Diagnosis of *Babesia equi* in horses belonging to resource-poor farmers in the northeastern Free State, South Africa

by

MakhosazanaYvonne Motloang

Student Number: 1997823008

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Supervisor: Prof. P.A. Mbati

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DEDICATION

This thesis is dedicated to Freddy Martin Mokoena for his undying belief in me and that I can make it. My son, Lehlohonolo Motloang, his happy face is what keeps me going all the time, my grand mother Ellen Motloang and my mother Adelaide Motloang, for making sure that I get a basic education, my sister Diatile Motloang for being there in hard times, my brother Tshediso Motloang for supporting me all the way, and above all I dedicate my deepest gratitude to God All Mighty for being there always.

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ABSTRACT

Babesia (Theileria) equi, a tick transmitted protozoan parasite and one of the causative organisms of equine babesiosis is widespread in South Africa. This work reports on a study that evaluated the usefulness of the polymerase chain reaction (PCR) for detection of *B. (Theileria) equi* in horses belonging to resource-poor farmers in the northeastern Free State, South Africa. Blood samples from a total of 99 horses and seven from previously known *B. (Theileria) equi* infected horses on Kaal plaas farm (Onderstepoort Veterinary Institute) were examined for the presences of *B. (Theileria) equi* using Giemsa-stained blood smears, immunofluorescent antibody test (IFAT) and PCR technique. Giemsa-stained blood smears and serum samples were prepared from blood samples on arrival at the university laboratory. Aliquots of blood samples from EDTA coated vacutainers were transferred into marked cryogenic vials and stored at -35°C for further use in PCR. A PCR system that amplifies a 664 bp target of region of the 16S rRNA gene and shown to specifically detect the genome of *B. (Theileria) equi*, was used in this study. Examination of the 99 blood smears from the northeastern Free State revealed no parasites whilst serological analysis showed 98% of animals were seropositive for *B. (Theileria) equi*, 48% were seropositive for *B. caballi* and 58% had mixed infections for both *Babesia* species. However, PCR was negative for all the 99 horses from the northeastern Free State. *Babesia equi* parasites were demonstrated in blood smears from 4 out of 7 horses from the Kaal plaas farm. All these animals were also seropositive for *B. (Theileria) equi* while 3 out of 7 were seropositive for *B. caballi*. Three out of seven horses were seropositive for both *B. (Theileria) equi* and *B. caballi*. All seven Kaal plaas farm horses were PCR positive for *B. (Theileria) equi* infection. Questionnaire results indicated that 100% of resource-poor black farmers in the northeastern Free State did not know about equine babesiosis, its cause, symptoms, diagnosis or control. Based on the negative blood smears and PCR results, and the high prevalence of antibodies against *B. (Theileria) equi* as demonstrated by IFAT, the conclusion drawn from this study is that the status of equine babesiosis in the northeastern Free state region is endemically stable.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Preamble

South Africa is often called a "world in one country", because of its diversity of people, plants and animal life, climatic regions and scenic splendor. It is one of the most geographically varied countries of the African continent, comprising territory that ranges from the rolling, fertile plains of the highveld and the wide-open savanna of Mpumalanga to the Kalahari Desert and the peaks of the Drakensberg Mountains. With its topographical and geographical differences, a large country ranging from tropical to temperate climates and from rain forests to the oldest desert on earth, South Africa presents countless diversity of animal species and an equally diverse parasitic fauna. Parasites of domestic animals were brought into South Africa by successive waves of immigrants (Penzhorn and Krecek, 1997). When Jan van Riebeeck arrived in the Cape in 1652, the only equids found in southern Africa then were the Quagga (*Equus quagga*) and the Cape Mountain Zebra (*Equus zebra*). There were no indigenous horses and the first horses to be introduced into South Africa were of Eastern, and not of European origin (De Waal, 1995).

From Harrismith to Fouriesburg with the beauty of Sterkfontein Dam, QwaQwa, Kestell, Golden Gate, Clarens and Bethlehem in between, one discovers two different worlds of agriculture, one being the commercial agriculture sector, dominated by white farmers and the other, backyard farmers, comprising the resource-limited black farmers (Saunderson, 1995).

1.2 Equine babesiosis

Equine babesiosis is a tick-borne protozoal disease affecting horses, mules, donkeys and zebras worldwide (De Waal, 2000). It may be difficult to diagnose equine babesiosis as it can cause variable and non specific clinical signs (Brüning, 1996). The disease can be acute, sub-acute and chronic and is characterised by fever, anaemia, icterus, hepatomegally, and splenomegally (Friedhoff and Soulé, 1996). Equine babesiosis is caused by two different protozoa called *Babesia (Theileria) equi* and *Babesia caballi.*

Horses infected with *B. (Theileria) equi* may remain carriers for long periods and act as a source of infection for ticks which inturn will act as vectors of the disease (De Waal, 2000). Zebras are considered as important reservoirs for infection in Africa (Brüning, 1996). In veterinary parasitology, ticks are considered the most important arthropods because of their ability to transmit diseases in both wild and domestic animals. The diseases they transmit are a major constraint to livestock improvement in many parts of the world (Mbati *et al.*, 2002). Fourteen species of ixodid ticks in the genera *Dermacentor, Rhipicephalus* and *Hyalomma* have been identified as trans-stadial vectors of the aetiological agents of babesiosis (Bashiruddin *et al.*, 1999), while eight of these species were also able to transmit *B. caballi* infections transovarially (De Waal, 2000).

1.3 Tick vectors of equine babesiosis

Walker (1991) reported that there are about 800 tick species recorded in the world and of these, about 80 ixodid tick species occur in South Africa. *Rhipicephalus evertsi evertsi* was found to be the most common tick species with a prevalence of 98.4% in

zebra) although cattle, sheep goats and a number of wild antelope species are also frequently parasitized (Norval, 1982; Horak, 1982).

Studies on seasonal abundance of adult ixodid ticks in the eastern Free State indicates that *Rhipicephalus e. evertsi* ticks are most active in summer although few ticks occur on animals throughout the year. These are reported to be the second most abundant tick species in this region comprising 44.74% of total tick burden (Hlatshwayo *et al*., 2001). The relative importance of the disease in South Africa was demonstrated in a survey which indicated that the number of cases treated exceeded those of every other infectiouse deseases of horses (Potgieter *et al.,* 1992).

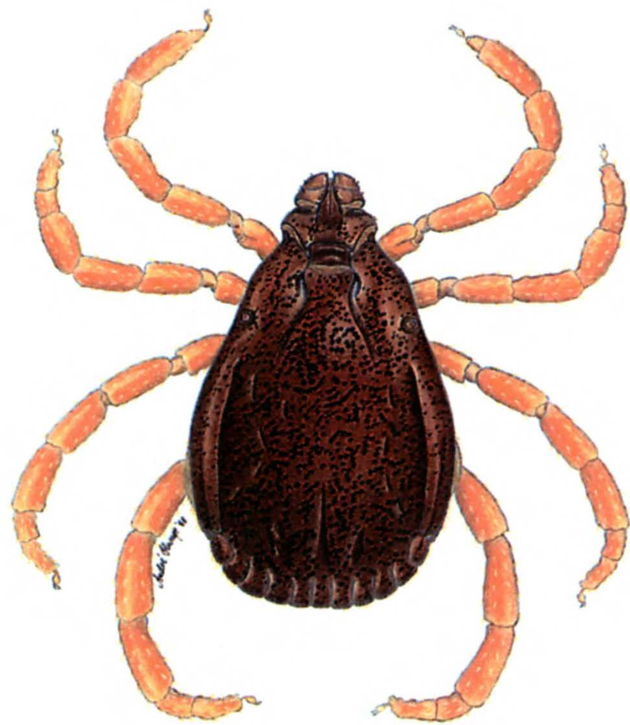


Plate 2: Adult male *Rhipicephalus evertsi evertsi* (Reproduced from Norval, 1994)

1.4 Classification and life cycle of *Babesia* species

The genus *Babesia* belongs to the phylum Apicomplexa, class Sporozoasida, subclass Coccidiasina, order Eucoccidiorida, suborder Piroplasmorina and family Babesiidae (Levine, 1985). Classification of the piroplasms has relied largely on microscopical, ultra-structural and life cycle observations. While these techniques allow some species to be assigned unequivocally to the theileras or babesias, the taxonomic status of several species remains unclear (Allsopp *et al.*, 1994). Molecular-genetic techniques provided another tool to demonstrate differences and similarities between these organisms. Molecular sequence evidence, for example, indicated that some species formerly assigned to the genus *Babesia* should be reassigned to different genera. *Babesia (Theileria) equi* has recently been reclassified as *Theileria equi* (Mehlhorn and Schein, 1998) as a result of 18S rRNA sequence analysis (Allsopp *et al.*, 1994), which confirmed previous observations of pre-erythrocytic stages in lymphocytes (Schein *et al.*, 1981; Moltmann *et al.*, 1983). Development in lymphocytes has also been reported for *Babesia microti* (Mehlhorn *et al.*, 1986) and 18S gene sequence evidence suggests that this organism should be removed from the genus *Babesia*. Doubt has also been raised on the assignment of *Babesia rodhani* to this genus (Ellis *et al.*, 1992; Allsopp *et al.*, 1994; Mackenstedt *et al.*, 1994).

The Apicomplexa is a monophyletic group composed almost entirely of parasitic species. Apicomplexa, along with ciliates and dinoflagellates, form a higher order group known as Alveolata. A major defining characteristic of this group are flattened vesicle-like structures called cortical alveolae, which are found just underneath the plasma membrane. Electron microscopy revealed unique ultra structural features among the various sporozoa, which were subsequently used to redefine the groups. A defining

characteristic of the Apicomplexa is a group of organelles found at one end called the apical end of the organism. This 'apical complex' includes secretory organelles known as micronemes and rhoptries, polar rings composed of microtubules, and in some species a conoid, which lies within the polar rings. At some point during their life cycle, members of the Apicomplexa either invade or attach to host cells. It is during this invasive (and/ or motile) stage that these apical organelles are expressed as well as the subpellicular membranes. The subpellicular membranes are actually cortical alveoli. The apical organelles play a role in interaction of the parasite with the host cell and the subsequent invasion of the host cell. Motile forms of Apicomplexa crawl along the substratum in a non-amoeboid fashion known as gliding motility. Many apicomplexan species have flagellated gametes.

The Apicomplexa have complex life cycles that are characterized by three distinct processes, sporogony, merogony and gametogony. Sporogony occurs immediately after a sexual phase and consists of asexual reproduction that culminates in the production of sporozoites. Sporozoites will develop into forms that undergo another asexual replication known as merogony. Merogony and the resulting merozoites are known by many different names depending on the species. Quite often there are multiple rounds of merogony and sometimes these multiple rounds involve a switch in host organism or a switch in the type of cell invaded by the parasite. Similarly, sporogony and gametogony can involve different hosts or cell types. As an alternative to asexual replication merozoites can become gametes through a process variously called gametogony, gamogony or gametogenesis. As in other types of sexual reproduction, the gametes fuse to form a zygote, which differentiates into a form yielding sporozoites. The sporozoites and merozoites are 'invasive' forms and possess the apical organelles. Although most

Apicomplexa exhibit this overall general life cycle, the details vary greatly between species (Figure 1).

