Molecular epidemiology of dourine, equine piroplasmosis and ehrlichiosis from donkeys and horses in South Africa

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DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my original work and that I have not previously in its entirety or in part submitted at any university for a degree. I furthermore cede copyright of the dissertation in favour of the North−West University.

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DEDICATION

To my family, friends, nephews, nieces and my daughter Rethabile Mlangeni. I thank you all for the support and for all you went through, especially my mother Jane Mlangeni and my brother Shadrack Mlangeni.

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ABSTRACT

Horses and donkeys are important domestic animals to human beings whereby historically and presently are used for transport and as drought animals. Furthermore, horses are used in different sporting types, and are important animals in police and military services. Therefore their health is of great importance.

Dourine is caused by *Trypanosoma equiperdum* and is the only trypanosome species that is transmitted sexually from infected to healthy animals, and to foals during birth and from maternal milk. Dourine is known to be present in South Africa with Eastern Cape being the most severely affected province whilst Western Cape, Free State and Limpopo have a comparatively low report case incidents. In addition to observation of clinical signs, laboratory diagnosis of dourine is done only by a serological technique called complement fixation test (CFT) in South Africa. In this study Polymerase chain reaction (PCR) and Loop−mediated isothermal amplification (LAMP) as well as Enzyme immune sorbent assay (ELISA) and Immunochromatographic test (ICT) were used to determine the occurrence of dourine in horses and donkeys in South Africa. The general aim of this study was to determine the occurrence of dourine, equine piroplasmosis, anaplamosis and erhlichiosis from horses and donkeys in provinces of South Africa.

In this study, a total of 256 blood samples were collected from equids (32 from donkeys and 224 from horses) from four provinces in South Africa, namely, Free State (FS), Mpumalanga (MP), Northern Cape (NC) and North West (NW). Out of 256 DNA samples screened by PCR, there was an overall prevalence of 14% (36/256) for dourine, with 15% [95% CI = 0.17± 0.29], 17% [95% CI = 1.61 ± 0.33], 11% [95% CI = 0.17 ± 0.21], and 12% [95% CI = 0.22 ± 0.23] for FS (n = 40), MP (n = 94), NC (n = 54) and NW (n = 68). LAMP was used to confirm that PCR positive samples are true positives. PCR positive samples were also sequenced and they matched with other *T. equiperdum* sequences on NCBI database. A phylogenetic tree constructed with 18S rRNA gene, the *T. equiperdum* correctly clustered with other trypanosomes of subgenus *Trypanozoon*.

There were 38 genital secretion samples collected by sterile swab in horses in the Free State province and only 1 sample was positive by PCR for the presence of *T. equiperdum* infections. The overall *T. equiperdum* prevalence by PCR was 8.8% (3/34) with 8.3% (1/12) and 10% (2/20) in NC and NW provinces in donkeys. There was no *T. equiperdum* detected in FS province.

The overall seroprevalence of dourine for all sampled equids was 18.4%, 15.6% and 2.4% by recombinant antigen ELISA (rELISA), crude antigen ELISA (caELISA) and ICT respectively. The seroprevalence was 17.6%, 16.6% and 2.8% by rELISA, caELISA and ICT respectively in horses. The seroprevalence was 23.5%, 8.8% and 0% in donkeys by rELISA, caELISA and ICT respectively. Polymerase chain reaction efficiently detected *T. equiperdum* infections as confirmed by sequencing and rELISA revealed higher detection sensitivity than caELISA. The detection efficiency of ICT for dourine was poor, and needs further improvements.

Equine piroplasmosis is one of the most important tick−borne diseases, with an economic worldwide impact on the horse industry and is endemic in equids in most of tropical and sub−tropical regions of the world where tick vectors are present. The disease is caused by the two hemoprotozoan parasites, *Theileria equi* and *Babesia caballi*. In South Africa information on the occurrence of equine piroplasmosis based on IFAT and PCR methods is available and recently there is a report based on real time PCR in some provinces such as Free State, Northern Cape, Western Cape, Eastern and KwaZulu−Natal. However there are no records obtained using modern molecular techniques for equine piroplasmosis in other provinces including Mpumalanga and North West. Furthermore, the disease prevalence in other equids such as donkeys in South Africa is not documented. Therefore this study used PCR to detect *T. equi* and *B. caballi* infections from blood samples (n = 256) collected from four Provinces of South Africa, namely, FS; MP, NC and NW.

The prevalence obtained by PCR for *T. equi* in horses for FS, MP, NC and NW was 35% [95% CI = 0.39 ± 0.31], 14.9% [95% CI = 0.39 ± 0.29], 7% [95% CI = 0.11 ± 0.14] and 6% [95% CI = 0.11 ± 0.11] respectively. The $\chi^2 = 19.83$ (df = 3) and $p > 0.05$. *Babesia caballi* prevalence in horses was 22.5% [95% CI = 0.43 ± 042], 5.3% [95% CI = 0.45 ± 0.10], 5.6% [95% CI = 0.15 ± 0.10] and 4.4% [0.15 ± 0.08] respectively [$\chi^2 = 16.35$ (df = 3) and $p > 0.05$]. Therefore there is a significant difference observed in the overall prevalence of *T. equi* and *B. caballi* in the sampled provinces. Loop mediated

isothermal amplification (LAMP) was used to confirm PCR positives. *Theileria equi* (35%) infections were more prevalent than *B. caballi* (8.2%) infections. In this study, *B. caballi* infections were detected in areas where it was not found by PCR previously. *Theileria equi* and *B. caballi* parasites appeared to be more prevalent in Free State, 35% (14/40) and 22.9% (9/40), respectively. Horses (14.9%) were more susceptible than donkeys (5.9%) for equine piroplasmosis.

Anaplasmosis and ehrlichisosis are tick−borne diseases caused by obligate intracellular bacteria of the genera *Ehrlichia* and *Anaplasma*. These organisms are widespread in nature and the reservoir hosts include numerous wild animals, as well as some domesticated species. In the past decade, ehrlichiosis has been recognized as a new zoonotic disease that is responsible for several thousand cases and several deaths annually. There is currently no information on the prevalence of equine anaplasmosis and ehrlichiosis in South Africa. The current study was aimed at determining the occurrence of equine anaplasmosis and ehrlichiosis by PCR in South African equids. In particular the study focused on detection of *Anaplasma phagocytophilum* and *Neorickettsia risticii* across the four sampled provinces.

Prevalence of *A. phagocytophilum* infections obtained was 68.5% [95% CI = 0.16 ± 0.04 in NC, 52.5% [95% CI = 0.24 ± 1.03] in FS, 20.6% [95% CI = 0.43 ± 1.43] in NW and 16.0% [95% CI = 0.17 ± 0.31] in MP {χ^2 = 57.37 (df = 3) and p < 0.05}. This was for the first time that *A. phagocytophilum* was detected in horses in selected provinces of South Africa using PCR.

The *N. risticii* prevalence in horses was 12.5% [95% CI = 0.32 ± 0.22], 3.2% [95% CI = 0.33 ± 0.02] and 1.9% [95% CI = 0.11 ± 0.03] for FS, MP and NC respectively. The χ^2 = 12.42 (df = 3) and p >0.05. There is a significant difference observed across the sampled provinces. None of the DNA samples from donkeys (*Equus asinus*) tested positive for *N. risticii* across all four sampled provinces. This support the fact that equine monocytic ehrlichiosis is the disease that is found in horses only.

Key words: *Anaplasma phagocytophilum, Babesia caballi, Neorickettsia risticii*, *Trypanosoma equiperdum*, PCR and ICT.

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ABBREVATIONS

µl: Microliter

AAT: Animal African Trypanosomiasis

BSA: Bovine Serum Albumin

BLAST: Basic Local Alignment Search Tool

cELISA: competitive−Inhibition Enzyme−Linked Immunosorbent Assay

caELISA: Crude Antigen Enzyme−Linked Immunosorbent Assay

CFT: Complement Fixation Test

DDW: Double Distilled Water

DDH_2O: Double Distilled Water

DNA: Deoxynucleic Acid

DNTPs: Deoxynucleotide Triphosphates

EDTA: Ethylenediaminetetraacetic Acid

EGA: Equine Granulocytic Anaplasmosis

EME: Equine Monocytic Ehrlichiosis

ELISA: Enzyme−Linked Immunosorbent Assay

EtOH: Ethanol

FS: Free State Province

HAT: Human African Trypanosomiasis

ICT: Immunochromatographic Test

IFAT: Indirect Fluorescent Antibody Assay

LAMP: Loop−Mediated Isothermal Amplification

MP: Mpumalanga Province

NaOAC: Sodium Acetate

NCBI: National Center for Biotechnology Information

NC: Northern Cape Province

NTC: No Template Control

NW: North West Province

OD: Optical Density

PCR: Polymerase Chain Reaction

PBS: Phosphate Buffered Saline

PBS−T: Phosphate Buffered Saline with Tween

rELISA: Recombinant Enzyme−Linked Immunosorbent Assay

RIME: Repetitive Insertion Mobile Element

RPM: Revolutions Per Minute

RT: Room Temperature

TAE: Tris−Acetate EDTA

TMB: Tetramethylbenzidine

CHAPTER 1

GENERAL INTRODUCTION

1.1. Dourine

Dourine is a chronic or acute contagious, venereal disease of horses and other equids (Clausen *et al.,* 2003; OIE, 2013; Hagos *et al.,* 2010a), which is caused by *Trypanosoma equiperdum* which is transmitted directly from animal to animal during sexual intercourse (Clausen *et al.,* 2003; Claes *et al.,* 2005; Hagos *et al.,* 2010a; Calistri *et al.,* 2013; Luciani *et al.,* 2013). Neurological signs, emaciation, and high mortality rates can result in this protozoal infection. *Trypanosoma equiperdum* does not survive very long outside its host and is not transmitted by fomites, therefore, parameters associated with resistance to physical and chemical actions (*i.e.* temperature, chemical/disinfectants, and environmental survival) are not meaningful (OIE, 2009). It is widely distributed in Africa, Asia; parts of Europe and Mexico, even though the cases of this disease have not been reported in various countries for years (Ricketts and McGladdery, 2011). Dourine may also exist in some areas where testing is not done, (OIE, 2009). Identification of dourine is a challenge, due to limited information about the parasite and host−parasite interaction following infection (Luciani *et al.,* 2013).

1.2. Equine piroplamosis

Equine piroplasmosis is one of the most significant tick−borne diseases, with an economic effect worldwide on horse production (Zobba *et al.,* 2008). The disease is caused by the two hemaprotozoan parasites, *Theileria equi* (formerly *Babesia equi*) and *Babesia caballi* (Rampersad *et al.,* 2003; Motloang *et al.,* 2008; Zobba *et al.,* 2008) which belong to the phylum Apicomplexa, order Piroplasmida (Zobba *et al.,* 2008) and is found in many wild and domestic animals. According to Piantedosi *et al.,* (2014), these parasites are spread from host to host via tick vectors and varied infections with both organisms has been frequently reported in equids. Approximately 14 species of Ixodid ticks of the genera *Dermacentor, Rhipicephalus*, and *Hyalomma* (Bhoora *et al.,* 2010a; Zobba *et al.,* 2008) are capable of transmitting *T. equi and B. caballi* (Zobba *et al.,* 2008). Ticks are well known vectors of protozoan, bacterial and viral diseases to

livestock. According to Piantedosi *et al.,* (2014), ticks represent a major risk of infections, and are recognised as one of the most economically significant parasites threatening horse industry worldwide. The disease is widespread in horse populations and is found in most tropical and sub−tropical areas of the world (Bhoora *et al.,* 2010b; Piantedosi *et al.,* 2014) where tick vectors are present (Zobba *et al.,* 2008) and temperate parts can be affected as well (Piantedosi *et al.,* 2014). A tick vector transmit the parasite from an infected to an uninfected horse. Transmission by simple contact is not conceivable (Brooks *et al.,* 1996). Both *B. caballi* and *T. equi* are infectious and capable of causing a disease in horses that is characterized by progressive anaemia, fever, icterus, and hepato− and splenomegaly (Kim *et al.,* 2008; Bhoora *et al.,* 2010a). Horses may remain life−long carriers when infected with *T. equi* parasites however *B. caballi* parasites, which are self−limiting, horses persist to be carriers for up to four years (Bhoora *et al.,* 2009), and act as a source of infection for ticks (Motloang *et al.,* 2008; OIE, 2008) which in turn act as vectors of the disease (Motloang *et al.,* 2008). The clinical signs are frequently variable and generic making it easy to complicate the disease with other conditions, thus complicating diagnosis. It is not possible to distinguish between *B. caballi* and *T. equi* infections based on clinical manifestations alone (Bhoora *et al.,* 2009). Several studies have documented mixed infections of *T. equi* and *B. caballi* (Bhoora *et al.,* 2009; Malekifard *et al.,* 2014). In South Africa, piroplasmosis in horses was first reported during the 19th century when it was initially described as ‘anthrax fever’, biliary fever, a bilious form of African horse sickness. In West Africa it was known as equine malaria, because the clinical signs of the hemoparasitic infection observed in equids were comparable to malaria infections (plasmodiidae) found in humans, hence it was referred to as equine malaria (Deepak *et al.,* 2014).

1.3. Anaplasmosis and ehrlichiosis

Ehrlichiosis and anaplasmosis are caused by members of the genera *Ehrlichia* and *Anaplasma*, respectively. *Ehrlichia* and *Anaplasma* genera contain small, pleomorphic, Gram negative, obligate intracellular (McQuiston *et al.,* 2003; OIE, 2013a) bacteria that reside and reproduce in membrane−bound vacuoles of eukaryotic cells. They belong to the family Anaplasmataceae, order Rickettsiales (McQuiston *et al.,* 2003) and they are categorized as alpha−proteobacteria. A number of *Ehrlichia* and *Anaplasma* species

affect animals. A limited number of these organisms have also been recognised in people (OIE, 2013a). According to Doudier *et al.,* (2010), most of the ehrlichioses and anaplasmoses are tick−borne zoonoses. Their agents are maintained in nature through enzootic ticks and wild and domestic animals. Mammals seem to play a major role in proliferation and as reservoir of these pathogens. Transovarial transmission is inefficient in ticks. Depending on the continent the following ticks: *Ixodes* spp.*, Dermacentor* spp.*, Rhipicephalus* spp.*, Hyalomma* spp. and *Haemaphysalis* spp. are vectors of *A. phagocytophilum* (Dzięgiel *et al.,* 2013).

Ehrlichiosis is a group of diseases, generally named according to the host species and the type of white blood cell most frequently infected. Canine monocytic ehrlichiosis is caused by *Ehrlichia canis* and, irregularly, *E. chaffeensis*. Canine granulocytic ehrlichiosis is caused by *Anaplasma phagocytophilum* and *E. ewingii*. *Anaplasma phagocytophilum* also causes a tick−borne fever, a disease of ruminants and Equine granulocytic ehrlichiosis (which is now called equine granulocytic anaplasmosis). Equine monocytic ehrlichiosis or Potomac horse fever is caused by *Neorickettsia risticii* (formerly *Ehrlichia risticii*) (Barlough *et al.,* 1998; Park *et al.,* 2003; Pusterla *et al.,* 2003; OIE, 2005; Ferrão *et al.,* 2007; Cicuttin *et al.,* 2013). Human monocytic ehrlichiosis is caused by *E. chaffeensis* and *E. ewingii*. Human granulocytic ehrlichiosis is caused by *A. phagocytophilumi* (OIE, 2005; Dumler *et al.,* 2005b; Rymaszewska and Grenda, 2008). Sennetsu fever is caused by *Neorickettsia sennetsu* (formerly *Ehrlichia sennetsu*) (OIE, 2005). Phylogenetic studies revealed taxonomic disorder amongst organisms broadly referred to as ehrlichiae during the process of classification of the human agent, and a careful reorganization now places those bacteria previously classified as *E*. *phagocytophila*, *E*. *equi*, and the HGE agent into a different genus as a single species, *A*. *phagocytophilum* (Dumler, 2005a).The diseases initiated by these pathogens have traditionally been characterized by the type of blood cell most usually infected. For instance, *E chaffeensis* and *E canis* reside mainly in monocytes, and the disease caused by these agents is normally called monocytic (or monocytotropic) ehrlichiosis. The infection is self−limiting in most horses, even though death can happen (McQuiston *et al.,* 2003). Table 1.1 below summarizes the cause of disease, mode of transmission, vectors and reservoir host.

Table 1.1: Venereal, tick−borne, bacterial and parasitic diseases, and their vectors and hosts

Disease	Causative agents	Arthropod vector	Transmission	Host	References
Dourine	*Trypanosoma equiperdum*	None	Venereal	Horses, donkeys, mules and zebras	(Brun *et al.,* 1998)
Equine piroplasmosis	*Babesia caballi*	*Rhipicephelus Hyalomma Dermacentor*	Transovarial and Transstadial (Nymph to adult)	Horses, donkeys, mules and zebras	(Uilenberg, 2006; Motloang *et al.,* 2008; Rothschild, 2013). (Bashirudinn *et al.,* 1999)
	Theileria equi		Transstadial		
Equine anaplasmosis	*Anaplasma phygocytophilum*	*Ixodes* spp.	Transstadial	Domestic animals, including horses and wild animals	(McQuiston *et al.,* 2003).
Equine monocytic ehrlichiosis	*Neorickettsia risticii*	Unidentified	Vertical and horizontal	Horses	(Greiman *et al.,* 2013)

CHAPTER 2

OBJECTIVES OF THE STUDY

2.1 Statement of the problem

Throughout the world, the one common factor leading to ill health, suffering and early demise of equines is the protozoan parasite, *Trypanosoma equiperdum* (Hagos *et al.,* 2010a). According to Clausen *et al.,* (2003) and Gillingwater *et al.,* (2007), *T. equiperdum* causes a venereal disease called Dourine in horses and other equids (OIE, 2009; Ricketts and McGladdery, 2011; Luckins *et al.,* 2004) and is morphologically indistinguishable to other *Trypanozoon* species (Brun *et al.,* 1998). Dourine was once widespread, but it has been eradicated in many countries (OIE, 2009; Ricketts and McGladdery, 2011). Currently, the disease is endemic in parts of Africa and parts of Asia including Russia (Brun *et al.,* 1998; OIE, 2009; OIE, 2013; Ricketts and McGladdery, 2011). Different approaches such as, Coplement fixation test (CFT) as the prescribed test for international trade, Enzyme linked immunosorbent assays (ELISAs) and agar gel immunodiffusion tests (AGID), have been employed to detect *T. brucei, T. equiperdum* and *T. evansi* infections (Brun *et al.,* 1998; Ricketts and McGladdery, 2011). However, there are no effective treatments or control methods that have been found yet. Based on the Department of Agriculture, Forestry and Fisheries (DAFF) data, dourine is known to be present in South Africa, and Eastern Cape is the most severely affected province, which had 708 cases between 2000 and 2010. The Western Cape, Free State and Limpopo have a comparatively low reported case incidents. In addition to observation of clinical signs, laboratory diagnosis of dourine is done only by serological technique called complement fixation test in South Africa (Epidemiology report, 2012).

Babesia caballi and *Theileria equi* parasites cause the disease called equine piroplamosis, which may be either acute or chronic with mortalities ranging from less than 10% up to 50%. Diagnosis of equine piroplasmosis relies on microscopic examination, serological assays such as complement fixation test (CFT), indirect fluorescent antibody test (IFAT), competitive−inhibition Enzyme−linked immunosorbent assay (cELISA), and molecular tools (Kim *et al.,* 2008). There are no drug therapies or vaccines currently available for the complete prevention or eradication of *T. equi* and *B.*

caballi infections. High international sero−prevalence suggests infection is widespread but unrecognized. In South Africa there is information on the occurrence of equine piroplasmosis based on IFAT and PCR methods (Motloang *et al.,* 2008) and recently reported by Bhoora *et al.,* (2010a & b) using real time PCR in Northern Cape, Western Cape, Eastern and KwaZulu Natal. There is still a need to document equine piroplasmosis in other provinces including Mpumalanga and North West. Furthermore, the disease prevalence also needs to be documented in other equids such as donkeys and mules in South Africa.

Over the past 20 years, *Anaplasma* and *Ehrlicha* have become increasingly recognized as emerging zoonotic infections that affects humans and animals. Genetic analyses of 16S rRNA genes, heat shock and surface protein genes have resulted in a reclassification of the genera *Anaplasma, Ehrlichia, Cowdria, Neorickettsia* and *Woolbachia.* As a result, the genus *Ehrlichia* is now named according to the disease they cause, the genus *Anaplasma* is now comprised of: *Anaplasma phagocytophilium* (previously *Ehrlichia equi, Ehrlichia phagocytophilia* or the human granulocytic ehrlichia, i.e. (the HGE agent) (Loewenich *et al.,* 2003; OIE, 2013a) and some species have been transferred to the genus *Neorickettsia* (OIE, 2013a). According to Woldehiwet (2010) and M'ghirbi *et al.,* (2012), Equine granulocytic ehrlichiosis, which is now reported as equine granulocytic anaplasmosis (EGA), was first recognized as a disease of horses in California and the disease was subsequently found in parts of US and Europe. Apart from America and Europe, the bibliographical data also report the occurrence of equine anaplasmosis in Asia and Africa (Dzięgiel *et al.,* 2013). *Neorickettsia* (formerly *Ehrlichia*) *risticii,* the causative agent of Potomac horse fever (PHF), causes a significant febrile gastrointestinal disease of horses in North America, Canada and Europe. Defining the epidemiology of *N. risticii* has been the subject of intensive research for years and, in spite of many investigations, no evidence has been found for transmission of the disease by haematophagous arthropod vectors such as ticks (Pusterla *et al.,* 2003). Serological diagnosis utilizing the indirect fluorescent antibody technique (IFAT) is currently recommended for confirming a diagnosis of ehrlichiosis. To our knowledge, no information is available regarding the presence of *A. phagocytophilum* and *Neorickettsia risticii* in horses and ticks in South Africa.

2.2 Aims of the study

This study sought to document the prevalence of dourine, equine piroplasmosis, ehrlichiosis and anaplasmosis in horses and donkeys from different provinces of South Africa. Furthermore, this study introduces the use of different molecular based assays for diagnosis of the above−mentioned equine diseases.

2.2.1 Specific aims

- To determine the occurrence of dourine in equids in South Africa.
- To determine the occurrence of equine piroplasmosis in equids in South Africa.
- To determine the occurrence of *Anaplasma/Ehrlichia* complex in equids in South Africa.

2.3 Objectives

2.3.1 General research objectives

This study was aimed at determining the current status of equine diseases including dourine, equine anaplasmosis, ehrlichiosis and piroplasmosis in equids around South Africa. The main focus was to identify and record the occurrence of *Trypanosoma equiperdum, Anaplasma* spp., *Rickettsia* spp., *Babesia caballi* and *Theileria equi* affecting equines within the sampled provinces. Objectives of this were fulfilled by using DNA based techniques, Polymerase chain reaction (PCR) and Loop−mediated isothermal amplification (LAMP) and serological methods including Enzyme−linked immunosorbent assay (ELISA) and Immunochromatographic test (ICT) whereby both employ the use of recombinant antigens.

2.3.2 Specific objectives

- Molecular techniques (PCR & LAMP) and sero−diagnosis (ELISA & ICT) were used to detect *Trypanosoma equiperdum* infections.
- Molecular techniques were used to detect equine piroplasmosis parasites (*Babesia caballi* and *Theileria equi*) infections.
- PCR was used to detect of *Anaplasma/Ehrlichia* species infections.

CHAPTER 3

MOLECULAR AND SERO−DIAGNOSIS OF *TRYPANOSOMA EQUIPERDUM* INFECTIONS IN EQUIDS IN SOUTH AFRICA

3.1. Introduction

Dourine has been known since ancient times but its nature was only established only in 1896 when Rouget discovered trypanosomes in an infected Algerian horse. It is also called or known as covering disease mal de coit, syphilis du cheval, el dourin, morbo coitale malign, Beschalseuche, slapsiekte, and sluchnaya bolyezni (OIE, 2009; OIE, 2013). It is a disease that affects equines and is caused by flagellate protozoan called *T. equiperdum* of the genus *Trypanozoon*. It is a chronic or acute contagious, venereal disease of horses and other equids, which is transmitted directly from animal to animal during sexual intercourse (Clausen *et al.,* 2003; Claes *et al.,* 2005; OIE, 2009; Hagos *et al.,* 2010a, b, & c; Luciani *et al.,* 2013). It differs with other trypanosomes because it is a tissue parasite that is rarely detected in blood and is the only member of the genus that is not transmitted by the insect vector (Hagos *et al.,* 2010a, b, & c; Pascucci *et al.,* 2013).*Trypanosoma equiperdum* is spread through sexual intercourse (Hagos *et al.,* 2010a; Luciani *et al.,* 2013) and from infected to healthy animals. There are literature reports of transmission of *T. equiperdum* to foals during birth and through maternal milk (Pascucci *et al.,* 2013). Infected equids are the only known natural reservoir for this parasite and is found in the genital secretions of both infected females and males (Claes *et al.,* 2005).

Trypanosoma equiperdum infections are lethal if left untreated and are considered incurable in terms of chemotherapy, where administered drugs can reach parasites within blood, however not necessarily accessing parasites hidden in certain tissues (Gillingwater *et al.,* 2007). The incubation period, severity, and duration of the disease vary considerably. It is often fatal, however, it was claimed that spontaneous recoveries do occur and latent carriers do exist (OIE, 2013). The occurrence of dourine is notifiable in the European Union (Ricketts and McGladdery, 2011). Dourine was once widespread, however, it has been eradicated from many countries.It is still seen in horses in Asia and southern and eastern Europe, as well as outside the tsetse belt in

North and South Africa. Currently the disease is endemic in parts of Africa and parts of Asia including Russia (OIE, 2009; Ricketts and McGladdery, 2011).

3.1.1 Etiology

Trypanosoma equiperdum is classified as follows (Stevens and Brisse, 2004):

Kingdom: Protista

Subkingdom: Protozoa

Phylum: Sarcomastigopha

Class: Kinetoplastea

Order: Trypanosomastida

Family: Trypanosomatidae

Genus: *Trypanosoma*

Species: *T. equiperdum*

3.1.2 Species affected

In nature infections with *T. equiperdum* are restricted to equines (horses, donkeys, mules and Zebras) (Brun *et al.,* 1998). These species appear to be the only natural reservoirs for *T. equiperdum*. According to Claes *et al.,* (2005) and OIE (2013), there is no known natural reservoir of this parasite other than infected equids. Dogs, rabbits, rats and mice can be infected experimentally (OIE, 2009). Rodents, such as rabbits, rats, and mice can be used to maintain strains of the parasite indefinitely (OIE, 2013) and to prepare antigen for diagnostic tests. Dourine signs have been reported in sheep and goats that were inoculated in murine−adapted strains, but ruminants do not seem to be susceptible to the isolates from equids. (OIE, 2009)

3.1.3 Life cycle

Trypanosoma equiperdum is monomorphic (Brun *et al.,* 1998; Luckins *et al.,* 2004). It is generally transmitted sexually from infected to healthy animal (Pascucci *et al.,* 2013) by direct transmission without an intermediate host. Slender trypomastigotes occurs in mucus secretions of the reproductive organs with a free flagellum, although pleomorphic, stumpy forms are recognised. Typical strains of the parasite range in length from 15.6 to 31.3 µm (OIE, 2013); its size is within the same range as of *T. evansi* (Brun *et al.,* 1998).

3.1.4 Clinical signs

Dourine is characterised mainly by swellings and local oedema of the genital organs Brun *et al.,* (1998) and OIE (2009) during the first stage of the disease and in mares there is a discharge from the vagina. The second stage includes cutaneous plaques and this stage is pathognomonic for dourine and neurological signs can develop as the third stage (Claes *et al.,* 2005; Ricketts and McGladdery, 2011). Clinical signs are marked by periodic exacerbation and relapse or tolerance, which differs in duration and which may transpire once or several times before death (OIE, 2013). Fever, local oedema of the genitalia and mammary glands, cutaneous eruptions, incoordination, facial paralysis, ocular lesions, anemia, weight loss, abortion and emaciation may all be observed (Brun *et al.,* 1998; Fikru *et al.,* 2010; Claes *et al.,* 2005; OIE 2013). Urticaria−like plaques called dollar spots occur on the skin in some forms of the disease. Acute disease lasts only 1−2 months, or exceptionally, one week. A chronic, usually mild, form of the disease may persist for several years (Claes *et al.,* 2005). Clinical signs consist of intermittent pyrexia, weight loss and a purulent urethral or vulvar discharge in the first stages. As the disease progresses, round coin−sized plaques appear on the skin. Depigmentation develops as the plaques vanish. Finally, neurological manifestations of the disease appear and death eventually follows (Metcalf, 2001).

3.1.5 Diagnosis

It is extremely difficult to detect parasite in the body fluids of infected horses (Claes *et al.,* 2005); therefore, in practice, diagnosis is based on clinical evidence supported by

serology. Diagnosis of the disease becomes more difficult in an area where the causative agents of surra or nagana occur (Hagos *et al.,* 2010c). Traditionally, the complement fixation test has been used to identify carriers (Ricketts and McGladdery, 2011). Definitive diagnosis is by identification of the parasite; however, the organisms are extremely difficult to find. Furthermore, isolation of *T. equiperdum*, the causative agent of dourine in horses, by standard parasitological methods is usually challenging (Hagos *et al.,* 2010b & c), due to low numbers of parasites in the blood or tissue fluids (Hagos *et al.,* 2010c) and the frequent absence of clinical signs of disease (Hagos *et al.,* 2010b).

3.1.6 Treatment

There are no approved drugs to treat horses suffering from dourine, although some older published research reports mention experimental treatment of horses with naganol and neoarsphenamine or quinapyramine (Claes *et al.,* 2005). Evidence from *in vitro* drug sensitivity determination of *T. equiperdum* indicates that suramin, diminazene, quinapyramine and cymelarsan are effective against this trypanosome species, although no reports on clinical efficacy have been published (Brun *et al.,* 1998). Successful treatment with tyrpanocidal drugs has been reported in some endemic areas (OIE, 2009), however, same drugs which are used for *T. evansi* are available (Brun *et al.,* 1998).

3.1.7 Control

To prevent dourine from being introduced into a herd or region, new animals should be quarantined and tested by serology (OIE, 2009). Dourine can be controled by castration of seropositive stallions in order to prevent disease transmission. Seropositive equids must not be moved from one place to another. Good hygiene at assisted mating is also essential (Claes *et al.,* 2005).

3.1.8 Aims of the study

This chapter uses molecular techniques including PCR and LAMP as well as serological assays including ELISA and ICT to determine the occurrence of dourine in horses and donkeys in sampled provinces of South Africa.

3.2. Materials and methods

3.2.1 Location of the study areas

Blood samples were collected from horses and donkeys in four provinces, namely, Free State (FS), North West (NW), Mpumalanga (MP), and Northern Cape (NC) (Figure 3.1). In the Free State (FS) province samples were collected in May 2014 at Thaba bosiu S 28° 40'04.5"; E 028°51'16.2" and Tsheseng S 28°35' 19.2"; E 028°56' 16.7". In North West (NW) samples were collected from Mafikeng and Luchtenburg. Samples were also collected at Middelvlei abattoir in Gauteng S 26 21'640", E 27 37'282" between June 2015 and September 2015. The provinces for which samples were collected from the abattoir were Mpumalanga (MP), North West (NW) and Northern Cape (NC).

