SEROLOGY, MOLECULAR EPIDEMIOLOGY AND STRAIN DIVERSITY OF EQUINE PIROPLASMS IN SOUTH AFRICA

By

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DECLARATION

I, Tshoanelo Portia Moloi, declare that the thesis hereby submitted for the Master of Science degree at University of the Free State is an original work under the supervision of Dr. Michael Cunningham. The thesis has not been submitted in any form to another University. I therefore cede copyright of this dissertation infavour of the University of the Free State.

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ABSTRACT

Equine piroplasmosis is a protozoan disease of horses caused by two parasites, *Babesia caballi* and *Theileria equi*. Both parasites are transmitted by ixodid ticks belonging to the genera *Boophilus*, *Hyalomma*, *Dermacentor* and *Rhipicephalus*. Equine piroplasmosis has a worldwide distribution and is endemic in tropical and sub-tropical regions, including Central and South America, Africa, Asia and Southern Europe. The economic impact of equine piroplasmosis in South Africa is assumed to be millions of Rands due to a combination of direct losses, convalescence period and incidental costs such as vaccinations, treatment and veterinary fees. There is little information on parasite strains in South Africa. The objectives of this study were to determine (i) the prevalence of equine piroplasmosis in Free State (FS) and KwaZulu Natal (KZN) of South Africa, using molecular and serological techniques, and (ii) strain variation of equine piroplasmosis parasites, namely, *B. caballi* and *T. equi*, using 18S rRNA DNA sequences analysis. Diagnostic methods used in this study include microscopy (thin blood smears), Polymerase Chain Reaction (PCR), Indirect Fluorescent Antibody Test (IFAT) and Enzyme-Linked Immunosorbent Assay (ELISA). Blood samples were collected from a total of 534 horses in the Free State and KwaZulu-Natal (444 were collected from FS and 90 from KZN).

No *B. caballi* was detected from all samples collected from both provinces (FS and KZN) by microscopy and PCR. Of 507 serum samples tested for *B. caballi* by IFAT, a sero-prevalence of 61% was detected. A mean value of 34% of samples was positive for *T. equi* using a PCR test and sero-prevalence of 94% was detected by IFAT. Fifty two

percent of the 250 tested sera samples were positive for *T. equi* by ELISA. Together these results suggest high levels of exposure to parasites, high levels of current infections and uncertainty in current serological tests for these parasites. Sequencing and phylogenetic analysis of the 18S rRNA gene showed considerable diversity of *T. equi* strains in South Africa.

T. equi is highly prevalent in South Africa with the parasite appearing to be more prevalent in KZN than FS. This study also confirmed the distribution of this disease as described in previous studies, and the disease was found also in the area which previously was declared disease-free. There is considerable variation in *T. equi* genotypes in the country and no clear phylogeographic structuring of these genotypes. These results may indicate that there is much movement of infected carrier horses within the country and even to and from other countries. *B. caballi* prevalence is still not clear as only IFAT seems to detect antibodies to infection by this parasite. There is a need for the development of highly sensitive assays for the detection of *B. caballi*, thereby enabling determination of prevalence and strain diversity studies of this parasite.

OPSOMMING

Equine piroplamosis is ‘n perde siekte wat veroorsaak word deur twee eensellige parasiete, *Babesia caballi* en *Theileria equi*. Alby parasiete word oorgedra deur ixodied bosluise, wat behoort aan die genera *Boophilus, Hyalomma, Dermacentor* en *Rhipicephalus*. Equine piroplasmosis het ‘n wêreld wye distribusie en is endemies tot die tropiese en sub-tropiese gebiede, insluitend sentraal en suiderlike Amerika, Afrika, Asia en suiderlike Europa. Die ekonomiese impak van equine piroplasmosis in Suid Afrika word geskat om miljoene rande te beslaan, weens direkte verlies, lang herstel periodes en addisionele onkostes vir inenting, behandelings en veeartse. Daar is beperkte informasie beskikbaar oor die parasiet variante verantwoordelik vir equine piroplasmosis in Suid Afrika. Die doel van die studie was om (i) te bepaal die digtheid van voorkoms van equine piroplasmosis vir die Vrystaat (VS) en KwaZulu Natal (KZN) omgewings in Suid Afrika deur gebruik te maak van molekulêre en serologiese tegnieke, en (ii) om variasie te toets in die parasiete varieteit van *T. equi* en *B. caballi*. Diagnostiese metodes waarvan gebruik gemaak was sluit in mikroskopie (din bloed smere), Polimerase Ketting Reaksies (PKR), Indirekte Floreserende Teenliggaams Toets (IFTT) en Ensiem-Koppeling Immunoabsorbeurende Analiese (EKIA). Bloed monsters was ingesamel vir ‘n totaal van 534 perde van die VS en KZN omgewing (444 vir die VS en 90 vir KZN).

Geen *B. caballi* was waargeneem vir die monsters ingesamel van beide provinsies deur die mikroskopie en PKR toetse nie. Van die 507 bloed monsters getoets vir *B. caballi* deur gebruik te maak van IFTT, ‘n serum-digtheid van 61% was verkry. ‘n Gemiddelde van 34% monsters het positief getoets vir *T. equi* tydens die PKR toets, terwyld die IFTT

toets ‘n serum-digtheid van 94% getoon het. Twee en vyftig persent van die serum toetse het positief getoets vir T. Equi tydens die EKIA toets. Gesamentlik toon die resultate hoë vlakke van blootstelling tot *T.equi*, hoë vlakke van huidige infeksie en onsekerheid oor huidige serologiese toetse vir *T.equi*. Basis volgorde bepaling en filogenetiese analisis van die 18S rRNA geen toon merkwaardige diversiteit van *T. equi* variente vir Suid Afrika. *T. equi* toon ‘n hoë digtheid in Suid Afrika, met KZN digter as VS.

Die studie verder bevestig die distribusie van equine piroplamosis soos beskryf deur vorige studies en dat equine piroplamosis verder versprei het na vorige ongeafekteerde areas. Daar is ook gevind dat daar groot variasie in *T. equi* genotipes is in die land en geen duidelike filogeografiese grense beskik vir die genotipes nie. Dit mag aanduiding gee dat daar baie beweging van geinfekteerde perde is binne die land, en selfs moontlik na en van ander lande. *B. caballi* digtheid is onseker weens die rede dat die IFTT toets slegs aanduiding gegee het van infestasie deur die parasiet. Laastens is daar ‘n nodigheid vir die ontwikkeling van ‘n meer sensitiewe toets vir die waarneeming van *B. caballi* teenwoordigheid, wat sal lei tot die digtheid van voorkoms en variasie toetsing tussen die variente van die *B. caballi.*

CHAPTER ONE

1. INTRODUCTION AND LITERATURE REVIEW

1.1 Preamble

The first description of an equine disease referred to as 'biliary fever' was made by Hutcheon (1895) the colonial veterinary surgeon of the Cape Colony, South Africa. Later, Theiler (1901) observed intra-erythrocytic parasites in blood samples, which he attributed to equine malaria, which were later recognized by Laveran (1901) as intra-erythrocytic piroplasms. Subsequently, the cause of equine piroplasmosis was found to be two parasites, *Piroplasma equi* (now *Theileria equi*, Mehlhorn and Schein, 1998) and *Piroplasma caballi* (now *Babesia caballi*,Nuttall and Strickland, 1912).

In equine blood smears, *T. equi* trophozoites appear as round, elliptical, or spindle-shaped basophilic structures. These organisms have a small erythrocytic stage reaching only 1.5-2.5 µm with the merozoite stage appearing as two or four pyriform parasites. The trophozoites of *B. caballi* appear as round, oval or elliptical basophilic structures with the erythrocytic stage reaching 3 to 6 µm. The organisms occur in pairs forming an acute angle which are commonly found in a single erythrocyte (Edwards *et al*., 2005).

Both *T. equi* and *B. caballi* are transmitted by ixodid ticks belonging to the genera *Boophilus*, *Hyalomma*, *Dermacentor* and *Rhipicephalus*. Clinical manifestations of the disease caused by *B. caballi* are characterized by the destruction of parasitized erythrocytes and include fever, depression, anorexia and swelling of eyelids (Ogunremi *et*

al., 2008). *T. equi* infection is manifested by the swelling of the lymph nodes, anorexia, mucous discharge from eyes and nostrils, diarrhoea and dyspnea. The disease can be acute, chronic or sub-clinical, where the infected animal can aid in the transmission of the parasites (Rampersad *et al*., 2003). The severity of clinical response in individual horses is variable and in many cases spontaneous recovery may occur without showing hemoglobinuria or anemia (Vial and Gorenflot, 2006).

The morbidity and mortality rates of equine piroplasmosis are high (Ogunremi *et al*., 2007). Generally the mortality rate depends upon virulence of the organisms and the general immune status of the affected animals. A high mortality rate is expected in susceptible horses from disease-free areas introduced into endemic regions (Chahan *et al.,* 2006).

B. caballi and *T. equi* co-exist in geographical distributions in association with the presence of vector ticks. In such areas, an individual horse may be infected by both species (Huang *et al*., 2006). In South Africa, it was found that the number of cases of piroplasmosis treated animals exceeded that of every other infectious disease of horses, including infectious respiratory diseases and African horse sickness (Potgieter *et al*., 1992). Equine piroplasmosis is an important cause of wastage and economic losses especially in race-horses in South Africa (de Waal and van Heerden, 2004).

1.2 Classification

Piroplasms are protozoal parasites characterized by intra-erythrocytic forms of different shapes depending on the parasite species. They have apical complex organelles, a merozoite stage within the vertebrate host erythrocytes and sexual development with sporozoite formation within the tick vectors. There are two families within the order Piroplasmida (Eucoccidiorida), which are Babesiidae and Theileriidae; the primary distinction between them is usually defined as the absence of a schizogony cycle in *Babesia* and the absence of trans-ovarial transmission in *Theileria* (Homer *et al*., 2000).

The following taxonomic classification of equine piroplasms is obtained from de Waal and van Heerden (2004):

Class : Sporozoasida

Subclass : Coccidiasina

Order : Eucoccidiorida

Suborder : Piroplasmorina

Families : Theileriidae and Babesiidae

Genera : *Theileria* and *Babesia*

Babesia equi, Laveran, 1901 was transferred to *Theileria equi*, Mehlhorn and Schein, 1998, and thus transferred from one genus to another. This parasite shows characteristics of *Theileria*, which are:

- It has a pre-erythrocytic development within lymphatic cells (schizogony) of their hosts, this schizogony is lacking in *Babesia* species.

- The absence of transovarial transmission, where infection passes through the ovary and the egg to the next tick generation, which occurs in other *Babesia* species.
- *T. equi* has small erythrocytic stages reaching only 1.5-2.5 μm in length instead of the 3-6 μm long erythrocytic stages of *Babesia* species.
- The formation and shape of sexual stages of *T. equi* in the tick vector differ from those of typical *Babesia* species.
- Surface proteins of *T. equi* show more identity to those of *Theileria* species.

When examining *T. equi* and comparing its biological, morphological and developmental features, and genetic relationships with other members of the piroplasms, the transfer into the genus *Theileria* seems most reasonable (Mehlhorn and Schein, 1998).

1.3 Phylogenetic relationships

Classification of piroplasmids has largely relied on morphological and biological observations. However, the use of these techniques does not always result in exact assignation of some isolates to a definite species from this group. Recently, advances in methodologies such as automated DNA sequencing has made it possible to ascertain the phylogenetic relationships of species from their genes (Criado-Fornelio *et al.*, 2003). The 18S ribosomal RNA (18S rRNA) gene is commonly used to establish phylogenetic inferences and diagnoses, since its mutation rate has been shown to be slow (Kawamoto *et al*., 1996).

There are two families considered, Babesiidae and Theileriidae, although Allsopp *et al*. (1994) erected a new family, Nicollidae, and proposed to group *B. equi*, *Cytauxzoon felis* and *B. rodhaini* in this family. Other authors have found more than two groups after analyzing the 18S rRNA sequences of several isolates of *Babesia* and *Theileria*. For example, Zahler *et al*. (2000) proposed three groups of piroplasmids: Theilerids, Babesids and *B. microti*. The third group was assumed to belong to a separate genus, for which the name *Nicollia* was proposed. Although Zahler *et al*. (2000) supported the proposal for a new family by Allsopp *et al*. (1994), they differed, suggesting that the species *Cytauxzoon felis* and *B. equi* should belong to the Theilerids (Zahler *et al*., 2000). Penzhorn *et al*. (2001) described four groups; including a new cluster of *Babesia* spp. isolates from the West Coast of USA. Criado-Fornelio *et al*. (2003) proposed five groups; with a new group composed of only *Babesia* species from ungulates, which are *B. bovis*, *B. ovis*, *B. bigemina* and *B. caballi*. The status of those proposed groups has not been established yet, but they might be regarded as new piroplasmid families (Criado-Fornelio *et al*., 2003).

1.4 Distribution and transmission

Equine piroplasmosis is endemic in tropical and sub-tropical regions, including Central and South America, Africa, Asia and Southern Europe (Schein, 1988). *B. caballi* infection has recently been found in southern and eastern Europe, Asia, Africa, Middle East, Cuba, South and Central America as well as certain parts of the southern United States (Ogunremi *et al*., 2008). The prevalence of *T. equi* is high in tropical and subtropical regions of Africa; in South, Middle, and North America; in Asia; in countries

deriving from the former Soviet Union; and in all coastal countries of the Mediterranean. Thus it might be introduced into most countries worldwide (Mehlhorn and Schein, 1998).

In South Africa, equine piroplasmosis is widespread throughout the country and its distribution corresponds to the distribution of the vector (Zweygarth *et al*., 2002b). Animals which are low-level carriers, or which may act as reservoirs, pose a risk of introduction of these parasites to disease-free areas as a result of the increased movement of horses worldwide (Bashiruddin *et al*., 1999).

Ticks are the only known vectors for equine piroplasmosis. They represent a major risk of infections and are recognized as one of the most economically significant parasites threatening livestock production throughout the world. Ticks belonging to the genera *Boophilus, Hyalomma, Dermacentor* and *Rhipicephalus* transmit the disease worldwide (Nagore *et al*., 2004). *Rhipicephalus evertsi evertsi,* a two-host tick, is the only confirmed vector of *T. equi* in South Africa. It occurs throughout Limpopo, North West, Gauteng, Mpumalanga, KwaZulu-Natal, the northern part of the Free State, and along the coast of the Eastern Cape and Western Cape Provinces. Transmission occurs transstadially, i.e. passage from one stage of the life cycle to another, and therefore, in this case adults can transmit the disease (de Waal and van Heerden, 2004).

Two tick species have been confirmed to transmit *B. caballi* in South Africa, namely *R. e. evertsi* and *Hyalomma truncatum* (Potgieter *et al*., 1992). Transmission by *H. truncatum* occurs transovarially, i.e. infection passes through the ovary and the egg to the larvae and

transstadially by *R. e. evertsi. H. truncatum* is present throughout the western and northern parts of South Africa (de Waal and van Heerden, 2004). As a result of trans-ovarial transmission, the tick can become an important reservoir for *B. caballi* (Mehlhorn and Schein, 1998).

T. equi can undergo transplacental transmission in the mammalian host. There is no reliable evidence that *B. caballi* can pass from mare to foal through the placenta. Intra-uterine infection of the foal is fairly common, with *T. equi.* Equine piroplasmosis can also be transmitted by contaminated needles and syringes (Allsopp *et al*., 2007). After recovery, horses may become carriers for long periods of time. The incubation period for *B. caballi* is 10-30 days and 12 to 19 days for *T. equi* (de Waal and van Heerden, 2004).

1.5 Clinical signs

Clinical signs of equine piroplasmosis are often non-specific and can be misdiagnosed with other equine infections. *T. equi* infections are more severe and recovered animals become lifelong carriers. *B. caballi* causes a less severe disease (Irby, 2002). Most clinical cases of equine piroplasmosis in southern Africa are caused by *T. equi.* The disease can occur as per-acute, acute, sub-acute and chronic forms. The per-acute form of the disease where horses are found either dead or dying is rare. In the acute forms, signs include fever, usually exceeding 40ºC, varying degrees of anorexia and malaise, and elevated respiratory and pulse rate (de Waal and van Heerden, 2004). The signs in horses with chronic infections usually are not specific.

In general, clinical signs alone are often non-specific and the disease may be confused with a variety of other diseases such as equine influenza, encephalitis virus infections, equine infectious anemia and trypanosomiasis.

1.6 Diagnosis

Diagnosis of equine piroplasmosis can be made on the basis of typical clinical signs, evidence of exposure to infected ticks or history of blood transfusion. Examination of blood smears, serology and molecular techniques are all used in making the diagnosis of equine piroplasmosis. Clinical signs alone can not be used to differentiate between disease caused by *T. equi* and *B. caballi* infections (de Waal, 1992, de Waal and van Heerden 2004).

1.6.1 Identification of the piroplasms

Definitive diagnosis depends on the identification of the parasite in thin or thick blood smears stained with Giemsa or acridine orange using light microscopes (Nagore *et al*., 2004).

Direct parasitological verification of chronic *B. caballi* infection is almost impossible, but is occasionally successful with *T. equi*. In general, chronic or in-apparent infections can only be confirmed after transfusion of approximately 500 ml of blood into susceptible animals. The parasites are best demonstrated by staining blood smears with 10% Giemsa solution (Ali *et al*., 1996).

The carrier state in equine piroplasmosis commonly presents a special problem of diagnosis since no outward signs of the disease are evident (Knowles, 1988). Zweygarth *et al.* (2002a) used an *in vitro* tissue culture technique to isolate *T equi* parasites from Mountain zebra to demonstrate their carrier state. This technique was found to be sensitive and successfully confirmed the carrier animals.

In per-acute, acute and sub-acute infections it is often difficult to detect the parasites in thin smears due to the low level of parasitaemia, however, examination of thick blood smears may be a useful addition.

1.6.2 Serological diagnosis

Various serodiagnostic tests have been developed for the disease, such as the Complement Fixation Test (CFT), Indirect Fluorescent Antibody Test (IFAT) and Enzyme Linked Immunosorbent Assay (ELISA) (Brüning, 1996).

During the past decades, many attempts have been made to standardize serological diagnostic procedure of equine piroplasmosis (Todorovic, 1975). Various serological techniques have been used to identify infected animals. Most conventional immunodiagnostic methods are used for the measurement of the humoral response following natural or experimental disease. Each serological assay has some advantages and/or disadvantages depending on its level of sensitivity, specificity, simplicity and cost effectiveness (Ali *et al.,* 1996).

In recent studies, most researchers proposed the combination of at least two different assays to increase the reliability of the sero-diagnosis of equine piroplasmosis (Weiland, 1986). The value of serological methods for the demonstration of antibodies is, however, limited by the impossibility of diagnosing prepatent infections, the persistence of titers after natural recovery or chemotherapy, and the difficulty in interpreting low grade and/or cut-off titer. The exact state of the infection may, therefore, pass unrecognized (Ali *et al.*, 1996).

1.6.2.1 Complement fixation test

The complement fixation test (CFT) was developed by Hirato *et al*. (1945), and in 1969 it was accepted as the official test for equine piroplasmosis by the American Department of Agriculture and since then is being used worldwide (Friedhoff, 1982). The CFT has been used to test horses to be moved from endemic areas to countries with suitable parasite vectors but where the disease does not occur (Ogunremi *et al*., 2007). However, CFT has low sensitivity during early and latent stages of the disease, therefore the serological status of a horse may not accurately reflect the carrier status. On the one hand, a long standing carrier may test CFT negative and if such animal is to be moved it places other susceptible animals at risk. On the other hand, importation restrictions based on false-positive horses results in economic liability for the owner (Sahagun-Ruiz *et al*., 1997).

