2C.

Collection date	Lane		DNA purity		Camp	Species extracted from
-	Lane number	Identified	ng/ µl	260/280	-	-
November 2017	1	None	579.2	2.07	GAVIN HILL	*R. (B.) microplus*
	2	None	722	2.07		
	3	None	387.5	1.95		
	4	None	559.1	1.93		
	5	None	11.9	2.47		
	6	*B. bovis*	32.9	2.45	SHEDS	
	7	None	11	2.06		
	8	None	16.1	2.48	LOLWENI	
	9	None	37.6	2.06	SHEDS	*R. (B.) decoloratus*
	10	None	17.9	2.48		
	11	None	72.8	2.08	BUSHALT	
	12	None	1321	2	LOLWENI	
	13	None	431.1	2.09		
	14	None	539.4	2.06		
	15	*B. bigemina*	564.7	2.08	GAVIN HILL	
	16	None	251	2.05		
	17	None	3825.4	1.98		
	18	None	4564.1	1.96		
	19	None	1412.7	2.01		
	20	None	497.7	1.6		
-	Ladder	-	-	-	-	-

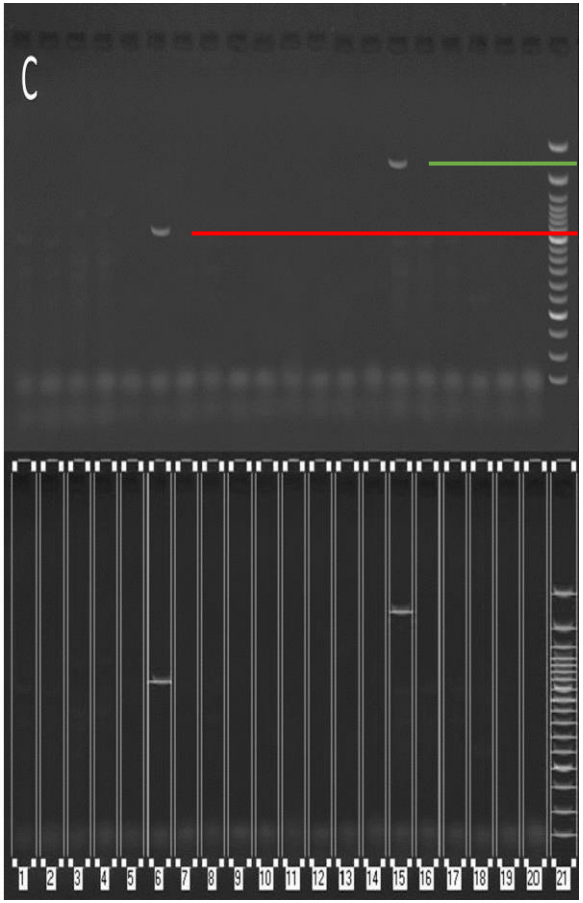

Figure 3.2C: Agarose gel of *Babesia* within adult ticks collected from cattle. Green *B. bigemina* (1124 bp fragment)*;* Red *B. bovis* (541 bp fragment).

Table 3.5D: Extracted adult tick DNA concentrations and PCR results from different camps and species collected during November 2017 and April 2018, with gel lane numbers corresponding to those indicated in Figure 3.2D.

Collection date	Lane		DNA purity		Camp	Species extracted from
-	Lane number	Identified	ng/ µl	260/280	-	-
-	Ladder	-	-	-	-	-
November 2017	2	*B. bigemina*	1967.8	2.04	GAVIN HILL	*R. (B.) decoloratus*
	3	None	766.4	1.95		
	4	None	930	2.01		
	5	None	44.7	2.09	MILKCOW	
	6	None	40.9	1.99		
	7	None	39.1	2.32		
	8	None	53.7	2.17		
	9	None	40.2	2.39		
	10	None	20.8	2.2		
	11	None	52.1	2.04		
April 2018	12	None	36.6	1.97	ARTHERS RESERVOIR	*R. (B.) decoloratus*
	13	None	157.7	1.93	BARBERS DAM	
	14	None	49.2	1.99	LANDS AND OLD ORANGE	
	15	None	73.3	1.91	MILK COW	
	16	None	47.6	2.02	ARTHERS RESERVOIR	*R. (B.) microplus*
	17	None	62.3	1.99	BARBERS DAM	
	18	None	13.2	2.01	LANDS AND OLD ORANGE	

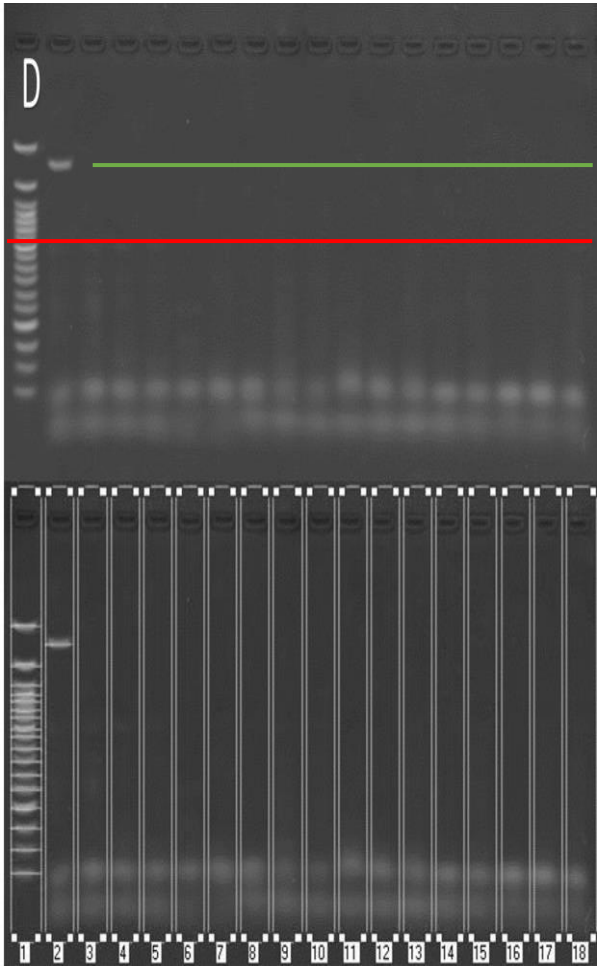

Figure 3.2D: Agarose gel of *Babesia* within adult ticks collected from cattle. Green *B. bigemina* (1124 bp fragment)*;* Red *B. bovis* (541 bp fragment).

Table 3.6: Extracted adult tick DNA with insufficient DNA concentrations and PCR results from different camps and species collected during April 201, November 2017 and April 2018.