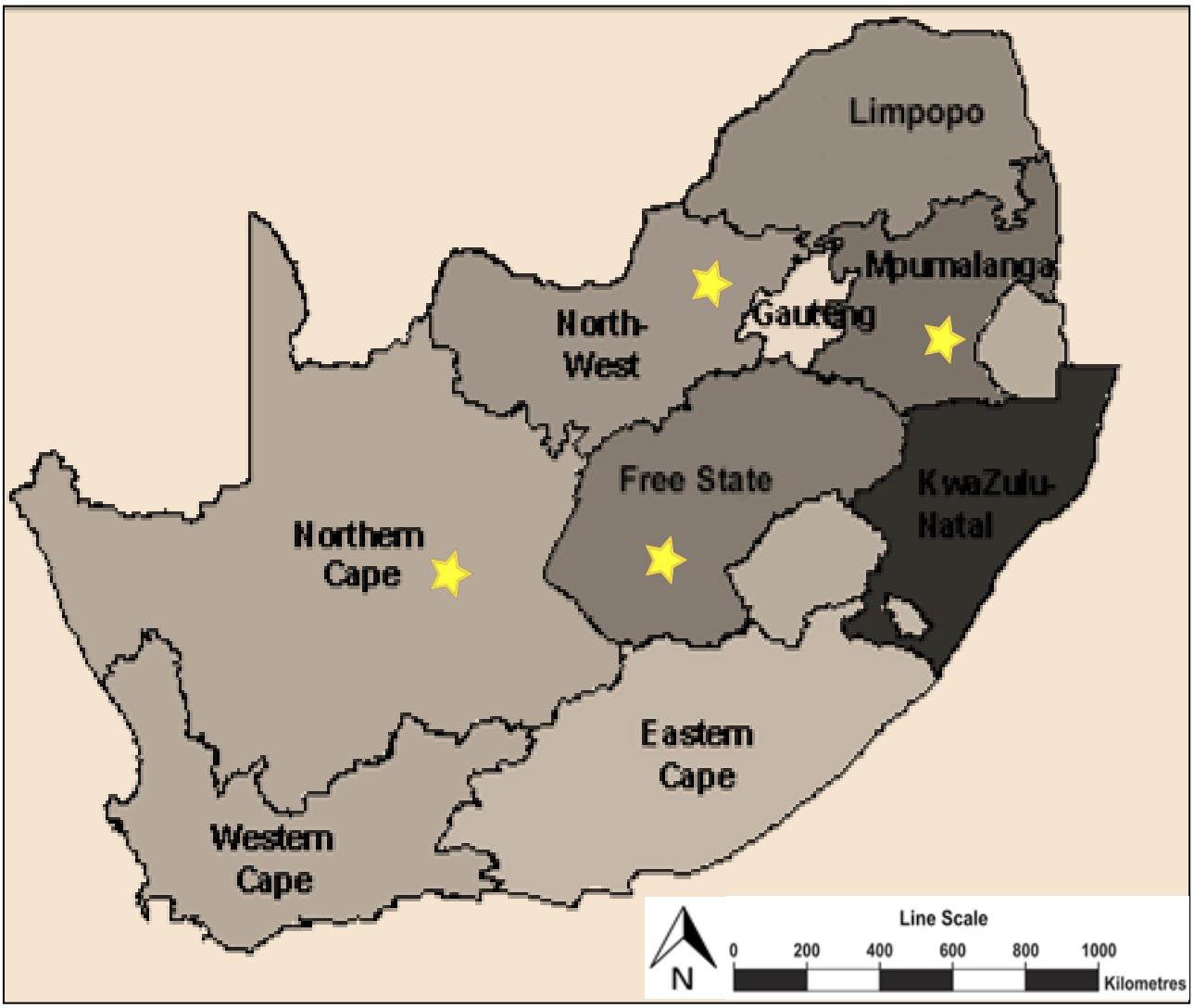

Figure 3.1: Map of South Africa with its nine provinces. The yellow stars indicate the sampled provinces. http://www.castserve.com/prodinfo.htm

3.2.2 Blood and serum samples

Blood samples were collected from the jugular vein of each horse and donkey using 18 gauge needles into EDTA vacutainers for DNA tests and silicone coated vacutainers for serological tests. The samples were placed in a cooler box and transported to the laboratory for further processing. Blood samples (n = 256) collected in EDTA vacutainers was stored at −20°C until DNA extraction was conducted. An equivalent number of samples collected in silicone−coated tubes were kept overnight at 4°C to allow clotting and then serum was harvested by centrifugation at 2500 rpm for 15 min. Sera was collected into cryogenic vials and stored at −20°C until used for the serological tests. Out of 256 samples, 34 were from donkeys, FS (n = 2), NC (n = 12), and NW (n = 20) and there rest were from horses with FS (n = 38), MP (n = 94), NC (n = 42), and NW (n = 48). The total number of serum samples from horses were 250, (n = 37), (n = 94), (n = 51) and (n = 68) in FS, MP, NC and NW.

3.2.3 Genital secretions

Genital secretions (n = 36) were collected from vagina and penis of each horse using sterile swabs and placed in sterile tubes. Two samples were collected from donkeys.

3.3. DNA extraction from blood and genital secretions samples

3.3.1 Modified salting out method

DNA extration for all samples collected from FS was done by salting out method (Nasiri *et al*., 2005). DNA was extracted from blood using 1.5 ml eppendorf tubes containing 50 microlitres of blood filled with 410 µl of extraction buffer [10 mM Tris−HCl pH 8.0], 10 mM EDTA, and 1% sodium dodecyl sulphate (SDS)]. Eighty microlitres of 10% SDS was added followed by 10 µl of Proteinase K (Pro−K). Swabs for genital secretions were immersed in 1.5 ml eppendorf tubes containing 500 µl of lysis buffer, thereafter DNA was extracted as described above.The samples from both blood and genital secretions were incubated at 55°C for 1 hour. Additional 10 µl of Proteinase K (Pro−K) was added after an hour, and the samples were incubated again at 55°C and left overnight to complete the digestion. On the following day, DNA was extracted by centrifuging samples for 5 minutes at 12 000 rpm. Six hundred microlitres of the supernatant

transferred to the second set of 1.5 ml sterile eppendorf reaction tubes and 180 µl of 5 M NaCl was added to the supernatant. Tubes were vortexed for 30 seconds, and centrifuged at 13 500 rpm for 5 minutes. 420 µl of ice cold isopropanol was added. The mixture was inverted 50 times followed by centrifugation at full speed (14 000 rpm) for 5 minutes at 4°C to precipitate the DNA. Subsequent to centrifugation, the supernatant was discarded, and pellet containing DNA was washed twice by 250 µl of 75% ethanol. Tubes were vortexed for 30 seconds followed by centrifugation at full speed for 5 minutes, and the supernatant was discarded. Washing was done twice to remove the excess cellular and chemical content that might inhibit PCR. The samples were left opened to air dry for an hour at room temperature to evaporate the 75% ethanol. Finally, the DNA pellet was dissolved in 200 µl of double distilled water (DDW) then incubated at 37°C for 30 minutes. The presence of DNA was confirmed by using Nano drop spectrophotometer (Thermo Fischer, USA) before storage at −35°C until further used.

3.3.2 DNA extraction by ZYMO DNA blood extraction kit

Blood samples collected at MP, NC and NW, were extracted with a Zymo DNA blood extraction kit according to manufactorer's instructions (Zymo, USA). Beta−mercaptoethanol (250 µl) was added to the Genomic Lysis Buffer. Then 200 µl of genomic lysis buffer was added on to 50 µl of blood samples and mixed completely by vortexing for 6 seconds, then left to stand at room temperature for 10 minutes. The mixture was transferred to a Zymo−Spin IIC^{TM}Column^{2} in a collection tube and centrifuged at 10 000 rpm for 1 minute. The supernatant was discarded, and 200 µl of DNA pre−wash buffer was added to the spin column and centrifuged at 10 000 rpm for 1 minute. Five hundred microlitres of g−DNA Wash Buffer was added to the spin column then centrifuged at 10 000 rpm for 1 minute. Spin columns were transferred to a clean micro centrifuge tubes. A 50 µl of DNA Elution Buffer was added onto each tube and then incubated at room temperature for 5 minutes then centrifuged at top speed (13 500 rpm) for 30 seconds to elute the DNA. The eluted DNA was stored at −20°C for molecular based applications.

3.4. Polymerase chain reaction (PCR)

3.4.1 PCR using Amplitaq Gold® 360 master mix with RIME and 18S rRNA primers

PCR was conducted in a total volume of 25 µl containing 12.5 µl of Amplitaq Gold® 360 Master Mix (Applied Biosystem, USA), 5 µl of primer mix (10 µM of each primer: RIME F3: CTG TCC GGT GAT GTG GAA C and B3: CGT GCC TTC GTG AGA GAG TTT C (Njiru *et al.,* 2008), 3 µl of template DNA and 4.5 µl DDW to adjust the volume. *Trypanosoma equiperdum* (Mongolia) and *T. evansi* (Tansui) were used as positive controls whilst DDW was used as negative control. PCR conditions were as follows: activation 95°C for 10 minutes, followed by 35 cycles at 95°C for 30 seconds, annealing at 62°C for 30 seconds, 72**°**C for 60 seconds and the final extension of 72°C at 7 minutes.

Nested PCR targeting the 18S rRNA gene was conducted using the primer set 18ST nF2 (CAA CGA TGA CAC CCA TGA ATT GGG GA) and 18ST nR3 (TGC GCG ACC AAT AAT TGC AAT AC) in the first reaction round and in the second reaction round, 18ST nF2 and 18ST nR2 (GTG TCT TGT TCT CAC TGA CAT TGT AGT G) (Mamabolo *et al*., 2009). The voume of the reaction mixture was described as above. PCR was performed with the following cycling conditions: activation 95°C for 10 minutes, followed by 35 cycles at 95°C for 30 seconds, annealing at 58°C for 30 seconds, 72**°**C for 60 seconds and the final extension of 72°C at 7 minutes for first and second reactions.

3.4.2 Agarose gel electrophoresis

All PCR amplifications were confirmed using a 1.5% agarose gel in 1 x TAE buffer (40 mM Tris, 20 mM Acetic acid, 1 mM EDTA, at pH 8.0) stained with 1 µg/ml Ethidium Bromide for visualisation under UV light. Five microliters of the PCR product and 1 µl of 6x Blue Loading Dye (Fermentas Life Sciences, US) were mixed, and loaded into wells. A 5 µl of 100 bp molecular weight marker (O'GeneRuler, Fermentas Life Sciences, US) was used to confirm the size of the amplification products. Electrophoresis was performed for 30 minutes at 100 V using a mini−sub cell GT electrophoreses system (Bio−Rad, UK). Gel images were captured using Gene Genius Bio Imaging System (Syngene, Synoptics, UK) GeneSnap (version 6.00.22) software.

3.4.3 Gel purification by QIAquick gel extraction kit protocol (QIAGEN, USA)

The bands of PCR positive samples were cut out from 1.5% agarose gel, weighed and final mass was measured and placed into eppendorf tubes, then QG buffer was added according to manufacturer's protocol. Samples were then heated for 10 minutes at 50°C. Isopropanol with same volume used on QG buffer was added, mixed then spun down using desktop centrifuge. The mixture was then added onto new column tubes and then centrifuged at full speed (13000 rpm for 1 minute). Supernatant was discarded. Seven hundred and fifty microliters of PE buffer was added then centrifuged at (13000 rpm for 1 minute), supernatant was discarded and then tubes were centrifuged to remove the extra PE buffer at (13000 rpm for 1 minute). The columns were placed in new eppendorf tubes and the lower part was discarded. To elute DNA, 30 µl of elution buffer (EB) was added straight on top of the white part of column and then samples were incubated at room temperature for 1 min and then centrifuged at 13000 rpm for 1 min. DNA was stored in −20°C. Gel electrophoresis was conducted as mentioned in 3.4.2 to confirm the presence of DNA after purification.

3.4.4 Sequencing

3.4.4.1 Sequencing PCR protocol

The reaction mixture for sequencing PCR was prepared as follows: One microliter of purified PCR product, 1 µl (10 µM) primer forward or reverse and 0.5 µl Big Dye (Applied Biosystem USA), plus 2 µl of sequencing buffer (80 mM Tris, pH 9.0, 2mM $MgCI_2$) in PCR tubes. The total volume 10 µl volume was adjusted by adding double distilled water. PCR for sequencing was conducted with the following conditions: 96°C for 1 minute, followed by 30 cycles at 96°C for 30 seconds, 50°C for 5 seconds, 60°C for 1 minute and 4°C hold.

3.4.4.2 Ethanol precipitation

Two microliters of 125 mM EDTA, 2 µl sodium acetate and 50 µl 100% ethanol (EtOH) and 10 µl DDW were added onto 10 µl of sequencing PCR product and mixed well.These were centrifuged at maximum speed for 30 minutes at room temperature. Ethanol was removed by adding 70 µl of 70% EtOH and centrifuged at maximum speed

for 15 minutes at room temperature then dried up. Fifteen microlitres of HiDi formamide was added then heated at 98°C for 3 min. The samples were put on ice for 2 minutes then transferred to the wells of sequencer plates. Samples were sequenced on ABI PRISM 3100 Genetic Analyzer (Applied biosystems USA) at the National Research Center for Protozoan Diseases, Obihiro University of Agricultre in Japan and other samples were sent for sequencing at Inqaba Biotech Pretoria, South Africa. The resulting sequences were identified using basic local alignment search tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST).

3.5. Loop−mediated isothermal amplification (LAMP)

3.5.1 LAMP reaction using PFR A primers

Loop−mediated isothermal amplification reaction was carried out in a total volume of 25 µl reaction mixture containing 12.5 µl of 2X LAMP buffer [RM] with 2.6 µl of primer mix (Table 3.1) 40 pmol each [forward inner primer (FIP) and backward inner primer (BIP), 20 pmol of LF and BF and 5 pmol of F3 and B3], 6.9 µl of DDW and 1 µl of DNA template, *Bst* DNA polymerase and Fluorescent dye (FD) respectively. The reaction mixture was incubated at 65°C for 1 hour in a real time turbidimeter (EIKEN CHEMICAL, CO, LTD, Japan). The results were observed by naked eyes during the amplification and after the completion.

Table 3.1: LAMP primer sequences (Thekisoe *et al.,* 2009)

Name	Sequences
BIP	CGC AAG TTC CTG TGG CTG CAT TTT TTC CCA AGA AGA GCC GTC T
FIP	TCA GAA GCG TCG AGC TGG GAT TTT ATC GAC AAT GCC ATC GCC
F3	TCA CAA CAA GAC TCG CAC G
B3	GGG CTT TGA TCT GCT CCT C
LF	CAG TTC GTC TTC GAT TTT CTC CAG
BF	GAT GAA CGT GGC TGT TGT GC

3.5.2 LAMP reaction using Loopamp™ *Trypanosoma brucei* detection kit

Loopamp *Trypanosoma brucei* Detection kit (Eiken chemical.Co.Ltd, Japan) was used to confirm PCR and ELISA positive results. A total volume of 25 µl reaction mixture, contained a 20 µl master mix of LAMP reagents (buffer, primers, Bst DNA polymerase and fluorescent detection reagent) and 5 µl of DNA template. The reaction mixture was mixed thouroghly by inverting the tubes 5 times. The LAMP mixture was incubated in a Loopamp™LF−160 incubator (Eiken Chemical Co. Ltd, Japan) or Genie II incubator (Optigene, UK) at 65°C for 40 minutes. The results were visualized under LED or UV light.

3.6. Serological methods

3.6.1 Enzyme−linked immunosorbent assay (ELISA)

Crude (TeCA) 10 µl/ml and recombinant (TeMG6−4r) 2 µl/ml antigens were obtained from (Obihiro University of Agriculture and Veterinary Medicine, Japan) including negative and positive controls. Antigens were reconstituted with 10 ml of coating buffer (50mM carbonate bicarbonate buffer, pH 9.6). The test was carried out in 96−well F−shaped microliter ELISA plates (Greiner GmbH, Fricken hausen, Germany). All wells were coated with 100 µl of diluted antigens. Then the plates were covered and incubated for 4 hours at room temperature. After incubation, antigens were discarded and the plates were washed in tap water. A 200 µl of blocking buffer phosphate buffered saline (PBS) with 0.05% Tween 20 and 1% bovine serum albumin (BSA) was added to each well. The plates were covered and incubated overnight at 4°C. The blocking buffer (PBS−T 1% BSA) was discarded and plates were washed five times with PBS−T 0.1% BSA, and once with PBS/PBS−T. Serum samples (n = 250) were diluted 200 times in dilution buffer (duplicate or triplicate) including positive (obtained from Japan horse serum) and negative controls (obtained from a disease free horse in Japan) for which a volume of 100 µl was added into the wells. The plates were covered and incubated for 2 hours at room temperature. The fluid was discarded and washed five times with PBS−T and once with PBS/PBS−T. Horse raddish peroxidase−conjugated protein G (Life Technologies Eugene OR, USA) which was diluted 5000 times in dilution buffer was added into the well with the amount of 100 µl per well. The plates were covered and incubated for 1 hour at room temperature and washed five times with PBS−T and once

with PBS/PBS−T using automatic washer machine. After drying, 100 µl of substrate tetramethylbenzidine (Kirkegaard & Perry) was added into the wells.The stop solution was added after 5 minutes, and the optical density (OD) values were determined by measuring OD at 450 nm or at 620 nm using a Multiskan^{TM} FC Microplate Photometer (Thermo Fisher Scientific, Shangai, China) (Nguyen *et al.,* 2012).

3.6.2 Immunochromatographic test (ICT)

Antigen (non−tag) was diluted into 300 µg/ml with PBS and 1000 µl of purified antigen was gently mixed (drop by drop) with 1 ml of gold colloid by inverting (British BioCell International, UK) (1:10) and incubated at room temperature for 10 minutes. Hundred microlitres of 0.05% PEG 20,000 (5% stock,) was added into 1000 µl 1% BSA (10% stock) and mixed by followed by incubation for 1 minute. The mixture was centrifuged at 10,000 rpm, 4°C for 20 min. The supernatant was removed and 50 µl were left in the well. The pelleted was suspended by tapping the tube, and sonicated in water bath for 5 min. Ten microliters of PBS containing 0.05% PEG 2000 [100 µl of 5% stock and 0.5% BSA (500µl of 10% stock)] was used for washing, mixed by inverting and centrifuged at 10,000 rpm, 4°C for 20 min. The supernatant was removed, the pellet was suspended by tapping the tube as mentioned before. The concentration of the conjugate was adjusted to reach 5 of absorbance at 520 nm (diluted 1:20, detect OD_{520}), the solution was kept at 4°C for stock. The conjugate was diluted in dilution buffer into OD 1.5 then sprayed on the glass fibre (Schleicher & Schuell, NH, USA) and dried in a vacuum overnight (air dried in drawer). The antigen TeMG6−4r was restrained at the test line, and anti−TeMG6−4r polyclonal antibodies were restrained at the control line on the ICT strips. TeMG6−4r was conjugated with gold colloid (BBI Solutions, UK) for the colour indication and sprayed on the conjugated portion. Ten microliters of serum samples were diluted with 40 µl of PBS and the sample part of ICT strips was dipped in the solution to test the samples. After 10−20 min the ICT strips were removed for observation by naked eye (Nguyen *et al.,* 2015). The ICT strips were designed and synthesized at Obihiro University of Agriculture and Veterinary Medicine, Hokkaido, Japan.

3.7. Statistical analysis

Positive samples were summarized as percentages for molecular and serological techniques. Confidence Interval (CI) of the mean at 95% was used to determine the prevalence of dourine in the sampled provinces. Pearson's chi square (χ^2) test was used to determine the distribution of dourine. Enzyme−linked immune sorbent assay (ELISA) cut off values [mean + 3SD] were also calculated for both antigens. Sequences obtained from Inqaba Biotechnical Industries were retrieved and edited using molecular evolutionary genetics analysis version 6 (MEGA 6). Sequences were first converted from AB1 format to FASTA format and the mixed bases (R, Y, M, S, W, H, B, V, D, and N) were also converted to their appropriate base pairs (A, C, G, and T) (Hall 2008; Tamura *et al*., 2011). They were subjected to BLAST to determine which *Trypanosoma* strains they represented, and to confirm that they are true positives. Additional homologous sequences of other related species were downloaded from National Center for Biotechnology Information (NCBI), and added to Alignment Explorer. Using MEGA 6 the names of the nucleotide sequences were changed to represent the sample batch number of the positive sample as well as the name of the province and the trypanosome species involved. Thereafter, the alignment was done in Clustal W then phylogenetic tree was constructed by neighbor joining method at 1000 bootstrap values.

3.8. Results

3.8.1 Polymerase chain reaction (PCR)

A total of 294, [256 DNA samples were obtained from blood, FS (n = 40), MP (n = 94), NC (n = 54), and NW (n = 68) and 38 DNA samples from genital secretions in the FS (n = 38)]. DNA samples were screened for the presence of *Trypanosoma equiperdum* parasite and the amplification revealed the positive bands between 200 and 300 bp for target gene (RIME). The overall infection rate obtained with *T. equiperdum* was 14% for the DNA samples screened with RIME gene for all sampled provinces (Table 3.2). Out of 256 DNA samples screened, 36 (14%) DNA samples were amplified with the prevalence of 15% [95% CI = 0.17± 0.29], 17% [95% CI = 1.61 ± 0.33], 11% [95% CI = 0.17 ± 0.21], and 12% [95% CI = 0.22 ± 0.23] respectively [χ^2 = 1.41, (df = 3) and $p < 0.05$]. There was no significant difference in the overall distribution of dourine in the sampled provinces.

The infection rate obtained with *T. equiperdum* in horse population was 14.9% and 8.8% (3/34) for donkeys. Table 3.2 below shows the infection rate of horses which differs per province. Mpumalanga province showed higher prevalence (17.0%) amongst all screened provinces, followed by FS (15.8%), NW (12.5%) and NC (11.9%) with the lowest prevalence (Figure 3.2). Only 8.8% prevalence was obtained from the total of 34 donkey DNA samples screened. About 8.3% (n = 12) and 10% (n = 20) prevalence were obtained for NC and NW provinces respectively. None of donkey samples tested positive for *T. equiperdum* in Free State province. Of the 40 DNA samples from horse blood, 15.0% were positive by PCR and only one out of 38 (2.6%) DNA samples from genital secretions was positive by PCR in Free State Province. None of the trypanosome infections were detected (0%) from donkey genital secretions DNA samples in FS.

The results were positive when the specific product size of 179 bp was observed for RIME PCR (Plate 3.1). To confirm the positive results, DNA amplified with *T. equiperdum* were submitted to direct sequencing using the RIME primers both forward and reverse. GenBank was used to identify the amplified sequences by using BlastN searches (http://www.ncbi.nlm.nih.gov/BLAST). BlastN searches revealed that 6 of 15 (40%) sequences were RIME gene sequences matched with *Trypanosoma brucei* accession number K01801.1 and EF567426 with 99% identity and e−value of 0.0.

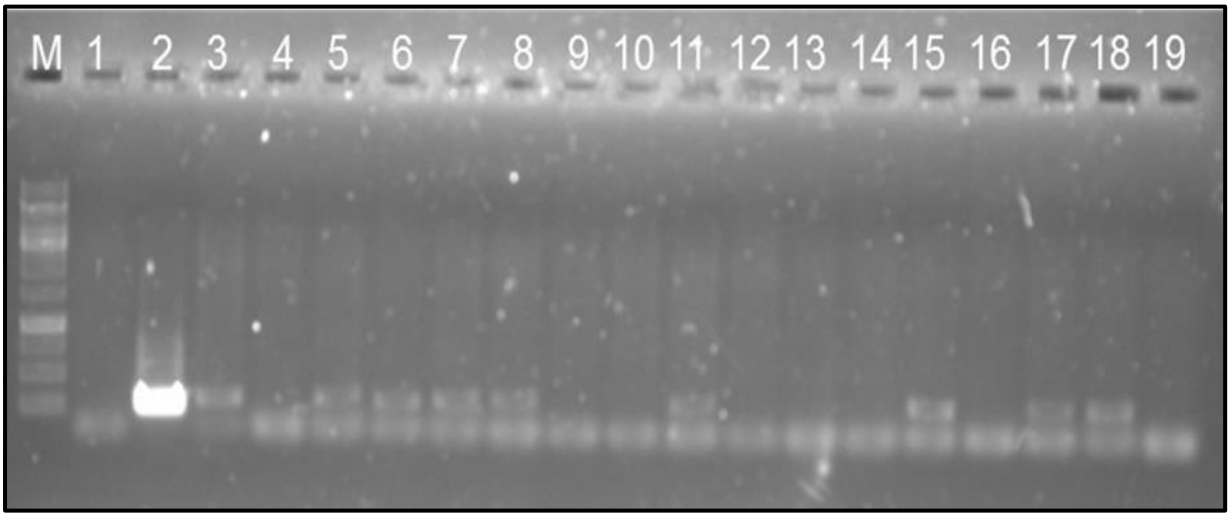

Plate 3.1: Gel image of 1.5% agarose gel electrophoresis 1 µg/ml of *T. equiperdum* amplicon size of amplified PCR product 179 bp: M: 100 bp (O"GeneRulerTM) DNA ladder, Fermentas Life Sciences, US). 1 DDW as negative control, 2 *T. equiperdum* as positive control, 3, 5, 6, 7, 8, 11, 15, 17 and 18 indicate positive results and 4, 9, 10, 12, 13, 16 and 19 indicate negative results.

Table 3.2: Summary of *Trypanosoma equiperdum* overall infections in horse and donkey DNA tested samples by PCR

Province	Total number of samples	PCR +ve (%)	Number of horses	PCR +ve (%)	Number of donkeys	PCR +ve (%)
Free State	40	6 (15)	38	6 (15.8)	2	0 (0)
Mpumalanga	94	16 (17)	94	16 (17.0)	–	–
Northern Cape	54	6 (11)	42	5 (11.9)	12	1 (8.3)
North West	68	8 (12)	48	6 (12.5)	20	2 (10)
Total	256	36	222	33	34	3
Percentage (%)		13.8	–	14.3	–	8.8

3.8.2 Phylogenetic analysis

Subject to 18S rRNA gene, 8 DNA samples were amplified with a band size of 800−900 bp then samples were sent for sequencing. Two (25%) of 8 sequences matched with the sequences available on Genbank by BLAST search http://www.ncbi.nlm.nih.gov/blast/). The sequences were 99% identical (e−value 0.0) with the published sequences of *T. equiperdum* (GenBank accession no: AJ009153.1).

The 18S rRNA sequences downloaded from the Genbank were aligned with *T. equiperdum* sequences generated in the current study using Clustal W with default parameters on MEGA6. A neighbour−joining tree with 1000 bootstrap values was constructed to determine the relatedness of the 18S rRNA sequences from this study to those on the Genbank. As observed in figure 3.3, four major clades were resolved from the tree with high bootstrap support values. The two sequences from this study, *Trypanosoma equiperdum* from MP and *T. equiperdum* from NW, formed a clade with corresponding subspecies from the *Trypanozoon* with 100% bootstrap support. The subsequent sequences also clustered with corresponding congener and they all had high bootstrap support values. The other resolved clades were *Nanommonas, Dutonella* and *Megatrypanum* respectively.

3.8.3 Enzyme−linked immunosorbent assay (ELISA)

The serum samples from horses and donkeys were demonstrated by crude (TeCA) and recombinant antigens (TeGM6−4r) in ELISA assays. OD value is equal to the average of OD values – blank (BL). Then cut off values for each ELISA tests were calculated using negative controls (mean of OD value +3SD). The cut off value of recombinant antigen (TeGM6−4r) and crude antigen (TeCA) ELISAs based assays calculated were 0.93 and 1.3 respectively. Then OD values were filtered by means of the cut off values of TeGM6−4r and TeCA. Samples were considered positive only when the OD value is equal to or greater than 0.93 and 1.3 for TeGM6−4r and TeCA antigens. Figure 3.4 elaborate the OD values for TeGM6−4r antigen for all sampled provinces. Most of the samples were scattered between 0.93 and 1.0 OD values for TeGM6−4r. Samples from NW were the most dominant between those values. Figure 3.5 expresses the OD values for TeCA antigen, samples were most scattered between 1.3 and 1.5 OD values. NW samples were still dominant between those values (1.3 and 1.5).

Horses obtained higher seroprevalence 34.3% (n = 216) than donkeys 32.4% (n = 34). In horse population the overall seroprevalence by TeGM6−4r ELISA was 17.6% (Table 3.3) which was slightly higher than 16.6% detected by TeCA ELISA. In FS TeGM6−4r and TeCA ELISA seroprevalence obtained was 5/35 (14.3%) and 3/35 (8.6%), MP obtained 12/94 (12.8%) and 19 (20.2%), NC obtained 7/39 (17.9%) and 3/39 (7.7%), and NW obtained 14/48 (29.2%) and 11/48 (22.9%) respectively. For donkey population (Table 3.3), TeGM6−4r ELISA obtained the overall seroprevalence of 23.5% while crude (TeCA) ELISA obtained 8.8%. No seropositive samples (0%) were detected in donkeys from FS (n = 2) for TeGM6−4r and TeCA ELISA, NC (n = 12) showed seroprevalence of 58.3% and 25%, in NW (n = 20) 5% and 0% respectively. No donkey serum samples were collected from MP. Northern Cape Province obtained highest seroprevalence in donkeys for both ELISA assays (TeCA and TeGM6−4r) while NW obtained higher seroprevalence for both ELISA assays (TeGM6−4r and TeCA) in horses. The combined overall seroprevalence from horses and donkeys for both antigens were 34% (n = 250). Blue colour change was also observed for both antigens on the ELISA plate (Plate 3.2).