Babesia exhibits a typical apicomplexan life cycle characterized by merogony, gamogony and sporogony. The vertebrate host acquires the infection when sporozoites are transferred during tick feeding. These sporozoites invade erythrocytes, except in some *Babesia* species and *Theileria*, which undergo a pre-erythrocytic cycle in lymphocytes. The mechanism of invasion is similar to other Apicomplexa. In contrast to *Plasmodium*, the parasitophorous vacuolar membrane disintegrates after invasion and the parasite is in direct contact with the host erythrocyte cytoplasm. The trophozoites divide by binary fission and produce merozoites, which infects additional erythrocytes and reinitiates the replication cycle.

In equine infection, a tetrad, referred to as a Maltese cross, is the character that differentiates *B.(Theileria) equi* from other Apicomplexa. In the tick gut, trophozoites develop into gametocytes, or gamonts, which are responsible for initiating the infection in the tick vector. The gametocytes undergo morphological changes within the tick's gut and develop into ray bodies. Two ray bodies or gametes fuse to form a zygote, which then develops into a kinete. The kinete penetrates the peritrophic membrane and intestinal epithelium to gain access to the hemolymph. Sporogony is initiated when kinetes invade the salivary glands. The parasite expands, fills a hypertrophied host cell and develops into a multinucleated sporoblast. Mature sporozoites, possessing apical organelles, will bud from this undifferentiated sporoblast when the tick feeds again on a new host. Five to ten thousand sporozoites can be produced by a single sporoblast. The

sporozoites will then be injected into the host with the saliva, thus completing the life cycle.

Holbrook *et al.*, (1968) described the intra-erythrocytic developmental stages of *B. (Theileria) equi* and studies indicated that, unlike most *Babesia* species (Plate 3 and 4 respectively), *B. (Theileria) equi* apparently also has a stage of schizogony in lymphocytes in the vertebrate host (Moltmann *et al.*, 1983; Schein *et al.*, 1981). Merozoite formation in the lymphocytes takes at least 12 to 14 days after infected ticks first attached to susceptible animals before being released to invade erythrocytes.

According to Schein *et al.*, (1981), inoculated into horses via a tick bite invade the lymphocytes and undergo development in the cytoplasm of these lymphocytes and eventually form *Theileria*-like schizonts. Merozoites released from these schizonts enter other red blood cells. Further support for the close relation between *B.(Theileria) equi* and *Theileria* species has come from the homology found between 30 and 34 kDa *B. equi* surface proteins and similar sized proteins of various *Theileria* spp. (Kappnmeyer *et al.*, 1993; Knowles *et al.*, 1992).

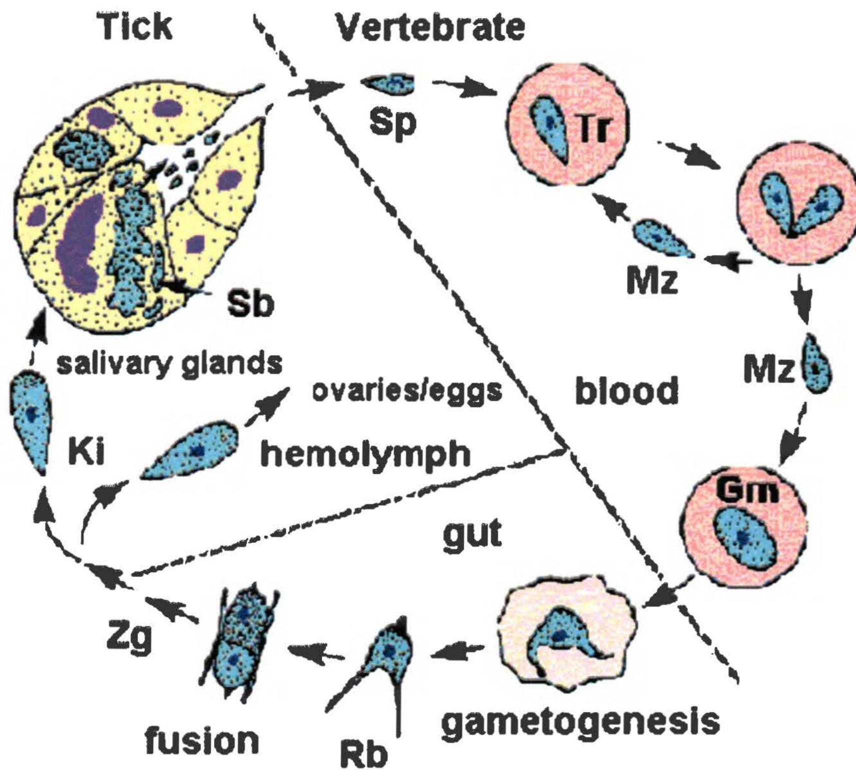


Figure 1: *Babesia* life cycle in the tick vector and vertebrate host (Reproduced from http:/www.Tulane.edu/~wiser/protozoology/notes/api.html)

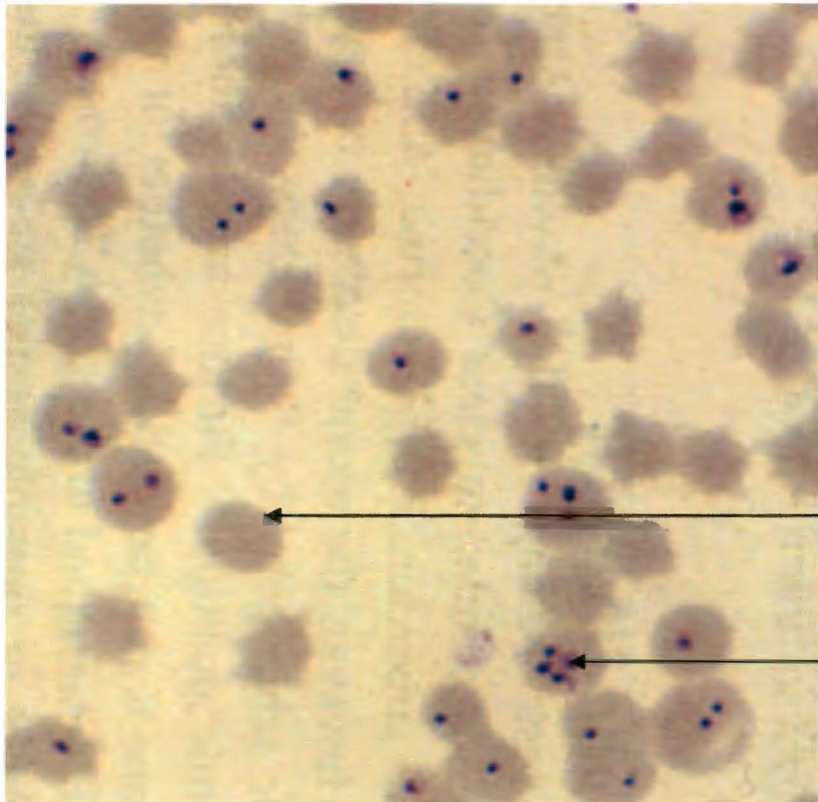


Plate 3: A photo showing *B. (Theileria) equi* infected red blood cells (Reproduced from Norval, 1994)

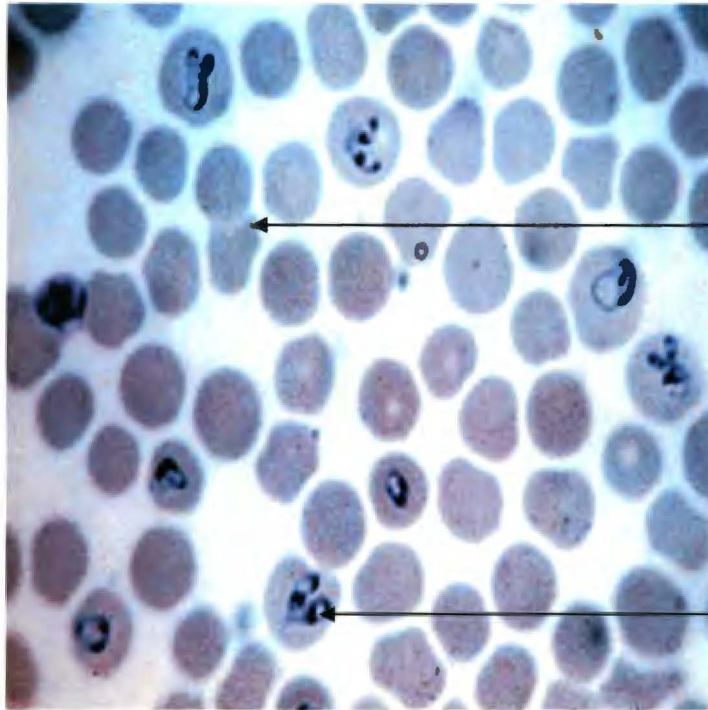


Plate 4: A photo showing *Babesia caballi* infected red blood cells (Reproduced from Norval, 1994)

chronic inappetence, poor performance, loss of body weight, pale-pink mucous membrane and mild tachycardia. Splenomegally is usually present in these cases. Clinical signs may include fever, anorexia, apathy and severe anaemia. Mortality may range from less than 10% to as high as 50% (Levine, 1985). Bilirubinuria and haemoglobinuria may be present in the later stages of the disease. Pregnant animals may abort as a result of these infections (De Waal and Potgieter, 1987). Once infected, horses may become carriers of the parasites and are potential disseminators of the disease into unaffected areas (Brüning *et al.*, 1997). Those animals that survive may remain subclinical carriers for life (Holman *et al.*, 1993). After recovery from the acute phase of babesiosis, the infection may assume a subclinical, chronic course and these animals could become carriers of the protozoans. Often clinical signs of babesiosis are too general to allow specific diagnosis and may be confused with other diseases especially in endemic areas. In carrier horses that may act as reservoirs of infection, parasites are present in very low numbers in the blood and generally may not be detected in Giemsa-stained blood smears (Böse *et al.*, 1995).

Acute cases are characterized by a fever usually exceeding 40°C, varying degrees of anorexia and malaise, and elevated respiratory and pulse rates. Fever is often accompanied by sweating, congestion of mucous membranes, tachycardia and palpitating cardiac sounds. The faecal balls are smaller and drier than normal and are yellowish green. Chronic cases usually present with a history of non-specific clinical signs including mild inappetance, poor performance and drop in body mass.