During the last 10 years, the indirect fluorescent antibody test has been used in conjunction with CFT to obtain more reliable results, especially for import/export of horses (Ali *et al.*, 1996).

1.6.2.2 Indirect Fluorescent Antibody Test

The indirect fluorescent antibody test (IFAT) has been successfully applied in the differential diagnosis of *T. equi* and *B. caballi* infections (Madden and Holbrook, 1968). The test is considered specific and sensitive. The cutoff titer for the determination of positive results varies from laboratory to laboratory; some report titers above 1:64 to be diagnostic (Homer *et al*., 2000).

Although the IFAT is more sensitive than the CFT (e.g. Böse *et al*. (1999) reported sensitivity of 98% for the IFAT and 47% for CFT), and rarely renders false negative results, standardization is difficult considering the subjectivity of the reader in assessing results (Baldani *et al*., 2007). As for several IFA tests, the recognition of a strong positive reaction is relatively simple, but the differentiation between weak positive and negative reactions requires considerable experience in interpretation (Madden and Holbrook, 1968). Moreover, other researchers have encountered inherent problems of IFAT that include cross-reactivity and impracticability especially for testing large numbers of samples (Bakheit *et al*., 2007).

1.6.2.3 Enzyme-Linked Immunosorbent Assay

Serological cross-reaction between *T. equi* and *B. caballi* in the IFAT and false-negative or false-positive serological reactions in CF and IFA tests may be encountered (Bruning *et al*., 1997). To overcome the problems associated with cross-reaction and antigen purity, ELISA based on recombinant antigens and monoclonal antibodies have been developed for both *T. equi* and *B. caballi* (de Waal and van Heerden, 2004).

Recombinant antigens for use in ELISAs have been described for *T. equi* (EMA-1, EMA-2, Be82 and Be158) and *B. caballi* proteins (RAP-1, Bc48 and Bc134) and have been produced in *Escherichia coli* (Hirata *et al*., 2005) or in insect cells by baculovirus (Xuan *et al*., 2001). Recombinant antigens produced in *E. coli* or by baculovirus have the advantage of avoiding the need to infect horses for antigen production, and of eliminating the cross-reactivity that has previously been experienced with the crude ELISA antigens. They also provide a consistent source of antigen for international distribution and standardization (Anonymous, 2008).

Competitive-inhibition ELISA using recombinant antigens was also developed for the detection of *T. equi* (Knowles *et al*., 1992) and *B. caballi* (Kappmeyer *et al*., 1999) infections. Recently, an ELISA that could specifically detect anti-*B. caballi* equine antibodies by using a recombinant BC48 protein was developed. The ELISA using this recombinant antigen could clearly distinguish between *B. caballi*- and *T. equi*-infected horse sera (Tamaki *et al*., 2004).

1.6.3 Molecular diagnosis

Conventional techniques including serology and microscopy do not always meet the requirement for sensitive diagnosis. Low levels of parasitaemia are usually not detected by blood smears, while antibodies detected by serological assays usually appear later during infection, thus making serological tests more suitable for surveillance rather than the diagnosis of acute cases of equine piroplasmosis. Southern hybridization with DNA probes for the detection of *T. equi* and *B. caballi* in blood have been developed. Probes

designed to detect certain *T. equi* and *B. caballi* genes have been shown to detect parasitaemias of 0.0016% (Posnett and Ambrosio, 1989, 1991).

Polymerase chain reaction (PCR), which was introduced in 1985, has been used to find and identify micro-organisms in the environment, in samples of soil, sediments and water, and in the diagnosis of several micro-organisms. It can amplify a fragment of DNA in a sample over 200,000-fold and could result in even more sensitive probing assay. PCR tests routinely detect as little as 1 pg of DNA (Ali *et al*., 1996). In the diagnosis of equine piroplasmosis, the diagnostic sensitivity of PCR was proved by Ali *et al.* (1996), where they tested *T. equi* in naturally and experimentally infected horse blood. PCR detected 10 pg of purified *T. equi* template DNA in the reaction and could also amplify *T. equi* DNA in horse blood with 0.001% parasitaemia. *T. equi* DNA could be detected by nested PCR in blood samples with parasitemia as low as 0.000083% (Nicolaiewsky *et al*., 2001).

Used in conjunction with serology, PCR can facilitate accurate diagnosis of the disease and carrier animals, allowing more efficient control of equine piroplasmosis. This is a very useful technique especially where horses are destined for exportation as parasite DNA is directly detected rather than the indirect detection of antibodies as is the case of serology.

Recently more molecular assays have been introduced, including loop-mediated isothermal amplification of DNA (LAMP) which has provided another rapid, sensitive

and specific diagnostic method (Notomi *et al*., 2000). The sensitivity of the LAMP methods for *B. caballi* and *T. equi* has been proved to be high (Alhassan *et al*., 2006). A sensitive and specific reverse line blot (RLB) hybridization assay has also been developed for detection of *Theileria* and *Babesia*, including piroplasmosis in horses. This assay enables the identification of mixed infections and has also proven to be a valuable tool in the identification of novel piroplasm species or genotypes (Nagore *et al*., 2004, Bhoora *et al.,* 2009).

1.7 Genetic variations in equine piroplasmosis

The use of PCR based diagnosis as well as gene sequencing, has prompted not only the discovery of a striking level of genetic diversity in those parasites, but also the finding of unexpected new hosts for piroplasms such as *Babesia canis*, *Theileria annae* and *T. equi* (Criado-Fornelio *et al*., 2004).

Due to the lack of morphological features that can help to identify genetic variants or even cryptic species of parasites, the best strategy for definitive diagnosis is the use of molecular methods such as species diagnostic PCR or DNA sequencing. Such an approach was employed by Criado-Fornelio *et al*. (2003) in a piroplasm survey in Spain by sequencing the 18S rRNA gene of some of the most frequent species. DNA-based diagnosis of piroplasmosis commonly employs the 18S rRNA gene as an amplification target due to the availability of 18S rRNA sequences in molecular databases for comparison with newly obtained sequences (Criado-Fornelio *et al*., 2006).

2. OBJECTIVE OF THE STUDY

2.1 Rationale

Equine piroplasmosis, caused by *Babesia caballi* and *Theileria equi*, is an important protozoan disease world wide from both a veterinary and an economic view point (Huang *et al*., 2006). The parasites are endemic in most tropical and sub-tropical areas of the world and also occur in some temperate areas. Due to the world wide distribution of the various tick vectors the introduction of carriers into non-endemic areas or countries must be prevented. Prior to importation to non-endemic areas or into countries, horses must test negative for equine piroplasmosis infection (Xuan *et al*., 2002).

The economic impact of the disease in South Africa is assumed to be in the millions of Rands due to a combination of direct losses, convalescence period and incidental cost such as treatment and veterinary fees (Naidoo *et al*., 2005). No vaccine is yet available for the prevention of equine piroplasmosis. Many countries forbid the importation of horses from South Africa because of the high prevalence of asymptomatic carrier animals in the southern Africa region. The South African thoroughbred racing industry is particularly badly affected by equine piroplasmosis, acute infections resulting in missed training sessions and races and loss through abortion in stud mares (Allsopp *et al.,* 2007).

Separate studies in South Africa have been conducted to study the prevalence of equine piroplasmosis. Most of the studies concentrated on the parasitological screening of blood smears and serological diagnosis of the disease. The use of molecular techniques is not

common and only reported in two recent publications (Motloang *et al*., 2008; Bhoora *et al*., 2009).

There is a need for the further use of molecular and immunological techniques to document the epidemiology of the disease in South Africa. Since there is no vaccine available for the disease, the determination of strain variation is needed. This will be of great importance in the development of effective vaccines and new control strategies for equine piroplasmosis.

2.2 General objective

To estimate the prevalence and strain variation of the causative agents of equine piroplasmosis, in two endemic provinces of South Africa, using molecular and serological techniques.

2.3 Specific objectives

2.3.1 To detect the presence of equine piroplasmosis parasites using Giemsa-stained thin smears.

2.3.2 To detect the presence of equine piroplasmosis antibodies in collected blood serum using indirect fluorescent antibody test (IFAT).

2.3.3 To estimate sero-prevalence of equine piroplasmosis antibodies in horse serum using enzyme-linked immunosorbent assay (ELISA).

2.3.4 To estimate the prevalence of equine piroplasmosis parasites in horses using the polymerase chain reaction (PCR).

2.3.5 To determine the strain variation in equine piroplasmosis parasites from South Africa using 18S rRNA gene sequence analysis.

CHAPTER TWO

MATERIALS AND METHODS

1. DESCRIPTION OF THE STUDY AREA

Horses were sampled from KwaZulu-Natal and Free State provinces of South Africa. *Rhipicephalus evertsi evertsi*, one of the known vectors of equine piroplasmosis is widely distributed in the study areas. The tick has been collected at altitudes varying from sea level to approximately 2500 m, and is commonest in regions receiving between 1200 mm and 2600 mm of annual rainfall. The adults and immature stages of the tick are present on host animals throughout the year (Walker *et al*., 2000).

1.1. Free State

The Free State, 26-30 ºS and 24-29 ºE, is situated on the flat boundless plains in the heart of South Africa, bordering six other provinces of South Africa as well as Lesotho. The rich soil and suitable climate allow a thriving agricultural industry. With more than thirty thousand farms, which produce over 70% of the country's grain, it is known locally as South Africa's breadbasket. The province is high-lying, with almost all land being above 1000 m. The Drakensburg and Maluti Mountains rise to over 3000 m in the east. The Province experiences a continental climate, characterized by warm to hot summers and cool to cold winters. Areas in the east experience occasional snowfalls, especially on the higher ranges, whilst the west can be extremely hot in summer. Almost all precipitation falls in the summer months as brief afternoon thunderstorms with aridity increasing towards the west (The World Factbook, 2007).

Samples were collected from the following areas, scattered across the Free State: Phuthaditjhaba (Witsieshoek), Sasolburg, Frankfort, Thabanchu, Petrusburg, Trompsburg, Springfontein, Bloemfontein, Smithfield, Philippolis and Fauresmith (Figure 1).

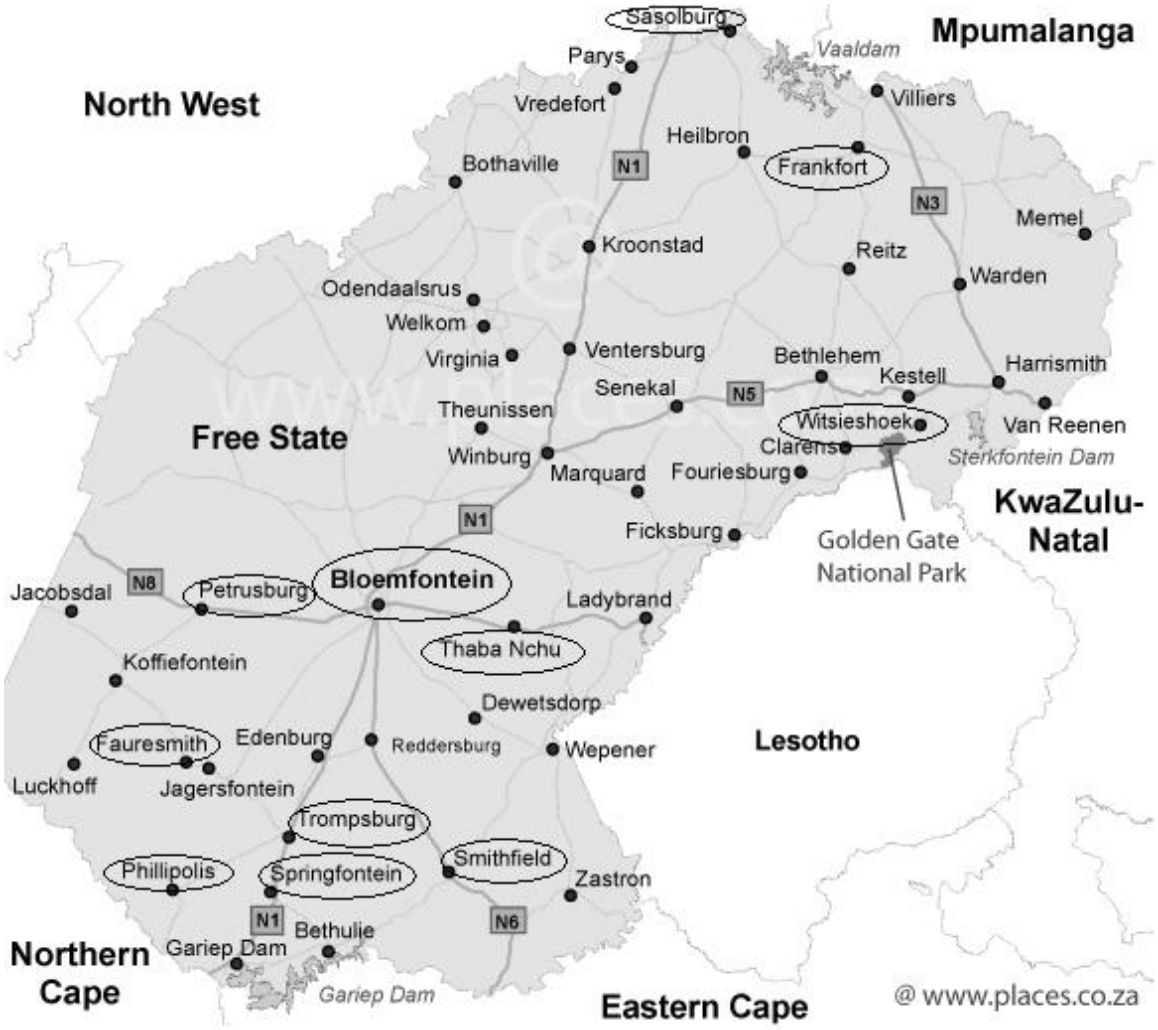


Figure 1: Location of study sites in the Free State Province. Map from www.places.co.za.

1.2. KwaZulu Natal

KwaZulu Natal province is located in the east of South Africa. It borders three other provinces and the countries of Mozambique, Swaziland and Lesotho, along with its shoreline on the Indian Ocean. The province has three different geographic areas. The lowland region along the Indian Ocean coast is extremely narrow in the south, widening in the northern part of the province. The central region is called the Midlands and is an undulating hilly plateau rising in the west towards the Drakensberg mountains. Generally, the coast is subtropical with inland regions becoming progressively colder. Durban on the south coast has an annual rainfall of 1009 mm, with daytime maxima peaking from January to March at 28 ºC (min: 21 ºC), dropping to daytime highs from June to August of 23 ºC (min: 11 ºC). Temperatures towards the hinterland are much cooler during winter (The World Factbook, 2007). The following study sites were selected as representative of the province: Hluhluwe, Mooi River and Vryheid as shown in Figure 2.

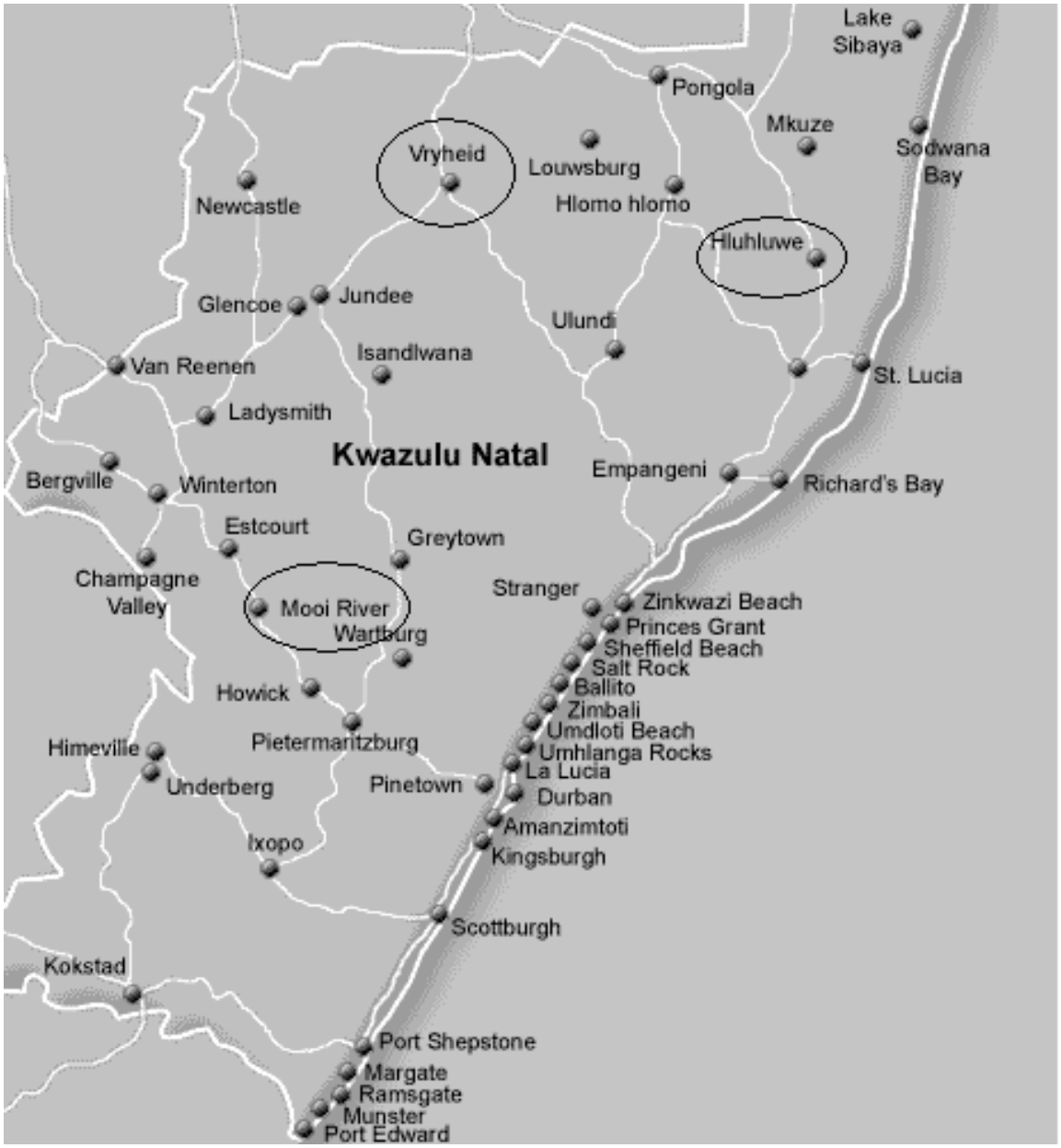


Figure 2: Location of study sites, Hluhluwe, Mooi River and Vryheid indicated by circles. Map from www.southafricarent.org.

2. Sampling of animals

Horses in the experimental sites were identified in terms of the farming systems, which are either communal or commercial. Most of the animals used in the experiment in the Free State were from communal farms, while most in KwaZulu Natal were from commercial farms. In the commercial farming system farmers use their animals for economic purpose, for example in Bloemfontein horses are used for racing. While in the communal farming system the animals are used to provide transport, for example in Phuthaditjhaba horses are used by the farmers to herd their livestock.