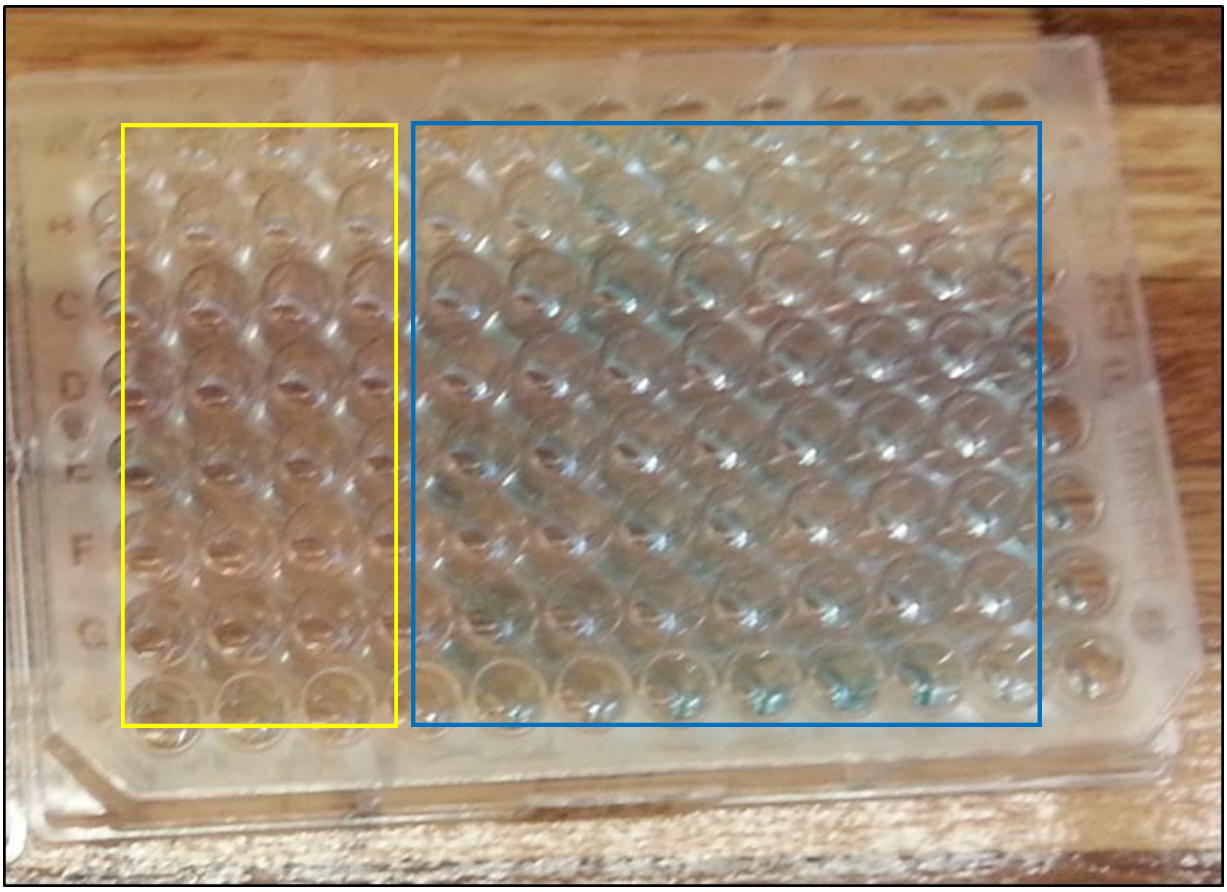

Plate 3.2: A blue line indicating colour change for seropositive and a in yellow line there was no colour change indicating negative sera results after the addition of a substrate TMB, on ELISA plate.

3.8.4 Immunochromatographic test (ICT)

The TeGM6−4r recombinant antigen was used for ICT assay. Sera results were illustrated positive or negative as shown in plate 3.3. Overall seroprevalence across all provinces by ICT in horses was 6/216 (2.7%) with 14.3% in FS (n = 35), 2.6% in NC (n = 39) and 0% in MP (n = 94) and 0% in NW (n = 48) as shown in (Table 3.3). There were no seropositve samples detected in donkeys across all four sampled provinces.

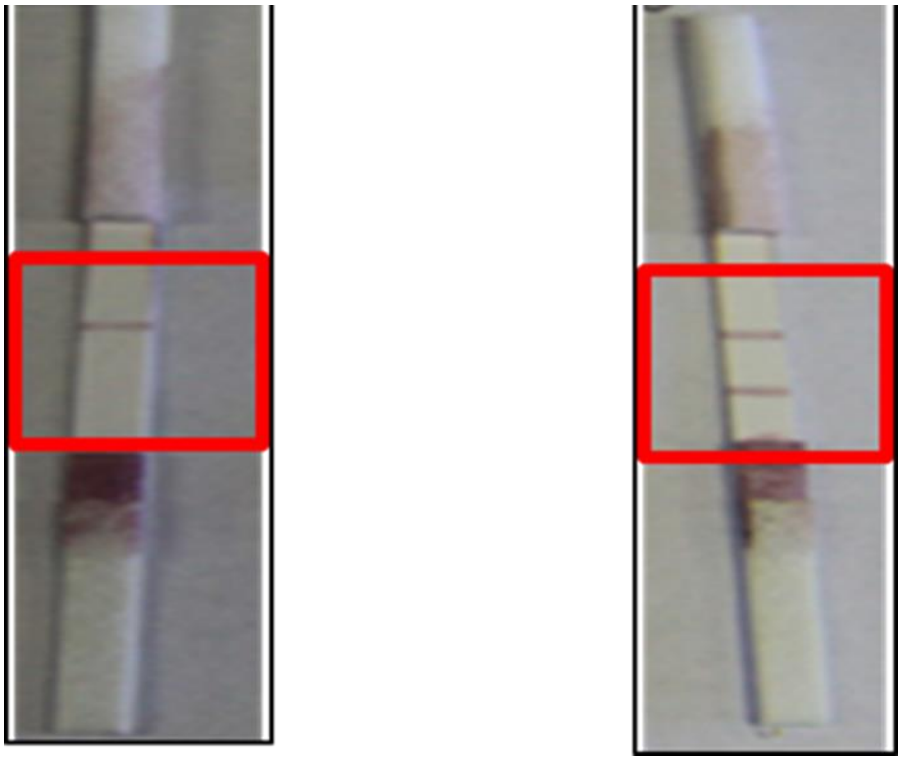

Plate 3.3: TeGM6-4r ICT strips with the red block indicating negative or positive. Results are negative when only one line appears a control line (CL) and positive when two lines appears a CL and test line.

Table 3.3: ELISA overall seroprevalence results from donkeys and horses serum samples

	Donkeys				Horses			
Province	**No. of samples**	**TeGM6−4r**	**TeCA**	**ICT**	**No. of samples**	**TeGM6−4r**	**TeCA**	**ICT**
FS	2	0	0	0	35	5	3	5
MP	−	−	−	−	94	12	19	0
NC	12	7	3	0	39	7	3	1
NW	20	1	0	0	48	14	11	0
Total	34	8	3	0	216	38	36	6
Percentage		23.5%	8.8%	0%		17.6%	16.7%	2.8%

Overall seropositivity of 18.4% for dourine was obtained by rELISA in sampled Provinces which was slightly higher than 15.6% obtained by caELISA, and higher than 2.4% ICT assay (Table 3.4). Eight point one per cent and 13.5% of samples were positive by ELISA in FS and were the lowest detection rate in both ELISA assays respectively. Figure 3.6 demonstrates ELISAs and ICT TeGM6−4r seroprevalence whereby rELISA based assay obtained higher (18.4%) prevalence than caELISA (15.6%) and TeGm6−4r ICT (2.4%). Of the 36 PCR positive samples, 8 were serologically positive by ELISA and 2 were ICT positives.

Table 3.4: Comparison of horse and donkey serum samples positive (+) and negative (−) results highlighted with a grey colour based on caELISA, rELISA and ICT assays

Name of province	Number of Samples	Techniques				
		caELISA		rELISA		ICT
		+ (%)	−	+ (%)	−	+ (%)
FS	37	3 (8.1)	34	5 (13.5)	32	5 (13.5)
MP	94	19 (20.2)	75	12 (12.8)	82	0 (0)
NC	51	6 (11.8)	45	14 (27.5)	38	1 (2.0)
NW	68	11 (16.2)	57	15 (22.1)	53	0 (0)
Total	250	39	211	46	205	6
Percentage (%)	−	15.6		18.4		2.4

3.8.5 Loop−mediated isothermal amplification (LAMP)

LAMP was used to confirm the positive results obtained by PCR. Thirty two PCR positive DNA and ELISA serum samples were screened for the presence of *T. equiperdum* infections by LAMP. The overall amplification detected was 81.3% (26/32) of the DNA samples. Only one PCR positive sample was negative. The results of LAMP were visualised under a UV light and normal light, Plate 3.4(a) and (b) respectively. LAMP with PFR A primers (Thekisoe *et al.,* 2009) was used to screen genital secretion DNA samples (n = 38), as a result two samples were positively (Plate 3.5). In a suspected horse (Plate 3.6) showing clinical signs, from Northern Cape Province all tests, molecular (PCR and LAMP) and serological (ELISA and ICT) confirmed the presence of *T. equiperdum* infections. Figure 3.7 shows overall prevalence obtained for all applied techniques in all sampled provinces (PCR, LAMP, ELISA and ICT).

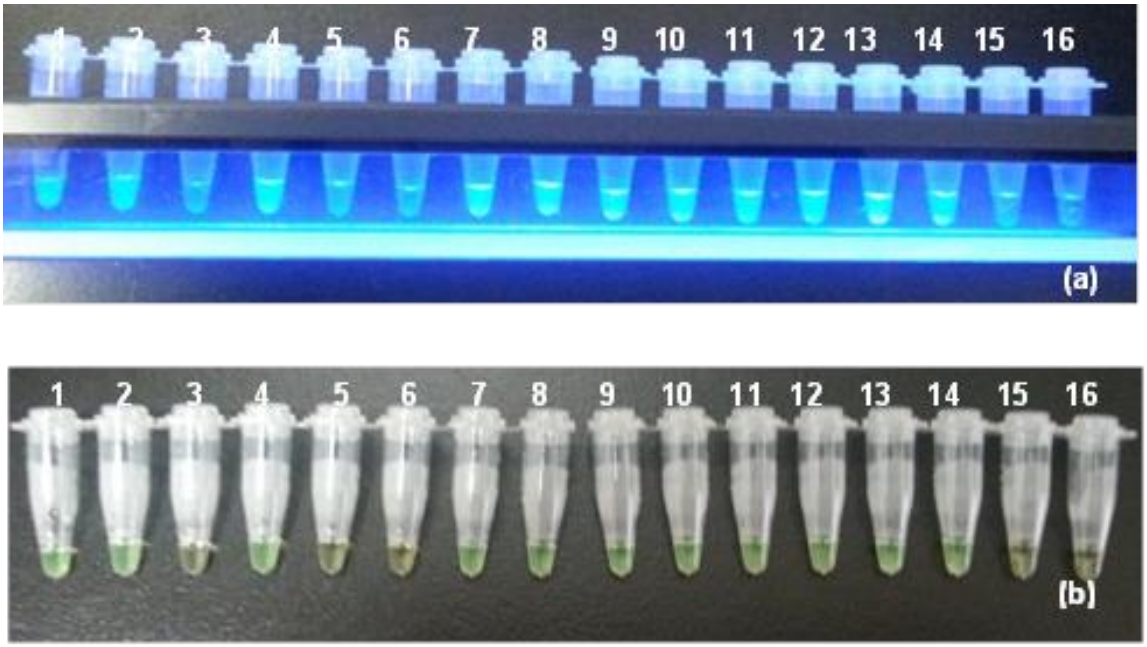

Plate 3.4: (a) Loopamp amplicons in the reaction tubes detected and visualised directly under UV light and (b) normal light. Samples 1, 2, 4, 7, 8, 9, 10, 11 & were positive while 3, 5 & were negative, 13 *T. evansi* as positive control (PC), 14 PC dHAT from the kit, 15 negative control (NC) form the dHAT kit and 16 DDW as a NC.

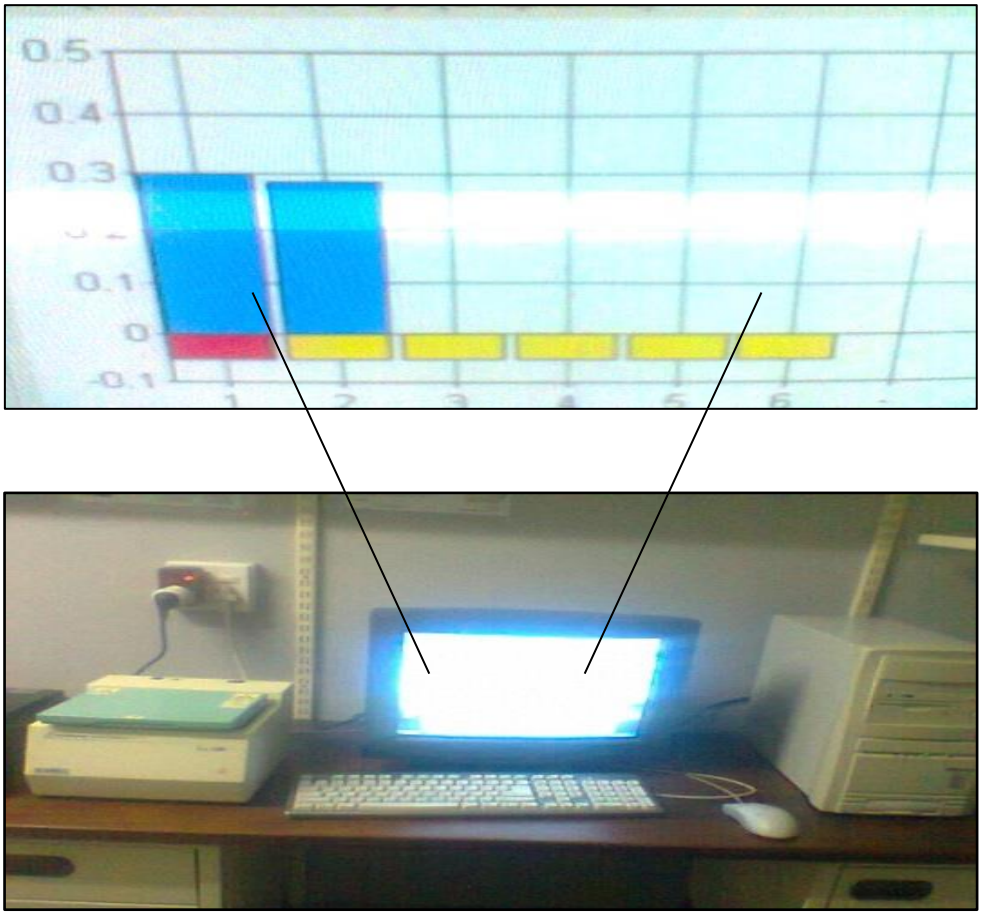

Plate 3.5: LAMP real time amplification results from genital secretions DNA samples. Lane 1 is1 positive control (*T. evansi*), Lane 2 indicative of positive sample and lane 3, 4, 5, 6, indicating negative results and lane 7 is DDW (negative control).

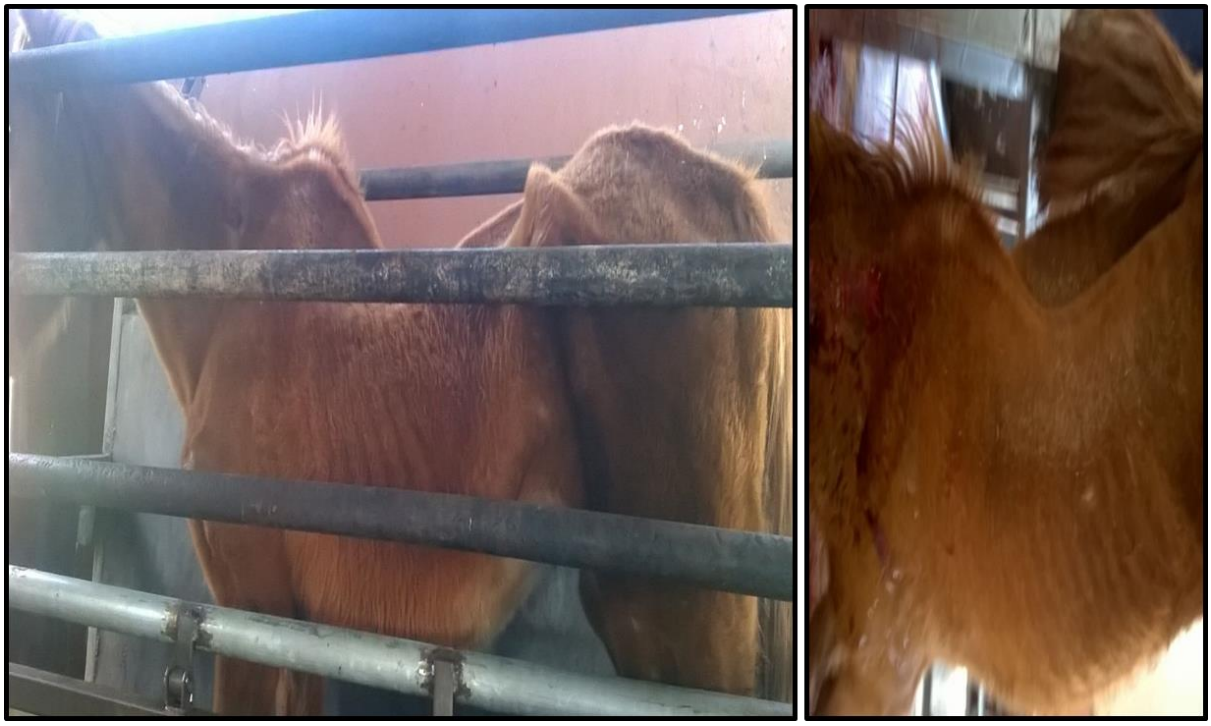

Plate 3.6: Sample horse from Northern Cape Province with clinical signs.

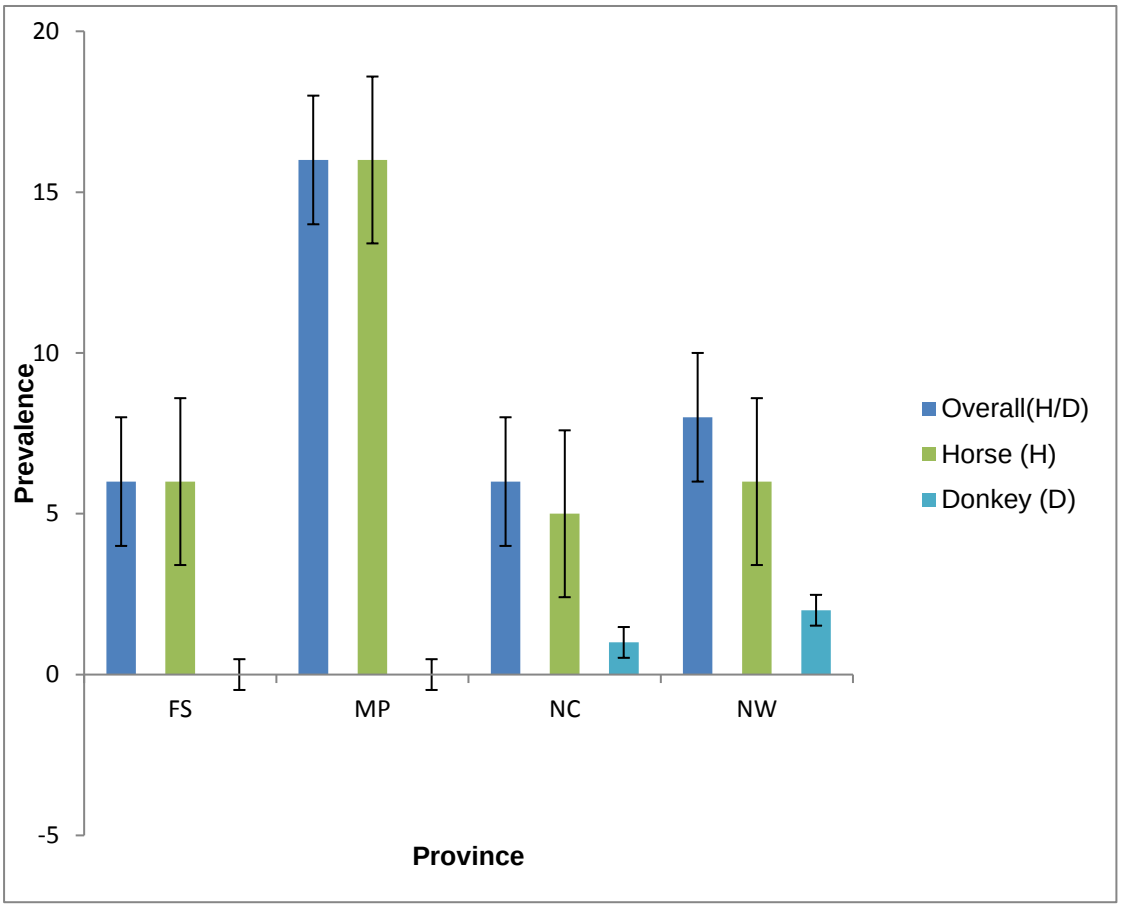

Figure 3.2: The overall *Trypanosoma equiperdum* prevalence obtained using RIME primers for all tested samples FS (n = 40), MP (n = 94), NC (n = 54) and NW (n = 68). Prevalence attained was 15%, 17%, 11% and 12% respectively. For horses only 15.0%, 17.0%, 11.9% and 12.5% prevalence was obtained respectively and for donkeys only 0% in FS, 8.3% in NC and 10% in NW was obtained. No donkeys DNA samples were obtained in MP.

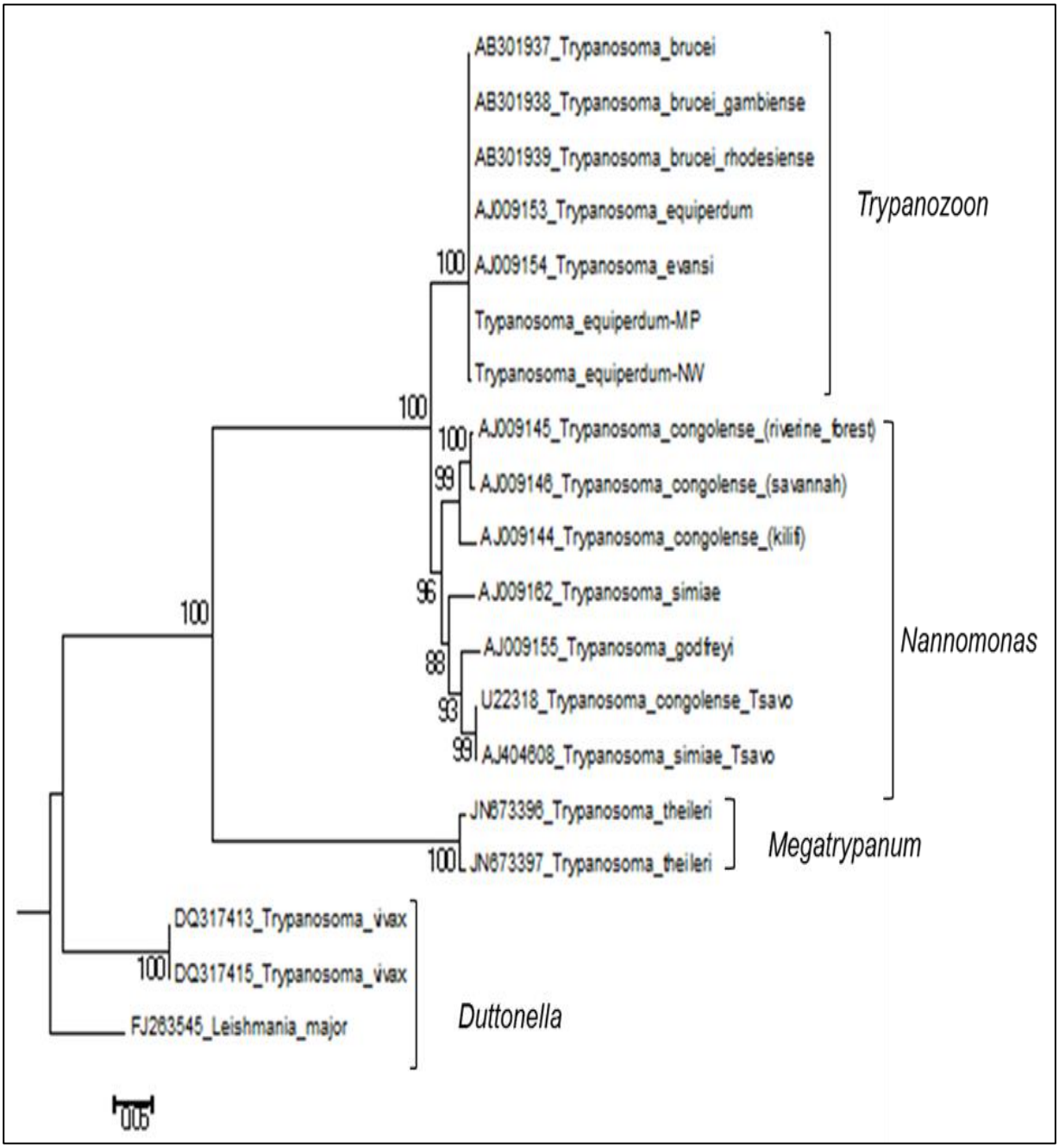

Figure 3.3: Phylogenetic tree constructed from the 18S rRNA of different trypanosomes. The analysis involved 19 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 487 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [Tamura *et al.,* 2013] with 1000 bootstrap values.

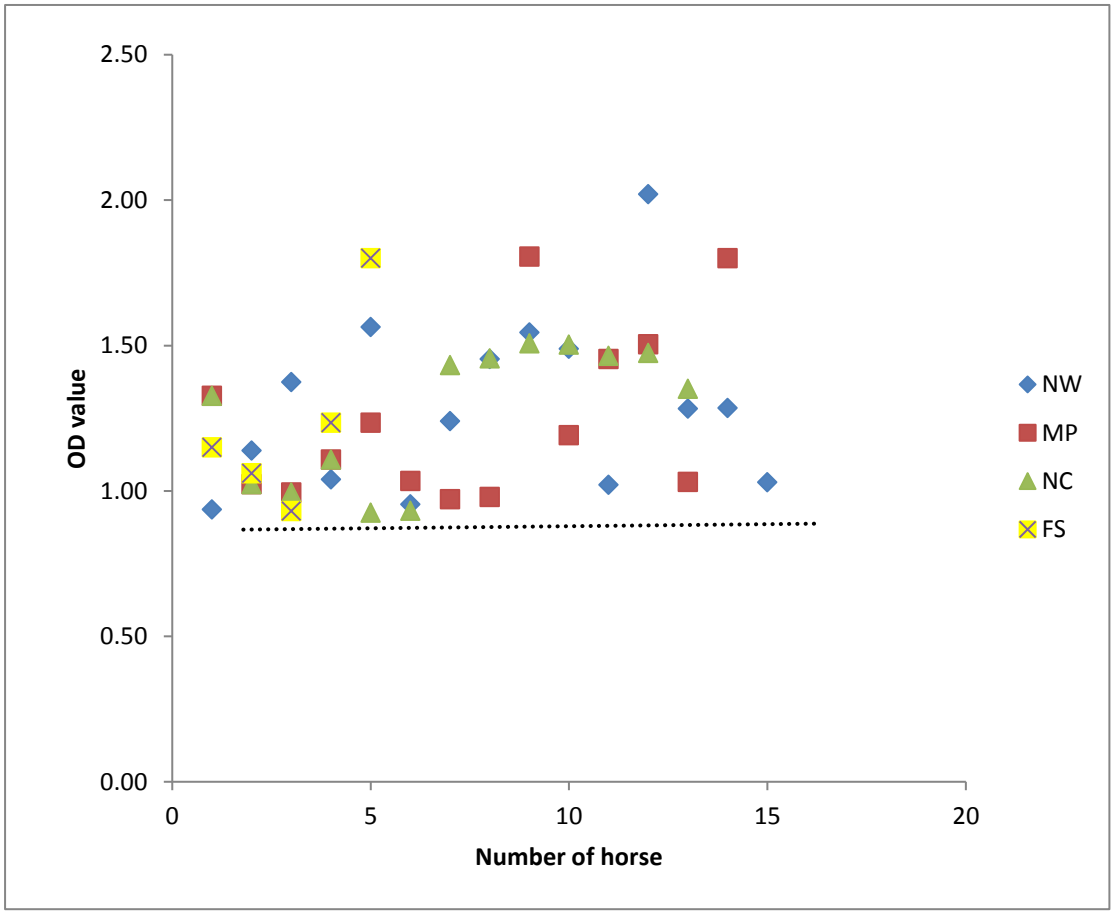

Figure 3.4: Sera OD (420 nm) values obtained from recombinant protein TeGM6-4r antigen-based ELISAs assay tested sera for four provinces (FS, MP, NC and NW). The total number of serum samples collected was 250, (n = 37), (n = 94), (n = 51) and (n = 68) respectively. Cut off value for TeGM6-4r obtained was 0.93 (shown by the broken lines).

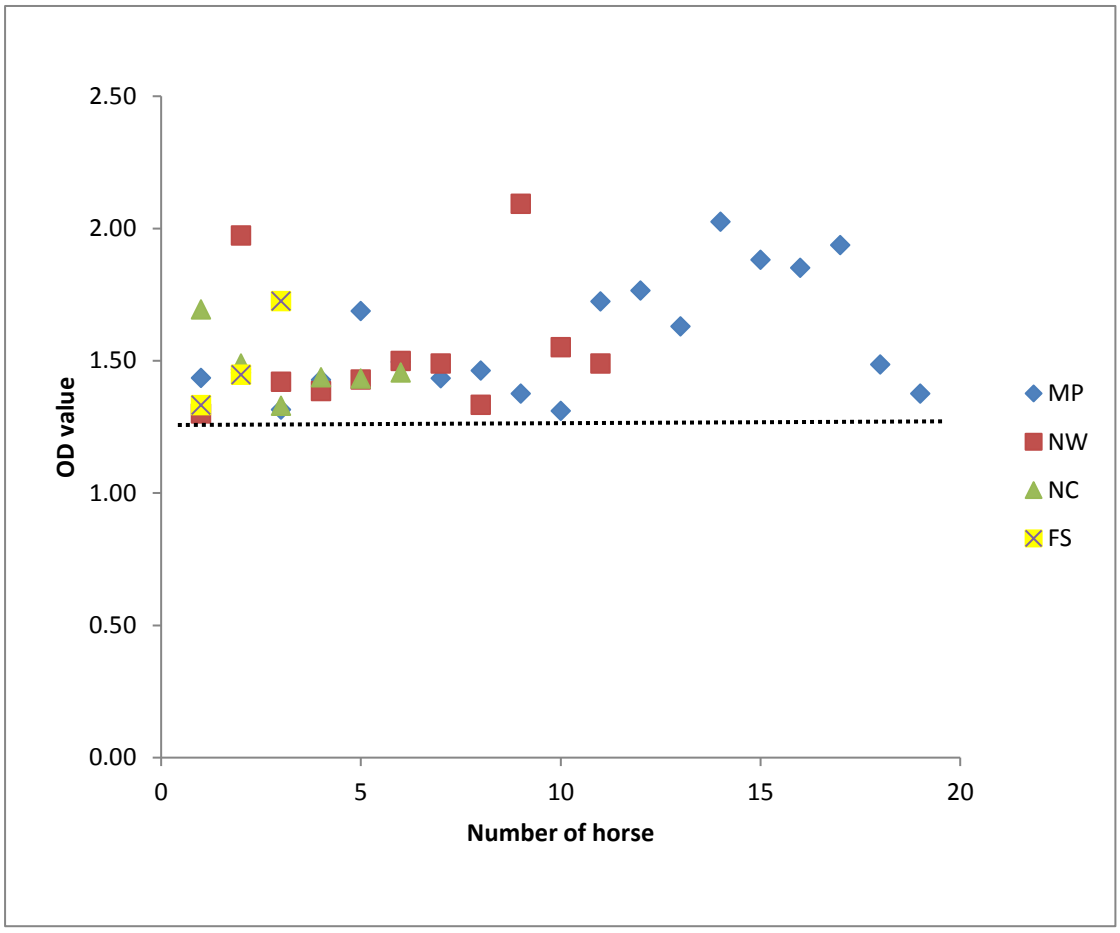

Figure 3.5: Sera OD (420 nm) values obtained from crude (TeCA) antigen ELISAs based assay tested sera for four provinces (FS, MP, NC and NW). The total number of serum samples collected was 250, (n = 37), (n = 94), (n = 51) and (n = 68) respectively. Cut off value for TeCA was 1.3 presented by the broken lines.