Collection date		DNA purity		Camp	Species extracted from
-		ng/µl	260/280	-	
April 2017	1	108.9	1.08	GUAVA	*R. (B.) microplus*
	2	12.5	3.73	GAALBOOM	
	3	100.4	2.73	QUARRY	
	4	52.3	3.64	QUARRY	*R. (B.) decoloratus*
	5	7.6	3.32	GUAVA	
	6	113.2	1.23		
	7	77.3	3.12	GAALBOOM	
November 2017	8	14.9	1.13	BUSHALT	*R. (B.) microplus*
	9	7.5	3.82	GAVIN HILL	*R. (B.) decoloratus*
	10	141.3	3.74		
	11	70.8	2.63		
	12	102.7	1.45	BUSHALT	
	13	65.2	2.58	MILKCOW	
	14	110.0	2.98		

3.3. Cattle blood samples

Blood samples were collected from a total of 130 cattle over all three collection periods. Blood samples were obtained from at least 10 animals, where possible, from each camp during each collection period (Table 3.7). These cattle grazed in the 11 camps in which tick drags were performed on.

Blood samples from 42 cattle during April 2017 consisted of 10 animals in camps Quarry, Bushalt, and Guava respectively. Gaalboom only had nine animals and Milk Cow had three. During November 2017 blood samples from 44 cattle that grazed within the camps Gavin Hill (15), Lolweni (3), Bushalt (15), Milk Cow (2) and Sheds (9) were obtained. None of these camps had cattle during these two periods that were infected with babesiosis as determined with blood smears and PCR evaluations (Table 3.7).

During April 2018, blood collections was collected from 44 cattle. They were representative of the camps Arthurs Reservoir with 15 animals, Milk Cow with two animals, Barbers Dam with 10 and Lands and Old Orange with 17 animals. One of the animals that was tested during all three collection periods (animal number 707), tested

positive for *B. bovis.* This animal had grazed in Lands and Old Orange camp during the April 2018 collection period. Another animal from Milk Cow camp that was not tested previously was positive for both *B. bigemina* and *B. bovis* when tested during the April 2018 collection (Table 3.7).

Table 3.7: Number of cattle from which blood was collected in each camp during April 2017, November 2018 and April 2018.

	April 2017	November 2017	April 2018
ARTHURS RESERVOIR	-	-	15
GAVIN HILL	-	15	-
LOLWENI	-	3	-
QUARRY	10	-	-
BUSHALT	10	15	-
MILKCOW	3	2	2#
GUAVA	10	-	-
SHEDS		9	-
BARBERS DAM		-	10
LANDS AND OLD ORANGE		-	17*
GAALBOOM	9	-	
Total	42	44	44

- : No collections were performed *: *B. bovis* infection #: *B. bigemina* and *B. bovis* infection.

3.3.1. Morphological identification

Three hundred and ninety blood smears, done in triplicate from blood drawn from 130 animals during all three collection periods, contained a single blood smear slide that was positive for *B. bovis* (Figure 3.3). Thirty of these blood collections were obtained from the same 10 animals during each fieldtrip. The positive blood smear identified was obtained during the April 2018 field trip from an animal, grazing in Lands and Old

Orange, with the number 707, which was one of the 10 cattle that was sampled during all three sampling dates. This indicated that this animal contracted the *B. bovis* during the study period (Figure 3.3).

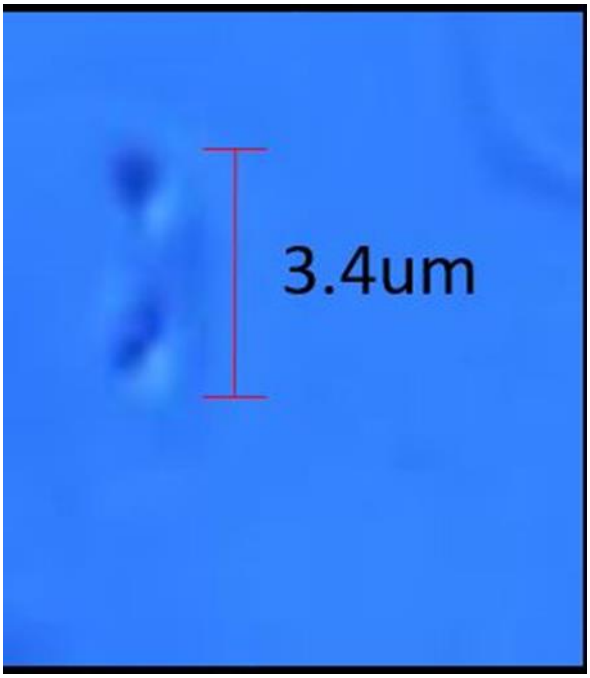

Figure 3.3: *Babesia bovis* with a paired length of 3.4 µm under 1000x magnification.

3.3.2. Polymerase chain reaction identification

Blood samples were collected from 42 animals during the April 2017 field trip. Twenty-eight of these samples yielded sufficient DNA for PCR. No *Babesia* PCR fragments were found in the 28 samples that were tested (Table 3.8A-3.8B, Table 3.8C; Lane 1-4) (Figure 3.4A-3.4C).

During the November 2017 collection period, 35 of the 43 blood samples collected yielded sufficient DNA for PCR. All November 2017 samples were negative for *Babesia* DNA (Table 3.8C; Lane 5-40 & Table 3.8D, Lane 1) (Figure 3.4C-3.4D).

During April 2018, blood was collected from 32 animals; 26 of the samples yielded sufficient DNA concentrations to perform PCR. Twenty-four of the 26 samples were negative for *Babesia* DNA fragments (Table 3.8D; lane 2-40) (Figure 3.4D). *Babesia* DNA was identified for two samples in this collection. One of the 10 cattle from which blood was collected during all three field trips, number 707, was positive for *B. bovis* (Table 3.8D; Lane 36) (Figure 3.4D) as also confirmed by means of a Giemsa-stained

blood smear obtained from this animal. The animal died a week after blood was obtained; babesiosis was confirmed by the local veterinarian.

One animal, from Milk Cow, also tested positive and was found to have a double infection of both *B. bigemina* and *B. bovis* (Table 3.8D; Lane 39) (Figure 3.4D). This animal was not previously included for blood collection and a blood sample was only obtained from this animal during April 2018. This animal showed no signs of illness before or after collection.

Twenty-eight blood samples obtained during the study period did not have sufficient DNA concentrations or purity for PCR (Table 3.9).

Table 3.8A: DNA concentrations extracted from blood and PCR results from different camps and species collected during November 2017 and April 2018, with gel lane numbers corresponding to those indicated in Figure 3.4A.