A total of 534 blood samples, 444 from Free State (Table 1A) and 90 from KwaZulu Natal (Table 1B), from various farms in each province, were collected into both plain and EDTA-coated vacutainers using sterile 21G needles as shown in Plate 3. Most animals were kept in a stable in the evening and during the day were grazed in the field together with other animals such as cattle, sheep and goats. In one of the farms in KwaZulu Natal, Mr. Forest's farm, the horses were kept together with zebra.

Blood collected into EDTA-coated vacutainers was immediately kept in ice and brought to the laboratory for preparation of blood smears and the rest stored in cryogenic vials at -35 ºC until used for DNA extraction and Polymerase Chain Reaction (PCR). Serum was harvested from the plain vacutainers and stored at -35 ºC until analyzed by Enzyme-Linked Immunosorbent Assay (ELISA) and Indirect Fluorescent Antibody Test (IFAT).

Table 1: Number of sampled animals per farm from both Free State (A) and KwaZulu Natal (B) and their sampling date.

A. Free State

Study area	Farming System	Farm name	Sample size	Sampling date
Phuthaditjhaba	Communal	Monontsha	16	27/05/06
		Monontsha	20	03/06/06
		Thaba-tshweu	62	01/07/06
		Lejwaneng	27	08/07/06
Sasolburg	Commercial	Roodepoortjie	14	14/03/07
		Scottsvalley	4	14/03/07
		Kronebloem	14	14/03/07
Frankfort	Commercial	Franfort	40	20/03/07
Thaba-Nchu	Communal	Longridge	6	02/04/07
		Paradys	7	02/04/07
		Ratabane	5	02/04/07
		Tiger river	8	02/04/07
		Sediba	2	02/04/07
Petrusburg	Commercial	Petrusburg	34	03/04/07
Trompsburg	Commercial	Trompsburg	32	04/04/07
Springfontein	Commercial	Kleinzuurfontein	8	05/04/07
		Springfontein	2	05/04/07
		Oranje	6	05/04/07
		Boshrand	5	05/04/07
		Hillside	4	05/04/07
		Kransfontein	3	05/04/07
Bloemfontein	Commercial	Waterborn	14	16/04/07
		Groenvlei	14	16/04/07
		Sherley stables	2	16/04/07
Smithfield	Commercial	Welgegund	16	17/04/07
		Hoogte	12	17/04/07
		Zandfontein	2	17/04/07
Philippolis	Commercial	Philippolis	22	18/04/07
		Donkerpoort	12	18/04/07
Fauresmith	Commercial	Fauresmith	4	19/04/07
		Riverside	16	19/04/07
		Brandfontein	11	19/04/07
Total			**444**	

B. KwaZulu Natal

Study area	Farming System		Sample size	Sampling date
Hluhluwe	Communal	Hluhluwe	20	22/08/06
Mooi River	Commercial	Bloemendal	11	25/10/06
		Mr Forest	14	25/10/06
		Ranches	11	25/10/06
		Croyden	14	25/10/06
Vryheid	Communal	Vaalkop	10	27/03/07
		Goedverwacht	10	27/03/07
Total			**90**	

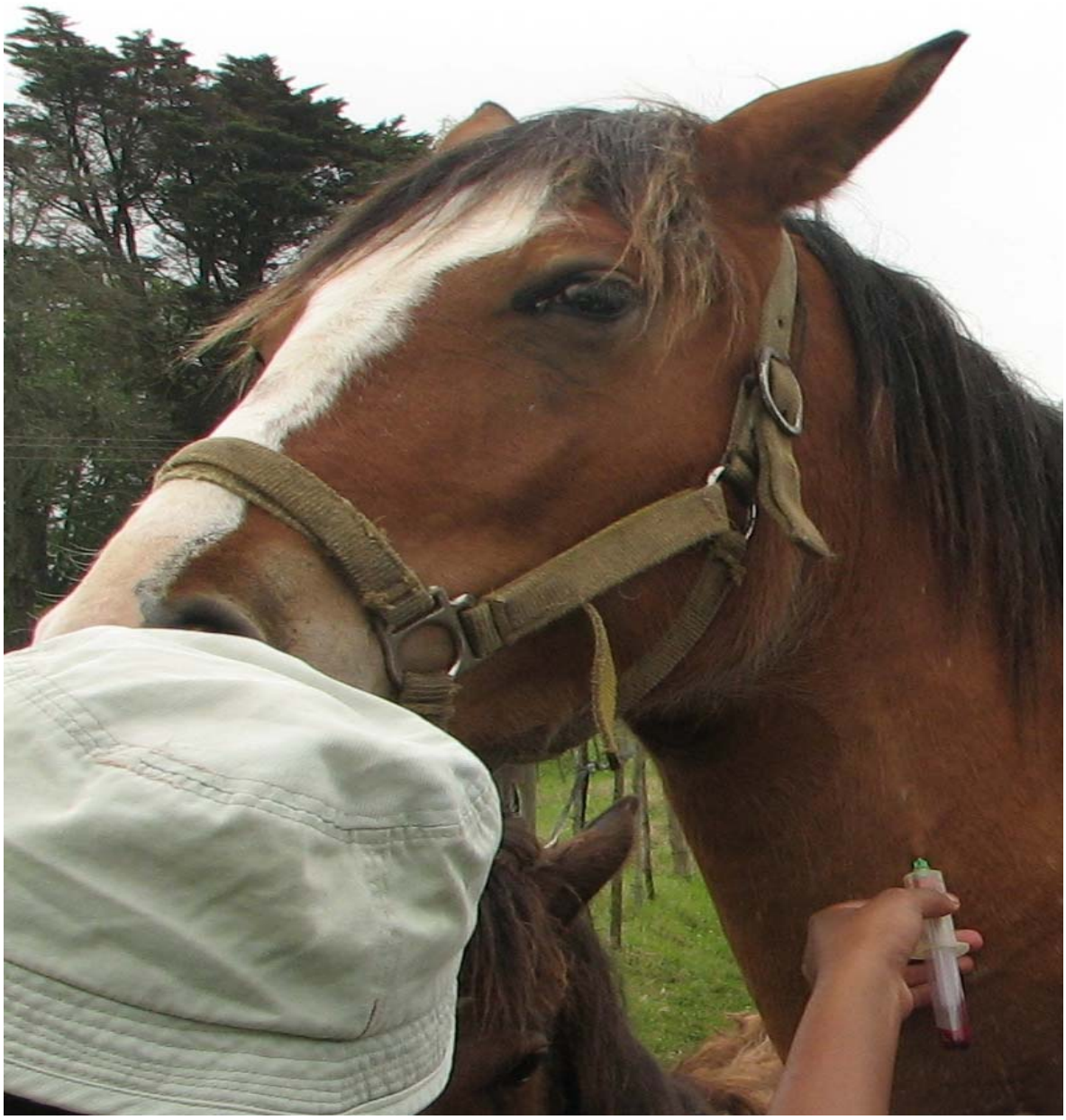


Plate 1: Collection of the blood samples from horses by jugular veni-pucture using 21G needles and a vacutainer. Photographed by M.A. Bakheit.

3. Tick management on the farms

Most samples were collected from commercial farming systems where tick control with commercial acaricides and traditional methods, such as the use of Jeyes fluid (disinfectant containing Tar acid as active chemical), was regularly practiced by the farmers. In communal farming systems, such as at Phuthaditjhaba and Thaba-Nchu, only the traditional methods were used to control the ticks.

4. Parasitological screening of equine piroplasms

The presence of equine piroplasms was investigated in red blood cells using Giemsa-stained thin blood smears from a total of 534 horses. Microscopic slides of thin blood smears were prepared, fixed using analytical grade absolute methanol (Shalom Laboratories, South Africa) and stained for 30 minutes using a 10% Giemsa solution. The smears were examined using a compound microscope (VWR International, France) at 1000x magnification, for the presence of parasite in the red blood cells, by placing a drop of 518C oil immersion (Zeiss Germany).

5. Serological diagnosis of equine piroplasmosis

Two serological techniques, the indirect fluorescent antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA), were used to detect the presence of anti-equine piroplasm antibodies present in the sera of tested animals. A total of 505 serum was harvested from collected samples in study areas, excluding does from Lejwaneng (n=27) in Phuthaditjhaba. IFAT was conducted in all 505 serum samples. For ELISA test, 250

serum samples were tested because the kit which was use for analysis contained reagents for 250 samples.

2.5.1. Indirect fluorescent antibody test (IFAT) for *T. equi* and *B. caballi*

The IFAT is a serological method used to detect antibodies against *B. caballi* and *T. equi*. Blood for antigen was obtained from horse that was infected with in vitro cultured parasite. The antigen was used to determine the presence of antibody in the serum. The antigen/antibody complex was visualized by staining the complex with secondary rabbit anti-horse antibody conjugated with a fluorochrome, fluorescence isothiocyanate (FITC). The cutoff titer for determination of positive results was taken in this study as 1/40 and 1/80 dilutions following Onderstepoort Veterinary Institute (OVI) IFAT SOP. World Organization for Animal Health (OIE) guidelines state that conjugates gave best results between 1/20-1/1280 dilutions.

The IFAT was performed at the OVI. Antigen slides were obtained from this laboratory. They were prepared by spreading 10 µl of cultured infected horse blood on each microscopic slide (Holman *et al.*, 1993, Holman *et al.*, 1994). The slides were covered with plastic pockets, to prevent the antigen from being damaged, and stored at -70 ºC until used. Antigen slides, test and control sera were allowed to thaw at room temperature (18 ºC to 25 ºC). Two-fold dilutions of the test and control sera of 1/40 and 1/80 were made using phosphate buffered saline (PBS), without Ca^{++} and Mg^{++}. Serum samples were marked numerically and written in the laboratory serological result book. The antigen slides were taken out of their protective plastic pockets covering, which was

placed after the slides were prepared, fixed in cold acetone for 1 minute, and allowed to air dry on the work bench. The slides were marked, *B. caballi*-c1 and c2, *T. equi* e1 and e2 (Figure 3), and with dates. A 25 µl drop of each of the 1/40 and 1/80 diluted sera were placed in individual wells on the antigen slide. Samples were loaded on the slide in serial dilution starting from the marked end of the slide. Each slide contained positive (sera from infected horse), first well, and negative (sera from uninfected horse), second well. Slides were incubated in a humid chamber at 37 ºC for 30 minutes. After incubation serum was flicked off from the slides and the slide was rinsed by dipping into a container with PBS. After rinsing, the slides were placed on a washing rack and immersed into a washing container with 300 ml PBS and washed on a magnetic stirrer set at very low revolutions for 10 minutes. The slides were then washed in distilled water for 5 minutes and dried by using a hot air blower. A drop of 25 µl diluted conjugate was placed on the slide to completely cover the well and then the slides were incubated in a humid chamber for 30 minutes at 37 ºC. After incubation they were rinsed in fresh PBS and washed in PBS for 10 minutes on a magnetic stirrer as before. A drop of 50% glycerin was placed on each slide and covered with a 24x50 mm cover-slip.

The slides were examined under a Fluorescent Microscope (Cosmo laboratory, India) using a 50 x water objective in a dark room by placing a drop of water on the cover-slip.

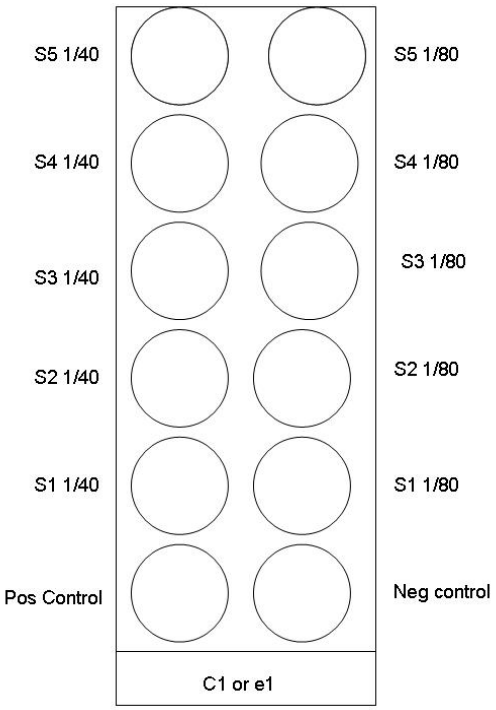


Figure 3: Different titer, controls and sera samples on the IFAT slide. c1= *B. caballi,* e1 = *T. equi,* S1 = Sample 1, S2 = Sample 2, S3 = Sample 3, S4 = Sample 4, S5 = Sample 5.

2.5.2. Enzyme-linked immunosorbent assay (ELISA) for *T. equi*

A competitive ELISA according to Knowles *et al*. (1991) that detects *T. equi* antibodies in equine sera was used in this study. In principle, the anti-*T. equi* antibodies present in the horse serum compete with and inhibit the binding of a primary IgG_1 monoclonal antibody against the *T. equi* merozoite antigen 1 (EMA-1). The binding of the primary monoclonal antibody to the antigen is detected by binding of a secondary antibody-horseradish peroxidase conjugate. Finally, the presence of the secondary antibody is detected by the addition of chromogenic enzyme substrate and subsequent colour development. Strong colour development indicates little or no inhibition of primary monoclonal antibody binding and therefore an absence of *T. equi* antibody in sample sera. Weak colour development, due to inhibition of the primary monoclonal antibody binding to the antigen on the solid phase, indicates the presence of *T. equi* antibodies in the sample.

The competitive ELISA test was performed using *Babesia equi* antibody test kit, manufactured by Veterinary Medical Research and Development Inc., USDA, following manufacture's protocol. The tests were performed at Onderstepoort Veterinary Institute. The positive and negative controls (produced by the kit), and test serum samples were diluted 1:2 with serum diluting buffer. Dilutions were made in a non-coated transfer plate. Using a multichannel pipette, 50 µl diluted controls and serum samples were transferred to the antigen-coated plate. The plate layout is shown in Figure 4. The plate was incubated for 30 minutes at room temperature. After incubation, the plate was washed using an EL X50 automated washer (Micro Plate Washer Thermo Electronic

Corporation, VWR International, New York) set to rinse 3X per well with washing buffer (PBS and 0.05% Tween 20). After washing, 50 µl diluted primary antibody was added to each well and incubated at room temperature for 30 minutes. The plate was then washed as in the first wash and after washing, 50 µl diluted secondary antibody-horseradish-peroxidase conjugate was added to each well. The plate was then incubated at room temperature for 30 minutes and then washed as in the first wash. After washing, 50 µl of the chromogenic enzyme substrate was added to each well and incubated for 15 minutes at room temperature. To stop the colour reaction, 50 µl of stop solution was added to each well.

Immediately after adding the stop solution, the plate was read using the Bio-Tek Model ELX800 Universal ELISA Microplate Reader (Multiskan Ascent Thermo Electronic Corporation, VWR International, New York) at 405 nm with reference filter of 620 nm at dual wavelength.

	1	2	3	4	5	6	7	8	9	10	11	12
A	NC	S1	9	17	25	33	41	49	57	65	73	81
B	PC	2	10	18	26	34	42	50	58	66	74	82
C	NC	3	11	19	27	35	43	51	59	67	75	83
D	NC	4	12	20	28	36	44	52	60	68	76	84
E	PC	5	13	21	29	37	45	53	61	69	77	85
F	NC	6	14	22	30	38	46	54	62	70	78	86
G		7	15	23	31	39	47	55	63	71	79	87
H		8	16	24	32	40	48	56	64	72	80	88

Figure 4: Sera samples and control in the ELISA plate. NC = Negative Control, PC = Positive Control, S (1-88) = Samples.

6. Molecular diagnosis of equine piroplasmosis using PCR

In the present study a PCR targeting the 18S rRNA gene was used to detect the presence of the equine piroplasmosis parasites in horses. The study reported by Salim *et al*. (2008) demonstrated that this method of PCR is rapid and sensitive for diagnosis of these parasites.

DNA was extracted from 200 µl of blood using a NucleoSpin® Blood QuickPure kit following the manufacturer's instructions. A final DNA product of 50 µl was eluted. Following extraction, DNA samples were stored at -20 ºC until analyzed by PCR.

PCR was performed according to the method of Alhassan *et al*. (2005). Primer pairs Bec-UF2/Cab-R and Bec-UF2/Equi-R (Table 2) were used to amplify the 18S rRNA gene of *B. caballi* and *T. equi,* respectively. Reactions were performed in 35 µl mixture containing distilled water, 1x PCR buffer including 15 mM $MgCl_2$, 0.2 mM dNTP, 0.2 µM of each primer, 2U *Taq* DNA polymerase (Southern Cross Biotechnology, South Africa) and 2 µl DNA template. Reactions were performed in an automated DNA thermal cycler (Eppendorf Mastercycler Gradient, Germany). After an initial denaturation step by incubation at 94 ºC for 3 minutes, 35 cycles of the following conditions were performed: denaturation at 94 ºC for 1 minute, annealing at 55 ºC for 1 minute and extension at 72 ºC for 90 seconds. A final extension step of incubation at 72 ºC for 7 minutes was included. The program ended by holding the reactions at 4 ºC. Five micro-liter amplified DNA was run on a 1.5% agarose gel (VWR International Gel Electrophoresis, Japan) for 45

minutes with 100 voltage and examined under UV light using a Bio Imaging System (Amersham Bioscience Gene Genius Bio Imaging System, USA).

Table 2: Sequences of primers and expected length of PCR-amplified products of *B. caballi* and *T. equi*

Parasite	Primer pairs	Sequence (5'-3')	Product size
B. caballi	Bec-UF2	TCGAAGACGATCAGATACCGTCG	540 bp
	Cab-R	CTCGTTCATGATTTAGAATTGCT	
T. equi	Bec-UF2	TCGAAGACGATCAGATACCGTCG	392 bp
	Equi-R	TGCCTTAAACTTCCTTGGAT	

7. Genetic determination of *Theileria equi* in the study area

PCR was carried out using the primer pair 990_AS: 5'TTGCCTTAAACTTCCTTG3' and 989_S: 5'AGTTTCTGACCTATCAG3' which amplifies the hyper-variable region V4 of the 18S RNA gene of *T. equi* (Allsopp *et al*., 1993). Reactions were performed in 50 µl mixture containing distilled water, 1x PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP, 0.2 µM of each primer, 2.6 U Super-Therm *Taq* DNA Polymerase (AbGene, USA) and 2 µl DNA template. After an initial denaturation step of 3 minutes at 94 ºC, 35 cycles of the following conditions were done: denaturation at 94 ºC for 1 minute, annealing at 55 ºC for 1 minute and extension at 72 ºC for 90 seconds; and final extension at 72 ºC for 7 minutes. The program ended by holding the reactions at 4 ºC. The amplified products of approximately 1080 bp were sequenced at Inqaba Biotec (Pretoria, South Africa). The sequences obtained were compared with those available in the international GenBank database by nucleotide sequence homology searches using BLAST (www.nih.nlm.ncbi.gov). The sequences were edited using FinchTV software (Finch Trace Viewer, Geospiza Inc., USA). DNA sequence alignment was performed using Mega 4.0 computer software Version 4 (2007), which uses Clustal W algorithm (Tamura *et al*., 2007). Sequence alleles, divergences and variable position with transitions and transversions were analyzed by pairwise distance calculation implemented in the software. A phylogenetic tree was constructed using the neighbor-joining method as implemented in Mega 4.0 software package (Tamura *et al*., 2007). This was the only available software, for sequence analysis, for this study.