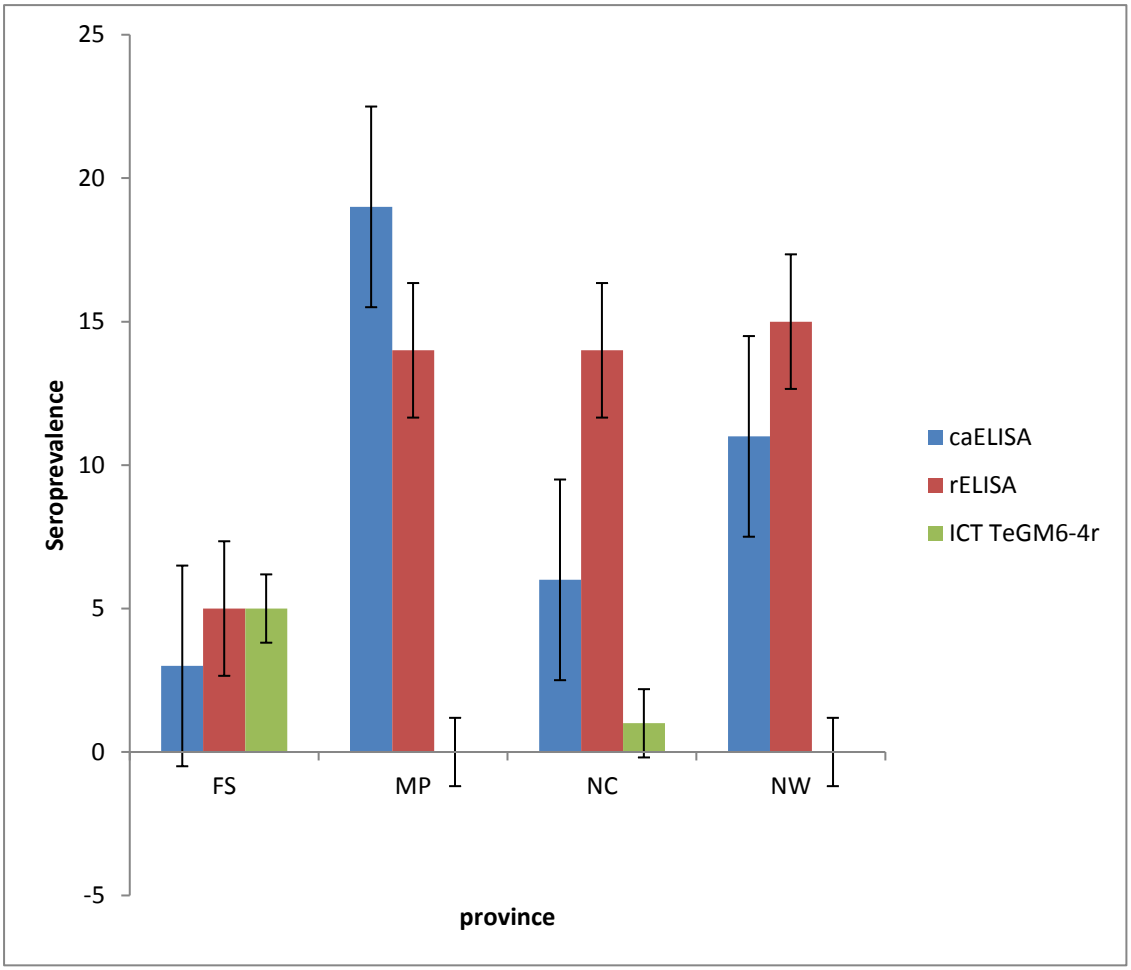

Figure 3.6: ELISA and ICT results of serum samples (n = 250) collected from equines (horse and donkey) in four sampled provinces FS (n = 37), MP (n = 94), NC (n = 51) and NW (n = 68) of South Africa. The seroprevalence obtained in caELISA was 8.1%, 20.2%, 11.8% and 16.2% respectively. In rELISA seroprevalence obtained was 13.5%, 12.8%, 27.5% and 22.1% respectively. The prevalence obtained in TeGM6-4r ICT was 13.5%, 0%, 2.0% and 0% respectively.

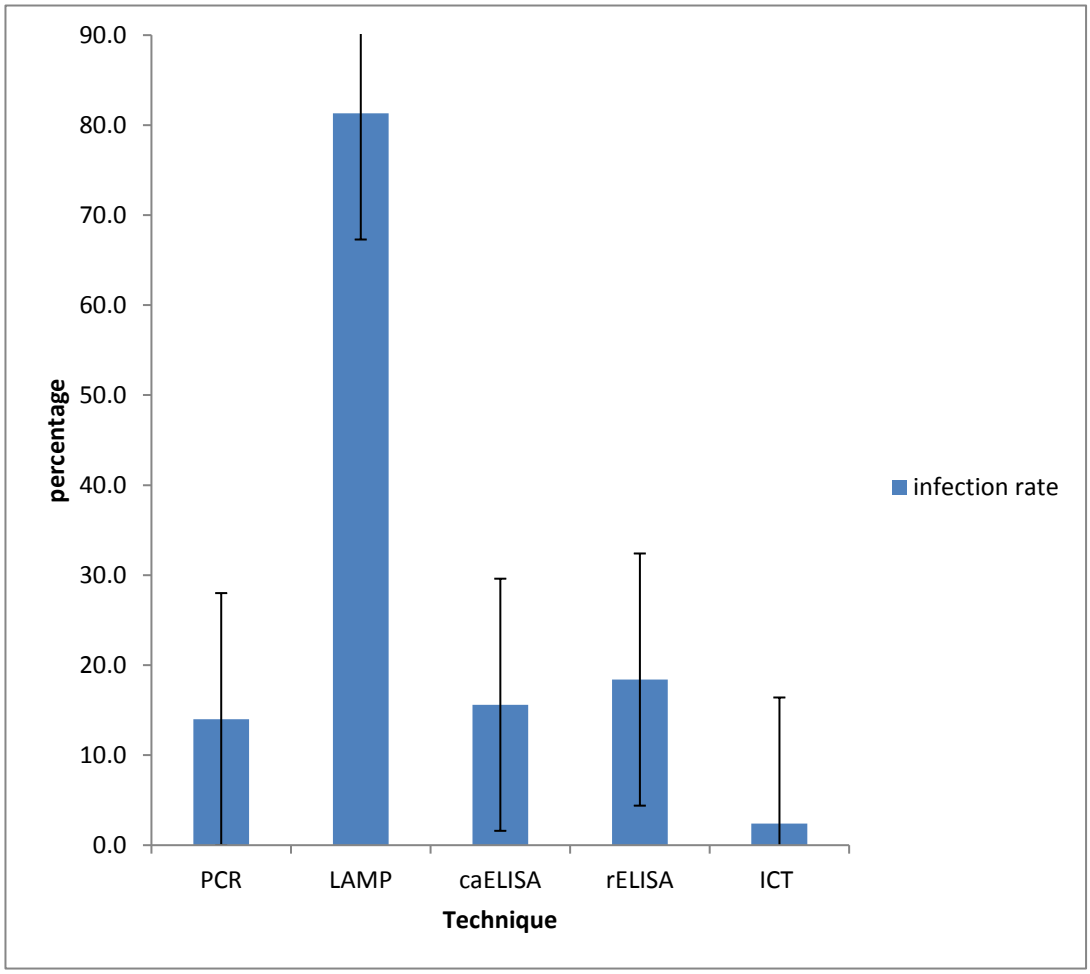

Figure 3.7: *Trypanosoma equiperdum* prevalence in selected provinces of South Africa by different diagnostic tests, PCR, LAMP, ELISA and ICT. PCR with 14%, LAMP with 81.3%, caELISA with 15.6%, rELISA with 18.4% and lastly ICT with 2.4%.

3.9. Discussion

Dourine is the sickness that affects equines under natural conditions (Brun *et al.,* 1998). In many countries it was eliminated years ago (OIE, 2009; Ricketts and McGladdery, 2011). According to DAFF report case dourine is known to be present in South Africa and internationally dourine was reported over past 7 years by 10 OIE associated countries, Asia, Africa, and Europe (Epidemiology 2012). The diagnosis was only fulfilled with the use of serological method (CFT). In this study 294 samples were screened for the presence of *Trypanosoma equiperdum* parasite infection which is known to be causative agent of dourine by using molecular techniques and serological assays which are more specific and reliable (Alemu *et al.,* 1997; Brun *et al.,* 1998; Clausen *et al.,* 1999; Clausen *et al.,* 2003; Hagos *et al.,* 2010b; Nguyen *et al.,* 2012), namely PCR, LAMP, ELISA and ICT.

KwaZulu Natal Province is the only province in South Africa known to have species of tsetse flies, namely, *Glossina austeni* and *G. brevipalpis* (Nguyen *et al.,* 2015). In this study the samples were collected from non−tsetse fly provinces which are: FS, MP, NC and NW, this means positive DNA samples amplified were true *T. equiperdum* parasite. The PCR targeting RIME gene detected the presence of *T. equiperdum* in horse and donkeys. The samples were confirmed positive by sequencing. The sequenced DNA samples matched with the strains of HAT trypanosomes available in Genbank with accession number K01801.1 (*T. brucei*) and EF567426.1 (*T. b. brucei*), species which belong to subgenus *Trypanozoon* together with *T. equiperdum* (Stevens and Brisse, 2004). Furthermore all trypanosome species in this subgenus are genetically closely related (Hide *et al.,* 1990). Due to the absence of tsetse vectors in the sampled provinces, the *T. b. brucei* sequences with sequences obtained in this study, support that the outcomes of the study are true *T. equiperdum*. South Africa and parts of Russia are the only countries where dourine is currently reported and often lie outside the distribution area of *T. evansi* (Claes *et al.,* 2005). Therefore the trypanosome infections detected in this study is *T. equiperdum*. Moreover there is no RIME gene sequences of *T. equiperdum* deposited in the database hence the sequences obtained in this study matched with *T. brucei* sequences only.

Dourine is a venereal disease whereby its causal agent, *T. equiperdum* is transmitted during coitus (Clausen *et al.,* 2003; Claes *et al.,* 2005; OIE, 2009). Molecular techniques (PCR & LAMP) were both able to detect *T. equiperdum* from extracted DNA of genital

secretions of horses in the Free State Province. The results obtained from PFR A LAMP (Thekisoe *et al.,* 2009) and Loopamp™ *Trypanosma brucei* detection kit assay (Eiken Chemical Co. Ltd, Japan) which are both designed to detect HAT trypanosomes infections (*T. b. gambiense* and *T. b. rhodesiense*) and other subgenus *Trypanozoon* species (*T. b. brucei, T. evansi* and *T. equiperdum)* have shown the presence of *T. equiperdum* in the sampled provinces. The low incidence obtained could be due to the fact that trypanosomes are not continually present in the genital tract and throughtout the course of the disease (OIE, 2013) and *T. equiperdum* generally parasitizes the tissue and causes local clinical symptoms of the genital organs at the beginning of infection (Clausen *et al.,* 2003). Since there are no records of human African trypanosomiasis in South Africa, therefore the findings of this study confirm the presence of *T. equiperdum* in South Africa. The 14% of dourine prevalence observed in the current study is higher than 6.2% obtained in Mongolia in related prevalence study (Clausen *et al.,* 2003), but less than 22.1% recorded in Ethiopia (Alemu *et al.,* 1997 and Clausen *et al.,* 1999). Similar with the studies conducted using the 18S rRNA gene Ethiopia achieved higher prevalence 47.6% (Fikru *et al.,* 2010) compared to the present study in South Africa with 25% prevalence. Difference could be due to the number of samples collected (Clausen *et al.,* 2003) and the fact that neither of two sereological tests nor the PCR test used in their study permit differentiation between *T. equiperdum* and *T. evansi*.

It has been reported that it is not easy to detect *T. equiperdum* from either blood or tissues or in discharges from the genitalia (Luckins *et al.,* 2004). In the current study the presence of *T. equiperdum* have been detected by PCR from DNA extracted from blood and genital secretions samples. The presence of *T. equiperdum* infections were mostly detected from blood samples than from genital secretions as shown in Table 3.2. Yet, *T. equiperdum* is primarily a tissue parasite that rarely invades the blood (Claes *et al.,* 2005). According to Luckins *et al.,* (2004), it is seldom found in peripheral blood and probably only uses the bloodstream as a means of transport from one side to another. The infection and pathogenicity dynamics of the South African *T. equiperdum* requires further studies as it appears to be circulating in the bloodstream. Though *T. evansi* and *T. equiperdum* are morphologically indistinguishable (Li *et al.,* 2005; Li *et al.,* 2007), but they differ greatly in their pathogenicity (Brun *et al.,* 1998). There are no records of *T. evansi* presence in South Africa. Therefore it can be concluded that all positive samples

in this study are true *T. equiperdum* infections and dourine is present in the sampled South Africa.

Phylogenetic tree constructed in this study has showed 4 trypanosome clades which are based on 4 trypanosome subgenera. The first clade namely the *Trypanozoon* clade had 100% bootstrap support observations were made previously using 18S rRNA whereby members of the subgenus *Trypanozoon* were clustered together with high bootstrap supports ranging between 90% and 100% (Stevens and Gibson, 1999; Hamilton *et al.,* 2007). Subgenus *Nannomonas* as the second clade*,* subgenus *Megatrypanum* was third clade and the fourth clade was subgenus *Duttonella.* The South African *T. equiperdum* MP and *T. equiperdum* NW obtained in the current study formed a well−supported cluster with other *Trypanozoon* species sequences from NCBI Genbank with 100% bootstrap support, indicating that indeed the South African trypanosomes are true positives. The *T. equiperdum* MP and *T. equiperdum* NW formed a monophyletic clade (Clade 1) Figure 3.3 along with previously published *Trypanozoon* sequences. The phylogenetic tree also indicated that there is no high genetic diversity amongst the subgenus *Trypanozoon* species.

Generally *T. equiperdum* is diagnosed with same techniques used for *T. evansi* (Brun *et al.,* 1998). In this study, rELISA, caELISA and ICT assays were used for detection of *T. equiperdum* antibodies in horses and donkeys from South Africa. It has been suggested that these assays can be used as diagnostic antigens for the infections of other species belonging to the same subgenus *Trypanozoon* (Nguyen et al., 2012), considering that TeGM6−4r antigen is 100% identical to *T. brucei brucei* GM6, and is highly conserved among salivarian trypanosomes (Nguyen *et al.,* 2015). Members of this genus (*Trypanozoon*) all share conserved cytoskeletal elements that aggravate a strong and cross−reactive serological response (OIE, 2013). The overall seroprevalence of 34% obtained in this study with 15.6% and 18.4% detected by caELISA and rELISA respectively. The 18.4% rELISA results obtained were higher as compared to 6.7% reported from Mongolia but less than 19.26% reported from Eithiopia. These seroprevalence difference could be credited to the difference in sensitivity and the specificity of the tests applied. Results from the current study indicate that both TeGM6−4r (ELISA and ICT) and TeCA diagnostic methods are capable of detecting *T. equiperdum* infections meaning that the antigen can be adopted for use in large epidemiological studies or surveys of dourine in South Africa and other affected nations.

The TeGM6−4r ICT was used for sero−diagnosis of animal trypanosomosis in South African cattle, goats and sheep (Nguyen *et al.,* 2015). This is the first time TeGM−6−4r assay has been used for sero−diagnosis of dourine. The low seroprevalence obtained when using TeGM6−4r is comparable to the findings of Nguyen *et al.,* (2015) were the TeGM6−4r assay was used. As a result, TeGM6−4r−ICT detection perfomance for *T. equiperdum* antigens was poor and needs further optimization.

Donkeys appeared to be less susceptible to *T. equiperdum* infections. Horses are the known reservoirs for *T. equiperdum,* thus appeared to be highly susceptible. This should be taken in consideration as in some areas they are still used as a means of transportation. The outcomes of the study proved that donkeys are resistant than horses. In donkeys, the disease passes often unperceived whereas its semen and vaginal secretions contain infective trypanosomes (OIE, 2013).

Trypanosoma equiperdum detection in equines is commonly dependent on clinical observation and the CFT (Alemu *et al.,* 1997). The CFT is the recommended test for international trade however it often has non−specific cross reactions in some uninfected animals, predominantly in donkeys due to anticomplementary activity of their serum, thus this makes results difficult to be interpreted (Ricketts and McGladdery, 2011; OIE 2013). Clinical diagnosis is not always conceivable due to the inconsistency and possible absence of apparent signs of dourine and laboratory diagnosis is essential to confirm diagnoses of dourine (Fikru *et al.,* 2010; Ricketts and McGladdery, 2011). According to Luckins *et al.,* (2004), in donkeys mostly, the strains of *T. equiperdum* tend to induce asymptomatic or hidden infections.

Clinical signs of dourine can provide a strong indication of the presence of the disease, but confirmatory diagnosis is required (Claes *et al.,* 2005). Therefore in the present study there was a horse from NC with an obvious clinical signs and all the diagnostics used confirmed it to have a dourine. It tested positive in molecular and serological tests conducted. As stated in the literature the second stage of the disease is pathognomonic. In this stage, distinctive cutaneous plaques or skin thickness can occur (Claes *et al.,* 2005). Poor body condition and compromised immune system due to poor nutrition and/or other tragedy may increase the chances of establishment of trypanososmiasis in such animals (Fikru *et al.,* 2010).

CHAPTER 4

MOLECULAR DIAGNOSIS OF *BABESIA CABALLI* AND *THEILERIA EQUI* INFECTIONS IN EQUIDS IN SOUTH AFRICA

4.1 Introduction

4.1.1 Theileriosis

Theileria, the tick−transmitted protozoa, is an obligate intracellular parasite that causes lymphoproliferative disease in mammalian host (Oladiran *et al.,* in press). There are many different types of *Theileria*, which cause different diseases in animals (all tend to be called theileriosis). Several species of *Theileria* infect cattle, sheep, goats, horses, small ruminants, throughout the world and are transmitted by Ixodid ticks acting as biological vectors. According to Shaw (1997), *Theileria* parasites are members of the phylum Apicomplexa, which comprises of several species of both medical (*e.g*. *Plasmodium* and *Toxoplasma*) and veterinary (*e.g. Babesia; Eimeria; Theileria*) significance. *Theileria* parasites are distinctive among protozoa in that certain species are proficient of immortalizing either mammalian lymphocytes or cells of the monocyte or macrophage lineage that they infect (Bishop *et al.,* 2004). *Theileria* have complex life cycles (Figure 4.1) that involve several morphologically distinct developmental stages in the vertebrate and invertebrate host cells (Shaw, 2003).

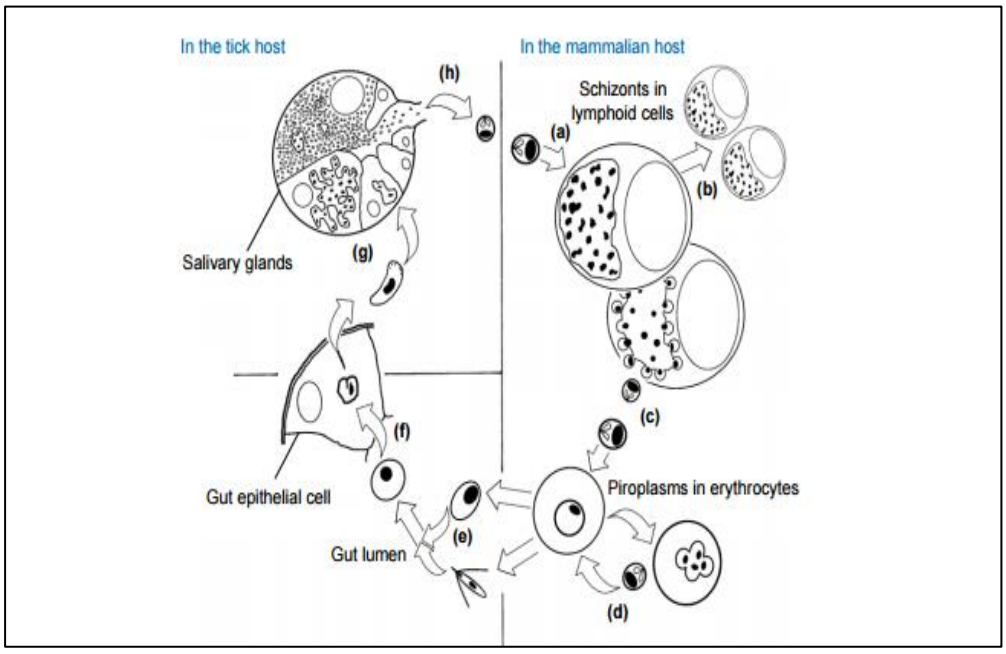

Figure 4.1: *Theileria* life cycle (Shaw, 2003)

4.1.2 Babesiosis

Hunfeld *et al.,* (2008) reported that the genus *Babesia* (phylum Apicomplexa) are the second most communal blood−borne parasites of mammals after the trypanosomes. They are tick−transmitted hemoparasites. Entirely species of *Babesia* are naturally transmitted by the bite of infested ticks (almost all ixodids rather than argasids) and the main life cycle variation amounts to the existence of transovarial transmission in some species (*Babesia* spp. sensu stricto) and not in others (*B. microti*−like). The parasites life cycles are very comparable (Figure 4.2).

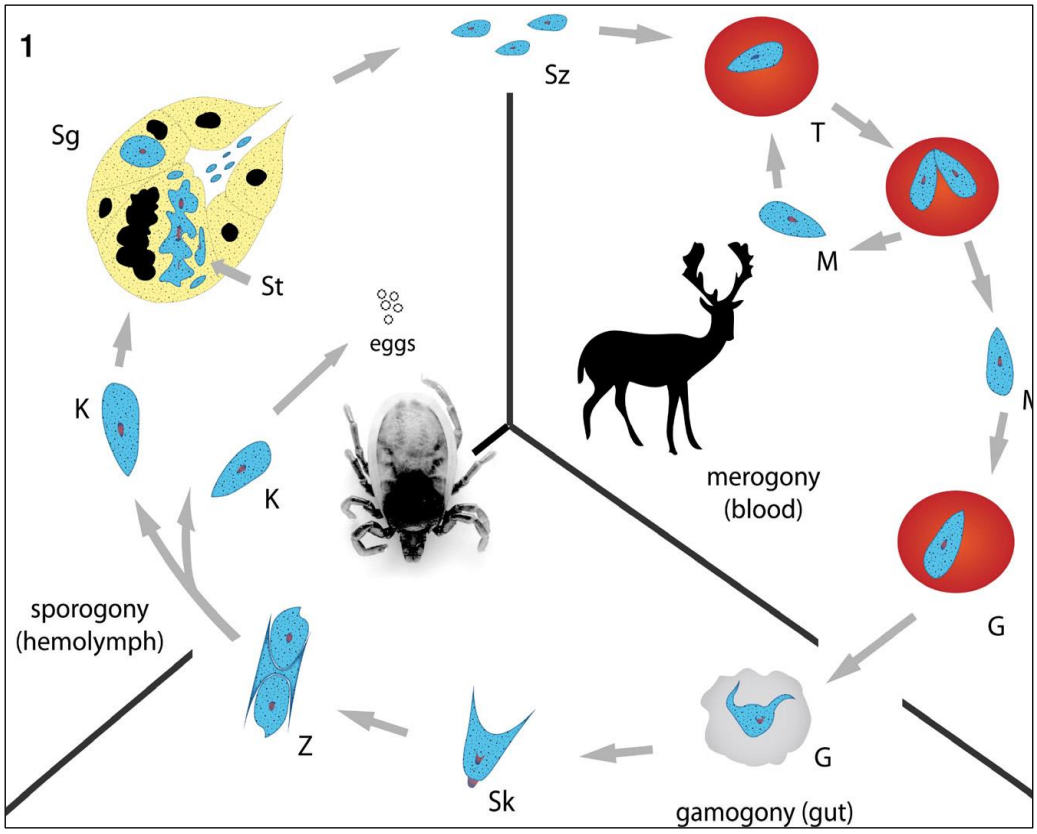

Figure 4.2: General simplified life cycle of *Babesia* spp. (Hunfeld *et al.,* 2008).

4.1.3 Equine piroplasmosis

Equine piroplasmosis is one of the most important tick−borne diseases (Sevinc *et al.,* 2008; Zobba *et al.,* 2008), with an economic worldwide impact on the horse industry (Zobba *et al.,* 2008). Two hemoprotozoan parasites, *Theileria equi* (formerly *Babesia equi*) and *Babesia caballi* (Sevinc *et al.,* 2008; Zobba *et al.,* 2008; Qablan *et al.,* 2013)

which belong to the phylum Apicomplexa, order Piroplasmida (Zobba *et al.,* 2008) are known to be the cause of the disease and are found in many wild and domestic animals. These parasites are transmitted from host to host via tick vectors. Mixed infection with both organisms has been frequently reported in equids (Bhoora *et al.,* 2009; Piantedosi *et al.,* 2014). Approximately 14 species (Zobba *et al.,* 2008; Bhoora *et al.,* 2010a) of Ixodid ticks of the genera *Dermacentor, Rhipicephalus*, and *Hyalomma* (Zobba *et al.,* 2008; Bhoora *et al.,* 2009; Bhoora *et al.,* 2010a; Mangana−V *et al.,* 2013, Qablan *et al.,* 2013) are able to transmit these parasites (Zobba *et al.,* 2008). Throughout the world ticks represent a major risk of infections and are recognised as one of the most economically significant parasites threatening horse industry. The disease is endemic in horse communities (Piantedosi *et al.,* 2014). It occurs in most tropical and sub−tropical regions of the world where tick vectors are present. A tick vector transmit the parasite from an infected to an uninfected horse. Transmission by simple contact is not possible (Brooks *et al.,* 1996). Both *B. caballi* and *T. equi* are infectious and capable of causing a disease in horses that is characterized by progressive anaemia (Bhoora *et al.,* 2010a). Fever, anaemia, icterus, lethargy are known characteristics of the disease and in some cases death may occur (Alhassan *et al.,* 2005; Qablan 2013; Rosales *et al.,* 2013). The clinical signs are often variable and nonspecific, making it easy to confuse the disease with other conditions, therefore complicating diagnosis. Based on clinical signs alone, it is also not possible to differentiate between *B. caballi* and *T. equi* infections (Rosales *et al.,* 2013). Several studies have documented mixed infections of *T. equi* and *B. caballi* (Malekifard *et al.,* 2014).

4.1.3.1 Etiology

According to Mehlhorn and Schein (1998), piroplasmas taxonomic status remains unclear, although they can be definitely assigned to the phylum Apicomplexa. The *T. equi* was formerly assigned to the genus *Babesia*, but as a result of molecular analysis and confirmation of pre−erythrocytic stages in its life cycle it was reclassified as *Theileria equi*. *Babesia caballi* and *T. equi* are two economically important species that may produce clinical disease and death in horses (Battsetseg *et al.,* 2002). Mixed infection with both organisms has been frequently reported in equids (Bhoora *et al.,* 2009; Piantedosi *et al.,* 2014 and these parasites are naturally transmitted between hosts via tick vectors (Piantedosi *et al.,* 2014). *Babesia caballi* and *T. equi* share many

of the same tick vectors, are usually present in the same geographic regions, and repeatedly co−infect horses (Rothschild, 2013). *Theileria equi* is considered a more pathogenic and more consistent cause of hemoglobinuria and death in equines, while *B. caballi* causes a more persistent syndrome characterized by fever and anaemia (Salim *et al.,* 2008). According to Rothschild (2013), *B. caballi* is regarded as a true *Babesia* because it replicates exclusively within erythrocytes in the vertebrate host while *T. equi* is considered to be a "small *Babesia*" where sporozoites do not readily infect red blood cells but initially penetrate a lymphocyte or macrophage (Hunfeld *et al.,* 2008). Most clinical cases of equine piroplasmosis in southern Africa are caused by *T. equi*, which usually shows a high prevalence, while *B. caballi* infections are usually not clinically discernable and usually has a lower prevalence. *Rhipicephalus evertsi evertsi,* the ixodid tick is the main vector of *T. equi* and *B. caballi* in South Africa (Motloang *et al.,* 2008).

Equine piroplasmosis is a tick−borne disease that affects equids (horses, donkeys, mules and zebras) (Motloang *et al.,* 2008; Short *et al.,* 2012; Mangana−V, *et al.,* 2013). Horses that recover from acute infections may remain reservoirs for ticks, which will transmit the infection to other susceptible horses or other equids (Heim *et al.,* 2007; Rüegg *et al.,* 2007). According to Rothschild (2013) a tick serves as a reservoir for *B. caballi* because the organism persists in ticks throughout several generations, with transstadial and transovarial transmission (although not in all tick species). Low−levels carriers constitute a risk introduction of these parasites disease−free areas as a result of the increased movement of horses' worldwide (Bashiruddin *et al.,* 1999)

Depending on the tick vector the incubation for *B. caballi* is 10−30 days up to 21 days for *T. equi* (Mehlhorn and Schein, 1998). Disease is usually more sensitive with *T. equi* than with *B. caballi*. Symptoms vary from chronic to per acute form. In per acute form horses are found dead or dying before apparition of premonitory signs (OIE, 2008).

4.1.3.2 *Babesia caballi*

The life cycle of *B. caballi* is typical of most *Babesia* species in that only erythrocytes are targeted in the mammalian host (Figure 4.3). Infection is initiated by feeding of infected ticks, most often *Dermacentor nitens*, on a naive equine host (Rothschild, 2013). Sporozoites initially enters the red blood cells (erythrocytes) where they are

converted into trophozoites which grow and cleave into two round, oval or pear− shaped merozoites. Merozoites infects new red blood cells and the division process is repeated (OIE, 2008; Short *et al.,* 2012). The merozoites measure approximately 2−5 µm long and 1.3−3 µm in diameter. Most parasites are destroyed within the tick's midgut when an uninfected tick subsequently feeds on the infected horse blood and ingests parasitized erythrocytes (Rothschild, 2013).