1.7 Diagnostic tests for *B. (Theileria) equi*

In 1969 the complement fixation test (CFT) was declared by OIE the official serodiagnostic test for the detection of equine *Babesia* infections. Gradually it has become apparent that, as well as presenting technical problems, the CFT does not detect

latent infections and in some cases gives false positive results (Joyner *et al.*, 1981; Tenter and Friedhoff, 1986). In borderline cases, the indirect immunofluorescent test (IFAT) has to be applied, which is time consuming and requires skilled interpretation (Brüning *et al.*, 1997). Furthermore, Böse *et al.*, (1995) and Brüning (1996) reported that the CFT and IFAT have commonly been used to detect *B. (Theileria) equi* infection. However, they discovered that the antibody detection limits and cross-reactivity generally restrict these serological tests. Besides CFT and IFAT, the enzyme-linked immunosorbent assay (ELISA) has been used for detection of antibodies against *B. (Theileria) equi*. However, the ELISA is hindered by a limiting antigen supply and poor specificity (Böse *et al.*, 1995; Brüning, 1996; Weiland, 1986).

In another study, Holman *et al.*, (1997) and Zweygarth *et al.*, (1997) used *in vitro* culture from horse blood for the identification of carrier animals with *B. (Theileria) equi*. Other workers found that direct detection of *B. (Theileria) equi* and *B. caballi* nucleic acid from horse blood with DNA probes detected a higher number of carrier animals than microscopic methods (Posnett and Ambrosio, 1989; Posnett *et al.*, 1991; Figueroa *et al.*, 1992). However, Böse *et al.*, (1995) argued that generally, probes are equivalent in sensitivity to Giemsa-stained thin blood films. Microscopic detection is still the best and most sustainable method for on-site diagnosis of acute diseases and is indispensable in every laboratory. It is more sensitive and less time consuming than commonly thought (Böse *et al.*, 1995).

1.7.1 Isolation of DNA from blood cells

The isolation of DNA is one of the commonly used procedures in molecular biology where purified DNA is required for applications such as studying DNA structure and chemistry, carrying out DNA hybridisation and PCR (Wortman, 1997). The application of molecular diagnostic techniques to the diagnosis of infectious diseases is based on

the identification of a unique signature sequence in the DNA or RNA of a pathogen (Persing, 1991). Mammalian DNA from horse blood is isolated by digestion of cells with proteinase K in the presence of EDTA and sodium-dodecyl-sulphate (SDS), followed by extractions with phenol (Sambrook *et al.*, 1989).

1.7.2 Purification of nucleic acids

The most basic of all procedures in molecular cloning is the purification of nucleic acids. It is necessary to purify DNA solutions prior to further enzymatic manipulations because DNA solutions containing contaminants inhibits restriction enzyme digestion or PCR amplification. One of the commonly used methods for de-proteinising a DNA solution is extraction with phenol, which efficiently denatures proteins and probably dissolves denatured protein. Iso-amyl alcohol is added to prevent foaming and aids in the separation of the organic and aqueous phases. The denatured protein forms a layer at the interface between the organic and aqueous phases and is thus isolated from the bulk of the DNA in the aqueous layer (Ausebel *et al.*, 1987).

1.7.3 Polymerase chain reaction (PCR) for the amplification of *B.(Theileria) equi* DNA

The application of molecular diagnostic techniques for the diagnosis of infectious disease is based on identification of unique "signature sequences" in the DNA or RNA of a pathogen. Polymerase chain reaction (PCR) is a molecular tool that allows scientists to amplify specific DNA sequence against the background of a complex genome. This is accomplished via a cyclic succession of incubation steps at different temperatures. The target DNA is heat-denatured to separate the two complementary strands to provide a template. Specific primers, short synthetic molecules of DNA complementary to both strands of the DNA flanking the target sequences, are then annealed to the single-stranded template at low temperature and extended with DNA

polymerase at an intermediate temperature. Once the polymerase has synthesized a new strand of DNA, the product separates from the template by heating to a higher temperature. These steps are called cycles and are repeated 20 to 40 times, yielding amplification of target DNA sequences (Bloom *et al.*, 1996). Figure 3 shows the three steps in PCR.

Bose *et al.*, (1995) explained that for applications where a high sensitivity is required, the very powerful PCR technique has largely superseded DNA probes. They also continued to clarify that the PCR test repeatedly amplifies a specific DNA sequence in the genome of the target organism to produce an easily detectable product. The high sensitivity and specificity of the PCR technique makes it useful for validating results from other diagnostic techniques and for applications such as exportation of livestock. In spite of their high sensitivity, false negative results are still observed (Hayden *et al.*, 1991).

The greatest problem facing the diagnostic application of PCR and other nucleic acid amplification methods is false positivity due to contaminating nucleic acids as cited by Persing (1991) from Kwok and Higuchi (1989). Kwok and Higuchi (1989) explained the exquisite sensitivity of the PCR proved to be its own undoing where the transfer of minuscule quantities of such sequences into a neighbouring tube resulted in false positive results. They further mentioned that nucleic acid contamination results from three sources. One source consists of clinical specimens containing large numbers of target molecules, which result in cross-contamination between specimens. The other source is contamination of reagents used in PCR by previously cloned plasmid DNA, a particularly aggravating problem for laboratories that have been studying a particular organism for many years. The third source is accumulation of PCR products in the laboratory by repeated amplification of the same target sequence.

1.8 Quantitation of DNA and RNA

There are two types of methods that are widely used to measure the amount of nucleic acid after extraction. If the sample is pure, that is, if the sample has no significant amounts of contaminants such as proteins, phenol, agarose, or other nucleic acids, spectrophotometric measurement of the amount of ultraviolet irradiation absorbed by the bases is simple and accurate. However, if the amount of DNA or RNA is very small and the sample contains significant quantities of impurities, the amount of nucleic acid can be estimated from the intensity of fluorescence emitted by ethidium bromide (Sambrook *et al.*, 1989).

1.9 Ethidium Bromide Fluorescent quantitation of the amount of double stranded DNA

Sometimes there is insufficient DNA, (less than 250 ng/ml) to assay spectrophotometrically, or the DNA may be heavily contaminated with other substances which absorb ultraviolet irradiation and therefore impede accurate analysis. In such cases, a rapid way to estimate the amount of DNA is to utilize the ultraviolet-induced fluorescence emitted by ethidium bromide molecules intercalated into the DNA. Because the amount of fluorescence is proportional to the total mass of DNA, the quantity of DNA in the sample can be estimated by comparing the fluorescent yield of the sample with that of a series of standards. As little as 1 to 5 ng of DNA can be detected by this method (Sambrook *et al.*, 1989).

CHAPTER 2

OBJECTIVES

2.1 Justification of the study

With the exception of the northeastern Free State, *Babesia (Theileria) equi*, a tick-transmitted protozoan parasite and one of the causative organisms of equine babesiosis (Zweygarth *et al.*, 1997) has been extensively studied in this country (De Waal, 1995). Prior to the present study, the only investigations conducted in this part of the province were the abundance of adult ixodid ticks infesting cattle (Hlatshwayo *et al.*, 2001) and sero-epidemiological surveys of blood parasites in cattle belonging to resource-poor farmers in the northeastern Free State (Mtshali *et al.*, 2004). *Rhipicephalus e. evertsi* was reported to be the second most abundant tick in this region comprising 44.5% of the tick burden being preceded by *Boophilus decoloratus,* which constituted 53% on average. It was therefore decided that a study be conducted to investigate the status of *B. (Theileria) equi* in horses in the northeastern Free State using blood smears, serology (immunofluorescent antibody test, IFAT) and the polymerase chain reaction, PCR. In a previous survey of thoroughbred mares in South Africa, it was found that 11% of abortions were due to *B. (Theileria) equi* (Coetzer and De Waal, unpublished observation, 1990).

2.2 General Objective

2.2.1 To diagnose *B. (Theileria) equi* in horses by conventional parasitological methods, (blood smear examination), serological method, (IFAT), and by molecular techniques, (target DNA amplification using PCR).

2.3 Specific Objectives

2.3.1 To optimize PCR conditions for the diagnosis of *B. (Theileria) equi.*

2.3.2 To diagnose *B. (Theileria) equi* in horses by Giemsa-stained blood smears, serology (IFAT) and PCR.

2.3.2 To conduct a questionnaire survey to assess perceptions, attitudes and expectations of farmers in the study area with regard to *B. (Theileria) equi* infection.

CHAPTER 3

MATERIALS AND METHODS

3.1 Description of study area

The northeastern Free State is characterised by very high mountains. It is located at an altitude of 1500 m to more than 3000 m above sea level (Kritzinger and Pieterse, 1987). It is situated between latitudes 28° and 30°S, 28° and 30°E. The neighbouring state on the southern border is Lesotho and Kwa-Zulu Natal on the eastern border.

Three areas, Harrismith, Kestell and QwaQwa were chosen as study sites because they are within a reasonable distance from the university campus and farmers were willing to allow the use of their animals for the survey. The distance from the university campus to Harrismith is 40km; from the university to Kestell is 28km and 5km to QwaQwa (Vrey and Smith, 1980). Harrismith is the center of one of the five wool-producing districts in Southern Africa established in 1849.

South Africa is divided into three main rainfall regions: a winter rainfall region in the southwestern Cape, an area with rainfall throughout the year along the southern coastal region and a summer rainfall area in the rest of the country. The northeastern Free State lies within the summer rainfall region of South Africa with more than 85 percent of the precipitation occurring during September to March mainly in the form of thunderstorms. The annual precipitation on the lower lying plateau in the Harrismith and Kestell localities is approximately 700mm per annum, increasing gradually to approximately 1340mm per annum in the QwaQwa Mountains (Kritzinger and Pieterse, 1987). Another feature is an increase in rainfall from the western to the eastern parts. Less than 125mm rainfall a year occurs along the eastern escarpment.

The temperature in the northeastern Free State Province may be described as cool to moderately warm. The average temperature during mid-winter is 7.4°C and 17.9°C during mid-summer. The average daily minimum temperature during winter is 4.6°C and average daily maximum temperature during summer is 29.9°C. Temperature decrease in excess of 5°C can occur 30 times a year, lasting up to two to three days at a time. Temperatures of 34°C also occur for four to five days per year. During September and October temperatures are regarded to be unstable, but during April and May to be quite stable (Kritzinger and Pieterse, 1987).

Figure 3: Free State map showing the three study sites, Harrismith; Kestell and QwaQwa.(http:/www.places.co.za/maps/free_state_map.htm)

3.2 Collection of blood samples from horses in the northeastern Free State

A total of 99 blood samples were collected from horses at various localities from the three study sites; 29 from (Harrismith, 21 from Kestell and 49 from QwaQwa). Blood was collected in 10ml of both plain and EDTA-coated vacutainers by venipuncture from the jugular vein. The samples were kept on ice and immediately brought to the university laboratory for preparation of blood smears and serum. Some of the blood samples in EDTA-coated tubes were transferred into sterile labelled cryogenic vials and stored at –35°C for later use in PCR.