8. Statistical error in estimation of results

Results were calculated as the number of positives and percentage positives with 95% confidence limits estimated using the Confidence Interval for Proportion Calculator from Dimension Research, Inc. (www.dimensionresearch.com/resources/calculators/conf_pr). Where either 0% or 100% of samples were positive, 95% confidence intervals were estimated using exact probabilities.

CHAPTER THREE

RESULTS

1. Prevalence of piroplasms

Neither *T. equi* nor *B. caballi* piroplasms were detected in the thin blood smears prepared from a total of 534 horses from both Free State and KwaZulu Natal. There were no enormous different in results obtained from commercial and communal systems. All the sampled animals appeared healthy and did not show any clinical signs of disease.

2. Serological examination

2.1. IFAT for *T. equi* and *B. caballi* in Free State and KwaZulu Natal

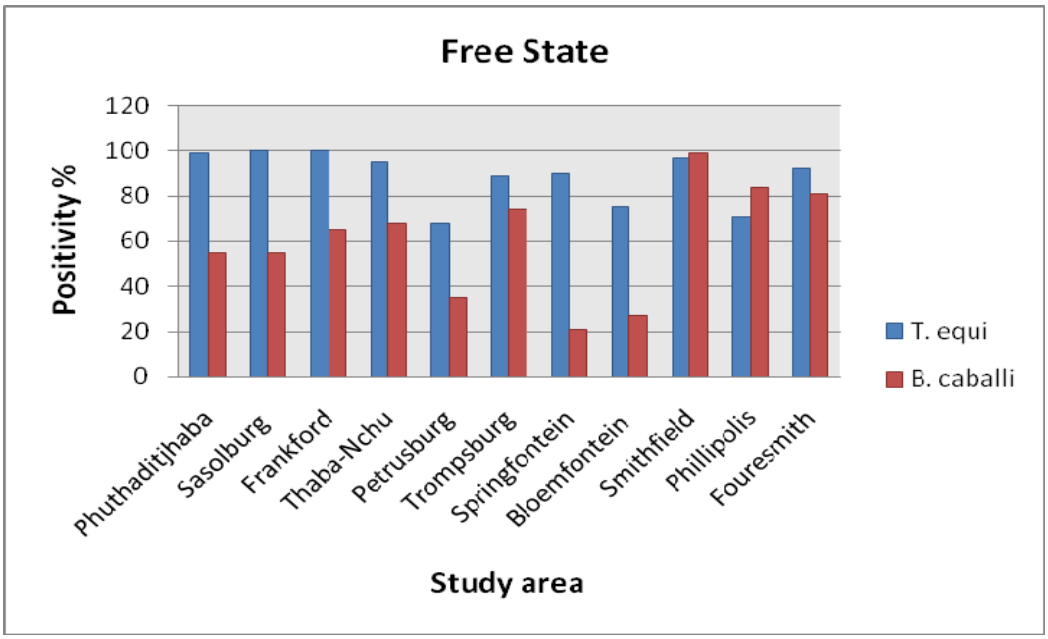
An overall seroprevalence of 61% and 60% was detected for *B. caballi* in the surveyed horses in the Free State and KwaZulu Natal, respectively. A prevalence of 89%, and 99% was also detected for *T. equi* antibodies in Free State and KwaZulu Natal, respectively. Generally, seroprevalence of *B. caballi* was less than that of *T. equi*. Several study sites in the Free State (Figure 5A) and the majority of the study locations in KwaZulu Natal (Figure 5B) showed a 100% seroprevalence for *T. equi* suggesting that large numbers of animals are exposed to the parasite in these regions.

2.2. ELISA for *T. equi* in Free State and KwaZulu Natal

A predetermined cut-off value of 40% was used below which tested sample were judged negative. Samples were considered positive only on inhibition values equal to or greater than 40%. Generally, differences in colour were clearly observed on the ELISA plate

(Plate 2). According to the ELISA results, a mean seroprevalence of 52±8% and 94±5% was determined for *T. equi* in the Free State and KwaZulu Natal, respectively, with detailed values obtained for the study locations shown in Table 3. It should be observed here that seroprevalence determined by ELISA in samples obtained from the Free State was generally less than that determined by IFAT. Thus, out of a total number of 160 animals examined by ELISA, 83 (52%) were found seropositive. However, the results obtained for KZN were comparable to those of the IFAT.

A. Free State



B. KwaZulu Natal

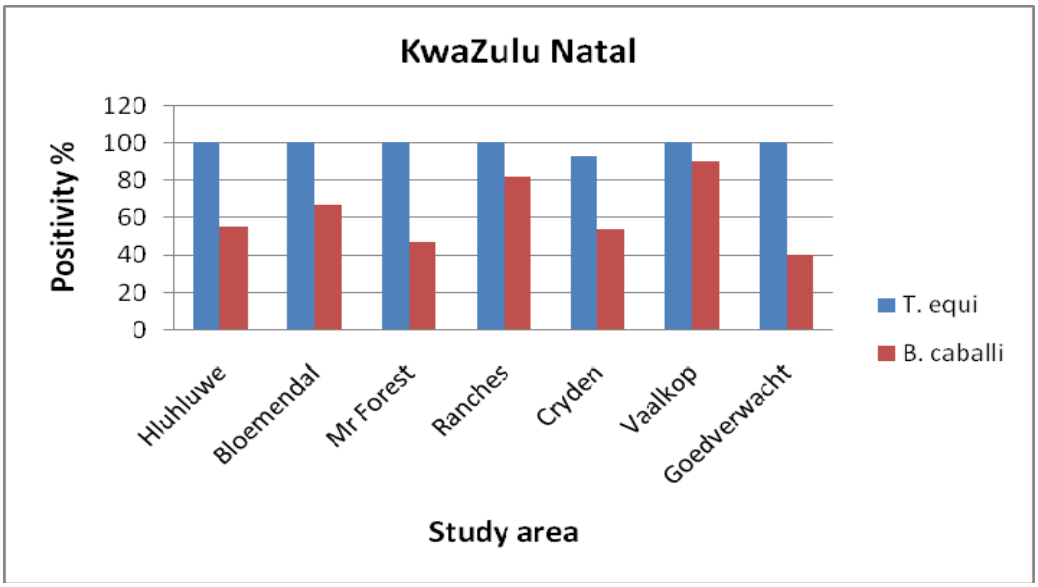


Figure 5: The sero-prevalence of *T. equi* and *B. caballi* in A. Free State and B. KwaZulu Natal, as determined by IFAT.

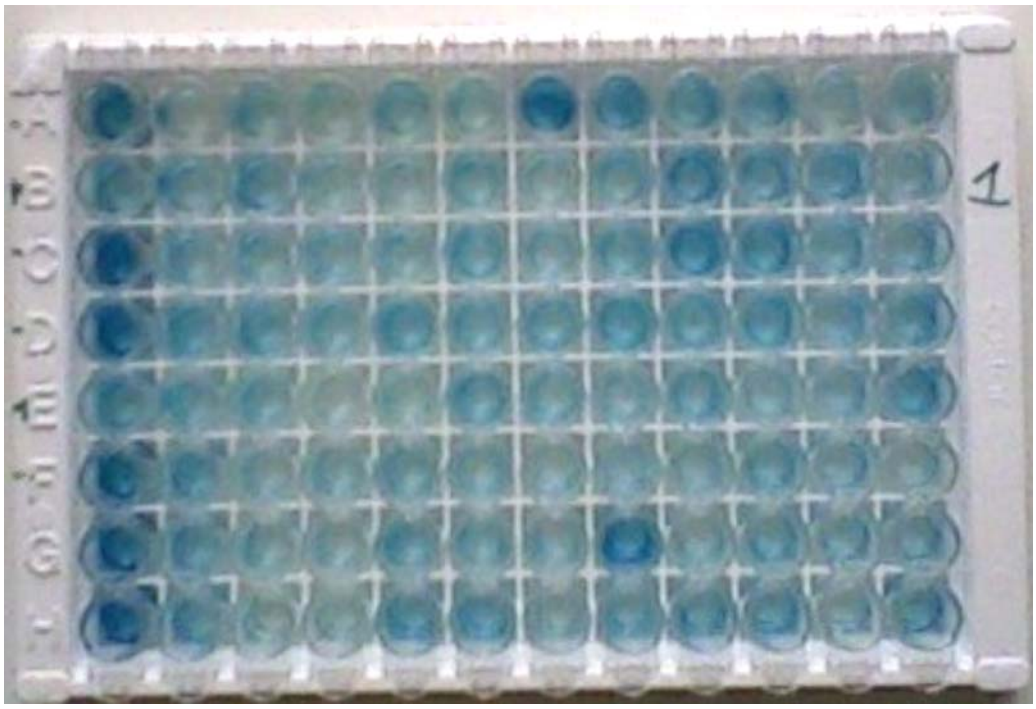


Plate 2: ELISA plates showing blue colour developed after addition of the enzymatic substrate.

Table 3: The sero-prevalence of *T. equi* in horses of Free State and KwaZulu Natal as determined by ELISA test.

A. Free State

Location	Farms	Number of tested animals	Number positive by ELISA (%)
Phuthaditjhaba	Monontsha	36	31 (86±11%)
	Thaba-tshoeu	62	30 (48±12%)
Sasolburg	Roodepoortjie	14	8 (57±26%)
	Scotsvalley	4	0 (0-52%)
	Kronebloem	14	6 (43±26%)
Bloemfontein	Waterborn	14	5 (36±25%)
	Groenvlei	14	2 (14±18%)
	Sherley Stable	2	1 (50±50%)
TOTAL		**160**	**83 (52±8%)**

B. KwaZulu Natal

Location	Farms	Number of tested animals	Number positive by ELISA (%)
Hluhluwe	Hluhluwe	20	20 (100-90%)
Mooi River	Bloemendal	11	11 (100-83%)
	Mr Forest	14	13 (93±13%)
	Ranches	11	10 (91±17%)
	Croyden	14	11 (79±21%)
Vryheid	Vaalkop	10	10 (100-81%)
	Goedverwacht	10	10 (100-81%)
TOTAL		**90**	**85 (94±5%)**

3. Molecular detection

No *B. caballi* parasite DNA was detected when using Bec-UF2 and Cab-R primers which target a segment of the 18S rRNA gene. For *T. equi*, a single band of about 392 bp was readily visualized for positive samples and no bands were observed for negative samples (Plate 3). A mean prevalence of 34±4% was calculated for *T. equi* using PCR. The parasite was found more prevalent in KwaZulu Natal (51±10%) than in the Free State (30±4%) (Table 4).

The overall prevalence of *T. equi* in the two provinces as determined by PCR (34±4%) was very low as compared to that determined by IFAT (89-99%) and ELISA (50%) (Figure 6). A total of about 57% was positive for *T. equi* in IFAT but negative on PCR. Likewise, a seroprevalence of 58% for *B. caballi* was obtained by IFAT but this parasite was not detected by PCR, as shown in Table 5.

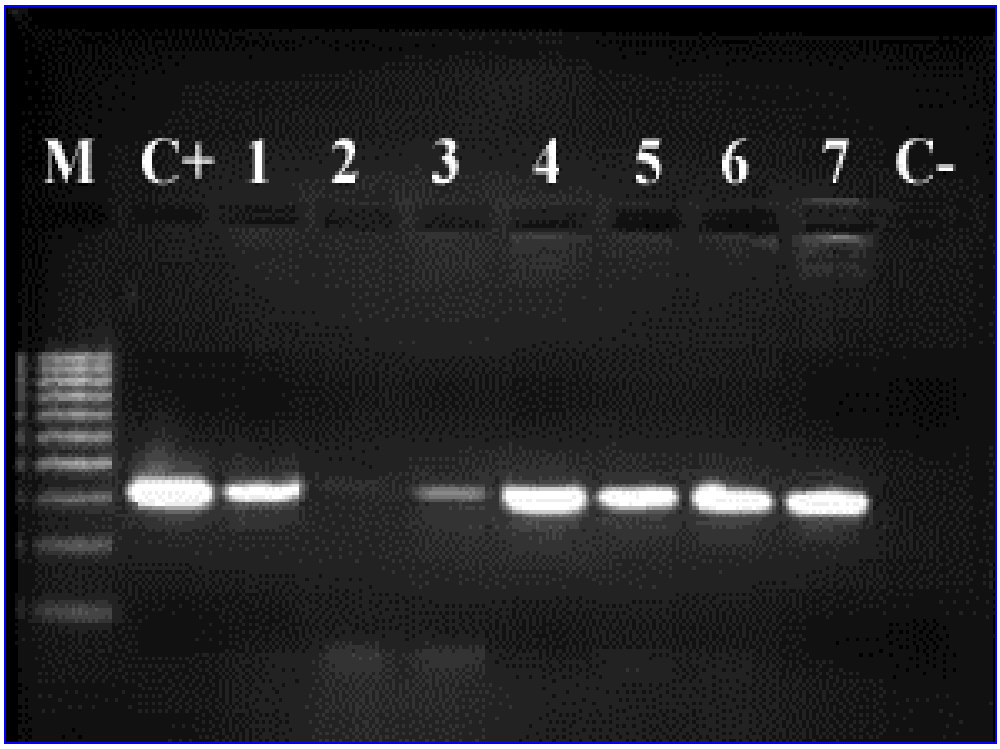


Plate 3: Agarose gel showing amplification of *T. equi* DNA. M = marker of 100 bp, C+ = positive control (DNA of infected animal) with 392 bp, 1-7= test samples, C- = distilled water control. Samples 1 and 5-8 show strong positive results; sample 3 shows a weak positive result; sample 2 result were below detection limit.

Table 4: The prevalence of *T. equi* in horses from Free State and KwaZulu Natal as determined by PCR.

A. Free State

Location within the Free State	Number of tested animals	Number positive by PCR
Phuthaditjhaba	125	85 (68±8%)
Sasolburg	32	5 (15±13%)
Frankfort	40	14 (35±15%)
Thaba-Nchu	28	4 (14±13%)
Petrusburg	34	8 (23±14%)
Trompsburg	32	3 (9±10%)
Springfontein	28	1 (4±7%)
Bloemfontein	30	4 (13±12%)
Smithfield	30	4 (13±12%)
Philippolis	34	2 (6±8%)
Fauresmith	31	5 (16±13%)
FS Total	**444**	**135 (30±4%)**

B. KwaZulu Natal

Location within Kwazulu Natal	Number of tested animals	Number positive by PCR
Hluhluwe	20	12 (60±21%)
Bloemendal	11	2 (18±23%)
Mr Forest	14	4 (28±24%)
Ranches	11	6 (54±29%)
Croyden	14	10 (64±24%)
Vaalkop	10	5 (50±31%)
Goedverwacht	10	8 (80±25%)
KZN Total	**90**	**47 (51±10%)**
TOTAL (FS and KZN)	**534**	**182 (34±4%)**

Table 5: Comparison of equine piroplasmosis prevalence as determined by PCR and IFAT.

N	*T. equi*		*B. caballi*	
Positive in PCR (n=534)	183	34%	0	0
Positive in IFAT (n=505)	469	93%	291	58%
Positive in PCR negative in IFAT	5	1%	0	0
Positive in IFAT negative in PCR	304	57%	272	54%
Positive in both IFAT and PCR	148	29%	0	0
Negative in both IFAT and PCR	33	6%	217	43%

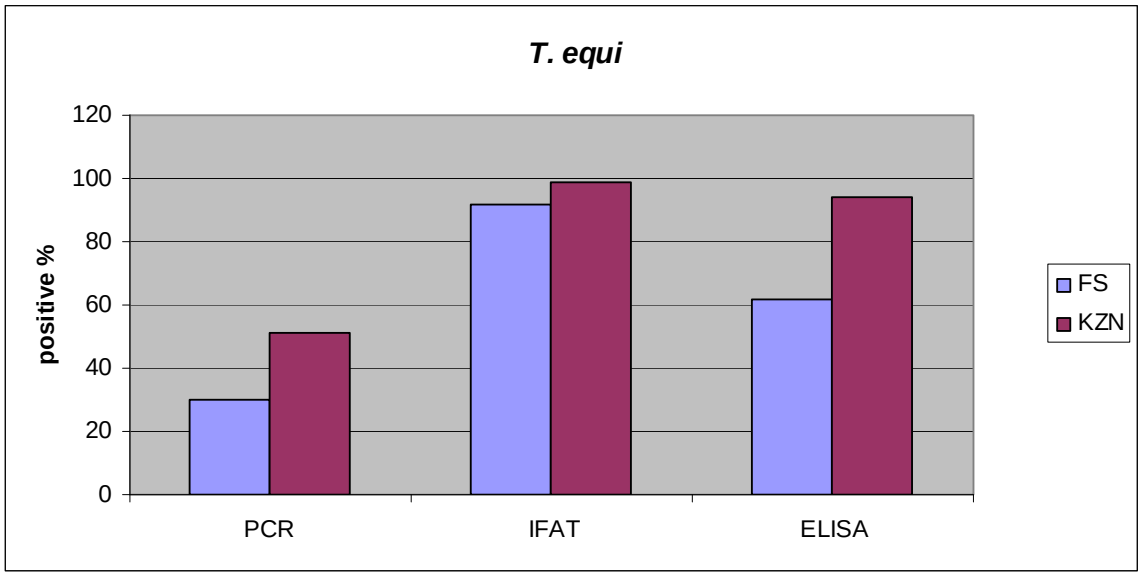


Figure 6: The prevalence of *T. equi* in both Free State and KwaZulu Natal as determined by PCR, IFAT and ELISA.

4. Genetic variations in *Theileria equi*

A Neighbour-Joining phylogenetic tree was constructed with 28 sequences of the 18S rRNA gene of *T. equi* and one *Babesia caballi* sequence as an outgroup. Fifteen of these sequences were obtained for this study from across six sampling locations (Figure 7). A further 13 sequences were obtained from GenBank, these included three sequences from Spain (Criado-Fornelio *et al*., 2003, Criado-Fornelio *et al*., 2006), and ten sequences from South Africa (Allsopp *et al.*,1994, Bhoora *et al*., 2009). Nucleotides were coded as unordered, discrete characters with five possible character states: A, C, G, T and N. Gaps were coded as missing data. The sequence length varied from 755 to 918 bp giving 919 bp of aligned sequences (Appendix III**)**. Five sequences had missing data of between 4-163 bp at the ends. There were no length mutations in the alignment. The aligned sequences had 35 variable positions with 21 transitions and 14 transversions. The study sequences together with Genbank sequences gave 16 sequence alleles (Table 6). Three different genotype groups were observed, Group A: a diverse and well supported group that is known only from South Africa, Group B: a poorly supported group that includes two sequences from Spain along with South African samples, and Group C: a group comprising the third Spanish sequence and a single *T. equi* sequence collected from a Mountain Zebra in the Western Cape by Bhoora *et al.* (2009). Sequence divergences varied from 0.0-2.5% (0-23 differences among samples) (Table 6), with a net divergence of 0.6% between groups.

Table 6: Uncorrected sequence divergences (A) in percentages and number of differences among sequences (B).