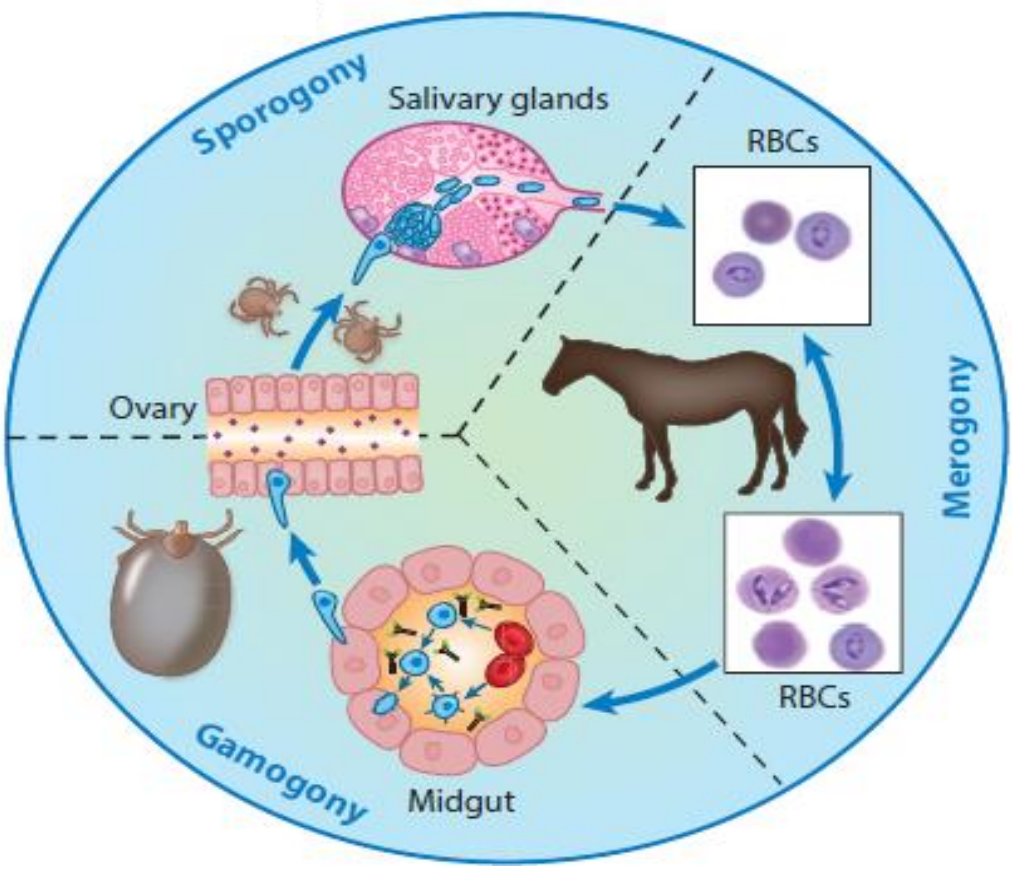

Figure 4.3: Life cycle of *Babesia caballi* (Scoles and Ueti, 2015)

4.1.3.3 *Theileria equi*

Details of the life cycle of *T. equi* (Figure 4.4) may vary depending on the tick species involved. Infected tick feeds on horses and inject *T. equi* sporozoites (Rothschild, 2013). The sporozoites penetrate into lymphocytes of the vertebrate host where formation of large macroschizonts (phase of nuclear reproduction) and microschizonts occur giving rise to merozoites (Mehlhorn and Schein, 1998). The merozoites released from schizonts invade red blood cells (erythrocytes) and reproduce by binary fission

(merogony) forming pyriform stages measuring approximately 2 – 3 µm. In erythrocytes, asexual division gives rise to four pear−shaped stages measuring approximately 2 µm and appearing as a “Maltese cross” form (Rothschild, 2013). After rupture of infected erythrocytes. Released merozoites enter other red blood cells, become spherical therein, and start another phase of asexual reproduction (Mehlhorn and Schein, 1998).

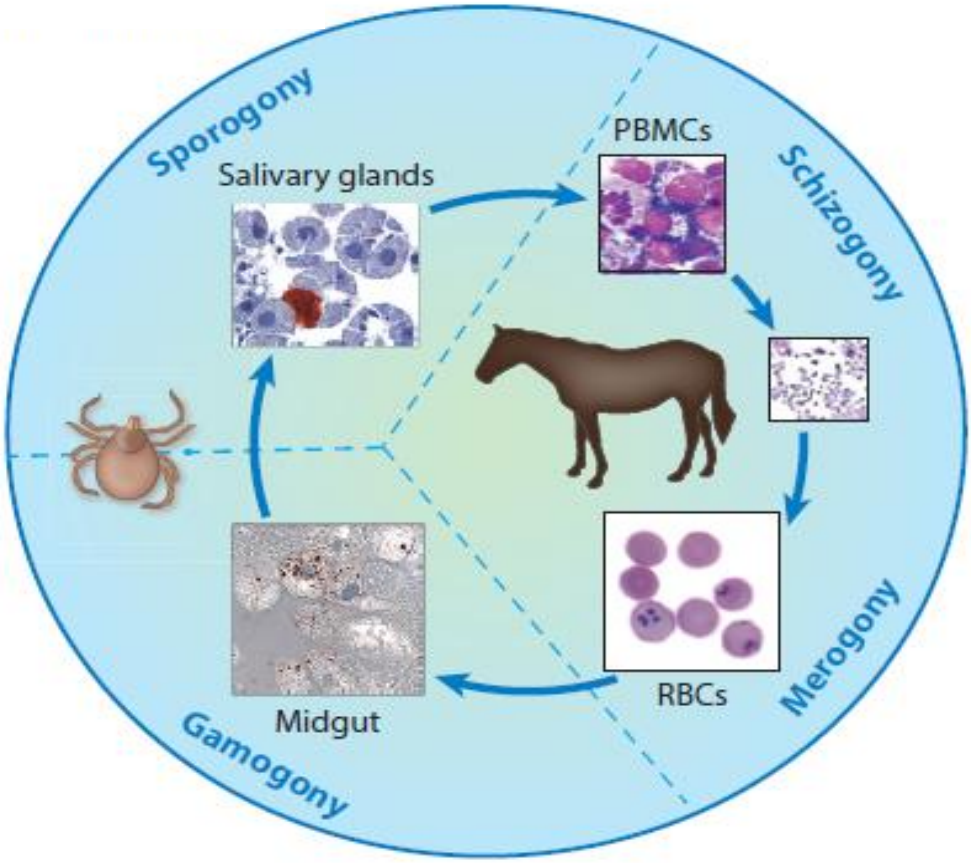

Figure 4.4: *Theileria equi* life cycle (Scoles and Ueti, 2015)

4.1.3.4 Geographic distribution

Equine piroplamosis is endemic in many parts of Europe, Africa, Asia (except Japan) (Brüning, 1996; Rüegg *et al.,* 2007), and America where suitable tick vectors are present (Zobba *et al.,* 2008) and may coexist where there is a common vector. In parts of southern Europe, infections caused by *T. equi* are more frequent than those due to *B. caballi* (Bashiruddin *et al.,* 1999). The prevalence of the *T. equi* is high in tropical and subtropical regions of Africa, in South, Middle, and North America; and in all coastal countries of the Mediterranean (Mehlhorn and Schein, 1998). Countries at risk of

becoming exposed to the parasites due to increasing movement of horses include the USA, Canada, Australia, New Zealand and Japan (Bruning, 1996).

4.1.3.5 Transmission

Twelve species of Ixodid ticks in the genera *Dermacentor, Rhipicephalus* and *Hyalomma* have been identified as trans−stadial vectors of *B. caballi* and *T. equi*, while eight of these species were also able to transmit *B. caballi* infections transovarially (OIE, 2008). Trans−stadial transmission occurs with *Theileria* spp. only (Allsopp and Allsopp, 2006). *Theileria equi* is transmitted transtadially where transmission occurs via bites of nymphs and adults; larvae are not pre−infected from ova (Mehlhorn and Schein, 1998). *Theileria equi* develop in salivary gland of tick vector and not found in other tick organs, this means it is not transmitted transovarially (egg to larva). *Theileria equi* can also be transmitted by *Boophilus microplus* (Bashirudinn *et al.,* 1999). Both pathogens can also be transmitted via used needles, syringes, and blood transfusions. Contaminated dental and tattoos equipment can also transmit equine piroplasmosis (OIE, 2008).

4.1.3.6 Clinical signs

Equine piroplasmosis clinical signs are categorized as per−acute, acute, and chronic forms. In subacute clinical signs cases are similar (OIE, 2008). Equine piroplasmosis has complicating diagnosis due to the fact that the clinical signs of piroplasmosis are often variable and nonspecific, making it easy to confuse the disease with other conditions (Bhoora *et al.,* 2010a). Affected animals normally show the following signs; loss of weight, fever is sometimes intermittent and mucous membranes vary from pale pink, pale yellow to bright yellow. Petechiae and/or ecchymoses may also be visible on the mucous membranes. Mild edematous swelling of the distal part of the limbs sometimes occurs as well (Chan *et al.,* 2010). Infections are characterized by fever, anaemia, icterus, haemoglobinuria, bilirubinuria and sometimes, death (Kouam *et al.,* 2010). In addition, intrauterine infections with *T. equi* may result in abortion and neonatal death (Rüegg *et al.,* 2007; Kouam *et al.,* 2010). In endemic areas clinical signs of infections are not pathognomonic (Kouam *et al.,* 2010).

4.1.3.7 Diagnosis

According to Bruning (1996), diagnosis can either be direct or indirect for clinical equine babesias or of subclinical infection with the parasites. Direct detection includes the clinical examination of a suspected animal, particularly for evidence of tick infestation and consideration of any recent travel. The clinical signs of equine piroplasmosis are often non−specific, complicating diagnosis. Therefore, it is not possible to differentiate between *T. equi* and *B. caballi* infections based on clinical signs alone and mixed infections also occur Bhoora *et al.,* (2009); Salim *et al.,* (2008). The severity of disease may vary depending on the strain of parasite and the susceptibility of the animal, clinical signs may be too general and not attributed to piroplasmosis but to another disease (Bruning, 1996).

Babesial infection is usually diagnosed by microscopic examination, which enables the detection of parasites from Giemsa or Wright stained blood films. Although this method is simple, it is insufficient for the accurate identification of *Babesia caballi* and *T. (Babesia) equi* during mixed infections and low parasitemias (Alhassan *et al.,* 2005). Equine piroplasmosis can be diagnosed by a number of different methods including Giemsa−stained blood smears, *in vitro* culture method, ELISA and PCR (Alhassan *et al.,* 2007a) where PCR has been shown to be sensitive, specific and useful diagnostic tool (Rampersad *et al.,* 2003).

4.1.3.8 Treatment

The treatment of Equine priroplasmosis is aimed to eliminate parasites from horses severely affected by the disease, or from horses travelling from endemic regions into disease free areas. Antitheilericidal drugs such as buparvaquone have been demonstrated to be effective in combatting disease due to *T. equi* and may in combination with imidocarb also eliminate the parasite (Bruning, 1996). Elimination of *T. equi* infections by chemotherapy is unsatisfactory (Kerber *et al.,* 2009). Drug sensitivity studies have shown that the exoerythrocytic schizonts of *T. equi* are highly susceptible to schizoticidal drugs such as halofuginone, parvaquone, and oxytetracycline used in the treatment of bovine theileriosis but not to the babesicidal drug diminazene diaceturate which is effective for the treatment of *B. caballi* (Bruning, 1996). Parvaquone

treatment alone is ineffective against *T. equi*, and parasites reappear in treated horses even after combined therapy (Allsopp and Allsopp 2006).

4.1.3.9 Control

The control of equine piroplasmosis in endemic countries is becoming increasingly important and plays a critical role in maintaining the international market open to the horse industry (Camacho *et al.,* 2005). Equine piroplasmosis must include effective tick control, sero monitoring of animals and the application of chemotherapy (Bruning, 1996). Tick control is an important aspect of transmission. No tick, no transmission (Brooks *et al.,* 1996). Piroplasmosis should also be controlled by the prevention of tick vector−equine host interaction such as by application of acaricides (Brüning, 1996)

4.1.3.10 Economic importance

Equine piroplasmosis is an economically important disease worldwide, for this reason some disease−free countries, including Austalia, Canada, Japan, and the USA, have established regulatory import restrictions in an attempt to prevent the entry of sero-positive animals (Piantedosi *et al.,* 2014). Cases of equine piroplasmosis are reported throughout the world where the tick vectors are present. Economic losses associated with equine piroplasmosis are significant and include the cost of treatment, especially in acutely infected horses, abortions, loss of performance and death (Leal *et al.,* 2011 and Rothschild, 2013). The costs associated with tick−borne diseases include both direct losses (from mortality and reduced production), and the costs associated with control and treatment (Minjauw and McLeod, 2003).

4.2 Aims of the study

This study was aimed at determining the prevalence of *Babesia caballi* and *Theileria equi* infections from horses in the Free State, Mpumalanga, Northern Cape and North West Provinces of South Africa.

4.3 Objectives

- To determine the prevalence of *T. equi* and *B. caballi* infections in equids in South Africa using PCR technique.
- To confirm PCR positive *T. equi* and *B. caballi* infections in equids in South Africa by LAMP technique.

4.4 Materials and methods

Blood samples were collected from horses and donkeys in four provinces of South Africa, namely Free State (FS), Mpumalanga (MP), North West (NW) and Northern Cape (NC) as mentioned from the previous chapter 3.2. Briefly blood samples were collected into EDTA−coated vacutainers which were immediately transported to the laboratory in a cooler box for DNA tests (PCR). Sera was also harvested for serological tests (ELISA).

4.5 DNA extraction

4.5.1. DNA extraction for FS samples by modified salting out method

DNA extration for all samples collected from FS was done by salting out method (Nasiri *et al*., 2005). DNA was extracted from blood using 1.5 ml eppendorf tubes containing 50 microlitres of blood filled with 410 µl of extraction buffer [10 mM Tris−HCl pH 8.0], 10 mM EDTA, and 1% sodium dodecyl sulphate (SDS)]. Eighty microlitres of 10% SDS was added followed by 10 µl of Proteinase K (Pro−K). Swabs for genital secretions were immersed in 1.5 ml eppendorf tubes containing 500 µl of lysis buffer, thereafter DNA was extracted as described above.The samples from both blood and genital secretions were incubated at 55°C for 1 hour. Additional 10 µl of Proteinase K (Pro−K) was added after an hour, and the samples were incubated again at 55°C and left overnight to complete the digestion. On the following day, DNA was extracted by centrifuging samples for 5 minutes at 12 000 rpm. Six hundred microlitres of the supernatant transferred to the second set of 1.5 ml sterile eppendorf reaction tubes and 180 µl of 5 M NaCl was added to the supernatant. Tubes were vortexed for 30 seconds, and centrifuged at 13 500 rpm for 5 minutes. 420 µl of ice cold isopropanol was added. The mixture was inverted 50 times followed by centrifugation at full speed (14 000 rpm) for 5

minutes at 4°C to precipitate the DNA. Subsequent to centrifugation, the supernatant was discarded, and pellet containing DNA was washed twice by 250 µl of 75% ethanol. Tubes were vortexed for 30 seconds followed by centrifugation at full speed for 5 minutes, and the supernatant was discarded. Washing was done twice to remove the excess cellular and chemical content that might inhibit PCR. The samples were left opened to air dry for an hour at room temperature to evaporate the 75% ethanol. Finally, the DNA pellet was dissolved in 200 µl of double distilled water (DDW) then incubated at 37°C for 30 minutes. The presence of DNA was confirmed by using Nano drop spectrophotometer (Thermo Fischer, USA) before storage at −35°C until further used.

4.5.2 DNA extraction for MP, NC and NW samples by ZYMO DNA blood extraction kit

Blood samples collected at MP, NC and NW, were extracted with a Zymo DNA blood extraction kit according to manufactorer's instructions (Zymo, USA). Beta−mercaptoethanol (250 µl) was added to the Genomic Lysis Buffer. Then 200 µl of genomic lysis buffer was added on to 50 µl of blood samples and mixed completely by vortexing for 6 seconds, then left to stand at room temperature for 10 minutes. The mixture was transferred to a Zymo−Spin IIC^{TM}Column^{2} in a collection tube and centrifuged at 10 000 rpm for 1 minute. The supernatant was discarded, and 200 µl of DNA pre−wash buffer was added to the spin column and centrifuged at 10 000 rpm for 1 minute. Five hundred microlitres of g−DNA Wash Buffer was added to the spin column then centrifuged at 10 000 rpm for 1 minute. Spin columns were transferred to a clean micro centrifuge tubes. A 50 µl of DNA Elution Buffer was added onto each tube and then incubated at room temperature for 5 minutes then centrifuged at top speed (13 500 rpm) for 30 seconds to elute the DNA. The eluted DNA was stored at −20°C for molecular based applications.

4.6 Polymerase chain reaction (PCR)

4.6.1 PCR for FS samples using Dreamtaq master mix

Three microliter of DNA template from collected samples (n = 40) were subjected to an initial amplification by PCR utilizing *T. equi* specific primers EMA1 R and EMA1 F (Table 4.1) with a product size of 743 bp for *T.equi* and BC48 R and BC48 F primers (Table 4.1) specific for *B. caballi* with a product size of 179 bp. PCR conditions for all primers were as follows: denaturation step at 94°C for 10 minutes, 35 cycles each at 94°C for 45 seconds, 60°C for 1 minute, 2 minutes at 72°C and followed by final extension at 72°C for 7 minutes. Reactions were performed with 12.5 µl Dreamtaq master mix (Thermo Scientific, USA), Primer mix containing 10 µM each oligonucleotide primer and 4.5 µl double distilled water (DDW) to adjust the volume to the total of 25 µl. The presence and size of amplified DNA in PCR reactions was determined by gel electrophoresis on a 1.5% agarose gel with a 100 bp DNA ladder. Reactions were scored as positive if amplified DNA was on the expected molecular weight as determined by gel electrophoresis. Selected PCR positive amplicons were sent for sequencing at Inqaba Biotechnology Company.

4.6.2. PCR for MP, NC and NW samples using Amplitaq® Gold master mix

DNA samples extracted from MP, NC and NW (n = 216) Provinces collected samples were subjected to PCR amplification using PCR primers shown in Table 4.1 which were reported by Alhassan *et al.,* (2005) for detection of *Theileria equi* and *Babesia caballi*. A 12.5 µl of Amplitaq Gold® 360 master mix (Applied Biosytems, U.S.A.) was mixed with (10 µM) each primer (Table 4.1) and 4.5 µl DDH_20 to adjust volume to 25 µl. Then 3 µl of template DNA was added. The following PCR conditions were used for the reactions: activation at 95°C for 10 minutes, followed by 35 cycles at 95°C for 30 seconds, 57°C for 30 seconds, 72°C for 60 seconds and the final extension of 72°C at 7 minutes.

To confirm the presence of *B. caballi* primer set Bec−UF2−TCG AAG ACG ATC AGA TAC CGT CG and Cab−R CTC GTT CAT GAT TTA GAA TTG CT; and for *T. equi,* Bec−UF2− TCG AAG ACG ATC AGA TAC CGT CG and Equi−R TGC CTT AAA CTT CCT GCG AT from Alhassan *et al.,* (2005) were used to screen only the positive DNA amplified when using *Alhassan et al.,* (2005) and Ikadai *et al.,* (1999) primer sets. PCR was conducted same as above.

4.6.3 Gel purification by QIAquick gel extraction kit (QIAGEN, USA)

The bands of PCR positive samples were cut out from 1.5% agarose gel, weighed and final mass was measured and placed into 1.5 ml eppendorf tubes, then QG buffer was added according to manufacturer's protocol. Samples were heated for 10 minutes at 50°C. Isopropanol with same volume used on QG buffer was added, mixed and spun down using desktop centrifuge. The mixture was then added onto new column tubes and then centrifuged at full speed (13000 rpm for 1 minute). The supernatant was discarded. Seven hundred and fifty microliters of PE buffer was added and centrifuged at (13000 rpm for 1 minute). The supernatant was discarded and the tubes were centrifuged to remove the extra PE buffer at (13000 rpm for 1 minute). The columns were placed in new 1.5 ml eppendorf tubes and the lower part was discarded. To elute DNA, 30 µl of elution buffer was added straight on top of the white part of column and then samples were incubated at room temperature for 1 minute and centrifuged at 13000 rpm for 1 minute. DNA was stored at −20°C. Gel electrophoresis was conducted as mentioned in 3.4.3 to confirm the presence of DNA after purification. The purified PCR positive samples were sent to Inqaba Biotechnical Co., Pretoria for sequencing.

Table 4.1: The nucleotide sequences of the primers used in this study

Pathogen	Sequences	Target gene	Reference
Theileria equi	F: GCATCCATTGCCATTTCGAG R: TGCGCCATAGACGGAGAAGC	EMA1	Alhassan *et al.,* (2005) and Motloang *et al.,* (2008)
Babesia caballi	F: GGCTCCCAGCGACTCTGTGG R: CTTAAGTGCCCTCTTGATGC	BC48	Alhassan *et al.,* (2005)
	F: CGGCTGCTATGGTTATTCAG R: AGAGTGCAACCGAGCAATGC		Ikadai *et al.,* (1999)

4.7 Loop−mediated Isothermal Amplification (LAMP)

4.7.1 LAMP reaction using Loopamp kit (Eiken Chemical Co., Japan)

LAMP was carried out in total volume of 25 µl reaction mixture containing 12.5 µl of 2X LAMP buffer, 2.6 µl Primer mix with 40 pmol of each FIP and BIP and 5 pmol of each F3 and B3), 6.9 µl DDW, 1 µl *Bst* DNA Polymerase and 2 µl of double−stranded target DNA. The mixture was incubated at 65°C for 1 hour and heating at 80°C for 10 minutes to terminate the reaction in LAMP real time turbidimeter (EIKEN CHEMICAL, CO, LTD, Japan). LAMP specific primers for *T. equi* and *B. caballi* (Table 4.2) described by Alhassan *et al*., (2007b) were used in this study.

Table 4.2: *Theileria equi* and *Babesia caballi* designed for LAMP by Alhassan *et al.,* (2007b)

Parasite	Primer set	Sequence
Theileria equi	BEQ 1	F3: TTGCCATTTCGAGCATCCT
		B3: ACGGTCTTTGGGGTATGTTC
		FIP:TGCTTGTCGATGGTGATGTGGTAGGAGGAGAAACCCAAGGC
		BIP: GTCCGAGGAGCACGTCGTCTAGTCTTGATGATGACGGAGTCGC
Babesia caballi	BCAB1	F3: TGTTTCCATCATGGCTCCC
		B3: GCGCTAACGGAAGCACTG
		FIB: GGCATTGGCAGCTGAGTCCAGGCGACGTTGACTAAGACCT
		BIP: AGCGATTACTTGTCGGCTGTGTCCCCTTAGGGACCTGACTG

4.8 Statistical analysis

Results were calculated as the number of positives and percentage positives. All data are given as absolute numbers, as percentages of means. Confidence Interval (CI) of the mean at 95% was used to determine the prevalence of equine piroplasmosis in the four sampled provinces. Pearson's chi square (χ^2) test was used to determine the distribution of *T. equi* and *B. caballi* across the four sampled provinces.

Hypothesis: the distribution of *T. equi* and *B. caball* in the four sampled provinces will not vary.

4.9 Results

4.9.1 Polymerase chain reaction

In the current study *T. equi* and *B. caballi*, causative agents of equine piroplasmosis were detected in all provinces by PCR. The PCR amplification revealed positive band size of 392 bp for *T. equi* (Plate 4.1). No amplification was observed when normal horse blood was used as DNA. Interestingly it was for the first time that *B. caballi* causing equine piroplasmosis were demonstrated by PCR in FS, MP, NC and NW provinces. Prevalence with *T. equi* infections using EMA1 primers in FS (n = 40), in MP (n = 94), in NC (n = 54) and in NW (n=68) were 35% [95% CI = 0.39 ± 0.31], 14.9% [95% CI = 0.39 ± 0.29], 7% [95% CI = 0.11 ± 0.14] and for 6% [95% CI = 0.11 ± 0.11] respectively. χ^2 = 19.83 (df = 3) and p > 0.05, there was significant difference in the overall distribution of *T. equi* per province sampled. Therefore the null hypothesis was rejected.

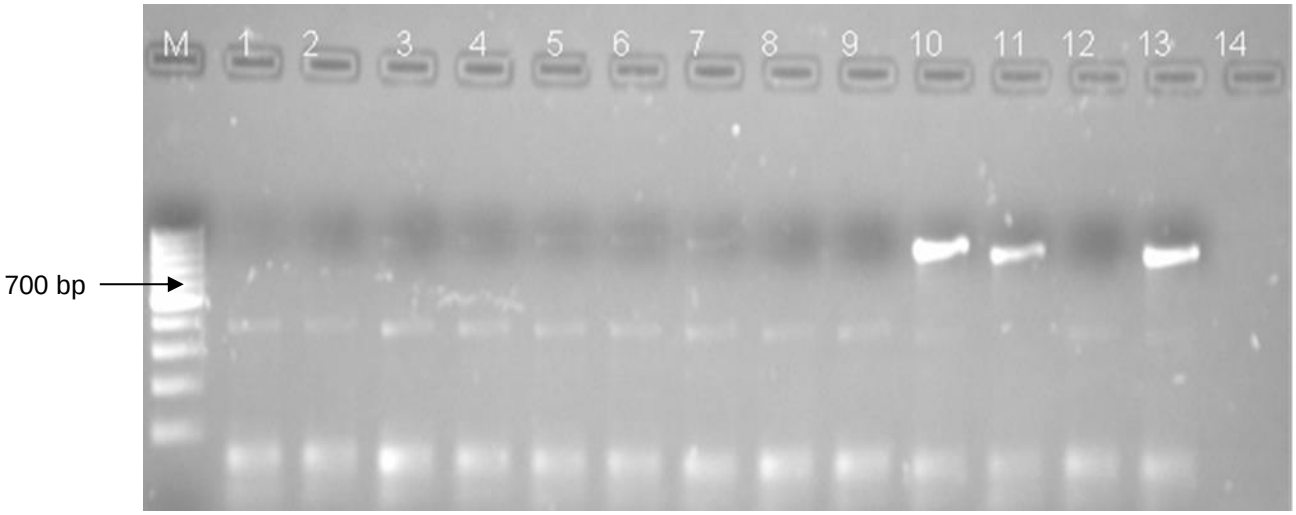

Plate 4.1: Gel electrophoresis (1.5% agarose, stained with ethidium bromide 1 µg/ml) of *T. equi* amplicon size of amplified PCR product 700-800 bp: M: 100 bp (O‟GeneRulerTM) DNA ladder, Fermentas Life Sciences, US). 1, 2, 3, 4, 5, 6, 7, 8, 9, & 12 amplified PCR product but not at expected size, 10 and 11 indicate positive with expected amplicon size 750 bp, 13 *B. equi* as positive control and 14 DDW as negative control (NC).

The PCR amplification revealed positive bands between 179 bp for *B. caballi* (Plate 4.2) *Babesia caballi* was also detected with an overall of 8.2% incidence. The prevalence obtained amongst the four sampled provinces FS (n = 40), MP (n = 94), NC (n = 54) and NW (n = 68) were, 22.5% [95% CI = 0.43 ± 042], 6.4% [95% CI = 0.45 ± 0.10], 5.6% [95% CI = 0.15 ± 0.10] and 4.4% [95% CI = 0.15 ± 0.08] respectively. χ^2 = 16.35 (df = 3) and p > 0.05. There was significant difference observed in the overall prevalence of *B. caballi* in the sampled provinces, and therefore null hypothesis was rejected.

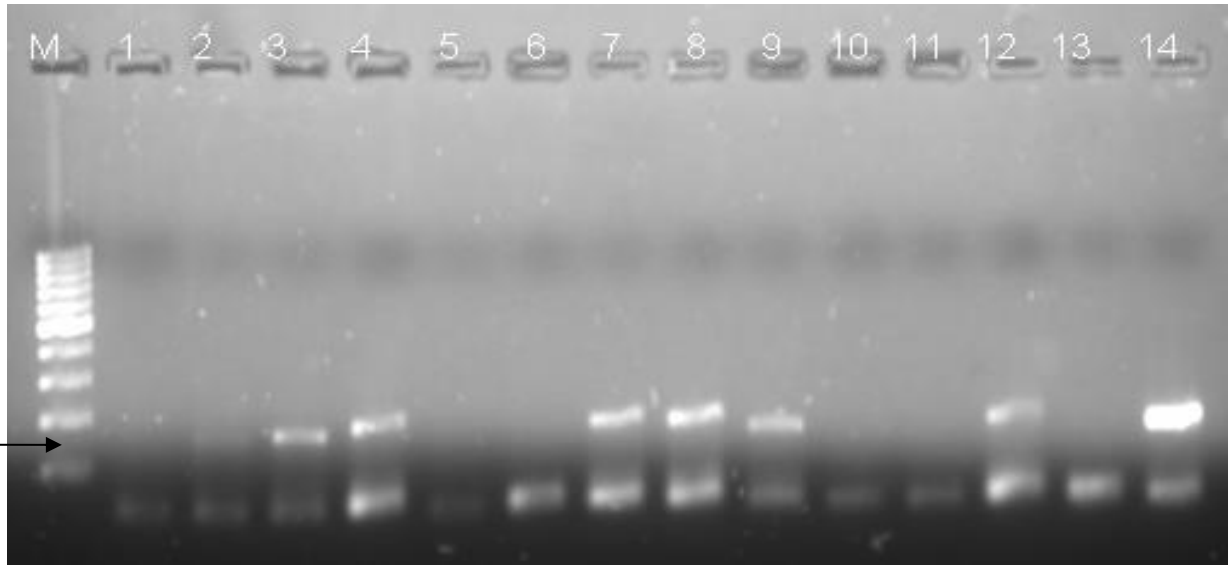

Plate 4.2: Gel electrophoresis of *B. caballi* amplified PCR product with amplicon size of 179 bp, M: DNA ladder (100 bp), 1 DDW & 13 *B. equi* as negative controls, 2, 3, 4, 7, 8, 9, and 12 showing positive results, 5, 6, 10 and 11 negative results and 14 *B. caballi* as positive control.

Horse samples

Free State Province (22.5%) obtained the highest prevalence for *B. caballi* and (35%) *T. equi*. Followed by MP, NC and NW as shown in Table 4.3. *Theileria equi* infection rate was higher than *B. caballi* in FS and MP Provinces.

Donkey samples

As shown in Table 4.3, infection rate in FS with *T. equi* was 50%, followed by 8.3% in NC, none were detected in NW and no samples were collected in MP Province. No infections with *B.caballi* were dected from all sampled Provinces.

The overall prevalence of equine piroplasmosis in horse and donkey samples using the primer set EMA1 and BC48 was 10.7% (55/256) with 13.7% *T. equi* infections, 8.2% *B. caballi* infections and with 10.5% mixed infections across all four sampled Provinces. In horse samples 33 (14.9%) DNA samples were amplified with *T. equi* infections and 9.5% were infections with *B.caballi*. Only two DNA samples from donkeys were detected 5.9% with *T.equi* parasite and none were detected with *B. caballi* parasite. High prevalence were obtained in horses than donkeys.

Table 4.3: Summary of the overall prevalence with *T. equi* and *B. caballi* infections in blood samples from horses and donkeys across the four sampled Provinces of South Africa

		PCR							
Province	**No.of horse & donkey samples**	***B. caballi***	***T. equi***	**No. of horse samples**	***B. caballi***	***T. equi***	**No. of donkey samples**	***B. caballi***	***T. equi***
FS	**40**	9 (22.5%)	14 (35%)	**38**	9 (23.7%)	13 (34.2%)	**2**	0 (0%)	1 (50%)
MP	**94**	6 (6.4%)	14 (15%)	**94**	6 (6.4%)	14 (15%)	**–**	–	–
NC	**54**	3 (5.6%)	4 (7.4%)	**42**	3 (7.1%)	3 (7.1%)	**12**	0 (0%)	1 (8.3%)
NW	**68**	3 (4.4%)	3 (4.4%)	**48**	3 (6.3%)	3 (6.3%)	**20**	0 (0%)	0
Total	**256**	21 (8.2%)	35 (13.7%)	**222**	21 (9.5%)	33 (14.9%)	**34**	0 (0%)	2 (5.9%)

To confirm the presence of *B. caballi* primer set Bec−UF2−TCG AAG ACG ATC AGA TAC CGT CG and Cab−R CTC GTT CAT GAT TTA GAA TTG CT; and for *T. equi,* Bec−UF2− TCG AAG ACG ATC AGA TAC CGT CG and Equi−R TGC CTT AAA CTT CCT GCG AT from Alhassan *et al.,* (2005) were used to screen only the positive DNA amplified when using Alhassan *et al.,* (2005) and Ikadai *et al.,* (1999) primer sets. Attained results are shown in (Plate 4.3 and 4.4) for *T. equi* and *B. caballi* respectively. PCR positive results, DNA amplified with *T. equi* and *B. caballi* were submitted to direct sequencing using the BEC−UF2 with EQUI−R and CAB−R primers with both forward and reverse. GenBank was used to identify the amplified sequences by using NCBI BLAST searches (http://www.ncbi.nlm.nih.gov/BLAST). BlastN searches revealed 92% identity and e−value of 0.0 with *Theileria equi* 18S rRNA gene partial sequence (GenBank accession number: KU 879051.1 and EU642510.1).