Collection date	Lane		DNA purity		Camp	Cattle ID number
-	Lane number	Identified	ng/ µl	260/280		-
-	Ladder	-	-	-		-
April 2017	2	None	395.1	1.65	Gaalboom	12
	3	None	35.7	1.94		38
	4	None	59.7	2.12		44
	5	None	62.5	2.19		51
	6	None	48.2	2.09		60
	7	None	37.2	2.01		76
	8	None	40.1	2.28	Quarry	158*
	9	None	15.8	2.49		218*
	10	None	45.6	1.69		228
	11	None	983.3	1.7		234*
	12	None	28.7	1.82		275
	13	None	20	2.17	Milk Cow	285

*: Blood was obtained from these animal during all three collection dates.

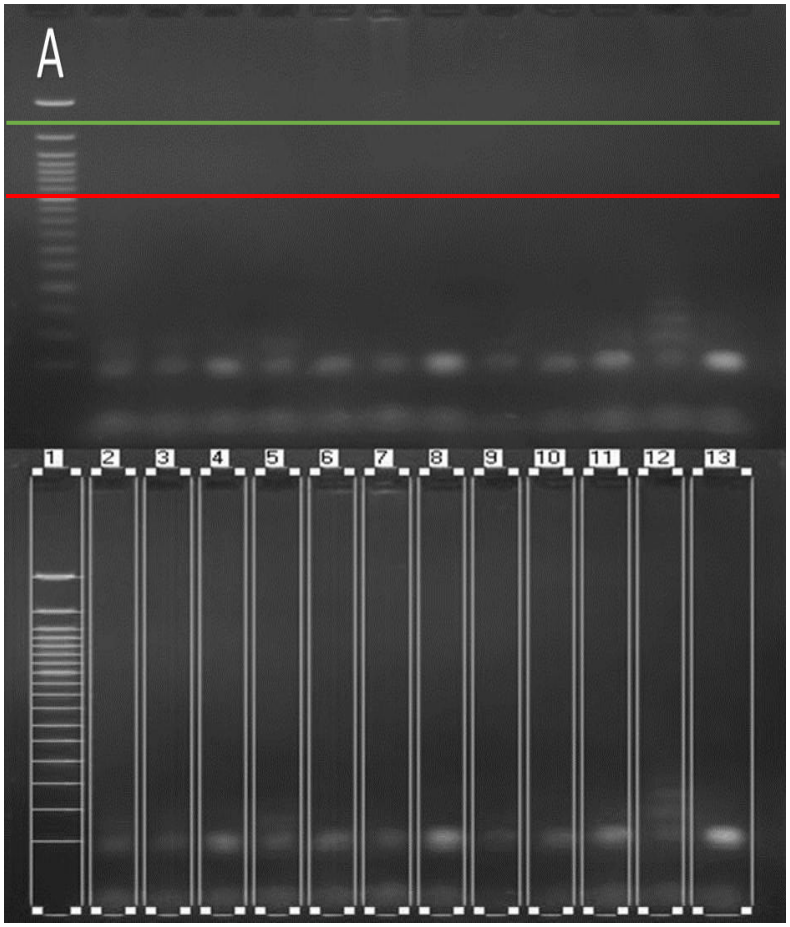

Figure 3.4A: Agarose gel of *Babesia* within blood of cattle. Green *B. bigemina* (1124 bp fragment)*;* Red *B. bovis* (541 bp fragment).

Table 3.8B: DNA concentrations extracted from blood and PCR results from different camps and species collected during November 2017 and April 2018, with gel lane numbers corresponding to those indicated in Figure 3.4B.

Collection date	**Lane**		**DNA purity**		**Camp**	**Cattle ID number**
-	Lane Number	Identified	ng/ µl	260/280		-
-	Ladder	-	-	-		-
April 2017	2	None	201.8	1.85	Milk Cow	295
	3	None	252	1.65		298
	4	None	41.7	1.91	Bushalt	301*
	5	None	37.1	1.96		309
	6	None	33.2	2.16		339
	7	None	40.4	2.18		360
	8	None	105.6	2		368
	9	None	39.3	2		390
	10	None	545.1	1.72		452
	11	None	381.4	1.79	Guava	475*
	12	None	449.8	1.79		707*
	13	None	63.1	1.84		779

*: Blood was obtained from these animal during all three collection dates.

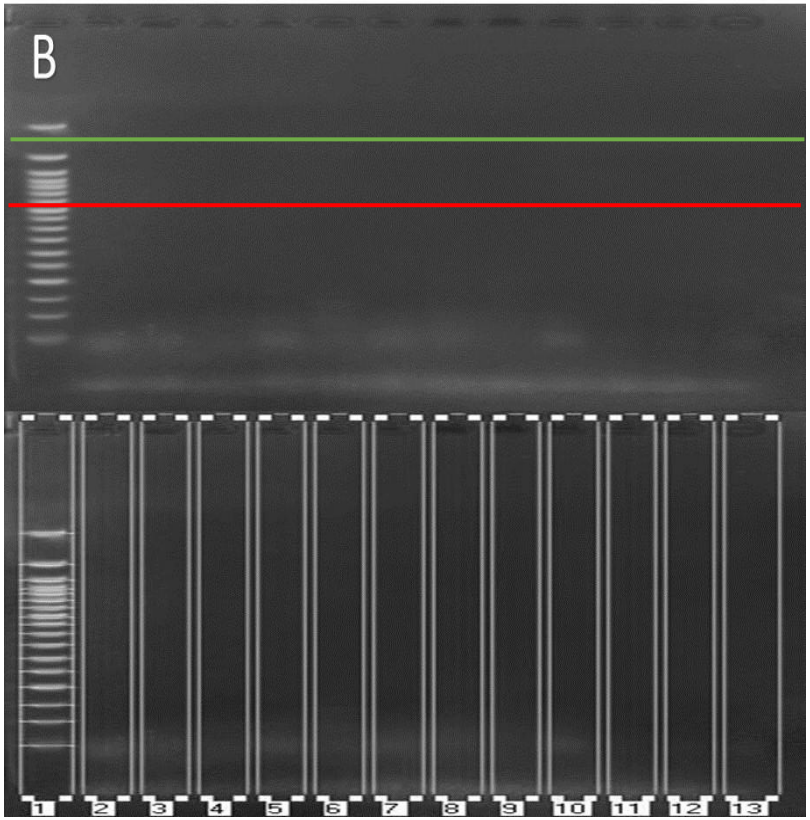

Figure 3.4B: Agarose gel of *Babesia* within blood of cattle. Green *B. bigemina* (1124 bp fragment)*;* Red *B. bovis* (541 bp fragment).

Table 3.8C: DNA concentrations extracted from blood and PCR results from different camps and species collected during November 2017 and April 2018, with gel lane numbers corresponding to those indicated in Figure 3.4C.