A.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1															
2	0.1														
3	0.1	0.3													
4	0.2	0.4	0.3												
5	0.5	0.8	0.7	0.8											
6	1.0	1.3	1.1	1.3	0.7										
7	0.4	0.7	0.5	0.7	0.5	1.2									
8	1.0	0.8	1.1	1.0	1.2	1.6	0.7								
9	1.3	1.2	1.2	1.4	1.5	2.0	1.0	0.3							
10	1.4	1.2	1.3	1.4	1.6	2.1	1.1	0.4	0.1						
11	1.2	1.1	1.3	1.3	1.4	1.9	0.9	0.2	0.1	0.2					
12	1.6	1.6	1.7	1.7	1.9	2.3	1.1	0.7	0.5	0.7	0.4				
13	1.4	1.3	1.5	1.5	1.7	2.1	1.0	0.5	0.4	0.5	0.3	0.3			
14	1.4	1.3	1.6	1.5	1.6	2.0	0.9	0.4	0.3	0.4	0.2	0.4	0.3		
15	1.6	1.7	1.7	1.6	1.6	1.9	1.5	1.3	1.4	1.5	1.3	1.7	1.5	1.6	
16	2.2	1.7	2.3	2.1	2.2	2.3	2.1	1.9	2.2	2.3	2.1	2.5	2.3	2.3	0.8

B.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1															
2	1														
3	1	2													
4	2	3	3												
5	5	6	6	7											
6	9	10	10	11	6										
7	4	5	5	6	5	11									
8	9	6	10	9	11	15	6								
9	12	9	11	12	14	18	9	3							
10	13	9	12	12	15	19	10	4	1						
11	11	8	12	11	13	17	8	2	1	2					
12	15	12	16	15	17	21	10	6	5	6	4				
13	13	10	14	13	16	19	9	5	4	5	3	3			
14	13	10	15	13	15	18	8	4	3	4	2	4	3		
15	15	13	16	14	15	17	14	12	13	14	12	16	14	15	
16	20	13	21	18	20	21	19	17	20	21	19	23	21	21	7

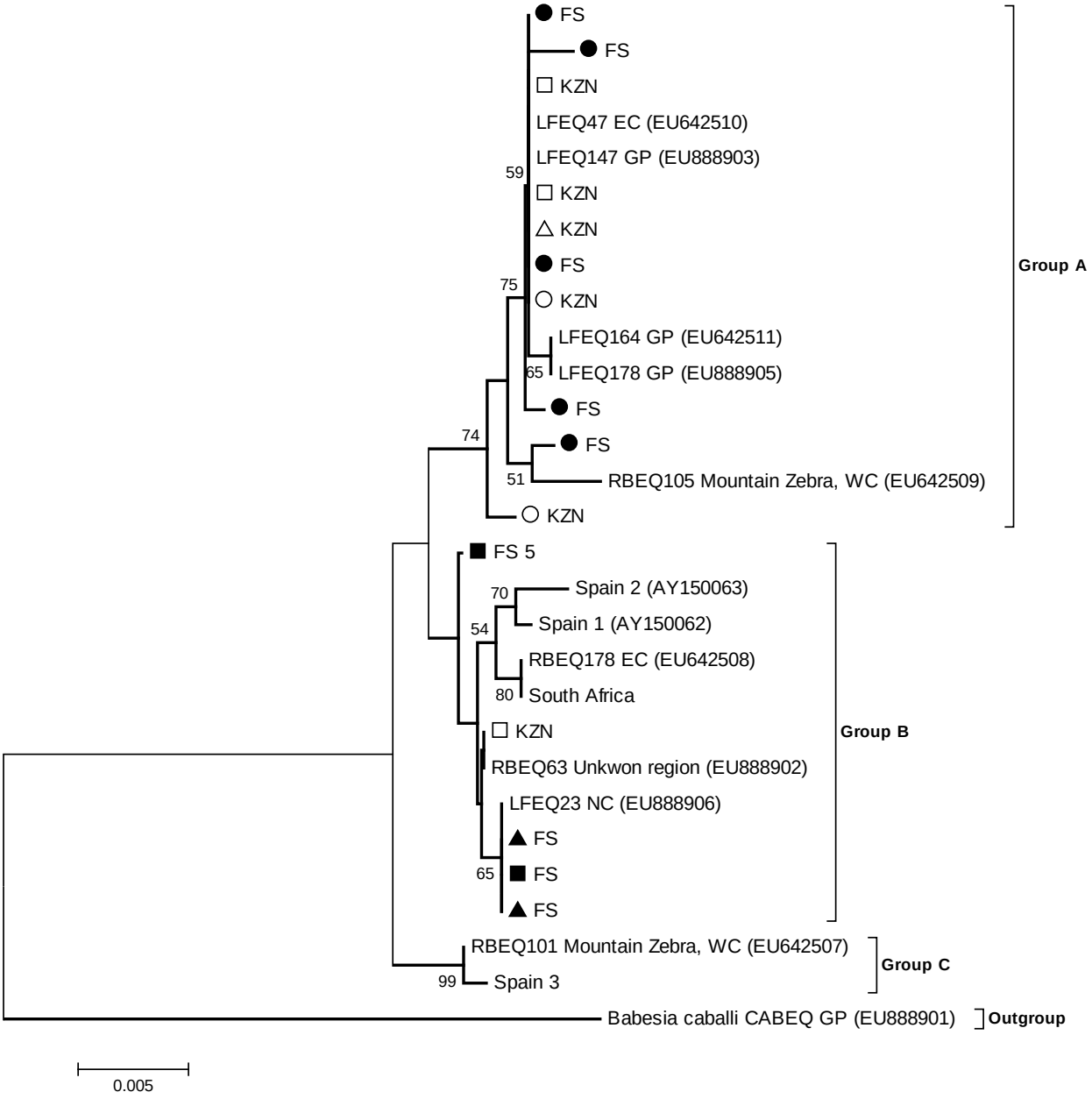


Figure 7: A Neighbour-Joining tree showing the relationship of *T. equi* 18S rRNA genotypes (GenBank accession numbers are given in brackets). Sample locations: ■Sasolburg, ▲Bloemfontein, ●Phuthaditjhaba, ○Vryburg, □Mooi River, Δ Hluhluwe.

CHAPTER FOUR

GENERAL DISCUSSION AND CONCLUSIONS

In this study, neither *T. equi* nor *B. caballi* parasites were detected in thin smears prepared from 534 horses. In acute *B. caballi* cases, parasitaemia varies from 0.1% up to 10% and in *T. equi* infections may exceed 20%, but levels of 1-5% are more common (Nagore *et al*., 2004). The finding of parasites in thin blood smears is often difficult, particularly in carrier animals where the parasitaemia is very low and likewise in acute cases at the onset of the disease (Nagore *et al*., 2004). As in other diseases, a negative result obtained by microscopic examination does not preclude infection (Ali *et al.*, 1996). Identification of equine piroplasmosis in carrier animals by means of blood smear examination is not only very difficult but also inaccurate and therefore this is not the preferred method of diagnosis. However, false-negative or/and false-positive reaction may be encountered in the course of serological tests (Tenter and Freidhoff, 1986). In such cases, the use of both serological and molecular techniques is needed to confirm the prevalence.

Motloang *et al.* (2008) did not detect any piroplasms in all smears of screened horses in Free State but found the exposure level to *B. caballi* (48%) and *T. equi* (98%) as detected by indirect fluorescent antibody tests (IFAT) were high. The present study confirms this result, indicating that equine piroplasmosis is endemic in Free State.

Because of difficulty of demonstrating circulating parasites, serological testing of animals is the preferred method of diagnosis especially for horses tested before their movement from endemic areas of equine piroplasmosis to countries free of the disease but with suitable tick vectors. In 2004, the World Organisation for Animal health prescribed the use of IFAT and enzyme-linked immunosorbent assays (ELISA) to qualify animals for movement (Ogunremi *et al*., 2007).

In the present study, IFAT showed higher seroprevalence for both *T. equi* and *B. caballi* antibodies. The test appears to be highly specific and sensitive in detecting horses exposed to equine piroplasmosis. Most samples were strongly positive while others were weakly positive or negative. The two titers, 1/40 and 1/80, were used as positives for IFAT. The lower titers are rarely associated with false positivity (Homer *et al*., 2000). Prevalence of 55% and 61%, for *B. caballi*, and 89% and 93%, for *T. equi,* were detected from 1/80 and 1/40 respectively. The 1/40 titer may results in occasional false-positive results while 1/80 may rarely result in false-positive.

The IFAT showed a very high prevalence of both *T. equi* and *B. caballi* antibodies as compared to polymerase chain reaction (PCR). The seropositivity of between 55% and 61%, for *B. caballi*, were detected from the population samples that were negative, as determined by PCR and on the examination of blood smears. Similar results were detected by Ogunremi *et al*. (2007) and Motloang *et al*. (2008) working in KwaZulu Natal and Free State, respectively, where a sero-prevalence of 99-100% was determined from the population that was declared parasite-free.

The seroprevalence, as determined by IFAT, of 94% for *T. equi* was also very high as opposed to 34±4% positivity by PCR. An estimated total of 57% of horses, as shown on Table 5, were detected positive for *T. equi* using IFAT and negative in PCR. According to Ali *et al.* (1996), after recovery from acute infection, horses become lifelong carriers of the disease, and these animals develop an infection-immunity-type of immune status with very low parasitaemia usually below the detection level.

A competitive inhibition ELISA (cELISA) using EMA-1, the major surface protein of *T. equi*, and a specific monoclonal antibody (MAb) that defines this merozoite surface protein epitope, have been used in cELISA for the detection of *T. equi* (Knowles *et al*., 1992). Xuan *et al*. (2001) reported a high degree of homology between amino acid sequences of EMA-1 from 19 *T. equi* strains from various countries, and therefore it is a suitable diagnostic candidate for the detection of antibodies to *T. equi*.

Fifty percent of the samples showed a very weak colour development, indicating inhibition of primary monoclonal antibody binding to the antigen and were positive for *T. equi* as determined by ELISA. Serum samples with strong colour, indicating little or no inhibition of primary monoclonal antibodies binding, were below the cut-off point and therefore were negative for *T. equi*. The high frequency of positive tests for the parasite indicated that high number of animals was exposed to equine piroplasmosis parasites.

When comparing the results obtained by ELISA and IFAT within the individual samples, they indicate that there are substantial differences between these two tests, in terms of

carrier detections. Of 250 tested individual samples, a total of 40% tested positive in IFAT but negative on ELISA. The low levels of fluorescence when observed under microscope make the test subjective and require an experienced eye to assess the results (Böse *et al*., 1995 and Bruning 1996). However, IFAT have been reported to have impracticality especially for testing large number of samples (Bakheit *et al*, 2007).

The competitive inhibition ELISA has solved many problems associated with antigen purity since its specificity depends solely on the monoclonal antibody used (Knowles *et al.*, 1995). The cELISA is not only specific, but also sensitive and the results can be understood easily with rarely false-negative and/or false-positive observations. Since there are high chances of false-negative and/or false-positive results in the IFAT, the use of ELISA is to be recommended as the serological diagnostic tool.

In this study, the species-specific polymerase chain reaction assay, which targets the 18S rRNA gene (Alhassan *et al*., 2005) was used. Additional molecular techniques for the detection of *T. equi* and *B. caballi* have been recently described. These methods include loop-mediated isothermal amplification (LAMP) (Alhassan *et al*., 2006), reverse line blot (RLB) assay (Schnittger *et al*., 2004, Bhoora *et al*., 2009), and real-time polymerase chain reaction (Kim *et al*., 2008). Probably the use one of these methods as an alternative test might have had better results.

The total prevalence of 34±4% for *T. equi* parasites was detected by PCR, with KwaZulu Natal horses (51±10%) showing higher prevalence than Free State (30±4%). The study

sites in the northern part of Free State, such as Phuthaditjhaba (68±8%) and Frankfort (35±15%), showed a higher prevalence of the parasite than other parts of the province. All study sites in KwaZulu Natal showed high prevalence with the highest prevalence in Vryheid (65±21%). According to de Waal and van Heerden (2004), the tick vector, *Rhipicephalus evertsi evertsi,* of *T. equi* is distributed throughout KwaZulu Natal and the northern part of the Free State. In this study, *T. equi* was detected in the southern part of Free State where the presence of the vector had not been confirmed, this may be because of the movement of the hosts from other provinces or within the province, or due to a recent movement and establishment of the vector in the southern part of the province.

No *B. caballi* parasites were detected when using PCR in any of the study sites. These results may be explained because infections with *B. caballi* are generally less severe and a smaller number of erythrocytes become infected and destroyed (American Veterinary Medical Association, 2006); hence making it difficult to detect parasite DNA. However, the parasite antibodies were detected when using IFAT. Both *T. equi* and *B. caballi* share the same vector, *Rhipicephalus evertsi evertsi*, but *T. equi* is generally more widely distributed than *B. caballi* (de Waal and van Heerden, 2004).

In the present report, the prevalence of equine piroplasmosis detected by 18S rRNA PCR diagnosis was lower than that of serological diagnosis. In previous studies, it has been reported that, although serological tests are more sensitive, they lack specificity and due to the occurrence of cross-reactivity false-positive results are possible (Bruning *et al*., 1997). PCR has been shown to have higher specificity compared with serological tests for

identification of *Trypanosoma* species in cattle (Geysen *et al*., 2003). The high prevalence obtained by the serological assays in this study may be related to various factors listed by Bruning *et al*. (1997) which include false-positivity, cross reaction or due to subjective interpretation of the serological results especially those from IFAT. It could, however, be possible to detect high antibody titers in recovered animals that are showing undetectable levels of parasitaemia.

Sequencing and phylogenetic results presented in this study demonstrated considerable genetic diversity of *T. equi* in South Africa. In the study done by Criado-Fornelio *et al*. (2003) there were two similar genotypes in Spanish isolates (Spain 1 (AY150062) and Spain 2 (AY150063)), which showed that there is some degree of polymorphism in parasite populations. They also suggested that an African isolate (Z15105) described by Allsopp *et al.* (1994) was ancestral to these Spanish isolates. These isolates are phylogenetically clustered with South African isolates described by Bhoora *et al.* (2009) from Northern Cape (EU888906), Eastern Cape (EU642508) and unknown region (EU888902) and also isolates from the Free State (Bloemfontein, Sasolburg) and KwaZulu-Natal (Mooi River) sequenced here. Few mutations separate these isolates and they probably evolved from a common ancestor. The Spain 3 isolate (DQ287951) described by Criado-Fornelio *et al*. (2006), and a Western Cape Mountain Zebra (EU642507; Bhoora *et al.,* 2009) genotypes were very different from the other isolates and appeared to be outside the study group.

A genotype from Phuthaditjhaba was identical to several sequences from KwaZulu Natal obtained in this study. Phuthaditjhaba is on the border of the Free State and KwaZulu Natal, and it is possible for the horses in these areas to be infected by the same *T. equi* isolate. Furthermore, the movement of horses between these areas may be relatively frequent. However, none of the sampled sites were in close proximity to each other and almost all movement of horses among these areas would require 2-3 hours of motorized transport.

Following the geographic distribution of *T. equi* in South Africa, the conclusion based on the genotype variation results may be stated as follows:

- Two divergent genotype clusters were found, each of which occurred in both the Free State and KwaZulu Natal, and other provinces as described by Bhoora *et al.* (2009) and are therefore widespread in South Africa.
- Only the two genotypes were shared among locations, one in each genotype cluster, and each of these shared genotypes was found in both KwaZulu Natal and the Free State.
- Multiple genotypes were detected within four of the six sample locations for sequences (Sasolburg, Phuthaditjhaba, Mooi River and Vryheid). Sample sizes were too small to exclude the presence of multiple genotypes in the other two sample sites.

More research is needed to clearly elucidate the genotype variation of *T. equi* in South Africa and elsewhere to help in understanding of the distribution of the parasite isolates.

It is possible that commercial farms, such as those sampled at Bloemfontein, are importing horses from other countries that introduce new genotypes in the country. Further sequencing of samples from such areas will give a clear understanding of genotype diversity in the country.

According to the results determined in this study, it can be concluded that *T. equi* is highly prevalent in South Africa. This study also confirmed the distribution of the disease as described by de Waal and van Heerden (2004), and the disease was found also in the area which previously was declared disease-free. There is considerable variation in *T. equi* genotypes in the country and no clear phylogeographic structuring of these genotypes. These may indicate that there is much movement of infected carrier horses within the country and even to and from other countries.

B. caballi prevalence is still not clear as only IFAT seems to detect antibodies to infection by this parasite. This confirms that single diagnostic techniques are not always accurate and require confirmation from other techniques. There is a need for the development of highly sensitive assays for the detection of *B. caballi*, thereby enabling determination of prevalence and strain diversity studies of this parasite.

In conclusion, this study discovered new distribution of equine piroplasmosis in South Africa. In previous studies, equine piroplasmosis parasites were not found in the southern part of Free State, and in the present study the parasites were found in this area. It was reported here that there is widespread sequence variation in the 18S rRNA gene within *T.*

equi and not clear phylogeographic structuring in South Africa. Sequencing of more samples is required in order to have a clear phylogeographic structure of the parasite in the country. The prevalence of *B. caballi* in South Africa appeared to be problematic since it was difficult to detect in the present and previous studies. The findings reported here show that there is a need for careful examination of present assays and also development of new assays which are more sensitive for the detection of *B. caballi*.

CHAPTER FIVE

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APPENDICES

Appendix I: Table showing results from individual samples in Free State. N = Negative sample, P = Positive sample, *P?* = Weak negative sample.