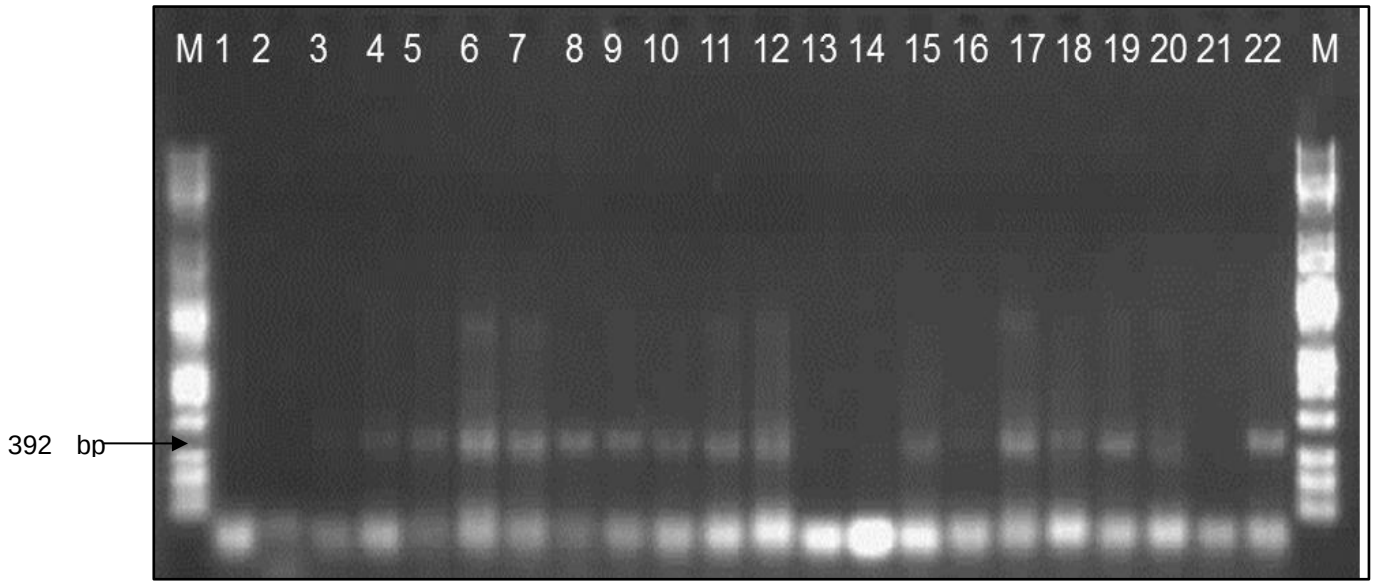

Plate 4.3: Gel electrophoresis of *T. equi* amplified PCR product with 392 bp amplicon size, M: 100 bp DNA ladder, 1 DDW and 2 *B. caballi* as negative controls, 4, 5, 6, 7, 8, 9, 10, 11, 12, 15, 17, 18, and 19 showing positive amplification, 3,13,14,16,20, and 21 negative results and 22 *B. equi* as positive control.

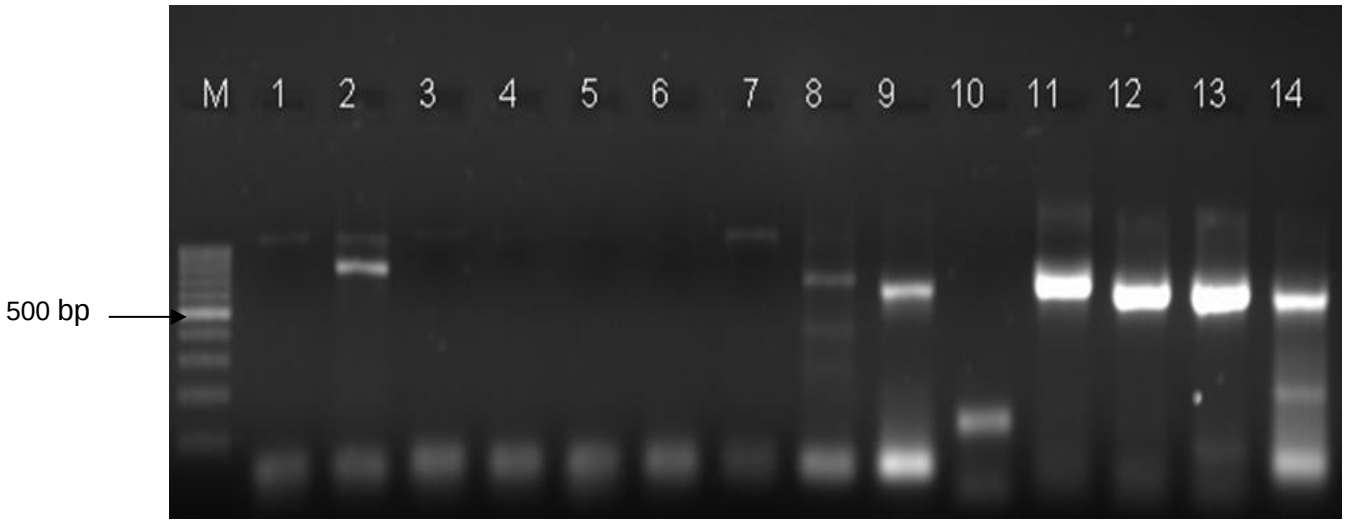

Plate 4.4: Gel electrophoresis (1.5% agarose, stained with ethidium bromide 1 µg/ml) of *B. caballi* amplicon size of amplified PCR product 500-600 bp: M: 100 bp (O"GeneRulerTM) DNA ladder, Fermentas Life Sciences, US). 1, 2, 3, 4, 7 & 10 amplified PCR product but not at expected size, 5 & 6 negative results, 8, 9, 11, 12, & 13 indicate positive results with expected amplicon size 540 bp, and 14 *B. caballi* as positive control.

4.9.2 Loop−mediated isothermal amplification

Loop mediated isorthermal amplification was used to confirm PCR positives. LAMP amplification products obtained were visualised under UV light (Figure 4.5) for *T. equi* and represent *B. caballi* (Figure 4.6). Ten DNA samples including positive and negative controls were screened for *T. equi* and only one PCR product were negative by LAMP (Table 4.4). Eighty percent of PCR products were positive when using LAMP amplification (Figure 4.5). For *B. caballi* only 10 DNA samples were screened and were also positive in LAMP (Figure 4.6) except negative control. One DNA sample was not amplified. DNA samples were chosen as representatives of each province from PCR positive samples, where LAMP was used only to confirm the PCR positive samples. Only very few samples were used for LAMP due to financial restrictions.

Table 4.4: LAMP amplification results for *T. equi* and *B. caballi* across the four sampled Provinces

Sample number	**Results for *T. equi***	**Sample number**	**Results for *B. caballi***
1. *B. caballi* (NTC)	−	1. FS (H5)	−
2. *B. equi* (Pc)	+	2. FS (H17)	+
3. FS (H29)	+	3. MP (45)	+
4. FS (P6)	+	4. MP (81)	+
5. MP (14)	+	5. NC (20)	+
6. MP (42)	+	6. NC (52)	+
7. NC (13)	+	7. NW (4)	+
8. NC (21)	+	8. NW (45)	+
9. NW (33)	+	9. *B. caballi*	+
10. NW (38)	−	10. *B. equi* (NTC)	−

*NTC: No template control, FS: Free State Province samples, MP: Mpumalanga Province samples, NC: Northern Cape Province samples and NW: North West Province samples

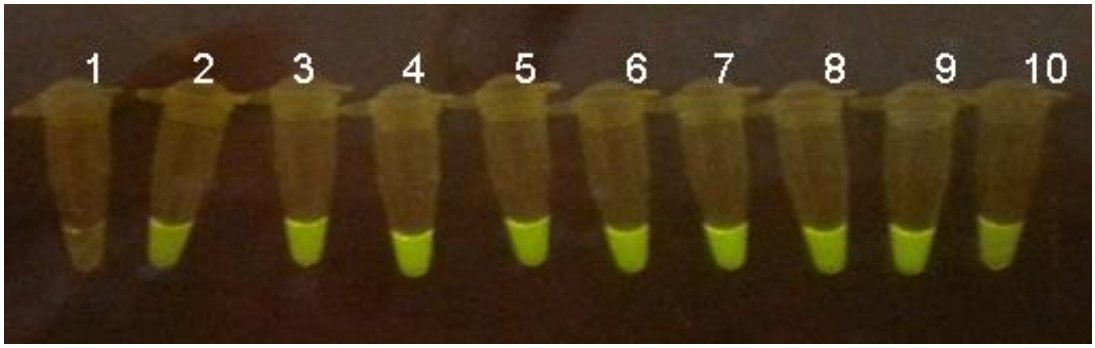

Plate 4.5: LAMP results visualised under UV light for *T. equi*: 1. *B. caballi* (NRCPD) as a negative control, 2. *B. equi* as a positive control, 3. (H29) and 4. (P6) samples from Free State, 5. (MP 14) and 6. (MP 42) samples from Mpumalanga, 7. (NC 13) and 8. (NC 21) samples from Northern Cape, 9. (NW 33) and 10. (NW 38) samples from North West.

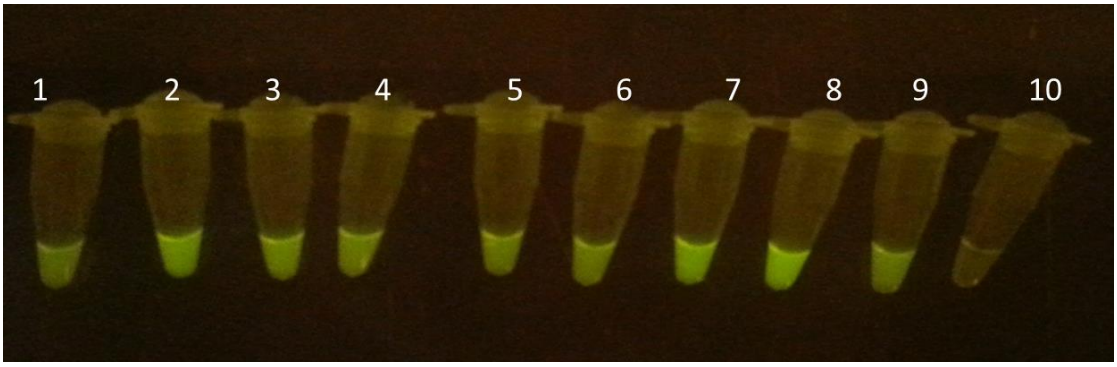

Plate 4.6: LAMP amplification results visualised under UV light for *B. caballi*: 1 (H5) and 2 (H17) samples from Free State, 3 (MP 45) and 4 (MP 81) samples from Mpumalanga, 5 (NC 20) and 6 (NC 52) samples from Northern Cape, and 7 (NW 4) and 8 (NW 45) sample from North West, 9 *B. caballi* as positive control and lastly 10 *B. equi* as a negative control.

4.9.3 Enzyme linked immune sorbent assay

Donkey and horse serum samples showed immunoreactivity in ELISA assays (Fig. 1). The cut−off values of antigens for Bc 134 lot1 (*B. caballi*) and EMA2R (*T. equi*) were 2.53 and 2.47 respectively. Most of the samples clustered between 2.5 − 2.6 OD values for *B. caballi* and *T. equi*. Out of 250 serum samples from these study's locations, overall seroprevalence of 42% was obtained for both donkey and horse samples combined, with 20% (50/250) and 22% (55/250) for *B. caballi,* and *T. equi* respectively, and 24.8% (26/105) had mixed infections. The FS had the highest seropositive *B. caballi* values at 57.8% followed by NW with 19.1%, NC with 12% and MP with 11%. For *T. equi*, FS had once again the highest sero−prevalence of 54.1%, followed by NW with 36.9%, MP with 7.5% and NC with 6%.

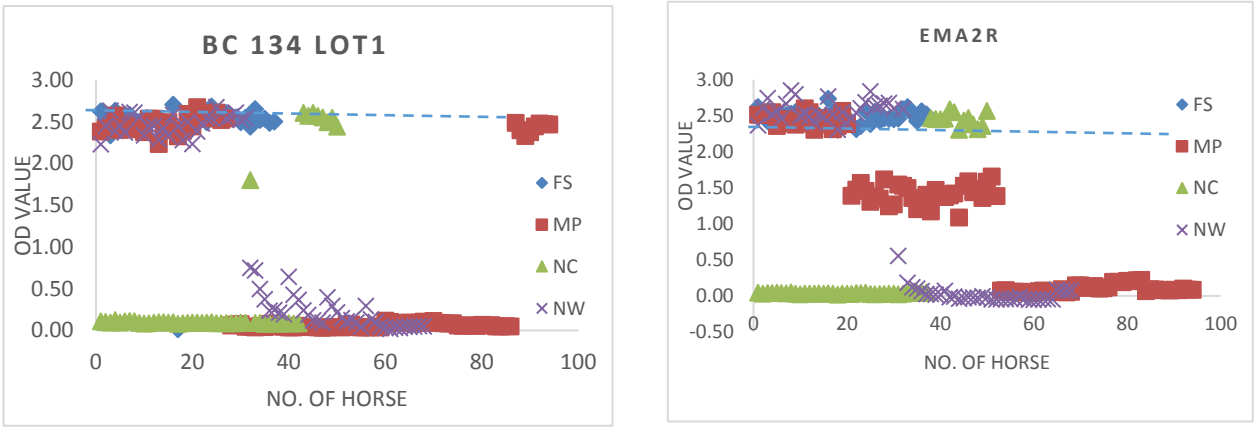

Figure 4.5: Demonstrates the cut-off values 2.53 and 2.47 (indicated by broken lines) for *B. caballi* and *T. equi* parasites respectively from different provinces of South Africa. A: Free State (FS) B: Mpumalanga, C: Northern Cape (NC), and Lastly D: North West (NW).

Horse serum samples

Babesia caballi

The seroprevalence of *B. caballi* was 19.9% (43/216) for horse samples by ELISA using reccombinant antigen Bc 134 lot1. Highest number of seropositive samples was from FS province with 54.3%, followed by NW with 18.8%, NC with 12.5% and MP with 11% (Table 2.1).

Theileria equi

As shown in table 2.1, ELISA with EMA2R antigen detected 21.8% (47/216) of antibodies against *T. equi*. The highest detection rate was in FS province with 54.3% sero−positives, followed by NW with 39.6%, MP with 7.5% and NC with 5.1%.

Donkey serum samples

Babesia caballi

Out of 34 donkey samples sero−prevalence of 21.9% was obtained for *B. caballi*. Free State had the highest sero−positives 100%, followed by NW (20%) and NC (8.3%). No serum samples were collected from Mpumalanga as shown in Table 4.1.

Theileria equi

Seroprevalence of 23.5% was obtained out of 34 serum samples. FS had higher seroprevalence of 50%, followed by NW (30%) and lastly NC (8.3%). No donkey serum samples were collected in MP.

Table 4.5: Detection of *Thelieria equi* and *B. caballi* antibodies in serum samples of horses and donkeys determined by ELISA assays

Province	No. of horse serum samples	ELISA		No. of donkey serum samples	ELISA	
		B. caballi	***T. equi***		***B. caballi***	***T. equi***
Free State	35	19 (54.3%)	19 (54.3%)	2	2 (100%)	1 (50%)
Mpumalanga	94	10 (11%)	7 (7.5%)	−	ND*	ND*
Northern Cape	39	5 (12.5%)	2 (5.1%)	12	1 (8.3%)	1 (8.3%)
North West	48	9 (18.8%)	19 (39.6%)	20	4 (20%)	6 (30%)
Total	216	43 (19.9%)	47 (21.8%)	34	7 (21.9%)	8 (23.5%)

*Not done (Donkey serum samples were not available)

4.10 Discussion

4.10.1 Polymerase chain reaction

Theileria equi and *Babesia caballi* are known to be the causative agents of equine piroplasmosis (Mehlhorn and Schein, 1998). These two hemoprotozoan parasites have biological differences but cause similar pathology and have similar life cycles and vector relationships (Scoles and Ueti, 2015). The current study revealed that equines from all the sampled provinces are exposed to equine piroplasmosis. *Theileria equi* and *B. caballi* parasites were detected among the sampled Provinces by PCR and ELISA techniques. Results of this study revelead that infections with *T. equi* (13.7%) were higher than *B. caballi* (8.2%), whilst overall this study data has showed higher prevalence than 11.0% of *T. equi* and 0% of *B. caballi* obtained by Motloang *et al.,* (2008). This is the first time that *B. caballi* is detected in the eastern Free State by PCR and in all three sampled provinces, as there are no records of detection *B. caballi* by PCR from previously conducted studies in South Africa (Motloang *et al.,* 2008 and Bhoora *et al.,* 2010b). Consistent with earlier findings of Motloang *et al.,* (2008), our study confirms that horses in four sampled Provinces are exposed to *T. equi*. In the recent years, there have been several reports on the detection of equine piroplasmosis by PCR worldwide (Alhassan *et al*., 2005, Motloang *et al.,* 2008, Bhoora *et al.,* 2010b and Malekifard *et al.,* 2014). Motloang *et al.,* (2008) did not detect *B. caballi* when using PCR but by Indirect Fluorescent Antibody Test (IFAT) which is used to detect antibodies showed the exposure levels of 48% for *B. caballi* and 98% for *T. equi* from screened horses in Free State. These could be due IFAT disadvantages such as low sensitivity for detecting latent infections and false−positive results (Deepak *et al.,* 2014). The present study confirms these results, indicating that equine piroplasmosis is prevalent in Free State province including Mpumalanga, Northern Cape and North West.

In the present study the prevalence of *B. caballi* was still low as compared to *T. equi*, this could probably be due to the low parasitaemia in *B. caballi* infections (Motloang *et al.,* 2008; Bhoora *et al.,* 2010b). The observations in the present study are similar to trends observed in parts of southern Europe where infections due to *T. equi* are more common than those caused by *B. caballi* (Bashiruddin *et al.,* 1999) and that *T. equi* is more frequent and pathogenic than *B. caballi* in endemic areas (Malekifard *et al.,* 2014). There is common agreement that *B. caballi* infections are mild and usually not clinically obvious (Gizachew *et al.,* 2013). As consequences of climate change over recent

decades have probably led to a wider spatial distribution of vector ticks and extensions in their periods of activity, the chances for transmission of *Babesia* are likely to be increased (Moretti *et al.,* 2010). A successful PCR detection in *B. caballi* (8.2%) infections increased prevalence might be due to expansion in vector distribution.

For donkeys 5.9% and 0% of *T. equi* and *B. caballi* results were lower than 17.4% and 3.6% for *T. equi* and *B. caballi* respectively reported in Italy (Laus *et al.,* 2015). In Brazil, Machado *et al*., (2012) reported 31.81% and 20.45% for *T. equi* and *B. caballi* respectively, whilst 50.94% for *T. equi* and 0% infections were reported for *B. caballi* in Iran (Abedi *et al.,* 2015).

The infection rate with *T. equi* observed in the current study was high in all sampled provinces (Table 4.2) as compared to previous studies carried out in South Africa and other different parts of the world using molecular methods (Alhassan *et al.,* 2005, Heim *et al.,* 2007, Motloang *et al.,* 2008, Zobba *et al.,* 2008, Bhoora *et al.,* 2010b, Leal *et al.,* 2011 and Malekifard *et al.,* 2014). The high prevalence for *T. equi* infections observed in the current study correspond to the ones described previously by Alhassan *et al.,* (2005) in multiplex PCR as the same primers were used in the current study but with conventional PCR. The result of this study supported the findings of Bashiruddin *et al.,* (1999) and Heim *et al.,* (2007) that PCR is highly sensitive in diagnosing piroplasmosis and is a useful tool in the detection and identification of *T. equi* and *B. caballi* in blood.

The high prevalence in *T. equi* infections than *B. caballi* also supported that *T. equi* and *B. caballi* are distinct parasites. They differ in a number of ways including the vectors capable of their transmission; their morphology and development within tick vectors and their differences in susceptibility to treatment (Knowles, 1996). Horses were most susceptible than donkeys for *T. equi* and *B. caballi* infections. This could be due the number of samples collected and that donkeys usually show asymptomatic form of the disease, with lower *T. equi* parasitaemia when compared to horses (Gizachew *et al* 2013; Salim *et al.,* 2013). In most instances, the horses become lifelong carriers due to immune evasion strategies of parasite (Deepak *et al.,* 2014).

In this study LAMP was used to confirm PCR positives. As shown in table 4.4 only one PCR positive sample was negative when using the species specific primers for *T. equi* and *B. caballi* pathogens. Successful LAMP amplification results of the current study were comparable with the findings of Alhassan *et al.,* (2007b) where LAMP was applied

in epidemiological studies of equine piroplasmosis on samples collected from China and South Africa. The current finding also proves that LAMP can be used to detect *T. equi* and *B. caballi*. It has been reported that because of its simplicity, rapidity and specificity, LAMP is appropriate to be used in the field and also at the point of care stations (Laohasinnarong, 2011). *Theileria equi* is thought to have a wider distribution than *B. caballi* (Deepack *et al.,* 2014). In regions where EP is considered endemic, *T. equi* infections are more prevalent than *B. caballi* infections and these observations have been recoderd from Morocco, South Africa, Madagascar, virtually all other parts of the African continent (Rothschild, 2013). This is due to poor management of equine populations, including inadequate veterinary care, poor nutrition, and overwork, and as a result these factors may worsen the impacts of infection (Scoles and Ueti, 2015).

Molecular detection of the parasites requires DNA isolation from parasites that are noticeably present in the blood sample to a measurable level above the sensitivity threshold of the particular detection method used. Regularly, *T. equi* parasites are not completely eliminated from the blood of horse after treatment or natural recovery as compared to *B. caballi* (Salim *et al.,* 2008).

The difference in the prevalence of equine piroplasmosis among countries may be due to differences in sensitivity of the diagnostic tests used, the occurrence and abundance of competent vectors, the activity of the equids and the presence and effectiveness of any control measure (Kouam *et al.,* 2010). The different prevalence of disease may be associated with the number of animals studied, the different diagnosis methods used, the geographic area, tick controls and difference in spread of vectors between these areas (Salim *et al.,* 2008; Karatepe *et al.,* 2009).

4.10.2 Enzyme linked immune sorbent assay (ELISA)

The objective of this study was to determine the prevalence of *T. equi* and *B. caballi* infections in donkeys and horses of South Africa using molecular (PCR) and serological (ELISA) methods. This study has confirmed that recombinant antigen ELISA is capable of detecting *T. equi* and *B. caballi* infections in South Africa**.** The results revealed that EMA2R antigen (*T. equi*) detected more sero positives than Bc 134 lot1 antigen (*B. caballi*). The seroprevalence reported for *T. equi* (22%) and *B. caballi* (20%) with 24.8% of mixed infections, indicated that animals are exposed to *Babesia* parasites.

Horses

Prevalence differed among provinces this could be due to the geographical distribution. *Theileria equi* infections were still more prevalent than *B. caballi* as it was previously found from earlier studies. However, the seroprevalence of antibodies against *T. equi* and *B. caballi* obtained were lower than 98% and 48% in a study of Motloang et al., (2008). This low seroprevalence could be due the sensitivity and specificity of the techniques used. The essential problems associated with IFAT which includes cross−reactivity and restriction by antibody detection limits (Xuan *et al*., 2001). Xuan *et al.,* (2002) findings in China, demonstrated 40.0% and 24.3% seroprevalence for *T. equi* and *B. caballi* respectively. In Mongolia prevalence of antibodies against both parasites were 72.8% and 40.1% for *T. equi* and *B. caballi* respectively, of which are higher than what is obtained in the current study. In South Africa *R. evertsi evertsi* is known to be the main vector (Motloang *et al.,* 2008). Kuoam *et al.,* (2010), reported seroprevalence of 11% for *T. equi* and 2.2% for *B. caballi,* which wereis low when compared to the current study. Abutarbush *et al.,* (2011), reported 14.6% for *T. equi* and none were detected for *B. caballi*. This could be due to the fact that *T. equi* is not completely removed from the horses' blood after treatment or natural recovery (Abutarbush *et al.,* 2011).

Donkeys

For most reports on EP conducted on horses, little is known about piroplasmosis in donkeys (Gizachew *et al.,* 2013). In donkeys, chronic cases of equine piroplasmosis with non−clinical signs, including mild appetite, poor work performance, or poor body weight gain are common (OIE, 2008; Gizachew, *et al.,* 2013). This study is the first to report on screening of donkey serum samples for *T. equi* and *B. caballi* using ELISA assays in South Africa. The results of this study, showed that antibodies against both parasites were detected in 15 (45.4%) of the examined 34 sera samples. Seroprevalence obtained for both parasites were 21.9% and 23.5% for *B. caballi* and *T. equi* respectively where 33.3% had mixed infections, which were higher compared to Tefera *et al.,* (2011), 2.08% and 1.04% for *T. equi* and *B. caballi* findings in Central Ethiopia but lower compared to Machado *et al.,* (2012), 73.86% and 93.2% for *T. equi* and *B. caballi*; In Sicily, Piantedosi *et al.,* (2013), reported 35.5% for *B. caballi* and

44.3% for *T. equi* while Gizachew *et al.,* (2013) reported 55.7% for *T. equi* and 9.9% for *B. caballi* in Central Ethiopia. Veronesi *et al.,* (2014), reported 39.3% and 47.5% for *T. equi* and *B. caballi* respectively, of which was higher compared to this study. This differences could be due to techniques used, body conditions which was found to create significant difference, season of sample collection, antigenic stimulation and some of these animals may act as parasite carriers (Tefera *et al.,* 2011; Gizachew *et al.,* 2013),

This the first report of seroprevalence by ELISA assays in these Provinces of South Africa. The variance in the incidence of equine piroplasmosis among countries may be due to differences in sensitivity of the diagnostic tests used, the occurrence and abundance of competent vectors, the movement of the equids, existence and effectiveness of any control measures (Tefera *et al.,* 2011; Gizachew *et al.,* 2013). In the same way, the reasons for the variation in prevalence levels among regions may be related to the host activity, the management practices and the difference in prevalence of suitable tick vectors (Kuoam *et al.,* 2010). Donkey's sera are frequently having capacity to remove or inactivate complement non−specifically (OIE, 2008). In this study prevalence of equine piroplasmosis in donkeys was found to be higher than in horses. *Theileria equi* infections were more prevalent than *B. caballi* in both animals, of which were in agreement with seroepidemiological studies conducted worldwide (Boldbaatar *et al.,* 2005; Xuan *et al.,* 2002; Zobba *et al.,* 2008).

Of 42% ELISA seropositives, 10.7% were PCR positives. The number of *T. equi* and *B. caballi* seropositive animals was higher than the number of PCR positive animals. This could be due to the fact that ELISA detects antibodies which is an indicator of expore to infction (Sigg *et al.,* 2010), which can be present infection or past infection as antibodies persist even when the pathogen had been eliminated. Lower number of positive animals in PCR assays could represent the presence of a low number of circulating parasite loads in the blood circulation (Machado *et al.,* 2012). Serological assays and molecular techniques are objective and accurate tools for the diagnosis of equine piroplasmosis (Abutarbush *et al.,* 2011).

CHAPTER 5

MOLECULAR DIAGNOSIS OF *ANAPLASMA PHAGOCYTOPHILUM* AND *NEORICKETTSIA RISTICII* INFECTIONS IN HORSES AND DONKEYS IN SOUTH AFRICA

5.1. Anaplasmosis and ehrlichiosis

Due to the changes in the taxonomy in 2001 (Gussmann *et al.,* 2014), some species of *Ehrlichia* have now been reclassified into the genera *Anaplasma* or *Neorickettsia*, and all were placed in the family Anaplasmataceae (OIE, 2005). The genera *Ehrlichia* and *Anaplasma* are widespread in nature. The reservoir hosts include numerous wild animals, as well as some domesticated species (OIE, 2013a). Phylogenetic studies showed taxonomic disarray among organisms broadly referred to as ehrlichiae, during the process of classification of the human agent and a careful reorganization which now places those bacteria previously classified as a different genus *E*. *phagocytophila*, *E*. *equi*, and the Human granulocytic ehrlichiosis agent into a single species, *A*. *phagocytophilum* (Dumler *et al.,* 2005b; De La Fuente *et al.,* 2005).

The diseases caused by these pathogens have traditionally been categorized by the type of blood cell most commonly infected (McQuiston *et al.,* 2003). *Anaplasma phagocytophilum* causes granulocytic anaplasmosis in dogs, cats, horses and humans and tick−borne fever (TBF) in ruminants (Murase *et al.,* 2010; Silaghi *et al.,* 2011). According to McQuiston *et al.,* (2003), the infection is self−limiting in most horses, although death can occur. *Ehrlichia chaffeensis, E ewingii, A. phagocytophilum,* and *N. sennetsu* are the zoonotic species. *Ehrlichia canis* also thought to be zoonotic but there is no evidence to confirm that, it still remains to be confirmed. *Ehrlichia chaffeensis E. canis* and *A. phagocytophilum* have worldwide distribution. Although *A. phagocytophilum* occurs worldwide, in Europe, India and South Africa only tick−borne fever has been reported. *Anaplasma phagocytophilum* has recently been classified based on genetic analysis in the genus *Anaplasma,* with *A. marginale,* which causes infectious anaemia in cattle by infecting erythrocytes, and *A. platys,* which causes canine cyclic thrombocytopenia by infecting platelets (OIE, 2005; Pusterla and Madigan, 2013). Horses with this condition may exhibit a range of debilitating clinical signs, with severe cases occasionally proving fatal (Lewis *et al.,* 2009; Chan *et al.,* 2010). The early

symptoms are nonspecific. Fairly common signs are gastrointestinal including nausea, vomiting, diarrhoea and abdominal pain (OIE, 2005).

5.2. Equine granulocytic anaplasmosis (EGA)

5.2.1 Etiology

Equine granulocytic anaplasmosis is one of a number of infectious rickettsial diseases that affect horses. It is noncontagious with seasonal activity (late fall to spring). Equine granulocytic anaplasmosis is an acute tick−borne infection caused by *Anaplasma phagocytophilum* and this disease used to be known as Equine granulocytic ehrlichiosis. The first cases of infection with the causal bacterium were observed in northern California in the late 1960s (Lewis *et al.,* 2009). Anaplasmosis is most frequently reported in the upper midwestern and northeastern USA; 6 states account for 88% of all reported cases of anaplasmosis, including New York, Connecticut, Massachusetts, Rhode Island, Minnesota, and Wisconsin (Clair and Decker, 2012). Apart from America and Europe, the bibliographical data also report the occurrence of equine anaplasmosis in Asia and Africa (Dzięgiel *et al.,* 2013). Tick vectors known as *Ixodes ricinus*, a sheep tick in Europe and subgroup of hard ticks, including the western black−legged tick (*I. pacificus*) in western North America, the deer tick (*I. scapularis*) in Europe which also cause the Lyme disease and in eastern North America, the taiga tick (*I. scapularis*) in Asia are known to transmit variants of *A. phagocytophilum* to the host species (Foley *et al.,* 2008; Chan *et al.,* 2010). According to Chan *et al.,* (2010), wild rodents, sheep, and deer have been suggested as reservoirs for *A. phagocytophilum*. Birds are believed to act as carriers of infected ticks, while they are feeding and resting at stopover sites along their routes, rather than serving as reservoirs themselves.