Collection date	Lane		DNA purity		Camp	Cattle ID number
-	Lane number	Identified	ng/ µl	260/280		-
April 2017	1	None	91.3	1.81	Guava	808*
	2	None	15.1	2.35		900*
	3	None	41.7	1.89		FF 5*
	4	None	93.8	1.78		KC 15*
November 2017	5	None	1462.1	1.86	Gavin Hill	228
	6	None	134.5	1.79		298
	7	None	35.1	1.79		808*
	8	None	15.6	1.86		290
	9	None	1639.3	1.82		312
	10	None	69	1.8		50
	11	None	111.7	1.85		707*
	12	None	108.5	1.74		218*
	13	None	640.6	1.84		301*
	14	None	39	1.94		44
	15	None	15.6	1.97		1412
	16	None	16	2.03		38
	17	None	65.2	1.82		34
	18	None	41.7	1.78	Milk Cow	KC 15*
	19	None	11.2	2.07		51
-	Ladder	-	-	-		-
November 2017	21	None	48.1	1.77	Sheds	158*
	22	None	30.4	1.75		900*
	23	None	233.5	1.7		234*
	24	None	50.1	1.84		861
	25	None	28.4	1.9		FF 5*
	26	None	37.7	1.66		347
	27	None	46.1	1.68		376
	28	None	69.9	1.71	Lolweni	475*
	29	None	184.2	1.72		452
	30	None	55.7	1.66		293
	31	None	52.4	2.21	Bushalt	430
	32	None	36.3	2.11		432
	33	None	16.2	2.22		441
	34	None	25.8	2.12		869
	35	None	13.8	2.37		916
	36	None	25	2.08		921
	37	None	25.7	2.31		936
	38	None	22.1	2.19		947
	39	None	20.5	2.19		961
	40	None	32	2.14		1000

*: Blood was obtained from these animal during all three collection dates.

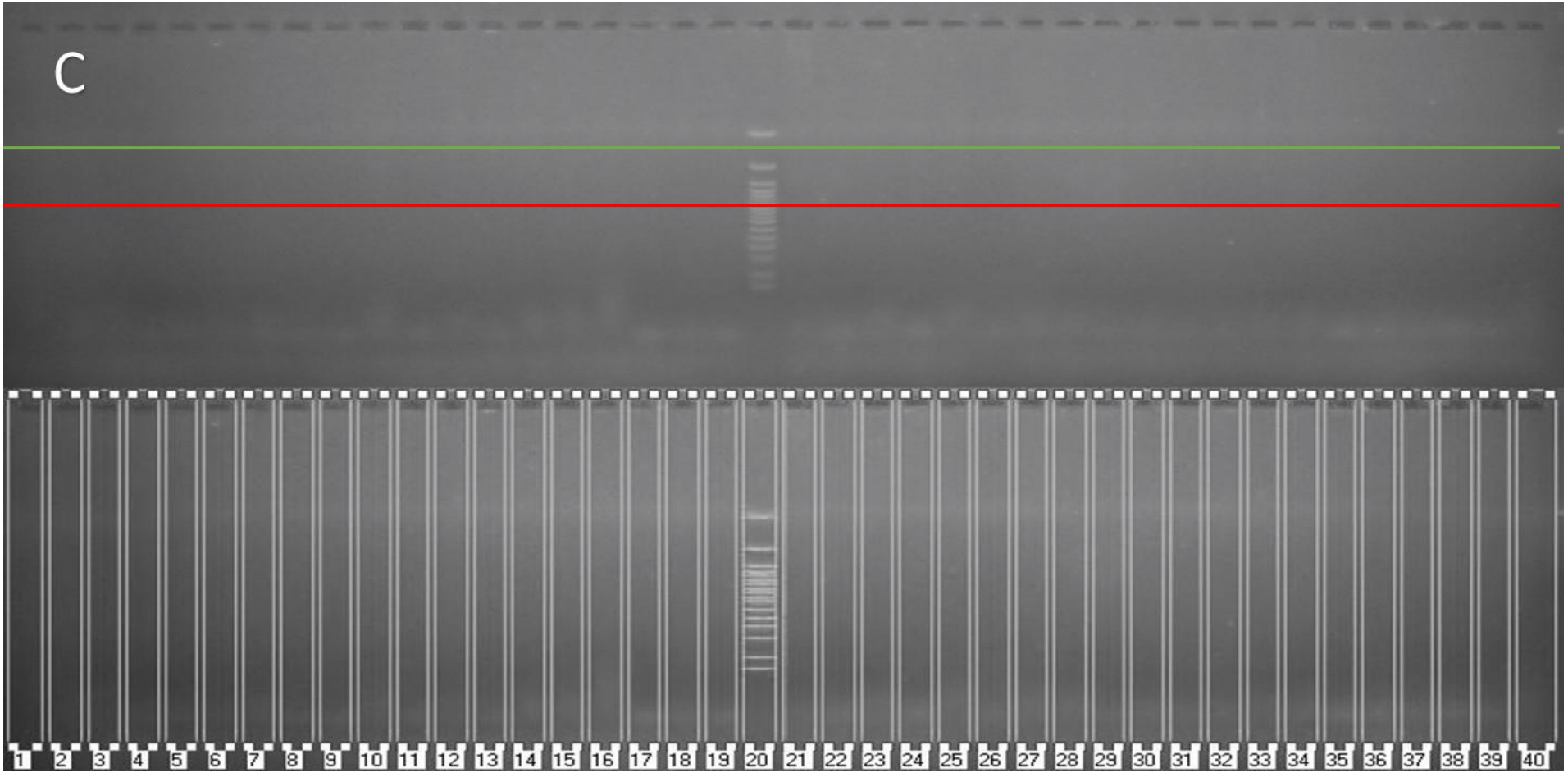

Figure 3.4C: Agarose gel of *Babesia* within blood of cattle. Green *B. bigemina* (1124 bp fragment)*;* Red *B. bovis* (541 bp fragment).

Table 3.8D: DNA concentrations extracted from blood and PCR results from different camps and species collected during November 2017 and April 2018, with gel lane numbers corresponding to those indicated in Figure 3.4D.