FREE STATE									
		T. equi					*B. caballi*		
Sample No.	**Sample Area**	**PCR**	**ELISA**	**IFAT**			**PCR**	**IFA**	
				1/40	**1/80**			**1/40**	**1/80**
	Qwa-Qwa								
1	Monontsha	**N**	**N**	P	P		**N**	**N**	**N**
2		P	P	P	P		**N**	**N**	**N**
3		P	P	P	P		**N**	P	P
4		P	P	P	P		**N**	P	P
5		P	P	P	P		**N**	P	P
6		**N**	**N**	P	P		**N**	P	P
7		**N**	P	P	P		**N**	P	P
8		P	P	P	*P?*		**N**	P	P
9		P	P	P	*P?*		**N**	P	P
10		P	P	P	P		**N**	P	P
11		**N**	P	P	P		**N**	P	P
12		P	P	P	P		**N**	P	P
13		**N**	P	**N**	**N**		**N**	**N**	**N**
14		P	P	P	**N**		**N**	P	P
15		P	P	P	P		**N**	P	P
16		**N**	**N**	P	P		**N**	**N**	**N**
17		**N**	P	P	P		**N**	P	*P?*
18		P	P	P	P		**N**	P	P
19		**N**	P	P	P		**N**	P	P
20		**N**	P	P	P		**N**	P	P
21		**N**	P	P	P		**N**	P	*P?*
22		**N**	**N**	P	P		**N**	**N**	**N**
23		**N**	P	P	P		**N**	**N**	**N**
24		**N**	P	P	P		**N**	P	P
25		**N**	P	P	P		**N**	**N**	**N**
26		**N**	P	P	P		**N**	P	P
27		P	P	P	P		**N**	P	P
28		P	P	P	P		**N**	P	P
29		**N**	P	P	P		**N**	P	P
30		**N**	**N**	P	P		**N**	**N**	**N**
31		**N**	P	P	P		**N**	P	P
32		**N**	P	P	P		**N**	*P?*	**N**
33		**N**	P	P	P		**N**	**N**	**N**
34		**N**	P	P	P		**N**	**N**	**N**
35		**N**	P	P	P		**N**	**N**	**N**
36		P	P	P	P		**N**	P	**N**
37	Thaba-tshoeu	P	P	P	P		**N**	P	*P?*
38		P	P	P	P		**N**	P	P
39		P	P	P	P		**N**	P	P
40		P	P	P	P		**N**	**N**	**N**

Sample No.	Sample Area	*T. equi*					*B. caballi*		
		PCR	**ELISA**	**IFAT**			**PCR**	**IFAT**	
				1/40	**1/80**			**1/40**	**1/80**
41	Thaba-tshoeu	P	P	P	P		**N**	P	P
42		P	P	P	*P?*		**N**	**N**	**N**
43		P	P	P	P		**N**	*P?*	**N**
44		P	P	P	P		**N**	P	P
45		P	P	P	P		**N**	P	P
46		**N**	**N**	P	P		**N**	P	P
47		**N**	P	P	P		**N**	**N**	**N**
48		**N**	P	P	P		**N**	P	P
49		**N**	**N**	P	P		**N**	P	P
50		**N**	P	P	P		**N**	P	P
51		P	P	P	P		**N**	P	P
52		P	P	P	P		**N**	**N**	**N**
53		P	P	P	P		**N**	**N**	**N**
54		P	P	P	P		**N**	**N**	**N**
55		P	P	P	P		**N**	P	*P?*
56		**N**	**N**	P	P		**N**	**N**	**N**
57		P	P	P	P		**N**	P	P
58		**N**	P	P	P		**N**	P	P
59		**N**	**N**	P	P		**N**	P	P
60		**N**	P	P	P		**N**	P	P
61		**N**	**N**	P	P		**N**	P	P
62		**N**	P	P	P		**N**	P	P
63		P	P	P	P		**N**	P	**N**
64		**N**	**N**	P	P		**N**	P	P
65		P	P	P	P		**N**	P	P
66		P	**N**	P	P		**N**	**N**	**N**
67		P	**N**	P	P		**N**	**N**	**N**
68		P	**N**	P	P		**N**	**N**	**N**
69		P	**N**	P	P		**N**	P	P
70		P	N	P	P		**N**	**N**	**N**
71		P	**N**	P	P		**N**	P	P
72		**N**	**N**	P	P		**N**	**N**	**N**
73		P	P	P	P		**N**	P	**N**
74		P	**N**	P	P		**N**	P	*P?*
75		**N**	**N**	P	P		**N**	P	P
76		P	P	P	P		**N**	**N**	**N**
77		**N**	**N**	P	P		**N**	P	P
78		P	**N**	P	P		**N**	P	P
79		P	**N**	P	P		**N**	P	*P?*
80		P	**N**	P	P		**N**	P	P
81		P	**N**	P	P		**N**	P	P
82		P	**N**	P	P		**N**	**N**	**N**
83		P	**N**	P	P		**N**	**N**	**N**
84		P	**N**	P	P		**N**	**N**	**N**
85		P	**N**	P	P		**N**	**N**	**N**
86		P	**N**	P	P		**N**	**N**	**N**
87		P	P	P	P		**N**	**N**	**N**

Sample No.	Sample Area	*T. equi*					*B. caballi*		
		PCR	**ELISA**	**IFAT**			**PCR**	**IFAT**	
				1/40	**1/80**			**1/40**	**1/80**
88	Thaba-tshoeu	**N**	**N**	P	P		**N**	**N**	**N**
89		P	P	P	P		**N**	**N**	**N**
90		P	**N**	P	P		**N**	**N**	**N**
91		P	**N**	P	P		**N**	**N**	**N**
92		P	**N**	P	P		**N**	**N**	**N**
93		P	**N**	P	P		**N**	**N**	**N**
94		P	**N**	P	P		**N**	**N**	**N**
95		P	P	P	P		**N**	**N**	**N**
96		P	**N**	P	P		**N**	**N**	**N**
97		P	P	P	P		**N**	**N**	**N**
98		P	**N**	P	P		**N**	**N**	**N**
99	Lejwaneng	P	*N/A*	*N/A*	*N/A*		**N**	*N/A*	*N/A*
100		P	*N/A*	*N/A*	*N/A*		**N**	*N/A*	*N/A*
101		P	*N/A*	*N/A*	*N/A*		**N**	*N/A*	*N/A*
102		P	*N/A*	*N/A*	*N/A*		**N**	*N/A*	*N/A*
103		P	*N/A*	*N/A*	*N/A*		**N**	*N/A*	*N/A*
104		P	*N/A*	*N/A*	*N/A*		**N**	*N/A*	*N/A*
105		P	*N/A*	*N/A*	*N/A*		**N**	*N/A*	*N/A*
106		**N**	*N/A*	*N/A*	*N/A*		**N**	*N/A*	*N/A*
107		**N**	*N/A*	*N/A*	*N/A*		**N**	*N/A*	*N/A*
108		P	*N/A*	*N/A*	*N/A*		**N**	*N/A*	*N/A*
109		P	*N/A*	*N/A*	*N/A*		**N**	*N/A*	*N/A*
110		P	*N/A*	*N/A*	*N/A*		**N**	*N/A*	*N/A*
111		P	*N/A*	*N/A*	*N/A*		**N**	*N/A*	*N/A*
112		P	*N/A*	*N/A*	*N/A*		**N**	*N/A*	*N/A*
113		P	*N/A*	*N/A*	*N/A*		**N**	*N/A*	*N/A*
114		P	*N/A*	*N/A*	*N/A*		**N**	*N/A*	*N/A*
115		P	*N/A*	*N/A*	*N/A*		**N**	*N/A*	*N/A*
116		P	*N/A*	*N/A*	*N/A*		**N**	*N/A*	*N/A*
117		P	*N/A*	*N/A*	*N/A*		**N**	*N/A*	*N/A*
118		P	*N/A*	*N/A*	*N/A*		**N**	*N/A*	*N/A*
119		P	*N/A*	*N/A*	*N/A*		**N**	*N/A*	*N/A*
120		P	*N/A*	*N/A*	*N/A*		**N**	*N/A*	*N/A*
121		P	*N/A*	*N/A*	*N/A*		**N**	*N/A*	*N/A*
122		P	*N/A*	*N/A*	*N/A*		**N**	*N/A*	*N/A*
123		P	*N/A*	*N/A*	*N/A*		**N**	*N/A*	*N/A*
124		P	*N/A*	*N/A*	*N/A*		**N**	*N/A*	*N/A*
125		P	*N/A*	*N/A*	*N/A*		**N**	*N/A*	*N/A*
	Sasolburg								
126	Rooderpoortjie	**N**	**N**	P	P		**N**	**N**	**N**
127		**N**	**N**	P	P		**N**	**N**	**N**
128		P	P	P	P		**N**	**N**	**N**
129		**N**	P	P	P		**N**	**N**	**N**
130		**N**	P	P	P		**N**	**N**	**N**
131		**N**	**N**	P	P		**N**	**N**	**N**
132		P	P	P	P		**N**	**N**	**N**
133		**N**	**N**	P	P		**N**	**N**	**N**

Sample No.	Sample Area	*T. equi* PCR	*T. equi* ELISA	*T. equi* IFAT 1/40	*T. equi* IFAT 1/80		*B. caballi* PCR	*B. caballi* IFAT 1/40	*B. caballi* IFAT 1/80
134	Rooderpoortjie	**N**	**N**	P	P		**N**	**N**	**N**
135		**N**	P	P	P		**N**	**N**	**N**
136		**N**	P	P	P		**N**	**N**	**N**
137		**N**	**N**	P	P		**N**	P	P
138		**N**	P	P	P		**N**	**N**	**N**
139		**N**	P	P	P		**N**	**N**	**N**
140	Scottsvalley	**N**	**N**	P	P		**N**	**N**	**N**
141		**N**	**N**	P	P		**N**	**N**	**N**
142		**N**	**N**	P	P		**N**	**N**	**N**
143		**N**	**N**	P	P		**N**	**N**	**N**
144	Kronebloem	**N**	**N**	P	P		**N**	P	P
145		**N**	**N**	P	P		**N**	P	P
146		**N**	P	P	P		**N**	**N**	**N**
147		**N**	**N**	P	P		**N**	**N**	**N**
148		**N**	**N**	P	*P?*		**N**	**N**	**N**
149		**N**	**N**	P	P		**N**	P	**N**
150		P	P	P	P		**N**	P	P
151		**N**	**N**	P	P		**N**	P	P
152		**N**	P	P	P		**N**	P	P
153		P	P	P	P		**N**	P	P
154		P	P	P	P		**N**	**N**	**N**
155		**N**	**N**	P	P		**N**	**N**	**N**
156		**N**	P	P	P		**N**	**N**	**N**
157		**N**	**N**	P	P		**N**	P	P
	Frankfort								
158		**N**	*N/A*	P	P		**N**	P	**N**
159		P	*N/A*	P	P		**N**	P	P
160		**N**	*N/A*	P	P		**N**	P	P
161		**N**	*N/A*	P	P		**N**	**N**	**N**
162		**N**	*N/A*	P	P		**N**	**N**	**N**
163		**N**	*N/A*	P	P		**N**	P	P
164		**N**	*N/A*	P	P		**N**	**N**	**N**
165		**N**	*N/A*	P	P		**N**	**N**	**N**
166		**N**	*N/A*	P	P		**N**	**N**	**N**
167		**N**	*N/A*	P	P		**N**	P	P
168		**N**	*N/A*	P	P		**N**	P	P
169		**N**	*N/A*	P	P		**N**	P	P
170		**N**	*N/A*	P	P		**N**	P	P
171		P	*N/A*	P	P		**N**	P	P
172		P	*N/A*	P	P		**N**	P	P
173		**N**	*N/A*	P	*P?*		**N**	P	P
174		**N**	*N/A*	P	P		**N**	P	P
175		**N**	*N/A*	P	P		**N**	P	P
176		**N**	*N/A*	P	P		**N**	P	P
177		P	*N/A*	P	P		**N**	P	P
178		**N**	*N/A*	P	P		**N**	P	P
179		**N**	*N/A*	P	P		**N**	P	P

Sample No.	Sample Area	*T. equi* PCR	*T. equi* ELISA	*T. equi* IFAT 1/40	*T. equi* IFAT 1/80		*B. caballi* PCR	*B. caballi* IFAT 1/40	*B. caballi* IFAT 1/80
180		**N**	*N/A*	P	P		**N**	P	P
181		**N**	*N/A*	P	P		**N**	P	P
182		**N**	*N/A*	P	P		**N**	P	P
183		**N**	*N/A*	P	P		**N**	P	P
184		**N**	*N/A*	P	P		**N**	**N**	**N**
185		**N**	*N/A*	P	P		**N**	P	P
186		P	*N/A*	P	P		**N**	**N**	**N**
187		P	*N/A*	P	P		**N**	**N**	**N**
188		P	*N/A*	P	P		**N**	**N**	**N**
189		P	*N/A*	P	P		**N**	*P?*	**N**
190		P	*N/A*	P	P		**N**	P	P
191		**N**	*N/A*	P	P		**N**	*P?*	**N**
192		P	*N/A*	P	P		**N**	**N**	**N**
193		**N**	*N/A*	P	P		**N**	**N**	**N**
194		P	*N/A*	P	P		**N**	**N**	**N**
195		P	*N/A*	P	P		**N**	*P?*	**N**
196		P	*N/A*	P	P		**N**	P	P
197		P	*N/A*	P	P		**N**	P	P
	Thaba-Nchu								
198	Longridge	**N**	*N/A*	P	P		**N**	P	P
199		**N**	*N/A*	P	P		**N**	*P?*	**N**
200		**N**	*N/A*	P	P		**N**	P	*P?*
201		**N**	*N/A*	P	P		**N**	P	P
202		P	*N/A*	P	P		**N**	**N**	**N**
203		**N**	*N/A*	P	P		**N**	P	*P?*
204	Sediba	**N**	*N/A*	P	P		**N**	**N**	**N**
205		**N**	*N/A*	P	P		**N**	**N**	**N**
206	Paradys	**N**	*N/A*	P	P		**N**	**N**	**N**
207		**N**	*N/A*	P	P		**N**	P	P
208		**N**	*N/A*	P	P		**N**	P	P
209		**N**	*N/A*	P	P		**N**	P	P
210		P	*N/A*	P	P		**N**	P	P
211		**N**	*N/A*	P	P		**N**	P	P
212		**N**	*N/A*	P	P		**N**	*P?*	**N**
213	Ratabane	**N**	*N/A*	P	P		**N**	**N**	**N**
214		**N**	*N/A*	P	P		**N**	P	P
215		**N**	*N/A*	P	P		**N**	*P?*	**N**
216		**N**	*N/A*	P	P		**N**	**N**	**N**
217	Tiger-river	P	*N/A*	P	P		**N**	**N**	**N**
218		**N**	*N/A*	P	P		**N**	P	P
219		P	*N/A*	P	P		**N**	P	*P?*
220		**N**	*N/A*	P	**N**		**N**	P	P
221		**N**	*N/A*	P	*P?*		**N**	P	P
222		**N**	*N/A*	P	**N**		**N**	**N**	**N**
223		**N**	*N/A*	P	**N**		**N**	P	P
224		**N**	*N/A*	P	P		**N**	P	P
225		**N**	*N/A*	P	P		**N**	P	*P?*

Sample No.	Sample Area	*T. equi* PCR	*T. equi* ELISA	*T. equi* IFAT 1/40	*T. equi* IFAT 1/80		*B. caballi* PCR	*B. caballi* IFAT 1/40	*B. caballi* IFAT 1/80
	Petrusburg								
226		**N**	*N/A*	P	P		**N**	**N**	**N**
227		**N**	*N/A*	**N**	**N**		**N**	P	P
228		P	*N/A*	P	P		**N**	P	P
229		**N**	*N/A*	**N**	**N**		**N**	P	*P?*
230		**N**	*N/A*	**N**	**N**		**N**	**N**	N
231		P	*N/A*	**N**	**N**		**N**	P	P
232		**N**	*N/A*	**N**	**N**		**N**	P	P
233		**N**	*N/A*	**N**	**N**		**N**	**N**	**N**
234		**N**	*N/A*	P	P		**N**	**N**	**N**
235		**N**	*N/A*	P	P		**N**	**N**	**N**
236		**N**	*N/A*	**N**	**N**		**N**	*P?*	**N**
237		**N**	*N/A*	**N**	**N**		**N**	*P?*	**N**
238		**N**	*N/A*	P	P		**N**	P	P
239		**N**	*N/A*	P	P		**N**	P	P
240		P	*N/A*	P	P		**N**	P	P
241		P	*N/A*	*P?*	**N**		**N**	**N**	**N**
242		**N**	*N/A*	P	**N**		**N**	**N**	**N**
243		**N**	*N/A*	P	P		**N**	**N**	**N**
244		P	*N/A*	P	P		**N**	**N**	**N**
245		**N**	*N/A*	P	P		**N**	**N**	**N**
246		**N**	*N/A*	*P?*	**N**		**N**	**N**	**N**
247		P	*N/A*	**N**	**N**		**N**	**N**	**N**
248		**N**	*N/A*	*P?*	**N**		**N**	**N**	**N**
249		**N**	*N/A*	P	P		**N**	**N**	**N**
250		**N**	*N/A*	P	P		**N**	**N**	**N**
251		**N**	*N/A*	P	P		**N**	**N**	**N**
252		**N**	*N/A*	P	P		**N**	**N**	**N**
253		P	*N/A*	P	P		**N**	**N**	**N**
254		**N**	*N/A*	P	P		**N**	**N**	**N**
255		**N**	*N/A*	P	P		**N**	**N**	**N**
256		**N**	*N/A*	P	P		**N**	P	P
257		P	*N/A*	P	P		**N**	P	P
258		**N**	*N/A*	P	P		**N**	**N**	**N**
259		**N**	*N/A*	P	P		**N**	P	P
	Trompsburg								
260		**N**	*N/A*	P	P		**N**	P	P
261		P	*N/A*	**N**	**N**		**N**	**N**	**N**
262		P	*N/A*	P	**N**		**N**	**N**	**N**
263		**N**	*N/A*	**N**	**N**		**N**	**N**	**N**
264		**N**	*N/A*	P	**N**		**N**	**N**	**N**
265		**N**	*N/A*	P	P		**N**	P	P
266		P	*N/A*	P	P		**N**	*P?*	**N**
267		**N**	*N/A*	P	P		**N**	P	P
268		**N**	*N/A*	P	P		**N**	P	P
269		**N**	*N/A*	P?	**N**		**N**	*P?*	**N**
270		**N**	*N/A*	P	P		**N**	P	P

Sample No.	Sample Area	*T. equi* PCR	*T. equi* ELISA	*T. equi* IFAT 1/40	*T. equi* IFAT 1/80		*B. caballi* PCR	*B. caballi* IFAT 1/40	*B. caballi* IFAT 1/80
271		**N**	*N/A*	P	P		**N**	P	P
272		**N**	*N/A*	P	P		**N**	P	P
273		**N**	*N/A*	P	P		**N**	P	P
274		**N**	*N/A*	P	P		**N**	*P?*	**N**
275		**N**	*N/A*	P	P		**N**	P	P
276		**N**	*N/A*	P	P		**N**	**N**	**N**
277		**N**	*N/A*	P	P		**N**	P	*P?*
278		**N**	*N/A*	P	P		**N**	P	P
279		**N**	*N/A*	P	P		**N**	**N**	**N**
280		**N**	*N/A*	P	P		**N**	P	P
281		**N**	*N/A*	P	P		**N**	**N**	**N**
282		**N**	*N/A*	P	P		**N**	P	P
283		**N**	*N/A*	P	P		**N**	P	P
284		**N**	*N/A*	P	P		**N**	P	P
285		**N**	*N/A*	P	P		**N**	P	P
286		**N**	*N/A*	P	P		**N**	P	P
287		**N**	*N/A*	P	P		**N**	P	P
288		**N**	*N/A*	P	P		**N**	P	P
289		**N**	*N/A*	P	P		**N**	P	P
290		**N**	*N/A*	P	P		**N**	P	P
291		**N**	*N/A*	P	P		**N**	P	P
	Springfontein								
292	Klein-zuurfontein	**N**	*N/A*	P	P		**N**	P	P
293		**N**	*N/A*	P	**N**		**N**	**N**	**N**
294		**N**	*N/A*	**N**	**N**		**N**	**N**	**N**
295		**N**	*N/A*	P	P		**N**	P	P
296		**N**	*N/A*	P	P		**N**	P	P
297		**N**	*N/A*	P	*P?*		**N**	**N**	**N**
298		**N**	*N/A*	P	P		**N**	**N**	**N**
299		**N**	*N/A*	**N**	**N**		**N**	**N**	**N**
300	Springfontein	**N**	*N/A*	P	P		**N**	**N**	**N**
301		**N**	*N/A*	P	P		**N**	P	P
302	Oranje	**N**	*N/A*	P	P		**N**	**N**	**N**
303		**N**	*N/A*	P	P		**N**	**N**	**N**
304		**N**	*N/A*	P	P		**N**	**N**	**N**
305		**N**	*N/A*	P	P		**N**	P	P
306		**N**	*N/A*	P	P		**N**	**N**	**N**
307		**N**	*N/A*	P	P		**N**	**N**	**N**
308	Boshrand	**N**	*N/A*	P	P		**N**	**N**	**N**
309		**N**	*N/A*	P	P		**N**	**N**	**N**
310		**N**	*N/A*	P	P		**N**	**N**	**N**
311		**N**	*N/A*	P	P		**N**	**N**	**N**
312		**N**	*N/A*	P	P		**N**	**N**	**N**
313	Hillside	**N**	*N/A*	*P?*	**N**		**N**	**N**	**N**
314		P	*N/A*	P	P		**N**	**N**	**N**
315		**N**	*N/A*	P	P		**N**	**N**	**N**