Plate 5: Collection of blood from the jugular vein of the horse.

3.3 <u>Collection of blood samples from horses on the Kaal plaas farm OVI , Pretoria</u>

Blood samples were collected from seven horses (168, 171, 176, 278, 300, 421 and 929) kept at Kaalplaas farm, the Onderstepoort Veterinary Institute Pretoria. These horses were known to have been infected previously experimentally with *B. (Theileria) equi.* Blood samples collected in 10ml EDTA-coated vacutainers were kept on ice and brought immediately to the laboratory for the preparation of smears. Aliquots of the remaining blood were transferred into labelled cyrogenic vials and stored at –35°C for later use in PCR. The samples in plain 10ml tubes were used to prepare sera. Blood smears, sera, and IFAT procedure were conducted as illustrated under section 3.2.

3.4 <u>Preparation of thin film blood smears</u>

In the laboratory, thin smears were prepared from the EDTA blood. The smears were stained using 10% Giemsa stain solution for 35 minutes. The slides were examined using a standard light microscope at 1000x magnification. Parasitaemia was estimated by counting parasitized red blood cells (RBC) in 8 fields containing an average of 800 RBC per field using the formula shown below. No. of infected RBC ÷ total No. of uninfected RBC x 100. A smear was considered negative after a five minutes examination revealed no parasites (De Waal and Potgieter, 1987).

3.5 <u>Preparation of thick film blood smears</u>

Thick blood smears were prepared by placing a fairly bigger drop of blood on the microscope slide. That drop of blood was dispersed in a circle along the middle of the slide with the corner of the spreader slide to facilitate the dispersal of the blood. The slide with the dispersed drop of blood was left to air dry and put into an immersion tank containing 10% Giemsa stain solution for 35 minutes. The slide was removed from the tank and allowed to air dry in a slide rack. A drop of 518C immersion oil (Zeiss Germany) was placed on both thin and thick smears. The slides were then examined

using a standard light microscope at 1000x magnification (eyepiece 10x; objective 100x), (De Waal, 1999).

3.6 Preparation of sera

Serum was prepared from blood samples collected into 10ml Vacutainer tubes and harvested into sterile cryogenic vials, (Plate 6). The blood was left for 24 hours at room temperature, before it was centrifuged at 200g for 5 minutes (De Waal *et al.*, 1987). The resulting sera was then harvested into labelled 5ml cryogenic vials and stored at –35°C until tested for the presence of antibodies against *B. (Theileria) equi.*

3.7 Immunofluorescent antibody test (IFAT)

3.7.1 Antigen preparation for the immunofluorescent antibody test (IFAT)

Antigen slides, conjugate and control sera were readily obtainable from the Onderstepoort Veterinary Institute, Pretoria.

3.7.2 IFA test procedure

Batches of antigen slides were removed from storage at -20°C and placed in an incubator at 37°C for 10 minutes. The antigen slides were fixed in cold acetone (-20°C) for one minute and then marked (e.g. *B. (Theilera) equi* = e_1, e_2 and so on and *B. caballi* = c_1, c_2...). The test sera were allowed to thaw at room temperature.

Two-fold dilutions, of the test and control sera, starting at 1/40, 1/80 and 1/160 were prepared using PBS (Tenter and Friedhoff, 1986). Test tubes were marked numerically to correspond with the sera numbers and placed in a test tube rack. Two milliliters of PBS was added in the first row where the 1/40 dilution of sera was to be prepared and 1ml was added in the second and third row of marked test tubes. Fifty microliters of sera was added to each corresponding front row test tube with a micropipette using a

clean tip for each sample. One milliliters of the resulting solution was added to the second row of corresponding test tubes containing 1ml of PBS and from this row 1ml of the resulting dilution was added to the third row of corresponding test tubes to make 1/160 dilution (Tenter and Friedhoff, 1986).

A drop (~ 4μl) of each diluted serum was pipetted into each respective circle of the antigen slides. The slides were incubated at 37°C for 30 minutes and then washed off with PBS for 10 minutes on a magnetic stirrer. The PBS was discarded and distilled water was added and put on a stirrer to wash for 5 minutes. After the slides were allowed to air dry, a drop (~ 2μl) of conjugate dilution was pipetted into each circle of the slides and incubated for 37°C for 30 minutes. The conjugate was then washed in PBS for 10 minutes on a magnetic stirrer. After air-drying, a drop of mounting fluid made from 50% glycerine and 50% PBS was placed on each slide and covered with a 24 x 50mm cover slip. Those slides were observed under a fluorescent microscope using a 50x water objective (Tenter and Friedhoff, 1986). Test readings were ranked as negative (-) for slides with no specific fluorescence when observed under fluorescent microscope, positive (+) for those that presented strong readings at serum dilution of 1/80 and positive/negative (±) for those that showed a weak fluorescence (OVI, 1999).

3.8 Polymerase chain reaction (PCR) *B. (Theileria) equi*

To perform a PCR, a small quantity of a target DNA was added to a buffered solution containing DNA polymerase, oligonucleotide primers, the four deoxinucleotide building blocks of DNA and the cofactor Mg^{++}. The PCR mixture was taken through 30 cycles of amplification; each cycle doubling the number of copies of the target region (Bloom *et al.*, 1996).

3.8.1 Genomic DNA extraction from horse blood

DNA was extracted from stored horse blood specimens by the following method: 400 μl of blood was treated with five units of TE (10 mM Tris-HCl, pH7.4, 1mM EDTA) and then centrifuged at 14,000 g for 2 min. The resulting pellet was washed five times in TE, removing the erythrocyte ghost layer with every wash, and re-suspended in 400 μl of distilled water. After incubation at 37°C for 1 hour with 0.1 mg of proteinase K, 1% SDS and 1% sarcosine, DNA was extracted with phenol-chloroform and precipitated over night in ethanol at -34°C. The DNA was then collected by centrifugation in a micro-centrifuge and re-suspended in 20 μl of distilled water. An amount of 5μl of the DNA suspension was used in a PCR reaction (Bashiruddin *et al.*, 1999). The 16S rRNA sequence (GenBank™ accession number Z15105) of *B. (Theileria) equi*, selected by alignment of the nucleotides and primers BEQF and BEQR for *B. (Theileria) equi*, was used for the experiment (Table 2).

Table1: Nucleotide sequences of primers and the expected length of PCR product of *B. (Theileria) equi* (Bashiruddin *et al.*, 1999).

Parasite	Primer	Sequence (5'-3')	Expected Product length (bp)
B. (Theileria) equi	BEQF	CATCGTTGCGGCTTGGTTGG	664
	BEQR	CCAAGTCTCACACCCTATTT	664

Eppendorf tubes were labeled 1-99 for each horse blood sample obtained. Up to 25 blood samples were treated per day. Frozen blood samples were thawed in a water bath at room temperature and after the blood had thawed, it was mixed gently and 400μl of blood from each sample was transferred to the respective labeled tubes. One ml of TE

buffer was immediately added to each labeled tube containing 400µl of blood. The tubes were tightly closed, mixed by vortexing and centrifuged at 4°C for 2 minutes at 14000g. This washing step was repeated five times as mentioned above. For the first two washes, the liquid was decanted form the pellet, but for the last three washes, a micropipette was used to remove all the liquid. After the final wash, all the liquid was removed without disturbing the pellet. The extraction buffer was prepared in a separate tube, (see appendix 1). An amount of 400µl of extraction buffer solution was added to each of the seven tubes containing a pellet. The pellet was re-suspended and the samples were incubated at 37°C overnight in eppendorf thermostat plus. After one hour, the pellet was dispersed with a micropipette to facilitate its dispersal. Although the samples could be incubated at 37°C for two hours, the incubation period can be extended overnight for a better degradation of proteins.

3.8.2 Phenol/chloroform extraction of DNA

The next day after incubation overnight, 400µl of phenol/chloroform was added to each of the seven, 1.5 µl tubes containing the extraction mixture. They were mixed on vortex until an emulsion was formed and centrifuged at 14 000g for two minutes at 4°C. Three separate phases were formed and the upper viscous aqueous phase transferred to clean labeled centrifuge tubes. To the clear solution in new-labelled tubes another 400µl of phenol-chloroform was added, vortex mixed and centrifuged at 14000g for 2 minutes at 4°C. Again, a clear upper supernatant was harvested to new-labelled tubes.

Working on ice, DNA was precipitated by adding 800µl of absolute ice-cold ethanol to each of the three tubes as well as 54µl of 2.2 M sodium acetate. These solutions were mixed and placed at -20°C for two hours. The centrifuge was pre-cooled and then the tubes were centrifuged at 14000 g for 5 min at 4°C. The solution from the DNA was discarded and 1 ml of 70 % ethanol was added to the pellet in the three tubes. The tubes

were re-centrifuged again at 14000g for 5 minutes at 4°C and the liquid was discarded. The tubes were placed upside down on a paper towel to remove the last drop of liquid. To dissolve the pellet, 200µl of double distilled and autoclaved water was added. This solution was left for 30 minutes on ice and then re-centrifuged. The clear supernatant was then transferred to new labelled tubes. Then, 10µl of this DNA mixture was mixed with 3µl of loading buffer and separated on a 1% agarose gel (Plate 7).

3.8.3 Optimization of PCR using pure *B. (Theileria) equi* DNA from Brazil

For the first PCR reaction, different concentrations of pure *Babesia* DNA were obtained and optimised. Because the concentration of ***B. (Theileria) equi*** from Brazil was not known, different dilutions of the DNA for the PCR reaction were prepared as follows: 0.01 µl, 0.05 µl, 0.1 µl and 0.5 µl.

3.8.4 Optimization of PCR conditions for the diagnosis of *Babesia equi*

3.8.4 (a) Denaturation of DNA

The denaturing of target DNA was conducted between 94°C and 96°C until a suitable temperature was obtained for this process (Bloom *et al.*, 1996).

3.8.4 (b) Annealing of primers

The initial annealing process was conducted for 5 minutes at 94°C during which the primers hybridised (by way of hydrogen bonds) to their complementary strands on either side of the target region (Bloom *et al.*, 1996).

The annealing conditions were optimised using an Eppendorf Mastercycler® Gradient. A PCR reaction for twelve PCR tubes was prepared using the positive control for the optimisation of the annealing temperature. The tubes were aligned in different columns

3.8.4 (c) Extension of a complementary DNA

This extension was conducted for 5 minutes at 72°C, during which the polymerase binds and extends a complementary DNA strand from each primer (Bloom *et al.*, 1996).