Collection date	**Lane**		**DNA purity**		**Camp**	**Cattle ID number**
-	Lane Number	Identified	ng/ µl	260/280		-
November 2017	1	None	28.5	2.25	Bushalt	1406
April 2018	2	None	5278.6	1.9	ARTHURS RESERVOIR	LS67
	3	None	31.9	1.77		475*
	4	None	49.4	1.81		979
	5	None	37.7	1.66		936
	6	None	56.3	1.75		347
	7	None	119.1	1.7		50
	8	None	101.2	1.76		FF 5*
	9	None	17.9	2.21		LS 49
	10	None	29.9	1.79		390
	11	None	103.9	1.84		76
	12	None	90.2	1.62		12
	13	None	86.9	1.85		441
	14	None	89.4	1.85		158*
	15	None	82.2	1.75		1412
	16	None	1845.7	1.81		LS41
	17	None	71.6	1.68	Barbers Dam	376
	18	None	284.8	1.72		916
	19	None	259.9	1.7		339
	20	None	28.1	1.92		KC 15*
	21	None	46.4	1.75		218*
-	Ladder	-	-	-		-
April 2018	23	None	115.2	1.59	Barbers Dam	953
	24	None	76	1.87		34
	25	None	62	1.6	Lands and Old Orange	1000
	26	None	251	1.63		900*
	27	None	111.1	1.71		808*
	28	None	115	2.12		362
	29	None	41.9	2.29		44
	30	None	156.2	1.73		961
	31	None	257.7	1.81		295
	32	None	99.9	1.78		301*
	33	None	515.1	1.7		234*
	34	None	540.4	1.91		745
	35	None	543.3	1.9		765
	36	*B. bovis*	46.1	2.01		707*
	37	None	397.8	1.91		1508
	38	None	366.4	1.91		1481
	39	*B. bovis & B. bigemina*	993.2	1.92	Milk Cow	MILK COW 1
	40	None	977.1	1.94		MC 1

*: Blood was obtained from these animal during all three collection dates.

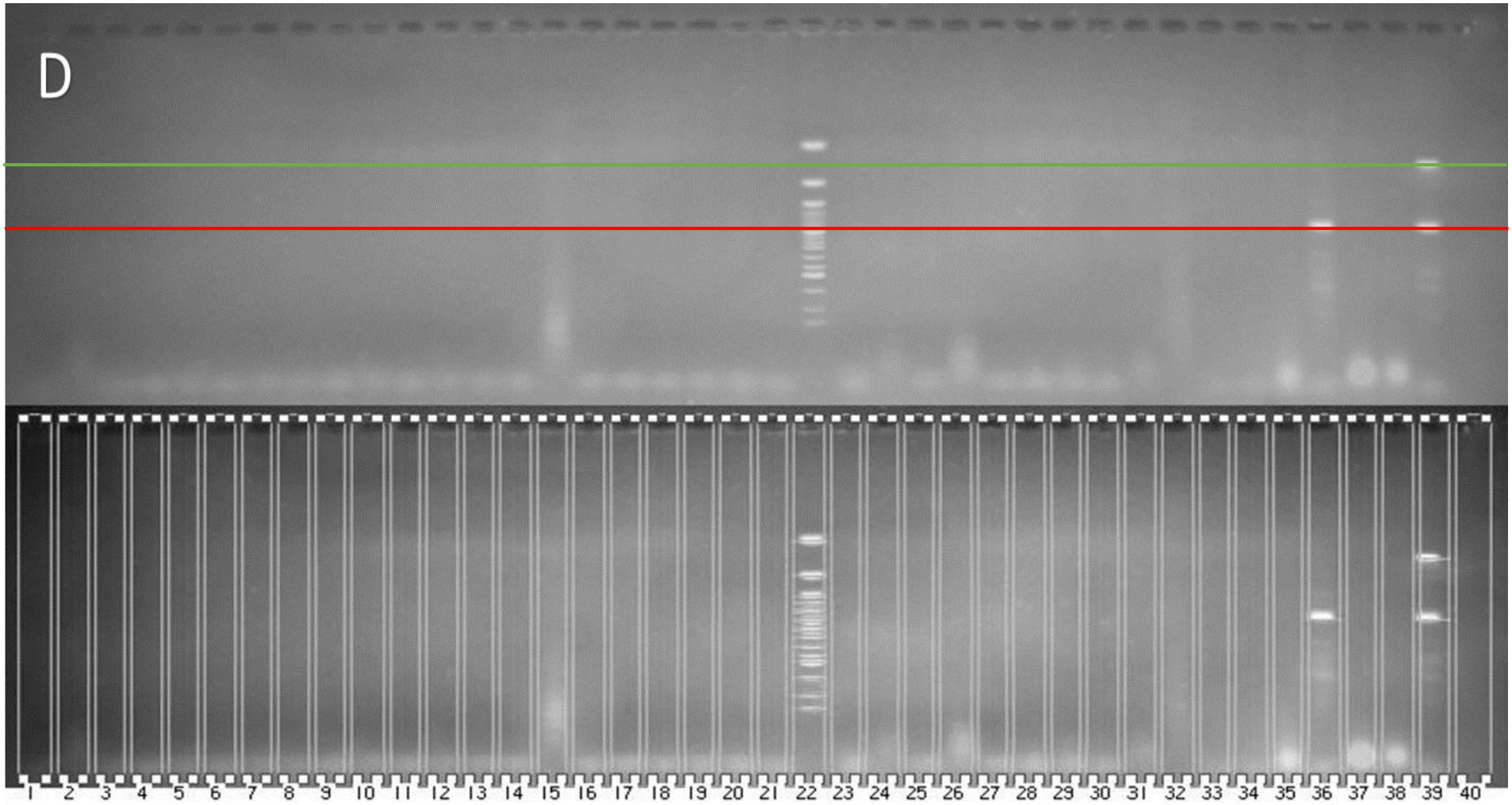

Figure 3.4D: Agarose gel of *Babesia* within blood of cattle. Green *B. bigemina* (1124 bp fragment)*;* Red *B. bovis* (541 bp fragment).

Table 3.9: Extracted DNA from Blood with insufficient DNA concentrations and PCR results from different camps and species collected during April 201, November 2017 and April 2018.

Collection date		DNA purity			Cattle ID number
		ng/ µl	260/280		-
- April 2017	1	151.1	3.43	Gaalboom	46
	2	47.7	2.71		250
	3	67.3	2.59		305
	4	137.1	3.56	Quarry	362
	5	177.5	3.51		430
	6	46.9	3.19		745
	7	118.3	2.96		765
	8	87.3	3.72		887
	9	8.5	1.65	Bushalt	921
	10	9.8	2.93		953
	11	232.4	3.12		987
	12	216.7	1.09	Guava	1406
	13	95.4	1.17		LS 49
	14	67.9	3.09		LS41
November 2017	15	2.2	1.54	Sheds	160
	16	187.2	2.53		313
	17	9.8	3.58	Gavin Hill	360
	18	235.7	2.50		880
	19	245.8	3.36	Bushalt	910
	20	3.1	3.74		987
	21	146.9	3.72		LS67
	22	5.3	1.63		MC 1
April 2018	23	227.5	2.66	Barbers Dam	368
	24	235.9	2.64		370
	25	205.8	3.39		432
	26	112.2	2.90	Lands and Old Orange	920
	27	150.8	1.31		931
	28	70.0	3.51		1482

3.4. Reference

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Chapter 4: Discussion

The objective of this study was to indicate the presence of *R. (B.) microplus* on a farm in the Eastern Cape province that was previously dominated by *R. (B.) decoloratus*. *Rhipicephalus (Boophilus) microplus* is a vector for the protozoan pathogen, *Babesia bovis.* The presence of this tick species would also be an indication that babesiosis, caused by *B. bovis* could have been introduced onto this farm. A report that *R. (B.) microplus* was found on this farm during a routine collection of *R. (B.) decoloratus* in 2014 (EMS van Dalen – Unpublished data) opened up the question of the current presence and prevalence of *B. bovis* on this farm. It was especially important to find out if the number of *R. (B.) microplus* increased on the farm, as *R. (B.) microplus* is still the only known vector of *B. bovis* in South Africa (Horak *et al.* 2018).