Sample No.	Sample Area	*T. equi* PCR	*T. equi* ELISA	*T. equi* IFAT 1/40	*T. equi* IFAT 1/80		*B. caballi* PCR	*B. caballi* IFAT 1/40	*B. caballi* IFAT 1/80
316	Hillside	**N**	*N/A*	P	P		**N**	**N**	**N**
317	Kransfontein	**N**	*N/A*	P	P		**N**	**N**	**N**
318		**N**	*N/A*	P	P		**N**	P	P
319		**N**	*N/A*	P	P		**N**	P	**N**
	Bloemfontein								
320	Waterborn	**N**	**N**	P	P		**N**	**N**	**N**
321		**N**	**N**	P	P		**N**	P	P
322		**N**	**N**	P	P		**N**	**N**	**N**
323		**N**	**N**	**N**	**N**		**N**	**N**	**N**
324		**N**	P	P	P		**N**	**N**	**N**
325		**N**	**N**	P	P		**N**	**N**	**N**
326		**N**	**N**	P	P		**N**	**N**	**N**
327		**N**	**N**	*P?*	**N**		**N**	**N**	**N**
328		**N**	P	P	P		**N**	**N**	**N**
329		**N**	**N**	P	P		**N**	P	P
330		P	P	P	P		**N**	**N**	**N**
331		**N**	P	P	P		**N**	**N**	**N**
332		**N**	P	P	P		**N**	**N**	**N**
333		**N**	**N**	P	P		**N**	**N**	**N**
334	Groenvlei	**N**	**N**	P	P		**N**	P	P
335		**N**	**N**	P	P		**N**	P	P
336		**N**	**N**	P	P		**N**	P	P
337		**N**	**N**	**N**	**N**		**N**	**N**	**N**
338		**N**	**N**	P	P		**N**	**N**	**N**
339		**N**	**N**	**N**	**N**		**N**	**N**	**N**
340		P	P	P	P		**N**	P	*P?*
341		P	P	**N**	**N**		**N**	**N**	**N**
342		**N**	**N**	**N**	**N**		**N**	**N**	**N**
343		P	**N**	P	P		**N**	P	P
344		**N**	**N**	P	P		**N**	**N**	**N**
345		**N**	**N**	**N**	**N**		**N**	**N**	**N**
346		**N**	**N**	P	P		**N**	**N**	**N**
347		**N**	**N**	**N**	**N**		**N**	**N**	**N**
348	Sherley Stable	**N**	P	P	P		**N**	**N**	**N**
349		**N**	**N**	P	P		**N**	P	P
	Smithfield								
350	Welgegund	**N**	*N/A*	P	P		**N**	P	P
351		**N**	*N/A*	P	P		**N**	P	P
352		**N**	*N/A*	P	P		**N**	P	P
353		**N**	*N/A*	P	P		**N**	P	P
354		**N**	*N/A*	P	P		**N**	P	P
355		**N**	*N/A*	P	P		**N**	P	P
356		**N**	*N/A*	*P?*	**N**		**N**	P	P
357		**N**	*N/A*	P	P		**N**	P	*P?*
358		**N**	*N/A*	P	P		**N**	P	P
359		**N**	*N/A*	P	P		**N**	P	P
360		**N**	*N/A*	P	P		**N**	P	P

Sample No.	Sample Area	*T. equi* PCR	*T. equi* ELISA	*T. equi* IFAT 1/40	*T. equi* IFAT 1/80		*B. caballi* PCR	*B. caballi* IFAT 1/40	*B. caballi* IFAT 1/80
361	Welgegund	P	*N/A*	P	P		**N**	P	P
362		P	*N/A*	P	P		**N**	P	P
363		**N**	*N/A*	P	P		**N**	P	P
364		**N**	*N/A*	P	P		**N**	P	P
365		**N**	*N/A*	P	*P?*		**N**	P	P
366	Hoogte	**N**	*N/A*	P	P		**N**	P	P
367		**N**	*N/A*	P	P		**N**	P	P
368		**N**	*N/A*	P	P		**N**	P	P
369		**N**	*N/A*	P	P		**N**	P	P
370		**N**	*N/A*	P	P		**N**	P	P
371		**N**	*N/A*	P	*P?*		**N**	P	P
372		P	*N/A*	P	*P?*		**N**	P	P
373		**N**	*N/A*	P	P		**N**	P	P
374		**N**	*N/A*	P	P		**N**	P	P
375		**N**	*N/A*	P	P		**N**	P	P
376		**N**	*N/A*	P	P?		**N**	*P?*	**N**
377		P	*N/A*	P	P		**N**	P	P
378	Zandfontein	**N**	*N/A*	*P?*	**N**		**N**	P	P
379		**N**	*N/A*	P	P		**N**	P	P
	Philippolis								
380	Philippolis	**N**	*N/A*	P	P		**N**	P	P
381		**N**	*N/A*	P	P		**N**	P	P
382		P	*N/A*	P	P		**N**	P	P
383		**N**	*N/A*	P	P		**N**	P	P
384		**N**	*N/A*	**N**	**N**		**N**	**N**	**N**
385		**N**	*N/A*	**N**	**N**		**N**	P	P
386		**N**	*N/A*	**N**	**N**		**N**	P	P
387		**N**	*N/A*	**N**	**N**		**N**	P	P
388		P	*N/A*	P	**N**		**N**	P	P
389		**N**	*N/A*	P	P		**N**	P	P
390		**N**	*N/A*	P	P		**N**	P	P
391		**N**	*N/A*	**N**	**N**		**N**	P	P
392		**N**	*N/A*	**N**	**N**		**N**	P	P
393		**N**	*N/A*	**N**	**N**		**N**	P	P
394		**N**	*N/A*	P	*P?*		**N**	P	P
395		**N**	*N/A*	P	*P?*		**N**	P	P
396		**N**	*N/A*	P	P		**N**	P	P
397		**N**	*N/A*	P	**N**		**N**	**N**	**N**
398		**N**	*N/A*	P	*P?*		**N**	*P?*	**N**
399		**N**	*N/A*	P	P		**N**	*P?*	**N**
400		**N**	*N/A*	**N**	**N**		**N**	P	P
401		**N**	*N/A*	**N**	**N**		**N**	P	**N**
402	Donkerpoort	**N**	*N/A*	P	*P?*		**N**	*P?*	**N**
403		**N**	*N/A*	P	*P?*		**N**	P	P
404		**N**	*N/A*	P	P		**N**	P	P
405		**N**	*N/A*	P	P		**N**	P	P
406		**N**	*N/A*	P	P		**N**	P	P

Sample No.	Sample Area	*T. equi* PCR	*T. equi* ELISA	*T. equi* IFAT 1/40	*T. equi* IFAT 1/80		*B. caballi* PCR	*B. caballi* IFAT 1/40	*B. caballi* IFAT 1/80
407	Donkerpoort	**N**	*N/A*	P	P		**N**	P	P
408		**N**	*N/A*	P	P		**N**	P	P
409		**N**	*N/A*	P	P		**N**	P	P
410		**N**	*N/A*	P	P		**N**	P	P
411		**N**	*N/A*	P	P		**N**	*P?*	**N**
412		**N**	*N/A*	P	P		**N**	**N**	**N**
413		**N**	*N/A*	P	P		**N**	P	P
	Faurismith								
414	Faurismith	**N**	*N/A*	P	P		**N**	P	P
415		**N**	*N/A*	P	P		**N**	P	P
416		**N**	*N/A*	P	P		**N**	P	P
417		**N**	*N/A*	P	P		**N**	P	P
418	River Side	**N**	*N/A*	P	P		**N**	P	**N**
419		**N**	*N/A*	P	**N**		**N**	*P?*	**N**
420		P	*N/A*	P	**N**		**N**	P	P
421		**N**	*N/A*	P	P		**N**	*P?*	**N**
422		**N**	*N/A*	P	P		**N**	P	P
423		P	*N/A*	P	P		**N**	**N**	**N**
424		**N**	*N/A*	P	P		**N**	*P?*	**N**
425		**N**	*N/A*	P	*P?*		**N**	**N**	**N**
426		**N**	*N/A*	P	P		**N**	**N**	**N**
427		**N**	*N/A*	P	P		**N**	**N**	**N**
428		**N**	*N/A*	P	P		**N**	P	P
429		**N**	*N/A*	**N**	**N**		**N**	P	P
430		**N**	*N/A*	P	P		**N**	P	P
431		**N**	*N/A*	P	P		**N**	P	P
432		**N**	*N/A*	P	*P?*		**N**	P	P
433		**N**	*N/A*	P	P		**N**	P	P
434	Brandfontein	**N**	*N/A*	P	P		**N**	P	P
435		P	*N/A*	P	**N**		**N**	P	P
436		P	*N/A*	P	*P?*		**N**	P	P
437		P	*N/A*	P	P		**N**	P	P
438		**N**	*N/A*	P	P		**N**	P	P
439		**N**	*N/A*	P	P		**N**	P	P
440		**N**	*N/A*	P	P		**N**	P	P
441		**N**	*N/A*	P	P		**N**	P	P
442		**N**	*N/A*	P	P		**N**	P	P
443		**N**	*N/A*	P	P		**N**	P	P
444		**N**	*N/A*	P	P		**N**	P	P

Appendix II: Table showing results from individual samples in KwaZulu Natal.

KwaZulu Natal									
		T. equi					*B. caballi*		
Sample No.	**Sample Area**	**PCR**	**ELISA**	**IFAT**			**PCR**	**IFAT**	
				1/40	**1/80**			**1/40**	**1/80**
	Hlabisa								
1		**N**	P	P	P		**N**	P	P
2		P	P	P	P		**N**	P	P
3		**N**	P	P	P		**N**	P	P
4		P	P	P	P		**N**	P	P
5		P	P	P	P		**N**	P	P
6		**N**	P	P	P		**N**	P	P
7		P	P	P	P		**N**	P	P
8		P	P	P	P		**N**	P	P
9		P	P	P	P		**N**	P	P
10		P	P	P	P		**N**	P	P
11		**N**	P	P	P		**N**	P	P
12		**N**	P	P	P		**N**	**N?**	**N**
13		**N**	P	P	P		**N**	**N**	**N**
14		**N**	P	P	P		**N**	**N**	**N**
15		**N**	P	P	P		**N**	**N**	**N**
16		P	P	P	P		**N**	**N**	**N**
17		P	P	P	P		**N**	**N**	**N**
18		P	P	P	P		**N**	**N**	**N**
19		P	P	P	P		**N**	**N**	**N**
20		P	P	P	P		**N**	**N**	**N**
	Mooi River								
21	Bloemendal	**N**	P	P	P		**N**	P	P
22		**N**	P	P	P		**N**	P	P
23		**N**	P	P	P		**N**	**N**	**N**
24		**N**	P	P	P		**N**	P	P
25		P	P	P	P		**N**	P	P
26		**N**	P	P	P		**N**	P	P
27		**N**	P	P	P		**N**	P	P
28		P	P	P	P		**N**	**N**	**N**
29		**N**	P	P	P		**N**	**N**	**N**
30		**N**	P	P	P		**N**	P	P
31		**N**	P	P	P		**N**	P	**N**
32	Mr Forest	**N**	P	P	P		**N**	**N**	**N**
33		**N**	P	P	P		**N**	**N**	**N**
34		P	P	P	P		**N**	**N**	**N**
35		**N**	P	P	P		**N**	P	P
36		**N**	P	P	P		**N**	P	**N**
37		**N**	P	P	P		**N**	**N**	**N**
38		P	P	P	P		**N**	**N**	**N**
39		P	P	P	P		**N**	**N**	**N**
40		P	P	P	P		**N**	P	P
41		**N**	**N**	P	P		**N**	**N**	**N**

Sample No.	Sample Area	*T. equi* PCR	*T. equi* ELISA	*T. equi* IFAT 1/40	*T. equi* IFAT 1/80		*B. caballi* PCR	*B. caballi* IFAT 1/40	*B. caballi* IFAT 1/80
42	Mr Forest	**N**	P	P	P		**N**	P	P
43		**N**	P	P	P		**N**	P	P
44		**N**	P	P	P		**N**	P	**N**
45		**N**	P	P	P		**N**	P	P
46	Ranches	**N**	P	P	P		**N**	P	P
47		P	P	P	P		**N**	**N**	**N**
48		**N**	P	P	P		**N**	**N**	**N**
49		**N**	P	P	P		**N**	P	P
50		**N**	P	P	P		**N**	P	P
51		P	P	P	P		**N**	P	P
52		P	P	P	P		**N**	P	P
53		P	P	P	P		**N**	P	P
54		P	P	P	P		**N**	P	P
55		P	**N**	P	P		**N**	P	P
56	Croyden	P	P	P	P		**N**	P	P
57		P	P	P	P		**N**	**N**	**N**
58		P	**N**	**N**	**N**		**N**	**N**	**N**
59		P	P	P	P		**N**	**N**	**N**
60		P	P	P	P		**N**	P	P
61		P	P	P	P		**N**	P	P
62		**N**	P	P	P		**N**	**N**	**N**
63		**N**	**N**	P	P		**N**	P	P
64		P	P	P	P		**N**	P	P
65		**N**	P	P	P		**N**	P	P
66		**N**	P	P	P		**N**	P	P
67		P	P	P	P		**N**	P	P
68		**N**	**N**	P	P		**N**	P?	**N**
69		P	P	P	P		**N**	**N**	**N**
70		P	P	P	P		**N**	**N**	**N**
	Vryhied								
71	Vaalkop	**N**	P	P	P		**N**	**N**	**N**
72		**N**	P	P	P		**N**	P	P
73		**N**	P	P	P		**N**	P	P
74		P	P	P	P		**N**	P	P
75		P	P	P	P		**N**	P	P
76		**N**	P	P	P		**N**	P	P
77		P	P	P	P		**N**	P	P
78		P	P	P	P		**N**	P	P
79		P	P	P	P		**N**	P	P
80		**N**	P	P	P		**N**	P	P
81	Goedverwacht	P	P	P	P		**N**	P	P
82		P	P	P	P		**N**	P	P
83		P	P	P	P		**N**	P	P
84		**N**	P	P	P		**N**	**N**	**N**
85		P	P	P	P		**N**	**N**	**N**
86		P	P	P	P		**N**	**N**	**N**
87		**N**	P	P	P		**N**	P	P

Sample No.	Sample Area	*T. equi*					*B. caballi*		
		PCR	**ELISA**	**IFAT**			**PCR**	**IFAT**	
				1/40	**1/80**			**1/40**	**1/80**
88	Goedverwacht	P	P	P	P		**N**	**N**	**N**
89		P	P	P	P		**N**	**N**	**N**
90		P	P	P	P		**N**	**N**	**N**

Appendix III: Aligned sequences of *Theileria equi* from study area and GenBank data base including an outgroup (*Babesia caballi*)