3.8.4 (d) Amplification of DNA

The primers, which were chosen from DNA sequences coding for the 16S rRNA of *B. (Theileria) equi* (Bashiruddin *et al.*, 1999), are described in Table 2. Amplification was performed with Sigma-reagents and *Taq* polymerase in 50µl reaction volumes using an Eppendorf Mastercycler® Gradient. The optimum conditions for amplification of *B. (Theileria) equi* were found to be as follows: 5 min at 94°C, 30 cycles each of 94°C for 30 seconds, 64°C for 30 seconds, and 72°C for 30 seconds. All cycles were finished by the addition of a final extension period of 5 minutes at 72°C. The PCR products were detected and their sizes estimated by co-electrophoresis of 5ml of the reaction mix and standard size markers of 50-2000bp (Sigma), on 1% agarose gels (Plate 8). A 0.01µl of pure *Babesia* DNA brought from Brazil was used as positive control. Distilled water was used as negative control and after electrophoresis gels were visualized by ultraviolet illumination of ethidium bromide stained DNA.

3.9 Physical condition of animals

The method adopted for scoring physical condition of tested animals was the one described by Van Niekerk and Louw (1990). The method used a four-point score where an extremely thin animal was given by a score of 1 and classified as poor, a moderately fed animal was scored 2 to 3 and regarded as fair while a score of 4 was given for an over fat horse (Table 3).

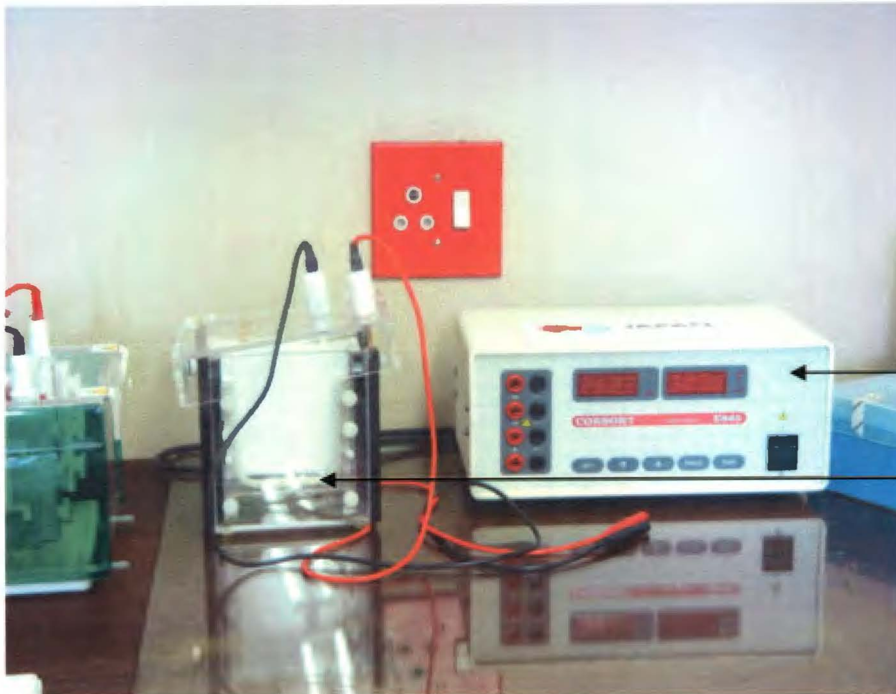


Plate 6: Apparatus used to run agarose gel

Table 2: Physical condition of horses in the northeastern Free State as described by Van Niekerk and Louw, 1990.

Score	Poor	Fair	Good
General feature of animals	Animal appear emaciated	Ideal condition	Over fat animal

3.10 Questionnaire survey

The questionnaire (Appendix 4) survey was conducted in the three study regions. The aim of the questionnaire survey was mainly to find out whether resource poor farmers in the northeastern Free State knew of *B. (Thileria) equi*, what causes it, whether they could recognize the symptoms of the disease and whether they knew how it should be treated. It was also intended to assess the employment status of the majority of the local emerging farmers and their dependents. Farmers were also asked whether they knew of any other parasites afflicting their animals and if so, how they reacted to the situation. The questionnaire involved direct and multiple-choice questions, assessments and personal opinions of farmers.

CHAPTER 4

RESULTS

4.1 Observation of thin and thick blood smears from the northeastern Free State

All the 99 blood smears from the northeastern Free State were found negative for *B. (Theileria) equi/ caballi* parasites. A summary of the results is given in Tables 4.

4.2 Observation of thin and thick blood smears from Kaal plaas, OVI Pretoria

Babesia (Theileria) equi parasites were detected microscopically in thin blood smears from 3 of the 7 blood samples (300, 421 and 929) collected at Kaal plaas farm, OVI (Table 5). They had detectable parasitaemias estimated at < 1/1000.

4.3 IFA test results from the northeastern Free State

Of the 99 blood samples tested, 98% were found to be positive for *B. (Theileria) equi*, 48% were positive for *B. caballi* and 58% had mixed infections (Table 4).

4. 4 IFA test results from Kaal plaas farm, OVI

The IFA results have shown that all the seven samples were seropositive for *B. (Theileria) equi* and three samples, 168, 278 and 300 were seropositive for *B. caballi* (Table 5). Three animals, number 171, 176, and 421 were seropositive for both *B. (Theileria) equi* and *B. caballi*.

Table 3: Microscopy, serology and PCR results for detection and diagnosis of *B. (Theileria) equi* and *B. caballi* from horse blood in the northeastern Free State.

Horse No.	Smear	IFAT (1/80)		PCR	Horse Physical condition
		B. (Theileria) equi	***B. caballi***		
1.	-ve	+ve	-ve	-ve	Fair
2.	-ve	+ve	+ve	-ve	Fair
3.	-ve	+ve	+ve	-ve	Fair
4.	-ve	+ve	-ve	-ve	Fair
5.	-ve	+ve	-ve	-ve	Poor
6.	-ve	+ve	+ve	-ve	Fair
7.	-ve	+ve	+ve	-ve	Fair
8.	-ve	+ve	+ve	-ve	Fair
9.	-ve	+ve	+ve	-ve	Fair
10.	-ve	+ve	+ve	-ve	Fair
11.	-ve	+ve	+ve	-ve	Fair
12.	-ve	+ve	+ve	-ve	Fair
13.	-ve	+ve	+ve	-ve	Fair
14.	-ve	+ve	+ve	-ve	Fair
15.	-ve	+ve	+ve	-ve	Fair
16.	-ve	+ve	+ve	-ve	Fair
17.	-ve	+ve	-ve	-ve	Fair
18.	-ve	+ve	-ve	-ve	Fair
19.	-ve	+ve	-ve	-ve	Fair
20.	-ve	+ve	-ve	-ve	Fair
21.	-ve	+ve	±ve	-ve	Fair
22.	-ve	+ve	-ve	-ve	Fair
23.	-ve	+ve	-ve	-ve	Fair
24.	-ve	±ve	±ve	-ve	Fair
25.	-ve	+ve	+ve	-ve	Fair
26.	-ve	+ve	+ve	-ve	Fair
27.	-ve	+ve	+ve	-ve	Fair
28.	-ve	+ve	+ve	-ve	Fair
29.	-ve	+ve	+ve	-ve	Fair
30.	-ve	+ve	-ve	-ve	Fair
31.	-ve	+ve	-ve	-ve	Fair
32.	-ve	+ve	-ve	-ve	Fair
33.	-ve	+ve	-ve	-ve	Fair
34.	-ve	+ve	-ve	-ve	Fair
35.	-ve	+ve	-ve	-ve	Fair
36.	-ve	+ve	-ve	-ve	Fair
37.	-ve	+ve	-ve	-ve	Fair
38.	-ve	+ve	+ve	-ve	Fair

Horse No.	Smear	IFAT		PCR	Horse Physical condition
		B. (Theileria) equi	***B. caballi***		
39.	-ve	+ve	+ve	-ve	Fair
40.	-ve	+ve	+ve	-ve	Fair
41.	-ve	+ve	+ve	-ve	Fair
42.	-ve	+ve	+ve	-ve	Fair
43.	-ve	±ve	+ve	-ve	Fair
44.	-ve	+ve	+ve	-ve	Fair
45.	-ve	+ve	+ve	-ve	Fair
46.	-ve	+ve	+ve	-ve	Fair
47.	-ve	+ve	+ve	-ve	Fair
48.	-ve	+ve	+ve	-ve	Fair
49.	-ve	+ve	+ve	-ve	Fair
50.	-ve	+ve	+ve	-ve	Fair
51.	-ve	+ve	+ve	-ve	Fair
52.	-ve	+ve	-ve	-ve	Fair
53.	-ve	+ve	-ve	-ve	Fair
54.	-ve	+ve	-ve	-ve	Fair
55.	-ve	+ve	-ve	-ve	Fair
56.	-ve	+ve	-ve	-ve	Fair
57.	-ve	+ve	-ve	-ve	Fair
58.	-ve	+ve	-ve	-ve	Fair
59.	-ve	+ve	+ve	-ve	Fair
60.	-ve	+ve	+ve	-ve	Fair
61.	-ve	+ve	+ve	-ve	Fair
62.	-ve	+ve	+ve	-ve	Fair
63.	-ve	+ve	+ve	-ve	Fair
64.	-ve	+ve	+ve	-ve	Fair
65.	-ve	+ve	+ve	-ve	Fair
66.	-ve	+ve	+ve	-ve	Fair
67.	-ve	+ve	+ve	-ve	Fair
68.	-ve	+ve	+ve	-ve	Fair
69.	-ve	+ve	+ve	-ve	Fair
70.	-ve	+ve	+ve	-ve	Fair
71.	-ve	+ve	+ve	-ve	Fair
72.	-ve	+ve	+ve	-ve	Fair
73.	-ve	+ve	+ve	-ve	Fair
74.	-ve	+ve	+ve	-ve	Fair
75.	-ve	+ve	+ve	-ve	Fair
76.	-ve	+ve	+ve	-ve	Fair
77.	-ve	+ve	+ve	-ve	Fair

Horse No.	Smear	IFAT		PCR	Horse Physical condition
		B. (Theileria) equi	***B. caballi***		
78.	-ve	+ve	-ve	-ve	Fair
79.	-ve	+ve	-ve	-ve	Fair
80.	-ve	+ve	-ve	-ve	Fair
81.	-ve	+ve	-ve	-ve	Fair
82.	-ve	+ve	-ve	-ve	Fair
83.	-ve	+ve	-ve	-ve	Fair
84.	-ve	+ve	-ve	-ve	Fair
85.	-ve	+ve	-ve	-ve	Fair
86.	-ve	+ve	-ve	-ve	Fair
87.	-ve	+ve	-ve	-ve	Fair
88.	-ve	+ve	+ve	-ve	Fair
89.	-ve	+ve	+ve	-ve	Fair
90.	-ve	+ve	+ve	-ve	Fair
91.	-ve	+ve	+ve	-ve	Fair
92.	-ve	+ve	+ve	-ve	Fair
93.	-ve	+ve	+ve	-ve	Fair
94.	-ve	+ve	+ve	-ve	Fair
95.	-ve	+ve	+ve	-ve	Fair
96.	-ve	+ve	-ve	-ve	Fair
97.	-ve	+ve	-ve	-ve	Fair
98.	-ve	+ve	-ve	-ve	Fair
99.	-ve	+ve	-ve	-ve	Fair
Total positive	**0**	**97**	**48**	**0**	

<u>Key:</u>

-ve = **Negative**

+ve = **Positive**

±ve = **Doubtful**

Table 4: Microscopy, serology and PCR results for detection and diagnosis of *B. (Theileria) equi* and *B. caballi* from horse blood from Kaal plaas farm, OVI.