Previous studies done in South Africa indicated possible displacement of *R. (B.) decoloratus* by *R. (B.) microplus*. Collections done by Nyangiwe *et al.* (2017) at 80 locations, in the Northern Cape, Free State, Western Cape and Eastern Cape provinces, showed *R. (B.) microplus* to be present at 64 locations (71.8%) and *R. (B.) decoloratus* at 47 locations (28.2%) investigated. Nyangiwe *et al.* (2017) further indicated that *R. (B.) microplus* was widely spread throughout most of the coastal areas of the Eastern Cape, western parts of the Western Cape as well as the north-eastern parts of the Northern Cape provinces. *Rhipicephalus (Boophilus) microplus* was only present within one area of the Free State.

Tønnesen *et al.* (2004) conducted a study within the Soutpansberg region in Limpopo and found that *R. (B.) microplus* had mostly replaced *R. (B.) decoloratus* in this area outnumbering *R. (B.) decoloratus* with 93.4% to 6.6%. *Rhipicephalus (Boophilus) microplus* was not found in the Soutpansberg region during studies conducted by Howell (1978) and De Vos (1979) but outbreaks of *B. bovis* was reported during 1980-1985, indicating that *R. (B.) microplus* might have spread to this region (Tønnesen *et al.* 2004).

Horak *et al.* (2009) documented that the previously dominant species, *R. (B.) decoloratus,* was displaced by *R. (B.) microplus* in many of the eastern regions of the Eastern Cape province. It was also found that the displacement was partial in some areas with complete displacement in other areas. In contrast with these findings the

farm under investigation, is located approximately 40 km west of Makhanda. This farm, located further west than the study area investigated by Horak *et al.* (2009), still did not show displacement of *R. (B.) decoloratus* by *R. (B.) microplus* four years after the initial *R. (B.) microplus* collection. This could be due to the fact that this is a commercial farm with a closed farming system and adequate chemical control. Tønnesen *et al.* (2004) indicated that displacement of *R. (B.) decoloratus* occurs more frequently on communal farms than commercial farms. Most of the other studies where displacement were indicated, were also done in communal grazing areas (Tønnesen *et al.* 2004, Nyangiwe *et al.* 2013).

4.1. Larvae

Rhipicephalus (Boophilus) decoloratus larvae, collected from field drags, were found to be the most abundant Blue tick species on the farm with an average 97.36% compared to *R. (B.) microplus* larvae at 2.64%. This is a strong indication that *R. (B.) microplus* is prevented from becoming established. *Rhipicephalus (Boophilus) decoloratus* larvae were present in all the collection camps that were dragged. Although not all collected larvae were allocated to this study to investigate for the presence of *Babesia* spp. a maximum of 30 larvae allocated for a camp, were an indication of high numbers found. This also gave an indication of increasing or decreasing larval numbers during this study.

In Milk Cow camp, where drags were done during all three collection periods, *R. (B.) decoloratus* larval numbers collected were consistently high, with the full allocation of 30 larvae provided for this study each time. *Rhipicephalus (Boophilus) microplus* larvae could only be found from drags made during the April 2017 collection period. The high tick numbers can be attributed to the fact that there were animals in this camp throughout the year. Although a low number of cattle were kept in this camp, the constant use and availability of suitable hosts, provided a stable food source for the ticks. The high number of ticks can also be attributed to the vegetation of the camp which consisted of open grass areas interspersed with trees and bushes that allowed sheltered places for the engorged females to oviposit.

In comparison to Milk Cow camp, the other camps like Quarry, Guava, Barbers Dam and Gaalboom that were also dragged for larvae during all three collection periods had a lower abundance of Blue tick species. This might be due to the fact that these camps were not used for cattle grazing all year round but cattle were rotated to other camps intermittently. The type of vegetation of Quarry camp, consisting of shrubs and short bushes with relatively few open grassland patches, could also have played a role in the overall low tick larval count in this camp, by preventing optimal questing conditions. *Rhipicephalus (Boophilus) microplus* was only found in this camp during April 2017 drags.

Rhipicephalus (Boophilus) decoloratus larval numbers increased in Guava camp and Barbers Dam from the first collection date to the last. This increase is likely due to some rainfall prior to collection, during April 2018, increasing the questing behaviour of tick larvae. The larval numbers however increased more dramatically in Guava camp where a full complement of 30 larvae was allocated to this study, for the last two collection periods. Only one *R. (B.) microplus* larva was collected in this camp during April 2017. In comparison Barbers Dam showed a tick larval increase from April 2017 (1) to November 2017 (4) and then again from November 2017 to April 2018 (15) but none of the collection periods provided a full allocation of 30 larvae for this study. No *R. (B.) microplus* larvae were however found in this camp during any of the collections. Gaalboom camp also showed a tick larval increase from one *R. (B.) decoloratus* larva found during April 2017 to the full complement of 30 during November 2017, but then drastically declined to only four larvae found in April 2018. This significantly lower larval collection during April 2018 could have been due to light rainfall the night before collection in that camp, resulting in damp vegetation and larvae more interested in water replenishment than questing during the collection (Hair *et al.* 1975). The same explanation can also be valid for Sheds camp that showed a decline in number of larvae collected from November 2017 to April 2018 although this camp was only dragged for larvae twice. No *R. (B.) microplus* larvae were found during any of the collections in these two camps. A higher November collection can be due to pervious reports that that questing by *R. (B.) decoloratus* is more likely during spring, September until November (Horak *et al.* 2011, Nyangiwe *et al.* 2011). Walker *et al.* (2003) however also indicated that blue ticks are found year round, explaining the presence of these ticks during the April collection months.

Gavin Hill, Lolweni and Bushalt camps were dragged for larvae during two of the three collection periods with an increase of larvae found on drags from the first to the second collection. No *R. (B.) microplus* larvae were found for Lolweni and Gavin Hill camps but they were found on both occasions for Bushalt camp.

Arthurs Reservoir and Lands and Old Orange camps were sampled once due to not being utilised for cattle grazing before the collection dates. Both indicated a fair number of *R. (B.) decoloratus* larvae present in the field but no *R. (B.) microplus* were found in these camps.