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Vryheid:         ----------------------------------------------------------------------------------------CATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTG
Phuthaditjhaba:  CGGGGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGASAAACGGCTACCACATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTG
Phuthaditjhaba:  CGGGGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTG
Sasolburg:       --GGGGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTG
Spain 3:         CGGGGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTG
Spain 2:         CGGGGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTG
Spain 1:         CGGGGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTG
South Africa:    CGGGGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTG
Mooi River:      CGGGGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTG
Sasolburg:       CGGGGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAWACGGCTACCACATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTG
Hluhluwe:        CGGGGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTG
Bloemfontein:    C--GGGAATTAGGGTTCGATTCCGGAGAGGGAGC--TGAGAAACGGCTACCACATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTG
Mooi River:      ------------------------------------------------------------GCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTG
Phuthaditjhaba:  ----------------------------------------------------------------------------------------------------------------------------TTACCCAATCCTG
Mooi River:      CGGGGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTG
Vryheid:         CGGGGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTG
Phuthaditjhaba:  ------------------------------------------------------------AGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTG
Bloemfontein:    CGGGGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTG
RBEQ101 WC:     CGGGGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTG
RBEQ178 EC:     CGGGGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTG
RBEQ105 WC:     CGGGGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTG
LFEQ47 EC:      CGGGGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAAAAACGGCTACCACATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTG
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RBEQ63:         CGGGGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTG
LFEQ189 GP:     CGGGGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTG
LFEQ178 GP:     CGGGGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTG
LFEQ23 NC:      CGGGGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTG
B. caballi:     CGGGGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTG
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Vryheid:         ACACAGGGAGGTAGTGACAAGAAATAACAATACGGGGCTTTAAGTCTTGTAATTGGAATGATGGGAATTTAAACCTCTTCCAGAGTATCAATT
Phuthaditjhaba:  ACACAGGGAGGTAGTGACAAGAAATAACAATACGGGGCTTTAAGTCTTGTAATTGGAATGATGGGAATTTAAACCTCTTCCAGAGTATCAATT
Phuthaditjhaba:  ACACAGGGAGGTAGTGACAAGAAATAACAATACGGGGCTTTAAGTCTTGTAATTGGAATGATGGGAATTTAAACCTCTTCCAGAGTATCAATT
Sasolburg:       ACACAGGGAGGTAGTGACAAGAAATAACAATACGGGGCTTGAAGTCTTGTAATTGGAATGATGGGAATTTAAACCCCTTCCAGAGTATCAATT
Spain 3:         ACACAGGGAGGTAGTGACAAGAAATAACAATACGGGGCTTGAAGTCTTGTAATTGGAATGATGGGAATTTAAACCCCTTCCAGAGTATCAATT
Spain 2:         ACACAGGGAGGTAGTGACAAGAAATAACAATACGGGGCTTGAAGTCTTGTAATTGGAATGATGGGAATCTAAACCCCTTCCAGAGTATCAATT
Spain 1:         ACACAGGGAGGTAGTGACAAGAAATAACAATACGGGGCTTGAAGTCTTGTAATTGGAATGATGGGAATCTAAACCCCTTCCAGAGTATCAATT
South Africa:    ACACAGGGAGGTAGTGACAAGAAATAACAATACGGGGCTTGAAGTCTTGTAATTGGAATGATGGGAATCTAAACCCCTTCCAGAGTATCAATT
Mooi River:      ACACAGGGAGGTAGTGACAAGAAATAACAATACGGGGCTTGAAGTCTTGTAATTGGAATGATGGGAATTTAAACCCCTTCCAGAGTATCAATT
Sasolburg:       ACACAGGGAGGTAGTGACAAGAAATAACAATACGGGGCTTGAAGTCTTGTAATTGGAATGATGGGAATTTAAACCCCTTCCAGAGTATCAATT
Hluhluwe:        ACACAGGGAGGTAGTGACAAGAAATAACAATACGGGGCTTTAAGTCTTGTAATTGGAATGATGGGAATTTAAACCTCTTCCAGAGTATCAATT
Bloemfontein:    ACACAGGGAGGTAGTGACAAGAAATAACAATACGGGGCTTGAAGTCTTGTAATTGGAATGATGGGAATTTAAACCCCTTCCAGAGTATCAATT
Mooi River:      ACACAGGGAGGTAGTGACAAGAAATAACAATACGGGGCTTTAAGTCTTGTAATTGGAATGATGGGAATTTAAACCTCTTCCAGAGTATCAATT
Phuthaditjhaba:  ACACAGGGAGGTAGTGACAAGAAATAACAATACGGGGCTTTAAGTCTTGTAATTGGAATGATGGGAATTTAAACCTCTTCCAGAGTATCAATT
Mooi River:      ACACAGGGAGGTAGTGACAAGAAATAACAATACGGGGCTTTAAGTCTTGTAATTGGAATGATGGGAATTTAAACCTCTTCCAGAGTATCAATT
Vryheid:         ACACAGGGAGGTAGTGACAAGAAATAACAATACGGGGCTTGAAGTCTTGTAATTGGAATGATGGGAATCTAAACCTCTTCCAGAGTATCAATT
Phuthaditjhaba:  ACACAGGGAGGTAGTGACAAGAAATAACAATACGGGGCTTTAAGTCTTGTAATTGGAATGATGGGAATTTAAACCTCTTCCAGAGTATCAATT
Bloemfontein:    ACACAGGGAGGTAGTGACAAGAAATAACAATACGGGGCTTGAAGTCTTGTAATTGGAATGATGGGAATYTAAACCCCTTCCAGAGTATCAATT
RBEQ101 WC:      ACACAGGGAGGTAGTGACAAGAAATAACAATACGGGGCTTGAAGTCTTGTAATTGGAATGATGGGAATTTAAACCCCTTCCAGAGTATCAATT
RBEQ178 EC:      ACACAGGGAGGTAGTGACAAGAAATAACAATACGGGGCTTGAAGTCTTGTAATTGGAATGATGGGAATCTAAACCCCTTCCAGAGTATCAATT
RBEQ105 WC:      ACACAGGGAGGTAGTGACAAGAAATAACAATACGGGGCTTTAAGTCTTGTAATTGGAATGATGGGAATTTAAACCCCTTCCAGAGTATCAATT
LFEQ47 EC:       ACACAGGGAGGTAGTGACAAGAAATAACAATACGGGGCTTTAAGTCTTGTAATTGGAATGATGGGAATTTAAACCTCTTCCAGAGTATCAATT
LFEQ164 GP:      ACACAGGGAGGTAGTGACAAGAAATAACAATACGGGGCTTTAAGTCTTGTAATTGGAATGATGGGAATTTAAACCTCTTCCAGAGTATCAATT
RBEQ63:          ACACAGGGAGGTAGTGACAAGAAATAACAATACGGGGCTTGAAGTCTTGTAATTGGAATGATGGGAATTTAAACCCCTTCCAGAGTATCAATT
LFEQ189 GP:      ACACAGGGAGGTAGTGACAAGAAATAACAATACGGGGCTTTAAGTCTTGTAATTGGAATGATGGGAATTTAAACCTCTTCCAGAGTATCAATT
LFEQ178 GP:      ACACAGGGAGGTAGTGACAAGAAATAACAATACGGGGCTTTAAGTCTTGTAATTGGAATGATGGGAATTTAAACCTCTTCCAGAGTATCAATT
LFEQ23 NC:       ACACAGGGAGGTAGTGACAAGAAATAACAATACGGGGCTTGAAGTCTTGTAATTGGAATGATGGGAATTTAAACCCCTTCCAGAGTATCAATT
B. caballi:      ACACAGGGAGGTAGTGACAAGAAATAACAATACAGGGCT-AATGTCTTGTAATTGGAATGATGGCGACTTAAACCCTCGCCAGAGTAACAATT
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Vryheid:        GGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAACTTGTTGCAGTTAAAAAGCTCGTAGTTGAATTTC
Phuthaditjhaba: GGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAACTTGTTGCAGTTAAAAAGCTCGTAGTTGAATTTC
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Sasolburg:      GGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAACTTGTTGCAGTTAAAAAGCTCGTAGTTGAATTTC
Spain 3:        GGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAACTTGTTGCAGTTAAAAAGCTCGTAGTTGAATTTC
Spain 2:        GGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAACTTGTTGCAGTTAAAAAGCTCGTAGTTGAATTTC
Spain 1:        GGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAACTTGTTGCAGTTAAAAAGCTCGTAGTTGAATTTC
South Africa:   GGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAACTTGTTGCAGTTAAAAAGCTCGTAGTTGAATTTC
Mooi River:     GGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAACTTGTTGCAGTTAAAAAGCTCGTAGTTGAATTTC
Sasolburg:      GGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAACTTGTTGCAGTTAAAAAGCTCGTAGTTGAATTTC
Hluhluwe:       GGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAACTTGTTGCAGTTAAAAAGCTCGTAGTTGAATTTC
Bloemfontein:   GGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAACTTGTTGCAGTTAAAAAGCTCGTAGTTGAATTTC
Mooi River:     GGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAACTTGTTGCAGTTAAAAAGCTCGTAGTTGAATTTC
Phuthaditjhaba: GGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAACTTGTTGCAGTTAAAAAGCTCGTAGTTGAATTTC
Mooi River:     GGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAACTTGTTGCAGTTAAAAAGCTCGTAGTTGAATTTC
Vryheid:        GGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAACTTGTTGCAGTTAAAAAGCTCGTAGTTGAATTTC
Phuthaditjhaba: GGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAACTTGTTGCAGTTAAAAAGCTCGTAGTTGAATTTC
Bloemfontein:   GGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAACTTGTTGCAGTTAAAAAGCTCGTAGTTGAATTTC
RBEQ101 WC:    GGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAACTTGTTGCAGTTAAAAAGCTCGTAGTTGAATTTC
RBEQ178 EC:    GGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAACTTGTTGCAGTTAAAAAGCTCGTAGTTGAATTTC
RBEQ105 WC:    GGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAACTTGTTGCAGTTAAAAAGCTCGTAGTTGAATTTC
LFEQ47 EC:     GGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAACTTGTTGCAGTTAAAAAGCTCGTAGTTGAATTTC
LFEQ164 EC:    GGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAACTTGTTGCAGTTAAAAAGCTCGTAGTTGAATTTC
RBEQ63:        GGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAACTTGTTGCAGTTAAAAAGCTCGTAGTTGAATTTC
LFEQ189 GP:    GGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAACTTGTTGCAGTTAAAAAGCTCGTAGTTGAATTTC
LFEQ178 GP:    GGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAACTTGTTGCAGTTAAAAAGCTCGTAGTTGAATTTC
LFEQ23 NC:     GGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAACTTGTTGCAGTTAAAAAGCTCGTAGTTGAATTTC
B. caballi:    GGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAACTTGTTGCAGTTAAAAAGCTCGTAGTTGAATTTC
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Vryheid:         TGCTGTATCGTTATCTTCTGCTT-GACAGTTTGATTTCGTTATGGCTTAGTTG---GGTCACTT------TGTGTCCCGGCGTTTACTTTGAGAAAATTA
Phuthaditjhaba:  TGCTGTATCGTTATCTTCTGCTT-GACAGTTTGATTTCGTTATGGCTTAGTTG---GGTCACTT------TGTGTCCCGGCGTTTACTTTGAGAAAATTA
Phuthaditjhaba:  TGCTGTATCGTTATCTTCTGCTT-GACAGTTTGATTTCGTTATGGCTTAGTTG---GGTCACTT------TGTGTCCCGGCGTTTACTTTGAGAAAATTA
Sasolburg:       TGCTGTTTCGTTGACTGC-GTTT-GGC--GTTTGTCATCGTTGCGGCTTGGTTGGGTTTCGATTATTCGTTTCCCGGCGTTTACTTTGAGAAAATTA
Spain 3:         TGCTGCATCGTGGTTCTTCGCTATGTCGAGTGATCTTCGTTGTGGCTTAGTTG--GGGCATGTTTTCATGACTCGACGTTTACTTTGAGAAAATTA
Spain 2:         TGCTGTTTCGTTGACTGC-GTTT-GGC-GTTT-GTCAT--GTTGCGGCTTGGTTGGGTTTCGATTATTCGTTTCCCGGCGTTTACTTTGAGAAAATTA
Spain 1:         TGCTGTTTCGTTGACTGC-GCTT-GGC-GTTTGTCAT-CGTTGCGGCTTGGTTGGGTTTCGATTATTCGTCTCCCGGCGTTTACTTTGAGAAAATTA
South Africa:    TGCTGTTTCGTTGACTGC-GCTT-GGC-GTTTGTCAT-CGTTGCGGCTTGGTTGGGTTTCGATTATTCGTTTCCCGGCGTTTACTTTGAGAAAATTA
Mooi River:      TGCTGTTTCGTTGA-T-TT-ACTT-GGT-GTTTGTCAT-CGTTGCGGCTTGGTTGGGTTTCGATTATTCGTTTCCCGGCGTTTACTTTGAGAAAATTA
Sasolburg:       TGCTGTTTCGTTGACTGC-GTTT-GGC--GTTTGTCATCGTT-----GC--TGGTTGGGTTTCGATTATTTGTTTCCCGGCGTTTACTTTGAGAAAATTA
Hluhluwe:        TGCTGTATCGTTATCTTCTGCTT-GACAGTTTGATTTCGTTATGGCTTAGTTGGGT—C--ATT-----TGTGTCCCGGCGTTTACTTTGAGAAAATTA
Bloemfontein:    TGCTGTTTCGTTGACTGC-GTTT-GGC-GTTTGTCATCGTTGCGGCTTGGTTGGGTTTCGATTATTCGTTTCCCGGCGTTTACTTTGAGAAAATTA
Mooi River:      TGCTGTATCGTTATCTTCTGCTT-GACAGTTTGATTTCGTTATGGCTTAGTTGGGT---CACTT-----TGTGTCCCGGCGTTTACTTTGAGAAAATTA
Phuthaditjhaba:  TGCTGTTTCGTTATCTTCTGCTT-GAC-------TCATTTCGTTATGGCTGAGTTGTTGGGACAATT--TGTGTCCCGGCGTTTACTTTGAGAAAATTA
Mooi River:      TGCTGTATCGTTATCTTCTGCTT-GACAGTTTGATTTCGTTATGGCTTAGTTG-----GGTCACTT--TGTGTCCCGGCGTTTACTTTGAGAAAATTA
Vryheid:         TGCTGTATCGTTT----------TTTGTTTCCCGGCGTTTACTTTGAGAAAATTA
Phuthaditjhaba:  TGCTGTATCGTTATCTTCTGCTT-GACAGTTTGATTTCGTTATGGCTTAGTTG------GGTCACTT--TGTGTCCCGGCGTTTACTTTGAGAAAATTA
Bloemfontein:    TGCTGTTTCGTTGACTGC-GTTT-GGC-GTTTGTCATCGTTGCGGCTTGGTTGGGTTTCGATTATTCGTTTCCCGGCGTTTACTTTGAGAAAATTA
RBEQ101 WC:     TGCTGCATCGTGGTCCTTCGCTATGTCGAGTGGTCCTCGTTGTGGCTTAGTTG-GGGCATGTTTTCATGACTCGACGTTTACTTTGAGAAAATTA
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LFEQ47 EC:      TGCTGTATCGTTATCTTCTGCTT-GACAGTTTGATTTCGTTATGGCTTAGTTG---GGTCACTT—TGTGTCCCGG----CGTTTACTTTGAGAAAATTA
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LFEQ178 GP:     TGCTGTATCGTTATCTTCTGCTT-GACAGTTTGGTATCGTTATGGCTT-GTTG---GGTCACAT—TGTGTCCCGG----CGTTTACTTTGAGAAAATTA
LFEQ23 NC:      TGCTGTTTCGTTGACTGC-GTTT-GGC-GTTTGTCATCGTTGCGGCTTGGTTGGGTTTCGATTATTCGTTTCCCGG--CGTTTACTTTGAGAAAATTA
B. caballi:     TGC-GTTGCGTTGTTCTT--GCTT----------TTTGCTTGATTTTCG--CTTCGCT----------TTTGTT----------TTTACTTTGAGAAAATTA
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B. caballi:      ATGGTTAATAGGAACGGTTGGGGGCATTCGTATTTAACTGTCAGAGGTGAAATTCTTAGATTTGTTAAAGACGAACTACTGCGAAAGCATTTG
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RBEQ63:         ACTTAAAGGAATTGACGGAAGGG-CACCACCAGGCGTGGAGCCTGCGGCTTAATTTGACTCAACACGGGGAAACTCACCAGGTCCAGACAGA
LFEQ189 GP:     ACTTAAAGGAATTGACGGAAGGG-CACCACCAGGCGTGGAGCCTGCGGCTTAATTTGACTCAACACGGGGAAACTCACCAGGTCCAGACAGA
LFEQ178 GP:     ACTTAAAGGAATTGACGGAAGGG-CACCACCAGGCGTGGAGCCTGCGGCTTAATTTGACTCAACACGGGGAAACTCACCAGGTCCAGACAGA
LFEQ23 NC:      ACTTAAAGGAATTGACGGAAGGG-CACCACCAGGCGTGGAGCCTGCGGCTTAATTTGACTCAACACGGGGAAACTCACCAGGTCCAGACAGA
B. caballi:     ACTTAAAGGAATTGACGGAAGGG-CACCACCAGGCGTGGAGCCTGCGGCTTAATTTGACTCAACACGGGGAAACTCACCAGGTCCAGACAGA
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Phuthaditjhaba: GGAAGGATTGACAGATTGATAGCTCTTTCTTGATTCTTTGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATTTGTCTGGTTAATTCC
Vryheid:        GGAAGGATTGACAGATTGATAGCTCTTTCTTGATTCTTTGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATTTGTCTGG----------
Phuthaditjhaba: GGAAGGATTGACAGATTGATAGCTCTTTCTTGATTCTTTGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATTTGTCTGGTTAATTCC
Phuthaditjhaba: GGAAGGATTGACAGATTGATAGCTCTTTCTTGATTCTTTGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATTTGTCTGGTTAATTCC
Sasolburg:      GGAAGGATTGACAGATTGATGGCTCTTTCTTGATTCTTTGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATTTGTCTGGTTAATTCC
Spain 3:        GGAAGGATTGACAGATTGATAGCTCTTTCTTGATTCTTTGGGTGGTGGTGCATGGCCGTTCTTATTTGGTGGAGTGATTTGCCTGGTTAATTCC
Spain 2:        GGAAGGATTGACAGATTGATAGCTCTTTCTTGATTCTTTGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATTTGTCTGGTTAATTCC
Spain 1:        GGAAGGATTGACAGATTGATAGCTCTTTCTTGATTCTTTGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATTTGTCTGGTTAATTCC
South Africa:   GGAAGGATTGACAGATTGATAGCTCTTTCTTGATTCTTTGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATTTGTCTGGTTAATTCC
Mooi River:     GGAAGGATTGACAGATTGATAGCTCTTTCTTGATTCTTTGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATTTGTCTGGTTAATTCC
Sasolburg:      GGAAGGATTGACAGATTGATRGCTCTTTCTTGATTCTTTGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATTTGTCTGGTTAATTCC
Hluhluwe:       GGAAGGATTGACAGATTGATAGCTCTTTCTTGATTCTTTGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATTTGTCTGGTTAATTCC
Bloemfontein:   GGAAGGATTGACAGATTGATGGCTCTTTCTTGATTCTTTGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTG-AGTGATTTGTCTGGTTAATTCC
Mooi River:     GGAAGGATTGACAGATTGATAGCTCTTTCTTGATTCTTTGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATTTGTCTGGTTAATTCC
Phuthaditjhaba: GGAAGGATTGACAGATTGATAGCTCTTTCTTGATTCTTTGGGTGGTGGT----------------------------------------------------------------------------------
Mooi River:     GGAAGGATTGACAGATTGATAGCTCTTTCTTGATTCTTTGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATTTGTCTGGTTAATTCC
Vryheid:        GGAAGGATTGACAGATTGATAGCTCTTTCTTGATTCTTTGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATTTGTCTGGTTAATTCC
Phuthaditjhaba: GGAAGGATTGACAGATTGATAGCTCTTTCTTGATTCTTTGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATTTGTCTGGTTAATTCC
Bloemfontein:   GGAAGGATTGACAGATTGATGGCTCTTTCTTGATTCTTTGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATTTGTCTGGTTAATTCC
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LFEQ47 EC:     GGAAGGATTGACAGATTGATAGCTCTTTCTTGATTCTTTGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATTTGTCTGGTTAATTCC
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LFEQ189 GP:    GGAAGGATTGACAGATTGATAGCTCTTTCTTGATTCTTTGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATTTGTCTGGTTAATTCC
LFEQ178 GP:    GGAAGGATTGACAGATTGATGGCTCTTTCTTGATTCTTTGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATTTGTCTGGTTAATTCC
LFEQ23 NC:     GGAAGGATTGACAGATTGATGGCTCTTTCTTGATTCTTTGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATTTGTCTGGTTAATTCC
B. caballi:    GGTAGGATTGACAGATTGATAGCTCTTTCTTGATTCTTTGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATTTGTCTGGTTAATTCC
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Phuthaditjhaba: GTTAACGAACGAGACCTTAACCTGCTAAATAGGGTGTTGGA
Vryheid:        ---------------------------------------------------------------------------------
Phuthaditjhaba: GTTAARGAACGAGACCTTAACCTGCTAAATAGGGTGTTGGA
Phuthaditjhaba: GTTAACGAACGAGACCTTAACCTGCTAAATAGGGTGTTGGA
Sasolburg:      GTTAACGAACGAGACCTTAACCTGCTAAATAGGGTGTGAGA
Spain 3:        GCTAACGAACGACACCTTAACCTGCTAAATAGGATGCGAGA
Spain 2:        GTTAACGAACGAGACCTTAACCTGCTAAATAGGGTGTGAGA
Spain 1:        GTTAACGAACGAGACCTTAACCTGCTAAATAGGGTGTGAGA
South Africa:   GTTAACGAACGAGACCTTAACCTGCTAAATAGGGTGTGAGA
Mooi River:     GTTAACGAACGAGACCTTAACCTGCTAAATAGGGTGTGAGA
Sasolburg:      GTTA--CGAACGAGACCTTAACCTGCTAAATAGGGTGTGAGA
Hluhluwe:       GTTAACGAACGAGACCTTAACCTGCTAAATAGGGTGTTGGA
Bloemfontein:   GTTAACGAACGAGACCTTAACCTGCTAA-TAGGGTGTGAGA
Mooi River:     GTTAAC----------------------------------------------------------------------
Phuthaditjhaba: ---------------------------------------------------------------------------------
Mooi River:     GTTAASGAACGAGACCTTAACCTGCTAAATAGGGTGTTGGA
Vryheid:        GTTAACGAACGAGACCTTAACCTGCTAAATAGGGTGT--------
Phuthaditjhaba: GTTAACGAACGAGA----------------------------------------------------
Bloemfontein:   GTTAACGAACGAGACCTTAACCTGCTAAATAGGGTGTGAGA
RBEQ101 WC:    GTTAACGAACGAGACCTTAACCTGCTAAATAGGATGTGAGA
RBEQ178 EC:    GTTAACGAACGAGACCTTAACCTGCTAAATAGGGTGTGAGA
RBEQ105 WC:    GTTAACGAACGAGACCTTAACCTGCTAAATAGGGTGTTGGA
LFEQ47 EC:     GTTAACGAACGAGACCTTAACCTGCTAAATAGGGTGTTGGA
LFEQ164 GP:    GTTAACGAACGAGACCTTAACCTGCTAAATAGGGTGTTGGA
RNEQ63:        GTTAACGAACGAGACCTTAACCTGCTAAATAGGGTGTGAGA
LFEQ189 GP:    GTTAACGAACGAGACCTTAACCTGCTAAATAGGGTGTTGGA
LFEQ178:       GTTAACGAACGAGACCTTAACCTGCTAAATAGGGTGTTGGA
LFEQ23 NC:     GTTAACGAACGAGACCTTAACCTGCTAAATAGGGTGTGAGA
B. caballi:    GTTAACGAACGAGACCTTAACCTGCTAACTAGCTTCCCTTT
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