Horse No.	**Smear**	**IFAT**		**PCR**	**Horse Physical condition**
	B. (Theileria) equi	***B. (Theileria) equi***	***B. caballi***	***B. (Theileria) equi***	
168	-ve	+ve	-ve	+ve	Fair
171	-ve	+ve	+ve	+ve	Fair
176	-ve	+ve	+ve	+ve	Fair
278	-ve	+ve	-ve	+ve	Fair
300	+ve (<1/1000)	+ve	-ve	+ve	Poor
421	+ve (<1/1000)	+ve	+ve	+ve	Poor
929	+ve (<1/1000)	+ve	±ve	+ve	Poor
Total positive (%)	3 (43%)	7 (100%)	3 (43%)	7 (100%)	

<u>**Key:**</u>

-ve = Negative

+ve = Positive

±ve = Borderline

Table 5: Results of serodiagnosis for *B. (Theileria) equi* and *B. caballi* using IFAT on horse blood from Harrismith, Kestel and Qwa Qwa

Sites	Number of sampled animals	Number of *B.(Theileria) equi* positive samples by IFAT at ≤ 1/80 dilution	Number of *B. caballi* positive samples by IFAT at ≤ 1/80 dilution
Harrismith	29	28/ 29 (96.5%)	5/ 29 (17.2%)
Kestel	21	20/ 21 (95.2%)	13/ 21 (61.9%)
Qwa Qwa	49	49/ 49 (100%)	30/ 49 (61.2%)
Total number of sampled animals	99	97/ 99 (98%)	48/ 99 (48%)

4.5 Polymerase chain reaction

4.5.1 Genomic DNA extraction from horse blood samples collected in the northeastern Free State

For all the extractions, the quality of extracted DNA was assessed by observation of the agarose gel under ultra violet light. A single solid band (Plate 8) was observed demonstrating good quality DNA.

4.5.2 Genomic DNA extraction from horse blood samples collected in OVI

Genomic DNA from the blood samples collected from OVI was extracted using the same procedure as in 3.8.1 and a single solid band was also observed and accepted as good quality DNA that was later used for PCR amplification procedure.

4.5.3 Optimization of pure *B. (Theileria) equi* DNA from Brazil

All four dilutions (0.01 µl, 0.05 µl, 0.1 µl and 0.5 µl) gave the correct band size, with no signal in the negative control lane. It was decided that a dilution of 0.01µl will be used for every PCR reaction and will serve as a positive control sample.

4.5.4 Optimization of PCR conditions for the diagnosis of *B. (Theileria) equi*

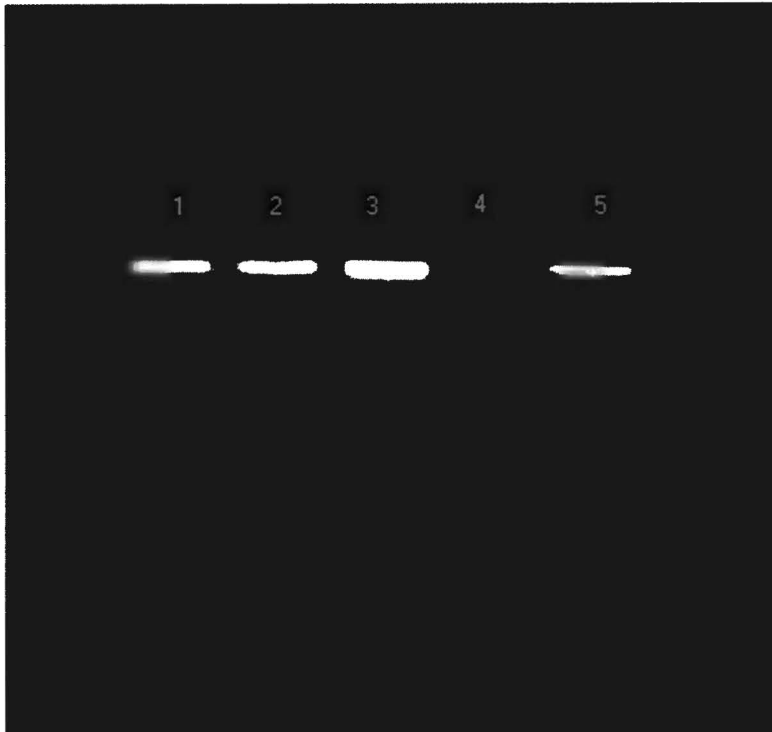
The suitable optimum temperature for the denaturing of target tissue in this study was obtained at 94°C and the optimum annealing temperature was observed at 64°C.

4.5.5 Amplification of DNA products from northeastern Free State samples by PCR

After amplification and agarose gel electrophoresis were completed, PCR products were observed under ultra violet light. All 99 horses from the northeastern Free State were PCR negative. The 16S rRNA strand of pure *B.(Theileria) equi* DNA from the positive control amplified as a single band of about 660 bp.

4.5.6 Amplification of DNA products from Kaal plaas farm samples by PCR

In this experiment all seven horses from OVI were infected. The 16S rRNA strand of *B. (Theileria) equi* from both the positive control and the seven infected samples amplified as a single band of about 660 bp with no band indicated in the lane that contained a negative control (Plate 7).



Lane 1: 0.5 µl

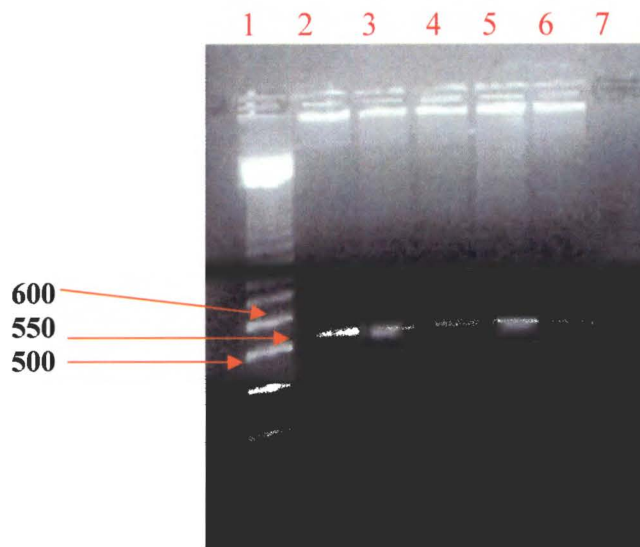
Lane 2: 0.05 µl

Lane 3: 0.01 µl

Lane 4: Negative control (water)

Lane 5: 0.1 µl

Plate 7: Agarose gel electrophoresis of the four DNA dilutions



Lane 1: Marker

Lane 2: 929

Lane 3: 168

Lane 4: 171

Lane 5: 421

Lane 6: Positive control

Lane 7: Negative control

Plate 8: Agarose gel showing amplification of 16S rRNA of *B. equi* in four horses from Kaal plaas farm.

4.6 Questionnaire Survey

4.6.1 Demographic information of the farmers in the three study areas

Of the 21 individuals issued with the questionnaires, 11 were pensioners, 6 were unemployed whilst 4 were self-employed (Figure 5).

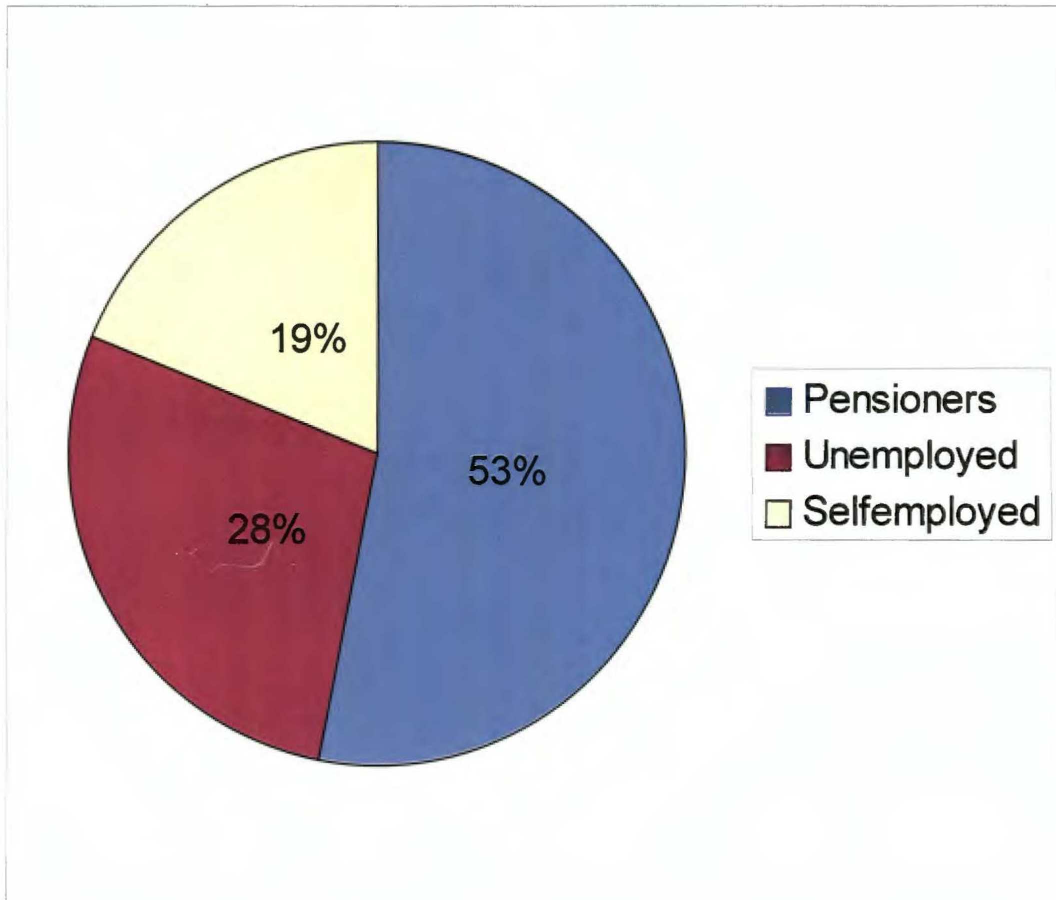


Figure 4: A pie chart showing employment status of the farmers

4.6.2 Composition of animals owned by farmers in the three study sites

Figure 6 shows the average percentage of different animal species owned by farmers. Of the animals owned, the number of horses was found to be the lowest, being only 8.6% on average. Cattle were the second lowest with an average of 19%; 20.9% were poultry, 24.8% were goats and the most commonly owned animals were sheep with an average of 27%.