According to Pusterla *et al.,* (2000), Granulocytic anaplasmosis is an infectious multi organ human and animal disease accompanied by thrombocytopenia. Micro−organisms are etiological agents of the disease previously classified as separate species of *Ehrlichia* but currently they are classified as single species, *A. phagocytophilum* (OIE, 2005; Adasczek and Winiarczyk, 2011). The *A. phagocytophilum* is an obligate intracellular bacterium (Loewenich *et al.,* 2003; Boni *et al.,* 2009; Siska *et al.,* 2013) that invades neutrophils, creating intravascular aggregates called morulae. Transmission to humans and horses occurs through tick bites (*Ixodes*). It induces a febrile disease (Boni

et al., 2009) and replicates in neutrophils (Gussmann *et al.,* 2014). *Ixodes ricinus* and *I. pacificus* have both been demonstrated to transmit the disease (Lewis *et al.,* 2009). It causes granulocytic anaplasmosis in numerous mammals, including humans, sheep, goats, horses, dogs, cattle, llamas, (Murase *et al.,* 2010; Silaghi *et al.,* 2011) and tick−borne fever (TBF) in ruminants (Silaghi *et al.,* 2011; Siska *et al.,* 2013). Symptoms vary depending on the infected species, the variant of *A. phagocytophilum* involved or the age, condition and the immune status of the host (Silaghi *et al.,* 2011).

5.2.2 Life cycle

The life cycle involves four distinct stages of their ticks egg, larva, nymph and adult (Lewis *et al.,* 2009) in this genus. A blood meal is required for transition from larva to nymph and from nymph to adult; adult ticks also take a blood meal to complete life cycle (McQuiston *et al.,* 2003). *Anaplasma phagocytophilum* cannot be passed effectively from infected adult *Ixodes* species, ticks to eggs. Thus, larvae are not infected and this is trans−stadial transmission. Ticks at the larval, nymphal, or adult stage acquire *A. phagocytophilum* strains through blood feeding on infected animals.

5.2.3 Transmission

Transmission occurs through the larvae and nymph (Ogden *et al.,* 1998). The immature ticks (larvae and nymph) must feed on infected animals to acquire and transmit infection to the successive life stages (nymphs and adults, respectively). Tick transmission is believed to be the only epidemiologically important means of acquiring infection. There is no evidence of transmission from horses to humans; this means direct infection from horses to humans has not been identified (McQuiston *et al.,* 2003). Infection can also be transmitted by blood transfusions, and mechanical transmission by biting insect has been suggested as a possible means of spread (OIE, 2005).

5.2.4 Clinical signs

Fever, depression, lack of appetite, leg swelling, reluctance to move and yellowish gums (M'ghirbi *et al.,* 2012) are most common symptoms. Lethargy and oedema of the

limbs, anorexia, and thrombocytopenia are also common clinical signs that develop over several days (McQuiston *et al.,* 2003). Weakness and ataxia can be severe, to the point that horses will sustain fractures after falling (Pusterla and Madigan, 2013). Clinical signs of infection vary with age of the horse; the severity of the disease ranges from mild to severe. In horses with low numbers of platelets, petechial haemorrhages may develop (McQuiston *et al.,* 2003). The clinical course of the disease ranges from 3 to 16 days (Pusterla and Madigan, 2013). The infection is self−limiting in most untreated horses, although death can occur. In young animals, clinical signs may be milder than those observed in old horses (McQuiston *et al.,* 2003).

5.2.5 Diagnosis

Anaplasmosis is often diagnosed by serological tests. Indirect immunoflourescent antibody tests are widely available and used at diagnostic laboratories (OIE, 2013a). Polymerase chain reaction (PCR) analysis is useful for the diagnosis of equine granulocytic anaplasmosis, particularly during the early stages, when number of organisms may be too small for diagnosis by microscopy and is considered to be highly sensitive and specific (Pusterla and Madigan, 2013). No reports have documented isolation or PCR detection of *Ehrlichia* spp. infection in horses worldwide (O'Nion *et al.,* 2015).

5.2.6 Treatment

According to OIE (2013a), only a limited number of antibiotics are effective for treating ehrlichiosis and granulocytic anaplasmosis. Tetracycline−class antimicrobials are the drugs of choice in humans and animals and are the most effective group of antibiotics in casual therapy of anaplasmosis (Dzięgiel *et al.,* 2013). Oxytetracycline is recommended for the treatment of horses with granulocytic anaplasmosis (ehrlichiosis) (McQuiston *et al.,* 2003). Supportive measures are recommended in severe cases, including fluid and electrolyte therapy, supportive limb wraps and stall confinement of severely ataxic horses to prevent secondary injury (Pusterla and Madigan, 2013). Many chemotherapeutics are not effective in fighting this infection because of *A. phagocytophilum* is an intracellular pathogen. Protective treatment of equine

granulocytic anaplasmosis (EGA) includes tick control and preventing them from feeding on the integuments of animals. Removal of a tick from the skin does not exclude the possibility of infection. There is no vaccine against EGA on the market currently (Dzięgiel *et al.,* 2013).

5.2.7 Prevention

Preventing tick bites can decrease the risk of infection (OIE, 2005). People who enter tick habitats should check frequently for ticks and remove them and also from pets as soon as possible (OIE, 2005; OIE, 2013a). The risk of infection can be increased when the tick removal techniques such as the use of hot matches or petroleum jelly may stimulate the tick to release additional saliva as the CDC warns (OIE, 2005; QLD, 2010). Acaricides, biological controls and control of tick habitats can decrease the populations of tick vectors in a community (OIE, 2005; OIE, 2013a).

5.3. Equine monocytic ehrlichiosis (EME) or Potomac horse fever (PHF)

5.3.1 Etiology

The order Rickettsiales embodied of a group of agents that are obligate intracellular coccobacilli (OIE, 2005) gram−negative bacteria transmitted by vectors (mainly arthropods) and include all family Rickettsiaceae and Anaplasmataceae (Cicuttin *et al.,* 2013). Intracellular endosymbionts of digeneans are bacteria found in the genus *Neorickettsia* (Greiman *et al.,* 2013). Equine monocytic ehrlichiosis or PHF is an equine disease caused by monocytotropic rickettsia, *Neorickettsia* (formely *Ehrlichia*) *risticii*, an important severe disease affecting horses, typically found in endemic countries during the warmest months, especially from mid to late summer (Cicuttin *et al.,* 2013). According to Park *et al.,* (2005), the disease was first discerned in 1979 in the areas along Potomac River in Maryland and Virginia. Since then, it has been diagnosed in most parts of the USA, Canada, Europe and other parts of the world. How *N. risticii* is transmitted to horses remained an enigma, for many years. It was then ascertained that *Neorickettsia helminthoeca* a trematode−borne bacterium; causative agent of salmon−poisoning disease in dogs was more closely related to the *N. risticii* than to tick−borne *Ehrlichia* spp. (Gibson *et al.,* 2005). Horses may be infected even if they do

not live directly by or near the bodies of water although PHF higher numbers may be found near the water (Withlock *et al.,* 2009).

The mode of transmission of *N. risticii* remained unidentified. There is no evidence for spread of the disease by arthropod vectors such as ticks (Pusterla *et al.,* 2000). Clinical signs can vary from mild depression and fever (Heller *et al.,* 2004). Abortion has been delineated related to fatal infection (Madigan *et al.,* 1999). The mortality rate is more than 30% in confirmed cases (Hollard *et al.,* 1985). In the environment *N. risticii* infects trematodes from genus *Acanthatrium* and *Lecithodendrium*. Although the lifecycles of these trematodes are not very clear, both unknown to involve several stages that range from free−living cercariae to forms that infects invertebrates. Certain species of bats and birds (swallows) have been suggested that they may act as wild reservoirs of *N. risticii* (Cucittin *et al.,* 2013).

5.3.2 Life cycle

Plagiorchis elegans has a life cycle typical for the Plagiorchiidae that involves an aquatic snail (Greiman *et al,* 2013). A trematode (fluke, parasite) that parasitizes snails as the first intermediate host, arthropod aquatic flies (May flies, caddis flies, dragon flies, dam flies and stone flies) as the second intermediate host, and bat intestines a vertebrate as the definitive host (Figure 5.1) is included in the life cycle of this organism. Insectivores, such as bats and birds, may serve as the definitive host of the trematode vector (Whitlock *et al.,* 2009; Pusterla *et al.,* 2003). Horses become infected through eating or drinking the fluke itself or the aquatic insects. Additionally, barn swallows and bats may eat flies and snails then pass the *N. risticii* in their faeces, which may eventually be ingested by horses (Whitlock *et al.,* 2009).

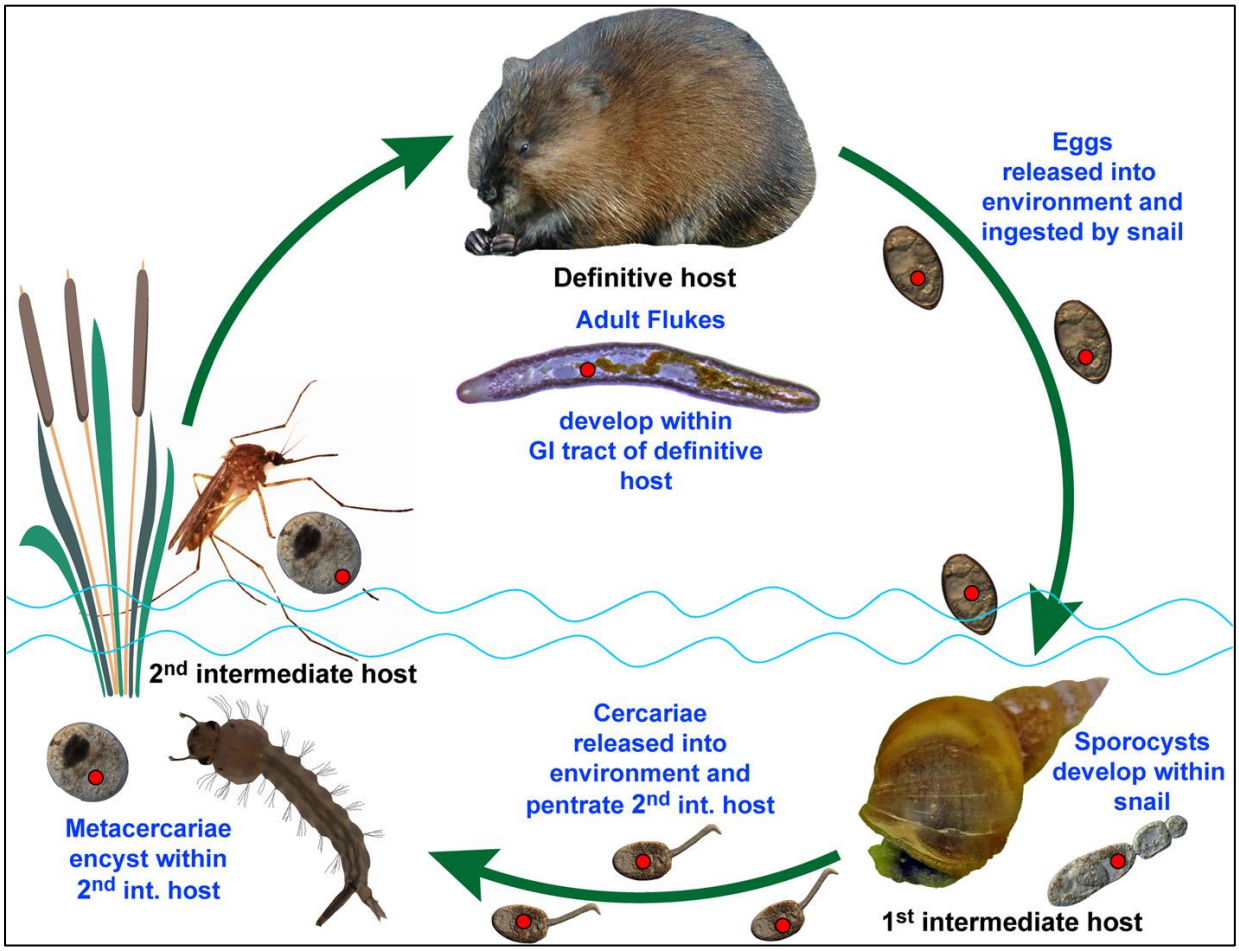

Figure 5.1: *Neorickettsia* infection is represented by a red dot. Natural life cycle of trematode *Plagiorchis elegans* (Grieman *et al.,* 2013)

Eggs are deposited and released with faeces into the external environment by an adult worm. Then eggs undergo embryonation in water and need to be ingested by snail (*Lymnae stagnalis*). In the next stage the mother sporocysts develops within the hepatopancreas of snails as the first intermediate host after miracidium process (where it hatches in the intestine of the snail, penetrates through the intestinal wall and reaches the hepatopancreas of the mollusk). Asexual reproduction commencing with mother sporocycysts that reproduce numerous daughter which in turn produce several free swimming cercariae. The cercariae are emitted into the aquatic environment by the mollusc whereby they may penetrate the second intermediate host which is larvae of aquatic insects. The cercariae moult and develop into metacercaria encysts within the

second intermediate host. Lastly, the metacercaria needs to be swallowed by an appropriate vertebrate host to mature into adult stage after some development (Greiman *et al.,* 2013).

5.3.3 Transmission

The transmission of *N. risticii* to horses and the organism's location in nature were previously unknown (Wright, 2004). The disease can be transmitted by oral inoculation, however, the causative agent has been identified in faeces (Barlough *et al.,* 1998*). Neorickettsia risticii* was found to be closely related to *N. helminthoeca,* the causative organism of salmon poisoning in dogs, which uses a helminth and aquatic snails as hosts (Heller *et al.,* 2004). Aquatic insects living in close association with snails in stream of water are infected with *N. risticii* carrying trematodes and transmit both trematodes and the rickettsial agent to potential hosts such as fish, amphibians, birds, reptiles, and mammals (Chae *et al.,* 2000). Transmission to horses is thought to occur through accidental ingestion of these insects while grazing pastures. No evidence suggests that horse to horse transmission occurs (Wright, 2004). Upon ingestion of *N. risticii* in the metacercarial stage of trematodes in aquatic insects by horses, *N. risticii* is horizontally transmitted from the trematodes to horses and replicates within inclusion bodies inside monocytes, macrophages, mast cells and intestinal epithelial cells (Lin *et al.,* 2009).

5.3.4 Clinical signs

The first observed sign of illness is often in a decreased appetite due to fever, which may then progress to more serious symptoms, including diarrhea, which may result in the need for hospitalization (Withlock *et al.,* 2009). According to Heller *et al.,* (2004), severity of clinical signs can range from mild (anorexia, fever, lethargy, and depression) to life threatening (laminitis, abortion, and diarrhea followed by severe dehydration) in horses (Gibson *et al.,* 2011). Colitis or diarrhea is the most well−known manifestation of the disease in horses affected with Potomac horse fever; however in clinical cases only about 60% of diarrhea is present (Withlock *et al.,* 2009). If untreated the disease causes abortions in pregnant mares and relatively high mortality approaching 30% (Greiman *et*

al., 2013). Some will also develop swelling of their lower limbs or body wall (QLD, 2010)**.**

5.3.5 Diagnosis

Diagnosis of Potmac horse fever usually relies on clinical signs, seasonality, and geographical occurrence of illness (Pusterla *et al.,* 2000; Heller *et al.,* 2004). Diagnosis of this disease is mainly done by indirect fluorescent−antibody (IFA) test based on *N. risticii* infected cells and by nested polymerase chain reaction (PCR) on blood samples (Gibson *et al.,* 2011), but due to the high percentage of false−positive test results when using the indirect immunofluorescence assay diagnosis is usually hampered (Pusterla *et al.,* 2000).

5.3.6 Treatment

The administration of tetracycline antibiotics at the early stage of infection is effective, in part by inhibiting bacterial protein synthesis and facilitating lysosome fusion with inclusions containing *N. risticii* (Gibson *et al.,* 2011).

5.3.7 Control and prevention

According to Lin *et al.,* (2009), Vaccine against Potomac horse fever has been marketed but Potomac horse fever still continues to cause widespread infections probably due to both the insufficient immunity developed by the vaccination and the antigenic variation of *N. risticii* strains in the field.

5.4. Economic Importance of equine anaplasmosis and equine ehrlishiosis

According to O'Nion *et al.,* (2015), ehrlichioses are important emerging zoonotic tick−borne disease that can affect both animals and humans worldwide (Dagnone *et al.,* 2009). It is the pathogens of veterinary importance responsible for tick−borne fever of ruminants and for granulocytic anaplasmosis of horses and dogs (Anderson *et al.,* 2005). Anaplasmosis, is a disease caused by various species of *Anaplasma,* and is

particularly important issue for animal breeders. *A. phagocytophilum* has been detected worldwide, particularly in North America and Europe as well as in South Africa, South America, and Asia; it infects humans, horses, ruminants, cats, dogs, and a variety of wildlife species, including rodents, deer, and carnivores (Strik *et al.,* 2007). The case fatality rate varies from 5 to 30% (Chae *et al.,* 2000), PHF remains a significant problem for horse owners (Park *et al.,* 2005).

5.5. Aims of the study

In this chapter the aim was to determine the rate of infection with *Anaplasma phagocytophilum* and *Neorickettsia risticii* amongst equids (Horses and donkeys) blood collected from the Free State, Mpumalanga, Northern Cape and North West Provinces of South Africa by PCR.

5.6. Materials and methods

5.6.1 Study areas

Blood samples were collected from horses and donkeys representing four provinces, namely, Free State, North West, Mpumalanga, and Northern Cape in May 2014. In the Free State Province sampled areas included Thaba bosiu S 28° 40’04.5”; E 028°51’16.2” and Tsheseng S 28°35’ 19.2”; E 028°56’ 16.7”. In North West samples were collected from Mafikeng and Lichtenburg. Samples were also collected at Middelvlei abattoir in Gauteng S 26 21’640”, E 27 37’282” between July and August 2015. The provinces which samples were collected from the abattoir were Mpumalanga (MP), North West (NW) and Northern Cape (NC). Equine blood samples were collected from jugular vein and were put into vials with EDTA and silicone vacutainers.

5.6.2 Isolation of DNA

The DNA concentration was determined on the basis of reading of A 260 and A 280 with a nanodrop machine GeneQuant *pro* spectrophotometer**.**

5.6.2.1 Salting out method (Nasiri *et al*., 2005) with modifications for FS samples

DNAs for all samples collected from FS were extracted by salting out method. For DNA extraction from blood, eppendorf tubes containing 50 µl of blood were filled with 410 µl of extraction buffer [10 mM Tris−HCl [pH 8.0], 10 mM EDTA, and 1% sodium dodecyl sulphate (SDS)]. Then, 80 µl of 10% SDS was added followed by 10 µl of Proteinase K (Pro−K). For genital secretions, swabs were immersed in 1.5 eppendorf tubes containing 500 µl of lysis buffer; thereafter DNA was extracted as described above. The samples from both blood and genital secretions were incubated at 55°C for 1 hour. After an hour additional 10 µl of Pro−K was added and the samples were incubated again at 55°C and left overnight to complete the digestion. On the following day, DNA was extracted as follows: Samples were centrifuged for 5 minutes at 12 000 rpm. Six hundred microlitres of the supernatant was transferred to the second set of 1.5 ml sterile Eppendorf reaction tubes and 180 µl of 5 M NaCl was added to the supernatant. The tubes were vortexed for 30 seconds, centrifuged at 13 500 rpm for 5 minutes. The supernatant was transferred to a new 1.5 ml Eppendorf tube where 420 µl of ice cold isopropanol was added to the supernatant. The mixture in the tubes was mixed by inverting the tubes 50 times followed by centrifugation at full speed (14 000 rpm) for 5 minutes at 4°C to precipitate the DNA. Subsequent to centrifugation, the supernatant was discarded and the pellet containing the DNA was washed twice by adding 250 µl of 75% ethanol. Tubes were vortexed for 30 seconds followed by centrifugation at full speed for 5 minutes and the supernatant was discarded. The wash was done twice to remove excess cellular and chemical content that might inhibit PCR. The samples were left opened to air dry for an hour at room temperature to evaporate the 75% ethanol. Finally, the DNA pellet was dissolved in 200 µl of double distilled water (DDW) then incubated at 37°C for 30 minutes. The presence of DNA was confirmed by using Nano drop spectrophotometer (Thermo Fischer, USA) before storage at −35°C until used.

5.6.2.2 DNA extraction for MP, NC and NW samples by ZYMO DNA blood extraction kit protocol (Inqaba biotec, PTA, South Africa)

For blood samples collected at MP, NC and NW, DNA was extracted with a Zymo DNA blood extraction kit according to manufactorer's instructions (Zymo, USA). Protocol is as

follows: Beta−mercaptoethanol (250 µl) was added to the Genomic Lysis Buffer. Then 200 µl of genomic lysis buffer was added on to 50 µl of blood samples and mixed completely by vortexing for 6 seconds (s), then left to stand at room temperature for 10 min. The mixture was transferred to a Zymo−Spin IIC^{TM}Column^{2} in a collection tube and centrifuged at 10 000 rpm for 1 minute. Then the supernatant was discarded. Two hundred microliters of DNA pre−wash buffer was added to the spin column and centrifuged at 10 000 rpm for 1 minute after spinning a 500 µl of g−DNA Wash Buffer was added to the spin column then centrifuged at 10 000 rpm for 1 minute. Then the spin columns were transferred to a clean micro centrifuge tubes. A 50 µl of DNA Elution Buffer was added onto each tube and then incubated at room temperature for 5 minutes then centrifuged at top speed (13 500 rpm) for 30 seconds to elute the DNA. The eluted DNA was stored at −20°C for molecular based applications.

5.6.3 Polymerase chain reaction (PCR)

5.6.3.1 PCR for *A. phagocytophilum*

The following primers were used for amplification of *A. phagocytophilum* DNA: forward EHR−747 GCA CTC ATC GTT TAC AGG GTG and reverse EHR−521 TGT AGG CGG TTC GGT AAG TTA AAG targeting 250 bp amplicons size (Welc−F *et al.,* 2009). The PCR was performed in a total of 25 µl reaction mixture using 12.5 µl Ampli *Taq* Gold Master Mix (Applied Biosystems, U.S.A). Primer mix (10 µM each primer), 8.5 µl double distilled water (DDW) and 2.0 µl template DNA. The DDW was used as a no template control. The PCR reaction conditions involved an initial denaturation at 95°C for 10 minutes followed by 35 cycles of 95°C for 30 secods, 56°C for 30 seconds and 72°C for 60 seconds and a final extension at 72°C for 7 minutes which were according to the Amplitaq Gold Master Mix protocol (Applied Biosystems, USA).

Nested PCR was also conducted using primer set ge3a CAC AAT GCA AGT CGA ACG GAT TAT TC and ge10r TTC CGT TAA GAA GGA TCT AAT CTC C as primary reaction and ge9F AAC GGA TTA TTC TTT ATA GCT TGC T and ge2 GGC AGT ATT AAA AGC AGC TCCC AGG as the second reaction (M'ghirbi *et al.,* 2012). The PCR mixture was prepared as above and the PCR conditions were the same as above except for the annealing temperature which was 55°C in both reactions.

5.6.3.2 Nested PCR for *Neorickettsia risticii* 16S rRNA

The following primer set was used: ER3 ATT TGA GAG TTT GAT CCT GG and ER2 GTT TTA AAT GCA GTT CTT GG (Pusterla *et al.,* 2000) in a first round of PCR, which amplifies 527 bp of the 16S rRNA and which are specific for the 16S rRNA gene of *N. risticii* (formerly *E. risticii*). A total of 256 blood samples were screened by PCR with the following conditions: initial denaturing of 5 minutes at 94°C, followed by denaturation at 94°C for 1 minute, annealing at 60°C for 2 minutes, and extension at 72°C for 1 minute, with 30 cycles in a PCR thermal cycler machine. Final extension followed at 72°C for 7 minutes. In a second PCR round, 1 µl of the PCR product of the first reaction was added as the template to a 25 µl reaction mixture containing 12.5 µl Amplitaq Gold Master mix (Applied Biosystems, USA), 2.0 µl of primer mix and 9.5 µl DDW. The following primers were used for the second round of PCR: ER 3a CTA GCG GTA GGC TTA AC and ER 2a CAC ACC TAA CTT ACG GG (Pusterla *et al.,* 2000), specific primers for *N. risticii*, were used in the nested reactions. PCR conditions for the nested reaction were identical to those for the first stage.

5.6.4 Product visualization

All PCR amplicons were visualized by electrophoresis on 1% agarose gel stained with gel red, under UV light and gel documentation was done using GeneSnap bio imaging system (Syngene, Synoptics, UK) GeneSnap (version 6.00.22) software.

5.6.5 Gel purification by QIAquick gel extraction kit (QIAGEN, USA)

The bands of PCR positive samples were cut out from 1.5% agarose gel, weighed and final mass was measured and placed into eppendorf tubes, then QG buffer was added according to manufacturer's protocol. Samples were then heated for 10 minutes at 50°C. Isopropanol with same volume used on QG buffer was added, mixed then spun down using desktop centrifuge. The mixture was then added onto new column tubes and then centrifuged at full speed 13000 rpm for 1 minute. Supernatant was discarded. Seven hundred and fifty microliters of PE buffer was added then centrifuged at 13000 rpm for 1 minute, supernatant was discarded and then tubes were centrifuged to remove the extra PE buffer (at 13000 rpm for 1 minute). The columns were placed in

new eppendorf tubes and the lower part was discarded. To elute DNA, 30 µl of elution buffer (EB) was added straight on top of the white part of column and then samples were incubated at room temperature for 1 minute and then centrifuged at 13000 rpm for 1 minute. DNA was stored in −20°C. Gel electrophoresis was conducted as mentioned in 3.4.3 to confirm the presence of DNA after purification. The purified PCR positive samples were sent to Inqaba Biotechnical Co., Pretoria for sequencing.

5.6.6 Statistical analysis

Confidence of the mean at 95% interval was used to determine the prevalence of equine anaplasmosis and equine ehrlichiosis across all four sampled provinces. X^2 to determine the distribution of *A. phagocytophilum* and *N. risticii* and the hypothesis which states that, the distribution of *A. phagocytophilum* and *N. risticii* will vary across the sampled provinces.

5.7. Results

Ehrlichia/Anaplasma species detection

Two hundred and fifty six (256) DNA samples collected from horses (n = 222) and donkeys (n = 34) in four provinces were screened for the presence of *Anaplasma phagocytophilum* and *Neorickettsia risticii* bacterial infections by PCR. The results of the gel electrophoresis with a product size of 250 bp are shown in (Plate 5.1). Using primer set Ehr 747− and Ehr−527 an overall of 87/256 (34%) of *A. phagocytophilum* infections were positive detected. The summary of the infection rates in horse and donkey DNA samples (Figure 5.2) with *A. phagocytophilum* for the four sampled provinces is given on Table 5.1. The highest prevalence of *A. phagocytophilum* infections obtained was 68.5% [95% CI = 0.16 ± 0.04 in NC, 52.5% [95% CI = 0.24 ± 1.03] in FS, 20.6% [95% CI = 0.43 ± 1.43] in NW and 16.0% [95% CI = 0.17 ± 0.31] as a least infection obtained. χ^2 = 57.37 (df = 3) and p > 0.05. There was significant difference observed in the overall prevalence, do not reject null hypothesis.

Northern Cape Province obtained the highest prevalence in *A. phagocytophilum* infections for horse DNA samples but obtained less prevalence of 25% in donkey DNA samples (Table 5.1). Followed by FS which obtained 65% in horse population with 0% in donkey DNA samples, MP obtained 16.3% and no DNA samples were obtained from donkeys, and NW obtained 10.3% and 35% respectively. North West Province obtained the highest infection of *A. phagocytophilum* for donkey DNA samples. There were no *Anaplasma* positive infections in donkey samples from FS. Same prevalence was obtained in nested PCR using primer set ge3a and ge10r first reaction and in a second reaction ge9f and ge2 primer set was used (M'ghirbi *et al.,* 2012).

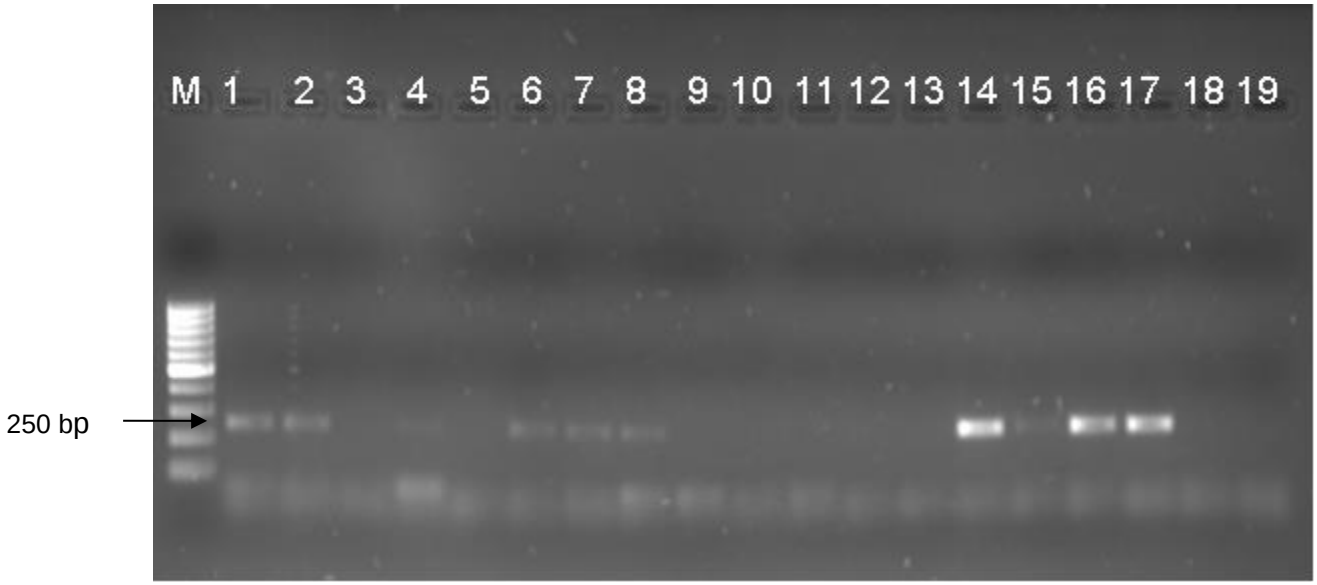

Plate 5.1: Gel electrophoresis (1.5% agarose, stained with ethidium bromide 1 µg/ml) of *A. phagocytophilum* amplicon size of amplified PCR product 250 bp: M: 100 bp (O"GeneRulerTM) DNA ladder, Fermentas Life Sciences, US). 1 positive control (PC) *A. phagocytophilum* 2, 4, 6, 7, 8, 14, 15, 16, 17 indicate positive results, 3, 5, 9, 10, 11, 12, 13, 18 indicate negative results and 19 negative control (NC).