DNA extractions from individual tick larvae to investigate the presence of *Babesia* DNA, was found to be unsuccessful in most cases due to extracted DNA concentrations that were too low to be used for DNA amplification. In cases were sufficient DNA was extracted the DNA was not sufficiently pure. Proteins like haemoglobin were found to interfere with PCR when DNA extraction were not pure (Schrader *et al.* 2012).

Complications were caused during DNA extractions due to the extraction reagents volume being too high for the amount of DNA present within tick larvae, therefore diluting the DNA too much. This may also further affect the PCR proses due to having insufficient parasite DNA to perform PCR successfully. In a pilot study performed in 2016 it was found that *Babesia* primers did not bind to tick DNA causing nonspecific binding and primer dimers to form during PCR (Johnston *et al.* 2019).

Other complications included the difficulty to crush individual tick larvae. Some DNA pallets was also lost due to the small size during DNA extraction, thus resulting in insufficient DNA for PCR testing.

4.2. Adult ticks

Adult ticks were collected from ten animals per herd. The cattle from each herd represented a different camp where they could have picked up the ticks as larvae.

Adult ticks identified as *R. (B.) decoloratus* were again found to be the dominant adult blue tick species on this farm with a total percentage of 97.64%, in contrast to 2.36% *R. (B.) microplus* collected. If compared to the larval percentages, the same ratio was found, likely indicating that larval and adult tick collection was accurately represented.

No statistical significant difference ($P > 0.05$) could be found between the numbers of adult *R. (B.) decoloratus* ticks collected during the different collection periods. The same was true for *R. (B.) microplus* ($P > 0.05$). In contrast, significantly more adult *R. (B.) decoloratus* than R. *(B.) microplus* ($P < 0.05$) were found during each collection period. *Rhipicephalus (Boophilus) microplus* was however found from cattle that grazed in all the camps except for the second and third collection made in Milk Cow camp during November 2017 and April 2018, respectively. This indicated that *R. (B.) microplus* was not successfully established in that camp.

Variation in adult tick numbers on the cattle in each camp can be due to various reasons. Tick loads in a camp can be low when the camp was not utilised for a period of time that allowed for the larvae to die off due to starvation and dehydration. Another influencing factor can be periods of low rainfall and drought that can also cause dehydration. Lower larval infestation of animals in a camp can be due to lowered questing activities of larvae occurring during autumn as discovered by Horak *et al.* (2011) and Nyangiwe *et al.* (2011). High tick burdens in a camp can be caused by increased rainfall during previous months. This is in correlation with the findings of Marufu *et al.* (2011) indicating that higher tick loads are found during dry-hot seasons with high humidity and temperatures increasing larval emergence.

Although Gaalboom Camp, during April 2017, had the least number of adult ticks collected, the numbers do not accurately represent the total number of adult ticks as the farmer had to do an emergency treatment before collection with Ivermax Gold, a long-lasting injectable, due to an excessive tick burden on the cattle. This however did not greatly influence results pertaining to babesiosis as ticks were still present during collection.

Larvae of adult ticks collected from cattle were used to allow for a greater number of progeny to be tested for the presence of *Babesia* as both species are transmitted transovarially (Bock *et al.* 2004, Oliveira *et al.* 2005). Larvae were pooled in groups of 100 for each adult tick to assure adequate DNA concentration after DNA extraction. A

higher number of larvae, used for DNA extraction, also increased the possibility to detect both *Babesia* species where present in a specific adult tick. Oliveira *et al.* (2005) indicated that due to transovarial transmission of 20% - 40% for *B. bigemina* and less than 14.5% for *B. bovis*, pooling of larvae was recommended.

During November 2017 progeny from two adult ticks, collected from animals grazing in Gavin Hill were found to have *B. bigemina* infection. It is unclear if both these ticks were collected from the same animal as ticks were selected from a pool of ticks collected from the group of animals in the camps. Unfortunately, no blood was collected from the animal that contained *Babesia* as no evidence of *Babesia* species was found in the blood samples collected from this group. It however gave an indication that carriers of *B. bigemina* were present in the group because no clinical signs were seen during or after collections. It is possible that this animal got infected with little clinical signs during the previous six months as animals become carriers of *B. bigemina* for at least six months as found by (Bock *et al.* 2004) and Saad *et al.* (2015).

Lolweni, with only five animals grazing in this camp, had the lowest number of *R. (B.) decoloratus* (55) adults collected with only one *R. (B.) microplus* found. Only the progeny of one of the adult *R. (B.) microplus* found from cattle grazing in the Sheds camp was infected with *B. bovis,* none of the animals were infected with *B. bovis* as confirmed by the lack of PCR fragments indicating the presence of *Babesia* during PCR of blood samples. No clinical signs were observed from the cattle thus indicating a possible carrier of *B. bovis* on this farm. It is possible for an animal to stay infective for four years as indicated by Bock *et al.* (2004).

Collections were done before the farmer treated the animals, thus lowering the risk of ticks spreading to new areas on the farm. The study of Pottinger (2019) from the concurrent study done on the same farm, indicated emerging resistance for Blue tick populations from some camps. This can increase the risk of ticks surviving and *Babesia* to be transferred to new camps that might have been uninfected as the farmer moves cattle to new camps after treatment.

During April 2018 the larvae were pooled per camp and species. This was done due to a complication that arose during the hatching of the eggs. The pooling did not influence the results as more tick larvae were used instead of the 100 used during

other collection dates. The low number of *Babesia* infections might not be a true indication of the presence of *Babesia* in vector ticks as only a small number of adults from each camp were collected.

4.3. Blood from cattle

Blood collected from the animals during each collection date represented approximately 10% of cattle on the farm. Ten cattle were selected to be followed throughout all three collection periods and blood was collected from them each time. This was done to follow *Babesia* infections occurring within this test group during this study period. Other cattle in this study were selected at random during each collection period until a total of 10 cattle per camp were utilised when available. The selection of random test animals was performed so that a bigger animal sample group could be utilised over the course of this study.

Morzaria *et al.* (1992) indicated that during the non-acute phase, parasitism is as low as one in 1000 RBC thus having an extremely low chance of detection with Giemsa-stained blood smears. During the acute phase the number of observation fields is less important as Morzaria *et al.* (1992) indicated that the parasite is easily observed in blood smears. The PCR however was found to be a more sensitive method for detecting *Babesia* DNA in vector DNA extractions (Chaudhry *et al.* 2010)

One blood smear was positive for the presence of *B. bovis*. It was also confirmed by the PCR evaluation. This discovery was surprising as no other parasites were found and the animal did not show signs of being infected during collection. This animal although healthy, at time of collection, suddenly died from *B. bovis* as diagnosed by the local veterinarian. The time laps corresponded to three to seven days, of the acute phase, after blood collection as indicated by Bock *et al.* (2004). It is likely that this animal was still in the first stage nearing the end of acute infection, making the identification of a positive infection possible on the blood smear. Polymerase Chain Reaction, which is more sensitive than blood smears can detect *Babesia* infections at far lower rates than blood smears (Morzaria *et al.* 1992, Zulfiqar *et al.* 2012).This animal did not have an infection prior to April 2018 as was indicated by PCR tests done during each collection period. It is likely that this animal contracted *B. bovis* from

R. (B.) microplus as these adult ticks were found in this camp. *Rhipicephalus (Boophilus) microplus* ticks collected from this camp did not indicate *B. bovis* infections within this time period likely due to the low tick sample size used.