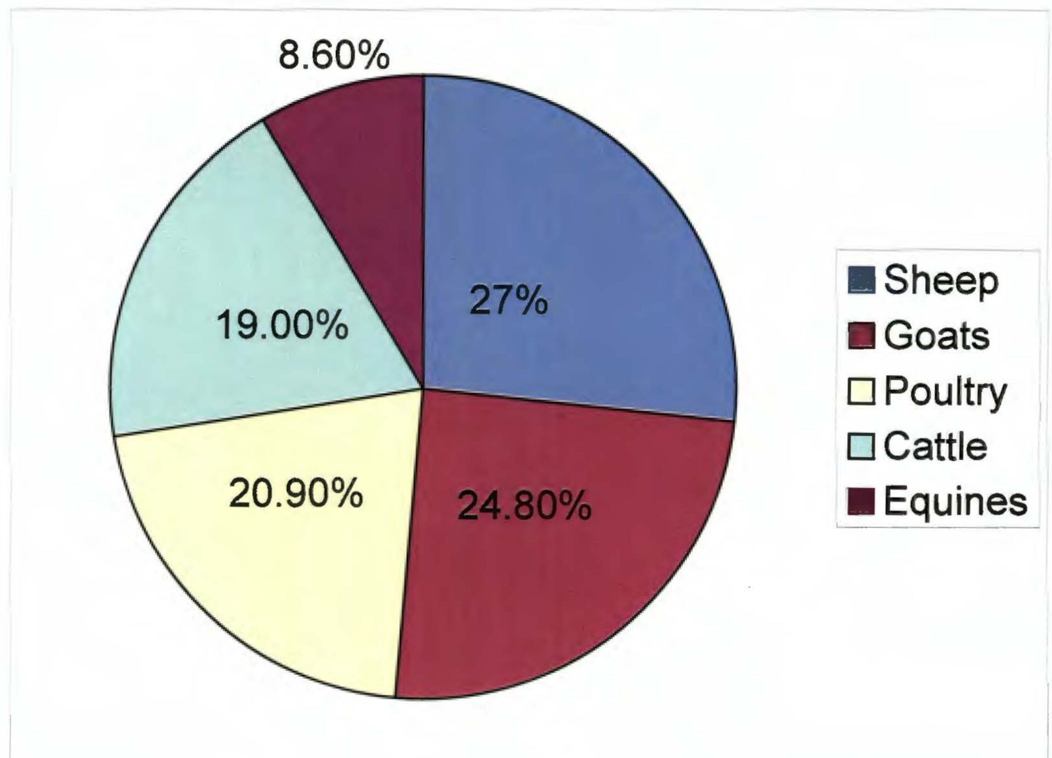


Figure 5: Average composition of animals owned by farmers

On most farms, horses, cattle, sheep and goats grazed together during the day. About 74% of the animals at all the study sites were released into the veld during the day and locked up in kraals at night whilst 26% were kept in the veld both day and night.

4.6.3 Perception of farmers on equine babesiosis

In the three study sites, livestock management was very poor due to lack of resources, lack information on tick and tick-borne diseases as well as other parasites infecting their livestock. In all of the sites studied, 100% of the farmers did not know about *B. (Theiliria) equi*, what causes it and how can it be prevented. After farmers had acquired information about the disease, 81% of them regarded equine babesiosis as a threat to their animals and were willing to get their animals tested. The remaining 19% thought the disease was not that important since most of them are interested in meat producing animals.

CHAPTER 5

5.1 DISCUSSION AND CONCLUSION

The parasites detected by microscopic analysis of smears in this study were only observed in thin blood smears from samples collected in Kaal plaas farm, OVI. Definitive diagnosis was by the demonstration of the parasites in peripheral blood smears. However, *B. caballi* is extremely difficult to detect in blood smears at any stage of the disease except the early acute phase of infection (Frerichs *et al.*, 1969). Small and round purple structures observed within red blood cells of the three horses were deduced to be *B. (Theileria) equi* trophozoites. All seven horses from Kaal plaas farm showed no signs of illness and their physical condition was scored as fair. Horses examined in the northeastern Free State showed no sign of illness except for one horse that was later reported dead. The cause of death was not identified.

In this study, diagnosis by blood smears, IFAT and PCR were performed three times at different time intervals and no parasites were detected in thin and thick smears. Beaver *et al.*, (1984) reported that thin blood films are useful for studying morphological changes of blood cells and blood parasites. However, the main disadvantage is that the sample volume is small, making the detection of low parasitaemia and carrier animals difficult (Ambrosio and De Waal, 1990).

Many immunodiagnostic tests have been developed for the detection of antibodies to *Babesia* species such as complement fixation test, IFAT and enzyme-linked immunosorbent assay. However, for the purpose of this study, IFAT was chosen because it has been the most widely used diagnostic test of all *Babesia* species and it is well suited to low technology laboratories. For all blood, samples collected both in the northeastern Free State and Kaal plaas farm, the IFA test was conducted at the Onderstepoort Veterinary Institute, Pretoria. Ideally, serology should allow

differentiation between recent and latent infections, and should be able to detect whether an animal is a carrier or not.

The present study shows wide prevalence of antibodies in horse blood obtained in the northeastern Free State. The drawback of serology in this case is that the demonstration of specific antibodies does not correlate with the present parasitological status of the host. The results of a serological test are therefore retrospective. Serological tests commonly used are based on the reaction of antibodies with antigenic parasite components (whole or soluble) (Ambrosio and De Waal, 1990) resulting in antigen-antibody complexes that are detected by the addition of antiglobulins coupled to fluorescent. Serological testing is not suitable for routine diagnosis because detectable antibody activity occurs after the development of parasitaemia (Frerichs *et al.*, 1969). To add to that, the serological status of a horse may not accurately reflect the current status of a *Babesia* infection in the animal and whether the horse would be a reservoir of infection for tick vectors (Frerichs *et al.*, 1969).

Genomic DNA extraction was prepared using the protocol brought by Professor Mbati from Brazil. Modifications were made to volumes of reagents and samples to cut down their excessive use. Stored and freshly obtained blood samples were robustly amplified and DNA was recovered from both. These was an indication of the validity of the DNA extraction technique and the stability of piroplasmal 16S rRNA in EDTA blood samples. The quality of extracted DNA was validated by the presence of a solid single band after agarose gel electrophoresis observed under ultraviolet light as described by Sambrook *et al.*, (1989). The primer set chosen for the PCR system described in this study was designed to separately amplify the 16S rRNA as *B.(Theileria) equi* exclusively; it was adapted from Bashiruddin *et al.*, (1999). The PCR conditions for this study were optimized until they were shown to be specific for the target DNA. The use

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the specific detection of *B. (Theileria) equi* is a new system that has not been reported in the northeastern Free State and comparison between standard detection techniques and PCR in the diagnosis of equine babesiosis is not yet available.

PCR has been shown to be a sensitive, specific and useful diagnostic tool (Böse *et al.*, 1995). A primary PCR, as applied in this study, has been shown to detect *B.(Theileria) equi* infection in horses; however, in the case of the three horses from Kaal plaas farm, the parasitaemia level was sufficiently high for the organism to be detected by microscopic examination (Bashiruddin *et al.*, 1999). In the present study, blood samples from 99 horses comprising of one apparently sick and 98 apparently healthy animals screened for the presence of *B. (Theileria) equi* by PCR were found to be negative. Although repeated DNA extractions and amplifications were performed, only the positive control amplified. Giemsa-stained blood smears were shown to identify *B. (Theileria) equi* in mostly clinically ill animals while PCR detected the organism in both ill and carrier healthy horses from Kaal plaas farm.

Although equine babesiosis is widespread in South Africa, very little is known about its epidemiology. Both *B. (Theileria) equi* and *B. caballi* occur in South Africa but *B. (Theileria) equi* is thought to be more widespread (Henning, 1949; Littlejohn, 1963) and is more commonly diagnosed in clinical cases (De Waal and Potgieter, 1987). Therefore for the purpose of this study *B. (Theileria) equi* was chosen as the target species for DNA amplification and detection. Results indicated that PCR with *B. (Theileria) equi* specific primers was more sensitive than microscopic analysis. As would have been expected (Rampersad *et al.*, 2003), microscopic analysis was only capable of detecting the presence of *Babesia* parasites in clinically sick animals while PCR amplified *Babesia* DNA in healthy horses. These results are in agreement with the relative sensitivity reported for these tests (Nicolaiewsky *et al.*, 2001; Bashiruddin *et al.*, 1999).

All samples obtained in the northeastern Free State were negative. These findings were not supportive because of the occurrence of the vector tick, *Rhipicephalus e. evertsi* (Potgieter *et al.,* 1992) in this area. Horses in this area belong to resource poor farmers are not well groomed and graze together with other animals such as cattle, sheep and goats which may act as second possible hosts for *Rhipicephalus e. evertsi*; and they are therefore susceptible to infection. Clinical condition of the horses was noted and of all 99 horses that were negative for *B. (Theileria) equi* by blood smears and PCR, one horse's condition was poor showing signs of sickness but tested negative by both microscopy and PCR. It was deduced then that the animal condition was not related to *B. (Theileria) equi*. Additional tests with experimentally infected horses and surveys of animals in endemic areas would be necessary for the calculation of diagnostic sensitivity of these systems.

Questionnaire results indicated that 100% of resource-limited farmers did not know what *B. (Theileria) equi* was, what causes it, what its symptoms were, how it is diagnosed and how it is treated. However, when asked about tick control, a common inexpensive method of de-ticking by those who own a relatively small number of livestock included burning of the veld in winter and dipping by those who owe larger numbers of animals. Of the 21 farmers issued with the questionnaires, 81% were interested in their horses being tested not only for *B. (Theileria) equi* but by other diseases as well. The main concern was that most of the farmers in these three study sites keep different animal species in the same yard thus allowing them to graze together during the day and locking them separately at night. Forty eight percent of farmers admitted observing symptoms such as nasal discharge where affected animals appear to suffocate from accumulation of mucous with occasional sneezes.

Serological testing is not routinely used for diagnosis because detectable antibody activity occurs after the development of parasitaemia (Frerichs *et al.*, 1969). Therefore the serological status of a horse may not accurately reflect the current status of a *Babesia* infection and whether the horse could be a reservoir of infection for tick vectors (Frerichs *et al.*, 1969; Friedhoff, 1982). The relatively large number of negative samples from the northeastern Free State was surprising considering the fact that the vector tick *Rhipicephalus e. evertsi* occurs in this area. It is therefore important in the future to also screen tick vectors for the presence of *B. (Theileria) equi*.

The absence of *B. (Theileria) equi* parasites from both thick and thin blood smear was probably due to very low parasitaemia from animals whose physical condition was also generally described as being fair. However, microscopy, though not so sensitive and more time consuming, is still necessary because of its excellent demonstration of morphological details of parasites. Böse *et al.*, (1995) verifies that this is currently the only technique that allows species identification, although specialist training may be needed to differentiate between species that appear to be morphologically similar.

The seven horses from the Kaal plaas farm were all previously known to have been infected with *B. (Theileria) equi*, and were all PCR positive for infection. The northeastern Free State area was shown to be highly endemic for both *Babesia* species judging from the prevalence of *B. caballi* and *B. (Theileria) equine* antibodies that ranged from 46.2% to 97.4%, respectively as determined by IFAT. Results from the negative Giemsa-stained blood smears, the high incidence of positive serological results, presence of the tick vector and no clinical symptoms from the northeastern Free State, gives the conclusion that the area is endemically stable for *B. (Theileria) equi* and *B. caballi*.

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APPENDICES

APPENDIX 1

1 Key to abbreviations

- Gametocytes (Gm)
- Kinete (Ki)
- Merozoites (Mz)
- Ray bodies (Rb)
- Sporoblast (Sb)
- Sporozoite (Sp)
- Trophozoite (Tr)
- Zygote (Zg)

2 Preparation of blood smears

2.1 10% Giemsa Stain

- 90 ml Giemsa Buffer mixed with 10 ml Giemsa Stain

2.2 Giemsa Buffer

- $Na_2HPO_4.2H_2O$ 104.41 g
- $NaH_2PO_4.2H_2O$ 19.2 g
- D-H_2O 1000.00 ml
- 518 C immersion Oil (Zeiss Germany)

3 Calculation of Parasitaemia

- 800 RBC/1 field of view = 800
- 800 RBC/8 fields of view = 6400
- Parasitaemia (%) = No. Of infected RBC/ No. Of total RBC X 100

4 Genomic DNA extraction

Materials

4.1 Chemicals

- Phenol chloroform Isoamyl alcohol (25: 24: 1)
- Lauryl sulphate SDS
- 100 bp standard size DNA markers
- Agarose powder
- Magnesium chloride (50 mM)
- Ethidium bromide solution
- Proteinase K
- Bromophenol blue
- Glycerol
- Tris – molecular weight 121014g/mol
- EDTA – disidium salt, dihydrate molecular weight 372.24
- Sarcosine
- Absolute ethanol
- Sigma Taq ploymerase
- 10 X PCR buffer
- *B. (Theileria) equi* primers
- dNTPs
- Acetic Acid glacial

4.2 Plastic ware

- Plastic holders f or 1.5 – 2 ml, 0.1 – 0.2 ml PCR tubes
- 1.5 and 2 ml PCR centrifuge tubes
- 200 µl PCR reaction tubes
- Pipette tips of various sizes ranging from 10µl – 1000 µl

4.3 Apparatus

- Vortex-Gene2®
- Eppendorf Centrifuge 5415R
- Fridge freezer
- Ice maker (fine ice)
- Eppendorf Mastercycler® Gradient
- Eppendorf thermostat plus incubator
- Agarose gel casting tray

5 Preparation of chemical reagents

- **1 M Tris**

Molecular weight:	121.11 g/mol
1 L of a 1 M solution:	121.11 g dissolved in 1 l water
50 ml of a 1 M Tris solution:	121.11 g /1 l = 1 M Tris solution
	Therefore x g / 50 ml = 121.11 g / 1000 ml
	x g = 6.055
	Therefore to prepare 50 ml of 1 M Tris, 6.055 g of Tris powder was dissolved in 50 ml of water

- **0.5 M EDTA, pH 8.0**

Molecular weight:	372.24 g/mol
1 l of a 1 M EDTA solution:	372. 24 g dissolved in 1 l water
1 l of a 5 M EDTA solution:	186. 12 g dissolved in 800 ml distilled water, solubilized and adjust the pH by adding NaOH pellets, and then filled up to 1000 ml

- **TE (Tris/ EDTA) buffer, pH 7.4**

There are two stock solutions, namely 1 M Tris and 0.5 M EDTA. To prepare a solution consisting of 10 mM Tris pH 8 and 1 mM EDTA pH 8.

Prepare a 50 ml solution containing the two solutions as follows:

VC = VC

= 50 ml * 10 mM Tris / 1 M Tris

= 0.5 ml of the 1 M Tris solution

VC = VC

= 50 ml * 1 mM EDTA / 0.5 M EDTA

= 0.1 ml of the EDTA solution

Take 0.5 ml of the Tris and 0.1 ml of the EDTA, put in a volumetric flask, make up to 50 ml, transfer to a Schott bottle, label with the concentration, pH and date of preparation.

5. Preparation of buffers

5.1 Extraction buffer 400 µl

10% SDS solution	40 µl (final concentration is 1%)
10 % Sarcosine	40 µl (final concentration is 1%)
Proteinase K	8 µl (final concentration is 0.2 mg)
Water	312 µl

5.2 70% Ethanol

	50 ml	100 ml
Ethanol (absolute)	35 ml	70 ml
PCR water	15 ml	30 ml

5.3 50 X TAE buffer

Tris base:	242 g
Acetic acid, Glacial:	57.1 ml
EDTA (0.5 M, pH 8.0):	100 ml
ddH_2O (autoclaved):	add to the 1000 ml mark

5.4 DNA extraction buffer for one sample

dH_2O:	312 µl
10% SDS:	40 µl
10% Sarcosine:	40 µl
Proteinase K:	8 µl
Total:	**400 µl**

Appendix 2: IFAT test sheet for *B. (Theileria) equi* and *B. caballi*

Sheet 1. ***B.*** *(Theileria)* ***equi***

REFERENCE NO.:	DATE TESTED:	OWNER:	TESTED BY:	ANIMAL SPECIES: Equines
Incubation time:	Wash:	Conjugate incubation:	Wash:	Time read:

SAMPLE NO.	ANIMAL NO.	TEST: IFAT				
		Antigen species: *B. (Theileria) equi*				
		Dilution:	1/40	1/80	1/160	1/320
+						
-						
1.						
2.						
3.						
4.						
5.						
6.						
7.						
8.						

Sheet 2. ***B. caballi***

REFERENCE NO.:	DATE TESTED:	OWNER:	TESTED BY:	ANIMAL SPECIES: Equines
Incubation time:	Wash:	Conjugate incubation:	Wash:	Time read:

SAMPLE NO.	ANIMAL NO.	TEST: IFAT				
		Antigen species: *Babesia caballi*				
		Dilution:	1/40	1/80	1/160	1/320
+						
-						
1.						
2.						
3.						
4.						
5.						
6.						
7.						
8.						

APPENDIX 3: PCR-reaction test sheet

Name:

Date:

Aim: Test amplification of *B.(Theileria) equi* DNA

Template: Purified DNA from horsre blood

Controls: Negative control (Water)

Positive control (Purified DNA from Babesia)

Primers: BeqF and BeqR

Taq-pol: Sigma Taq DNA polymerase

Number of reactions: 7

Reaction nr	1	2	3	4	5	6	7	8	Stock	Final
Name of reaction	4k1	4k2	4k4	4k5	4k6	4k7	Pos C	Neg C	Concentration	Concentration
DNA Template	5	5	5	5	5	5	5	0		10 ng
Buffer	5	5	5	5	5	5	5	5	10x	1x
Primer 1 (BeqF)	5	5	5	5	5	5	5	5	10 micro mol	1 micro mol
Primer 2 (BeqR)	5	5	5	5	5	5	5	5	10 micro mol	1 micro mol
dNTPs	1	1	1	1	1	1	1	1	10 mM	0.2 mM
Taq-pol	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	5 u/µl	1.25 units
Water	28.75	28.75	28.75	28.75	28.75	28.75	28.75	33.75		
	50	50	50	50	50	50	50	50		

Reaction conditions:	94oC for 5 min	1x
	94oC for 30 sec	30x
	64oC for 30 sec	30x
	72oC for 30 sec	30x
	72oC for 5 min	1x

Appendix 4: Sample of a questionnaire

A. Personal particulars of the farmer:

1. PERSONAL DETAILS (Mark with an X where applicable)

Surname ______________________________

First names ______________________________

Physical Address ______________________________

____

Population Group ______________________________

Dependents: Adults

Children ________________

____

Employment status: Pensioner ________________

Employed ________________

Unemployed ________________

B. Animal Health (Mark with an X where applicable)

1. Do you know what *B. (Theileria) equi* is? Yes: ________

No: ________

2. Do you know what causes it? Yes: ________

No: ________

3. Can you Identify symptoms of *B. (Theileria) equi?* Yes: ________

No: ________

4. Do you know how it is diagnosed? Yes: ________

No: ________

5. Do you know how it is treated? Yes: ________

No: ________

6. Have your horses tested for *B. (Theileria) equi* before? Yes: ________ No: ________

7. Would you like your animals to be tested for *B. (Theileria) equi*? Yes: ________ No: ________

8. How often would you like them to be tested?
Seasonally ________
Twice a year ________
Once a year ________

9. Will you be willing to pay for diagnostic services? Yes: ________ No: ________

10. How much would you pay per animal ________

11. How do you treat your animals when they are sick?
Call for professional help ________
Prepare traditional medicine ________
Don't know what to do ________

12. Do you know of any other parasites inflicting your animals Yes: ________ No: ________

13. If the answer above is "yes", name them:
1 ________
2 ________
3 ________

14. How do you treat the diseases in "13"?
____
____
____

C. Animal composition

1. Animal species owned

1.1 Number of animals owned

a. Horses ________

b. Donkeys ________

c. Cattle ________

d. Sheep ________

e. Goats ________

f. Poultry ________

1.2 What do you feed your animals? ____________________________

1.3 Where do you keep your animals? ____________________________

1.4 Are the animals listed in "1.1" kept together? ____________________________

D. Parasites

1. Do you observe any ticks on your animals? Yes: ________

   No: ________

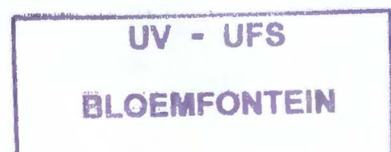
2. If the answer above is "yes", how often? ____________________________

3. During which season are they at the highest? ____________________________

E. Parasite Control

1. What do you use to control tick infestation? ____________________________

2. How often do you teat your animals for tick infestation? __________________


UV - UFS
BLOEMFONTEIN

This survey was prepared for a MSc study conducted by Ms.M. Y. Motloang under the supervisionof Prof. P. A.Mbati,

Parasitology Research Program, University of the Free State Qwa Qwa Campus