Another infection was found in an animal that was in Milk Cow camp. Blood smears from this animal did not indicate an infection but PCR indicated both *B. bigemina* and *B. bovis* infections. It is possible that this animal was infected during previous collections, but blood was not previously collected. This animal did not get sick after the collection indicating that this animal might be a carrier due to previous infections. It is possible that this animal contracted *B. bigemina* and *B. bovis* after November 2017 as *B. bigemina* does give some protection against *B. bovis* as indicated by Bock *et al.* (2004)

Limitations of this study that should be taken into consideration for similar future studies is that adult ticks collected from each animal should be pooled and kept separate from each other. This will enable the comparison of parasites found in ticks with DNA extraction from blood of each animal. More adult ticks should also be used for DNA extractions to get more reliable results.

4.4. Conclusion

Although both *R. (B.) decoloratus* and *R. (B.) microplus* were found in all camps investigated on this farm *R. (B.) decoloratus* are far from being displaced by *R. (B.) microplus* yet. Only three percent of the larvae collected from drags and two percent adult ticks, collected from cattle, were found to be *R. (B.) microplus*.

Both *B. bigemina* and *B. bovis* were found on this farm. Failure to extract sufficient DNA from larvae obtained through drags, caused results to be inconclusive with regard to the presence of *Babesia* in larvae collected from the field. Polymerase chain reactions from DNA obtained from adult blue ticks and blood collected from cattle indicated that *B. bigemina* was present in at least two camps, Milk Cow and Gavin Hill. Evidence for the presence of *B. bovis* was found in three of the 11 camps, Sheds, Milk Cow and Lands and Old Orange. This represents a 1.08% presence of *B. bigemina* and 1.85% of *B. bovis* from a sample pool of 146 adult ticks and 130 blood samples.

The small sample size and no larval data may not represent the full extent of the presence of *Babesia* species on the farm but is a clear indication that further monitoring is necessary to keep track of further invasion of *B. bovis* and its vector *R. (B.) microplus* on this farm.

4.5. Recommendations

Rhipicephalus (Boophilus) microplus numbers and the presence of *B. bovis* should be monitored for further dispersion on this farm. A breakdown in tick control must be followed up with scrutiny for the symptoms of babesiosis. Signs of infection and groups with infected animals should be treated with anti-*Babesia* chemotherapeutic agents to help prevent other animals from becoming infected and to treat infected animals.

Samples from animals that died of babesiosis should be sent for testing as to indicate the *Babesia* species and identify possible effected camps. Special care should then be taken to prevent spread of infected ticks to new camps by treating the affected group before movement. Affected camps can also be rested to starve larvae before moving cattle back into these camps.

Ticks larval loads can further be reduced by adding browsers like goats to the camps before introducing new cattle. Browsers will help to minimise tick larvae by removing microhabitats like the lower vegetation under trees and close to the ground as *Rhipicephalus (Boophilus)* species are usually found questing on grasses at body height of the cattle (Phalatsi *et al.* 2004)

The main method of controlling *B. bigemina* and *B. bovis* is still to control the vectors of these parasites. The farmer can keep on with current controlling management strategies as *R. (B.) microplus*, the vectors of *B. bovis* still seems to be present in low numbers.

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Appendix 1

Animal Research Ethics

19-Jul-2018

Dear Mr Abre Marais

Student Project Number: UFS-AED2017/0034

Project Title: The distribution of *Babesia bigemina* and *Babesia bovis* transmitted by *Rhipicephalus* spp. on a farm in the Eastern Cape.

Department: Zoology and Entomology (Bloemfontein Campus)

You are hereby kindly informed that, at the meeting held on 19-Jul-2018, the Interfaculty Animal Ethics Committee approved the above project.

Kindly take note of the following:

1.
A progress report with regard to the above study has to be submitted Annually and on completion of the project. Reports are submitted by logging in to RIMS and completing the report as described in SOP AEC007: Submission of Protocols, Modifications, Amendments, Reports and Reporting of Adverse Events which is available on the UFS intranet.

2.
Researchers that plan to make use of the Animal Experimentation Unit must ensure to request and receive a quotation from the Head, Mr. Seb Lamprecht.

3.
Fifty (50%) of the quoted amount is payable when you receive the letter of approval.

Yours Sincerely

Mr. Gerhard Johannes van Zyl
Chair: Animal Research Ethics Committee

Appendix 2

UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA

UFS·UV
NATURAL AND AGRICULTURAL SCIENCES
NATUUR- EN LANDBOUWETENSKAPPE

ZOOLOGY & ENTOMOLOGY

Consent for fieldwork collections

To whom it may concern:

I, Abré Marais, a Masters student, conducting a study, titled; *The distribution of Babesia bigemina and Babesia bovis transmitted by Rhipicephalus spp. on a farm in the Eastern Cape* as a requirement towards the completion of this degree at the department of Zoology and Entomology, Faculty of Natural and Agricultural Sciences, University of the Free State, Bloemfontein needs to collect the following specimens:

1. *Ticks from cattle*
2. *Blood from cattle*
3. ~~*Ticks from field*~~
4. Ticks from Field

from the farm/farms_____

during the following study periods 1 March 2017 till 31 December 2018

Student: Signature: [signature] Date: 01-3-2017

I Glynn Dixon,

owner of the farm/s, Claypits

hereby declare my willingness to partake in this study and give my consent by granting Abré Marais permission to access the farm, farm facilities and animals on the farm for specimen collection during the required study period as indicated above.

Producer: Signature: [signature] Date: 01-3-2017

Appendix 3

BLOOD COLLECTION FORM

Name of area (farm and district)										
CATTLE NR										
BREED										
AMOINT OF BLOOD COLLECTED										
SEX										
AGE (APROX)										
NAME OF COLECTOR										
DATE COLECTED										
GPS COORDANATES (IF AVALABLE)										
TIME BLOOD WAS PUT ON ICE										
SIGNUTURE OF DATA COLECTOR										

Appendix 4

Equipment name & number:

Description of material	Belong to & Contact number	In			Out		Remarks
		Date	Approx date out	Sign	Date	Par	