Table 5.1: Summary of the overall prevalence infections with *A. phagocytophilum* across the four sampled Provinces of South Africa

Province	No. of samples	PCR (+)	Overall %	No. of (H) only	PCR (+)	Overall %	No. of (D) only	PCR (+)	Overall %
Free State	**40**	21	52.5	**38**	21	55.3	**2**	0	0
Mpumalanga	**94**	15	16.0	**94**	15	16.0	**–**	–	–
Northern Cape	**54**	37	68.5	**42**	34	63	**12**	3	25
North West	**68**	14	20.6	**48**	7	10.3	**20**	7	35
Total	**256**	87		**222**	77		**34**	10	
Percentage			34			34.7			29.4

*No.: number of samples, %: percentage, Donkey (D), Horse (H), no DNA for donkey samples: (−)

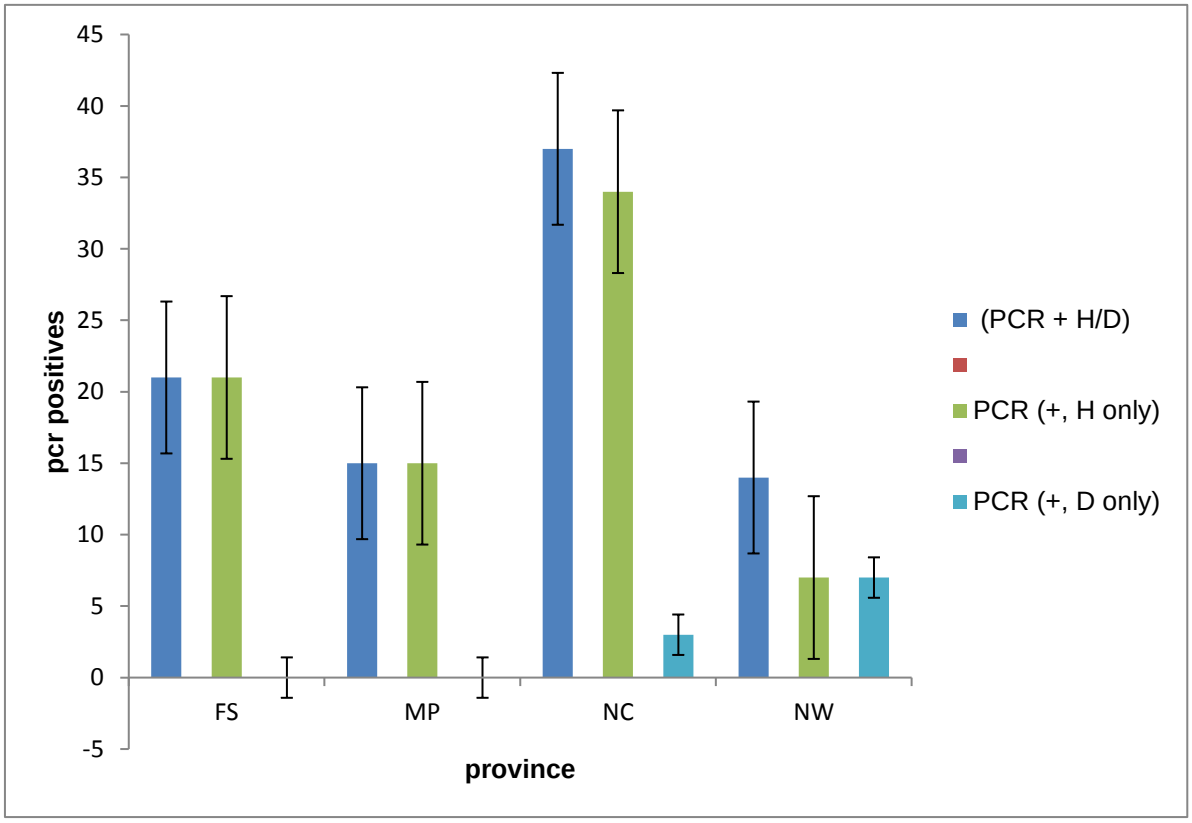

Figure 5.2: Illustrates the overall *A. phagocytophilum* detection by PCR from all tested samples FS (n = 40), MP (n = 94), NC (n = 54) and NW (n = 68). Overall PCR positive samples detected were 21, 15, 37 and 14 respectively. For horses only prevalence obtained was 21, 15, 34 and 7 respectively. For donkeys only prevalence was 0 in FS, 3 in NC and 7 in NW. No donkey DNA samples were collected in MP.

A nested PCR that amplifies 527 bp of the 16S rRNA gene of *N. risticii* (formely *Ehrlichia risticii*) was used to screen for the presence of *E. risticii* DNA in blood of horses and donkeys. Using primer set ER−2 and ER−3 for primary reaction and ER−2a and ER−3a in secondary reaction, samples tested positive for *N. risticii* gave an equivalent amplicons size of 527 bp during gel electrophoresis (Plate 5.2). Only 9 (3.5%) of 256 DNA were positively detected by PCR among the four sampled provinces from the horse samples. NW (n = 68) samples tested negative for this pathogen (0%), 12.5% infection in FS (n = 40) was observed followed by 3.2% in MP (n = 94) and 1.9% in NC (n = 54). Prevalence with 95% confidence interval was [95% CI = 0.32 ± 0.22], [95% CI = 0.33 ± 0.02] and [95% CI = 0.11 ± 0.03] for FS, MP and NC respectively. χ^2 = 12.42 (df = 3) and p >0.05. There was significant difference observed across the sampled provinces, therefore the do not reject null hypothesis. None of the DNA samples from donkeys (0%) tested positive across all four sampled Provinces.

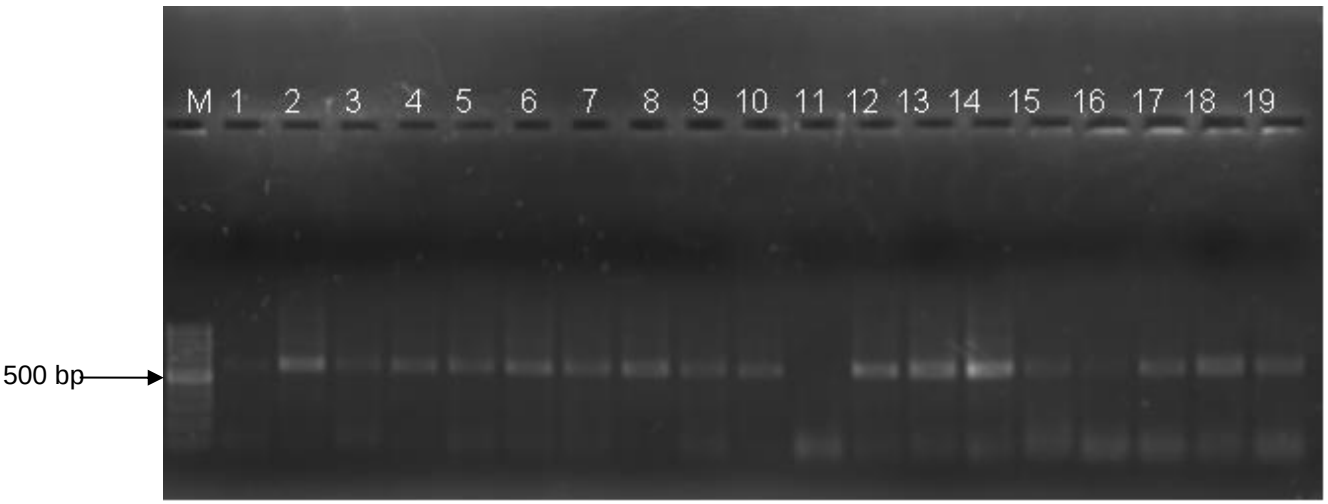

Plate 5.2: Gel electrophoresis (1.5% agarose, stained with ethidium bromide 1 µg/ml) of *N. risticii* amplicon size of amplified PCR product 500-600 bp: M: 100 bp (O"GeneRulerTM) DNA ladder, Fermentas Life Sciences, US). 1 DDW as negative control (NC), 2 *Ehrlichia*, 3, 4, 5, 6, 7, 8, 9, 10, 12, 13, 14,15, 17,18, and 19 indicate positive amplification, no amplification occurred in 11 and 16.

5.8. Discussion

The objective of this study was to evaluate the prevalence of equine anaplasmosis and equine ehrlichiosis in four sampled provinces of South Africa using DNA based technique. Obligate intracellular bacteria in the genera *Ehrlichia* and *Anaplasma* causes Ehrlichiosis and anaplasmosis tickborne diseases (OIE, 2013a). Ehrlichioses are considered emerging infectious diseases of animals and humans worldwide (O'nion *et al.,* 2015). In the current study the aim was to detect *Anaplasma phagocytophilum* and *Neorickettsia risticii* using PCR. Equine granulocytic anaplasmosis is one of several rickettsial diseases that affect horses. Molecular testing demonstrated the presence of *A. phagocytophilum* in horses and donkeys DNA samples in all four sampled provinces of South Africa. *Anaplasma phagocytophilum*, the causative agent of granulocytic ehrlichiosis affects numerous species of wild and domesticated mammals (OIE, 2013a), including horses.

The current study has revealed that *A. phagocytophilum* (*formely Ehrlichia equi* and *E. phagocytophila*) infects horses in South Africa. *Anaplasma phagocytophilum* is known to infect humans and many species of animals and is a very varied organism, and genetic variations can result in different in their virulence for host species. A possible zoonotic risk to humans from contact with a horses affected by equine granulocytic anaplasmosis has been suggested after a human granulocytic anaplasmosis (HGA) case was reported in the United States (Chan *et al.,* 2010)

Therefore, PCR confirmation of an active infection is considered the most reliable method of achieving a rapid and early diagnosis of equine granulocytic anaplasmosis (Dzięgiel *et al.,* 2013; Gussmann *et al.,* 2014). From 256 screened DNA samples by PCR, 34% shows that horses and donkeys are exposed to *A. phagocytophilum* across the four sampled provinces of South Africa. To date, there is no data available for *A. phagocytophilum* infecting equines in South Africa. For the first time using EHR−747 and EHR−521 primer set and ge3a and ge10r with ge9f and ge2 among the horse and donkey samples in South Africa, a successful PCR detected *A. phagocytophilum* when both primer set were employed. The PCR−positive from horse and donkey samples obtained in sampled provinces of South Africa were 34.7% and 29.4% respectively. The *A. phagocytophilum* infection rate reported in Europe was 17.9% (Dzięgiel *et al.,* 2013) and 14% in Sardinia (Alberti *et al.,* 2005), 13% in Tunisia (M'ghirbi *et al*., 2012) and 8%

in Central Italy (Alberti and Sparagano, 2006; Passamonti *et al.,* 2010). *Anaplasma phagocytophilum* infection has also been reported in horses (4.7%) and no infections in donkeys in Sicily (Giudice *et al.,* 2012). Torina *et al.,* (2008) reported prevalence of 0% from horses and 4% in donkeys In Sicily (Italy). PCR products obtained in this study revealed complete 92% − 100% sequence similarity with the 16S rRNA gene fragment in GenBank database, assecion number: KJ 865413.1, CP015376.1 Norway variant 2 complete genome, KT 986058.1.

To our knowledge no information regarding the *Neorickettsia risticii* in horses South Africa is currently available, thus this is for the first time horse blood samples were screened for *N. risticii* infections by PCR. This study provides circumstantial evidence that *N. risticii*, which is the causative agent of equine monocytic ehrlichiosis (EME), also known as Potomac horse fever or equine ehrlichial colitis (Dutra *et al.,* 2001) occurs in South Africa. Equine monocytic ehrlichiosis caused by the rickettsia *N. risticii* (formerly *Ehrlichia risticii*) is an acute enterocolitis of equidae (Dutra *et al.,* 2001). The identification of *N. risticii* in DNA extracted from horse blood suggested the potential importance of water borne infection. No clinical cases or any testing of PHF have been reported in South Africa.

The nested PCR for the detection of *N. risticii* described here relies on the specific amplification of the 16S rRNA gene from the parasite. The assay depends on two sets of oligonucleotide primers to amplify a 527 bp portion of the 16S rRNA gene of *N. risticii* (Barlough *et al.,* 1996). A nested PCR has many advantages such as higher specificity and enhanced sensitivity when compared to a single−round PCR. In United States and worldwide *N. risticii* is known to be most widespread member of the genus. There is no data available that shows the prevalence of *N. risticii* in horses across the world. Information available was more on to the detection of *N. risticii* from bats, ponds and snails by PCR targeting the 16S rRNA gene. The 3.5% incidence obtained in South Africa was equivalent to 3.5% obtained in Northern California (Barlough *et al.,* 1998), 0 to 3.3% (Pusterla *et al.,* 2000) and lesser to the findings of 8% by Greiman *et al* (2013), 10% (Cicuttin *et al.,* 2013), 13.1% (Park *et al.,* 2003) and 20% (Chae *et al.,* 2003). This difference can be attributed to geographical range, sample collection period and vectors. Dutra *et al.,* (2001) reported that swampy ecosystem near the Merin pond can be considerably related with peak incidence in March (summer). Clustering of cases

suggest that areas nearest the lake may be an ideal environment for the reseviors and hosts. The results of this study come to an agreement because of the incidence obtained in Free State Province, the samples were collected from areas that were near to marshy environment. The results of this study also sustain that, a compound aquatic ecosystem is involved in the epizootiology of *N. risticii* where snails, trematodes, aquatic insects, and unknown intermediate hosts interact or act as a source of infection for accidental hosts such as horses (Madigan *et al.,* 2000; Pusterla *et al.,* 2000; Park *et al.,* 2003). No infections of for *N. risticii* were detected by PCR infections from donkey samples in this study.

CHAPTER 6

GENERAL CONCLUSIONS AND RECOMMENDATIONS

6.1 General conclusions

The aim of the current study was to detect and determine the occurence of venereal, bacterial tick−borne pathogens previously known and unknown to occur in equids in South Africa by molecular (PCR and LAMP) and serological (ELISA and ICT). Samples were collected from donkeys and horses in different study areas across four provinces of South Africa i.e. Free State, Mpumalanga, Northern Cape and North West. A total count of 256 blood samples was obtained including 38 samples from genital secretions in Free State. The prevalence of infection of horses and donkeys with *A. phagocytophilum*, *Babesia caballi, Neorickettsia risticii, Theileria equi* and *Trypanosoma equiperdum* varied significantly from Province to Province and infection rates varied quite significantly. This study has detected dourine, equine piroplasmosis, anaplasmosis and ehrlichiosis in horses and donkeys in South Africa. Despite, the known occurrence of dourine and equine piroplasmosis in South Africa, this study has shown that other diagnostic techniques which have not been explored are capable of detecting their causal agents. Moreover this study has detected *Anaplasma* and *Neorickettsia* infections by PCR for the first time in South Africa, these findings open doors for other studies on these pathogens in South African equids.

6.1.1 Dourine

Dourine, a venereal disease caused by *Trypanosoma equiperdum* with natural transmission of the organism occuring during genital contact between animals (Brun *et al.,* 1998), as well as from stallion to mare or vice visa (Luckins *et al.,* 2004). Transmission of *T. equiperdum* by tsetse fly or other vector has not been reported (Luckins *et al.,* 2004; Gillingwater *et al.,* 2007). In South Africa the the available data on prevalence of dourine has been obtained through the use of CFT which is the only internationally recommended test (OIE 2013; Clausen *et al.,* 2003). The current study has used PCR, a DNA based test to determine the occurence of dourine in South Africa

and further used another DNA based test called LAMP to confirm the PCR positive samples as true positive. Serological tests, ELISA and ICT which detects the presence of antibodies were also used to determine the prevalence of dourine in horses and donkeys from sampled provinces of South Africa. All the methods used in the present study (molecular and serology) provided a valuable evidence that *T. equiperdum* occurs in South Africa. Phylogenetic tree with 18S rRNA sequences has also showed that *T. equiperdum* isolates in South Africa fall in the *Trypanozoon* clade which is represented by *T. b. brucei, T. b. gambiense, T. b. rhodesiense, T. evansi* and *T. equiperdum.* It is a known fact that *T. evansi* is absent in South Africa (Claes *et al.,* 2005) whilst the other *Trypanozoon* species are tsetse transmitted (Stevens and Brisse, 2004). Therefore this further confirms that the trypanosome positive equids in this study can only be infected by *T. equiperdum* as the samples were collected in tsetse free provinces. Thus in conclusion the present study showed that dourine is highly established in South Africa. The study has further showed lower *T. equiperdum* infections in donkeys than in horses which are an indication that they are less susceptible. This is similar to previous reports which state that donkeys and mules are more resistant to *T. equiperdum* infections than horses (OIE, 2013). This is first report on the evaluation of PCR, LAMP, ICT and recombinant and crude antigen ELISA assays for *T. equiperdum* prevalence conducted in South Africa. It is remarkable that PCR and ELISA assays can be considered for adoption as diagnostic assays for dourine due to their detection efficiency observed in this study.

6.1.2 Equine piroplasmosis

Equine piroplasmosis (EP) is considered as an economically significant tick−borne disease in horses (Alhassan *et al.,* 2007a & b; Zobba *et al.,* 2008; Malekifard *et al.,* 2014; Piantedosi *et al.,* 2014). EP is thought to be indigenous to Asia but due to the global transport of horses for personal transport, as draft animals and for equestrian sports (Rothschild, 2013), both parasites have become distributed throughout the tropical (Allsopp *et al.,* 2007) and subtropical areas of the world where suitable tick vectors are present (Brüning, 1996). Tick species, *Rhipicephalus evertsi evertsi* (Motloang *et al.,* 2008) and *Hyalomma* spp. (Leal *et al.,* 2011) have been identified as capable vectors of *T. equi* and *B. caballi* infections. Both species are widely distributed

throughout the South Africa however *T. equi* is generally more widely distributed than *B. caballi* (Bruning *et al.,* 1996). It is well know that EP ocurrs in South Africa (Motloang *et al.,* 2008). Findings from the current study have further confirmed the presence of EP in South African provinces using PCR and LAMP. It is remarkable that in the previous study of Motloang *et al.,* (2008), *B. caballi* infections could not be detected by PCR from horse samples collected in the Free State province, however, in the current study PCR detected the *B. caballi* infections, which an indication of the presence of the parasite circulating in infected horses. Only one donkey from Northern Cape Province tested positive for presence of *B. caballi* infections, whilst *T. equi* was not detected in all donkey samples from FS and NW. It can be highlighted that similar to previous reports (Alhassan *et al.,* 2007a & b; Motloang *et al.,* 2008), in this study *T. equi* had higher prevalence than *B. caballi*. In clinical cases *B. caballi* infections may be as low as 0.1% parasitemia, normally does not exceed 1% (Rothschild, 2013) which explains why there is always lower prevalence *B. caballi* than *T. equi.* It is concluded that *T. equi* and *B. caballi* infections are still prevalent among horses and donkeys in four sampled provinces of South Africa. The results of this study have demonstrated that recombinant antigen ELISA are capable of detecting *T. equi* and *B. caballi* infections in in horses and donkeys in South Africa. This study has further confirmed that PCR, LAMP and ELISA can be used efficiently for prevalence studies of EP and have the potential to be adopted for reference diagnosis of EP in South Africa.

6.1.3 Equine granulocytic anaplamosis

The A. phagocytophilum is the causative agent of equine granulycitic anaplasmosis. Most of the anaplasmoses are tick borne zoonosis; they can be transmitted from animals to humans (Mtshali *et al*., 2015). Their agents are maintained in nature through enzootic ticks and wild and domestic animals. *A*. *phagocytophilum* infects granulocytes and elicits febrile diseases in animals and humans. Horses infected with *A. phagocytophilum* may exhibit a range of debilitating clinical signs, and even death (Chan *et al.,* 2010). Using PCR, the current study revealed that the overall prevalence of equine anaplasmosis is 39.4% from all four sampled provinces of South Africa. It is concluded that equine anaplasmosis due to *A. phagocytophilum* occurs in horses and donkeys in South Africa. It is remarkable that this is the first report where *A.*

phagocytophilum and *N. ricsticii* has been detected in asymptomatic equids in South Africa using PCR.

6.1.4 Equine monocytic ehrlichiosis

Equine monocytic ehrlichiosis (EME) or Potomac horse fever (PHF) is caused by rickettsial pathogen formerly termed *Ehrlichia risticii*. In 1979 the disease was reported along the Potomac River in Maryland and has since been identified in other states and in Europe (Madigan *et al.,* 1999). There is no information regarding the equine monocytic ehrlichiosis prevalence or *N. risticii* infections in equids in South Africa. In order to find out whether equine monocytic ehrlichiosis or *N. risticii* infections occur is South Africa, the current study has applied nested PCR on DNA extracted from horse and donkey samples collected in four provinces (FS, MP, NC and NW).

With exception to NW, the *N. risticii* infections were detected by PCR in horses from FS, MP and NC, whilst no infections were detected in donkeys. This supports the notion that an *N. risticii* infection causes a significant febrile gastroinstestinal disease in horses only (Barlough *et al.,* 1998; Pusterla *et al.,* 2000; Pusterla *et al.,* 2003; Cicuttin *et al.,* 2013). It is remarkable that this is the first time that *N. risticii* infections are detected by PCR in South Africa.

6.2 Recommandations

− Prevalence studies of dourine using PCR, LAMP and ELISA assays should be extended to cover the rest of South African provinces.

− PCR detected *T. euiperdum* infections from DNA extracted from blood, this warrants further studies on isolation and experimental infections of South African *T. equiperdum* isolate(s) as this parasite is known to be very scarce in blood.

− There is a need for pathogenicity, assessemnet of therapeutic agents and economic impact studies of dourine in horses in South Africa.

− Large scale epidemiological studies for equine piroplasmosis need to be conducted using crude antigen ELISA as well as recombinant antigen ELISA and ICT assays as they will give true reflection of disease prevalence in South Africa.

− Further studies of mode of transmission of *A. phagocytohilum* and *N. risticii* infections in horses and donkeys in South Africa are required, particularly focusing on ticks and snails as they have been reported to play a role on transmission of these pathogens.

− There is also a need for phylogenetic studies of *A. phagocytohilum* and *N. risticii* in order to determine their relationship with other *Anaplasma* and *Ricketssia* species of other mammalian hosts.

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ANNEXURES

Table 1 to 4 represents samples collected from different provinces, Northern Cape, Free State, North West and Mpumalanga indicating which samples were positive and negative when using different techniques and species.

Northern Cape Province: Samples highleted with a yellow colour were collected from donkeys.

			Trypaosoma equiperdum					*Theileria equi*		*Babesia caballi*		*Anaplasma*	*Ehrlichia*
Sample name	Sex	Age	PCR	LAMP	rELISA	caELISA	ICT	*T. equi* PCR	*T. equi* LAMP	*B. caballi* PCR	*B. caballi* LAMP	*A.phagocytophilum* PCR	*Neorikettsia risticii* PCR
NC 1	F	8	pos	pos	pos	pos	neg	neg	neg	neg	neg	neg	neg
NC 2	M	2	neg	neg	neg	neg	neg	pos	pos	neg	neg	pos	neg
NC 3	F	5	neg	neg	pos	neg	neg	pos	pos	neg	neg	pos	neg
NC 4	F	6	pos	pos	neg	pos	neg	neg	neg	neg	neg	pos	neg
NC 5	M	4	pos	pos	pos	neg	neg	neg	neg	neg	neg	pos	neg
NC 6	F	8	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
NC 7	M	2	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg
NC 8	M	2	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg
NC 9	F	5	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg
NC 10	M	8	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
NC 11	F	4	neg	neg	neg	neg	neg	neg	neg	pos	pos	pos	neg
NC 12	F	5	neg	neg	neg	neg	neg	neg	neg	pos	neg	pos	neg
NC 13	F	8	neg	neg	neg	neg	neg	pos	pos	neg	neg	pos	neg
NC 14	M	4	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg
NC 15	M	5	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
NC 16	F	10	neg	neg	pos	neg	neg	neg	neg	neg	neg	pos	neg
NC 17	F	6	neg	neg	pos	neg	neg	neg	neg	neg	neg	pos	neg
NC 18	M	5	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg
NC 19	M	4	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg
NC 20	M	6	neg	neg	neg	neg	neg	neg	neg	pos	pos	pos	neg
NC 21	F	10	neg	neg	neg	neg	neg	pos	pos	neg	neg	pos	neg
NC 22	F	6	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg
NC 23	M	5	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg
NC 24	F	5	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg
NC 25	F	5	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
NC 26	M	8	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg

NC 27	M	5	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg
NC 28	F	0.5	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
NC 29	F	7	neg	neg	noserum	noserum	noserum	neg	neg	neg	neg	pos	neg
NC 30	F	6	neg	neg	pos	neg	neg	neg	neg	neg	neg	pos	neg
NC 31	M	8	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg
NC 32	F	8	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg
NC 33	M	8	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
NC 34	M	10	neg	neg	neg	pos	neg	neg	neg	neg	neg	neg	neg
NC 35	M	6	pos	pos	pos	pos	pos	neg	neg	neg	neg	neg	pos
NC 36	M	5	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg
NC 37	F	6	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg
NC 38	M	6	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg
NC 39	F	3	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg
NC 40	F	6	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
NC 41	F	5	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
NC 42	F	5	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg
NC 43	M	4	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg
NC 44	F	2	neg	neg	pos	neg	neg	neg	neg	neg	neg	pos	neg
NC 45	M	3	neg	neg	pos	neg	neg	neg	neg	neg	neg	pos	neg
NC 46	M	3	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg
NC 47	M	2	neg	neg	pos	pos	neg	neg	neg	neg	neg	neg	neg
NC 48	M	3	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
NC 49	M	3	neg	neg	pos	neg	neg	neg	neg	neg	neg	neg	neg
NC 50	M	2	neg	neg	pos	pos	neg	neg	neg	neg	neg	neg	neg
NC 51	F	3	neg	neg	pos	neg	neg	neg	neg	neg	neg	neg	neg
NC 52	M	3	neg	neg	pos	pos	neg	neg	neg	pos	pos	pos	neg
NC 53	F	3	neg	neg	noserum	noserum	noserum	neg	neg	neg	neg	pos	neg
NC 54	M	4	neg	neg	noserum	noserum	noserum	neg	neg	neg	neg	pos	neg
Total pos			4	4	14	6	1	4	4	3	3	37	1

Free State Province: Samples highleted with a yellow colour were collected from donkeys. Blood (B) and genital secretions (GS) samples

			Trypanosoma equiperdum							*Theileria equi*		*Babesia caballi*		*A.. phagocytophilum*	*N.. risticii*
Sample name	Age	Sex	(B) PCR	(GS) PCR	(B) LAMP	(GS) LAMP	caELISA	rELISA	ICT	*T. equi* PCR	*T. equi LAMP*	*B. caballi* PCR	*B. caballi* LAMP	PCR	PCR
H1	3	M	pos	neg	pos	neg	neg	neg	Pos	pos	pos	neg	neg	pos	pos
H2	4	F	pos	neg	pos	neg	neg	neg	Neg	pos	pos	neg	neg	pos	neg
H3	3	M	neg	neg	neg	neg	neg	neg	Neg	neg	neg	neg	neg	neg	neg
H4	4	M	neg	neg	neg	neg	neg	neg	Neg	neg	neg	pos	neg	neg	neg
H5	7	M	neg	neg	neg	neg	neg	pos	Neg	pos	neg	neg	neg	neg	neg
H6	1.5	F	neg	neg	neg	neg	neg	neg	Neg	neg	neg	pos	pos	pos	neg
H7	3	F	neg	neg	neg	neg	neg	neg	Neg	pos	neg	neg	neg	pos	neg
H8	5	F	neg	neg	neg	neg	neg	pos	Pos	pos	neg	neg	neg	pos	neg
H9	5	M	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg
H10	1.5	F	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	pos
H11	7	M	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg
H12	5	F	neg	neg	neg	neg	neg	neg	neg	pos	pos	neg	neg	pos	neg
H13	4	F	neg	neg	neg	neg	noserum	noserum	noserum	neg	neg	neg	neg	pos	neg
H14	6	M	pos	neg	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
H15	9	F	neg	neg	neg	neg	neg	neg	Neg	neg	neg	neg	neg	neg	neg
H16	15	M	neg	neg	neg	neg	no serum	no serum	no serum	neg	neg	neg	neg	neg	neg
H17	7	M	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	pos	neg	neg
H18	0.5	M	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
H19	12	M	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	pos
H20	10	F	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg	pos	neg
H21	7	M	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
H22	1.5	F	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
H23	1	F	neg	neg	neg	neg	pos	neg	neg	neg	neg	neg	neg	pos	neg
H24	1	F	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
H25	10	M	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg

H26	4	M	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	pos	neg	neg
H27	12	M	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
H28	6	F	neg	neg	neg	neg	neg	pos	neg	neg	neg	neg	neg	pos	neg
H29	5	F	neg	neg	pos	neg	no serum	no serum	no serum	pos	pos	neg	neg	neg	neg
H30	0.5	F	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	pos	neg	neg
H31	5	M	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos
H32	3	F	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
P1	3	F	neg	neg	neg	neg	pos	neg	neg	pos	neg	neg	neg	pos	neg
P2	0.5	M	neg	neg	pos	neg	pos	pos	neg	pos	neg	neg	neg	pos	neg
P3	9	F	pos	neg	pos	neg	neg	neg	pos	pos	pos	neg	neg	neg	pos
P4	8	M	neg	neg	pos	neg	neg	pos	pos	neg	neg	neg	neg	pos	neg
P5	2	F	neg	neg	neg	neg	neg	neg	neg	pos	neg	pos	neg	neg	neg
P6	2	M	neg	neg	neg	neg	neg	neg	neg	pos	pos	pos	pos	pos	neg
P7	15	M	neg	neg	neg	neg	neg	neg	neg	pos	neg	pos	pos	pos	neg
P8	3	F	pos	pos	pos	pos	neg	neg	pos	pos	pos	neg	neg	pos	neg
Total pos			5	1	8	1	3	5	5	14	7	9	7	21	5

North West Province: Samples highleted with a yellow colour were collected from donkeys.

		Trypanosoma equiperdum					*T. equi*			*B. caballi*	*A. phagocytophilum*	*N. risticii*
Sample name	Sex	PCR	ICT	caELISA	rELISA	LAMP	*T. equi* PCR	*T. equi* LAMP	*B. caballi* PCR	*B. caballi* LAMP	PCR	PCR
NW 1	F	pos	neg	pos	neg	pos	neg	neg	neg	neg	neg	neg
NW 2	M	pos	neg	pos	pos	pos	neg	neg	neg	neg	neg	neg
NW 3	F	neg	neg	pos	pos	neg	neg	neg	neg	neg	neg	neg
NW 4	F	neg	neg	pos	pos	neg	neg	neg	pos	pos	neg	neg
NW 5	F	neg	neg	neg	pos	neg	neg	neg	neg	neg	pos	neg
NW 6	M	neg	neg	pos	pos	neg	neg	neg	pos	pos	pos	neg
NW 7	F	pos	neg	neg	pos	neg	neg	neg	neg	neg	neg	neg
NW 8	F	pos	neg	neg	neg	pos	neg	neg	neg	neg	neg	neg
NW 9	M	neg	neg	neg	pos	neg	neg	neg	neg	neg	neg	neg
NW 10	F	neg	neg	pos	pos	neg	neg	neg	neg	neg	neg	neg
NW 11	F	neg	neg	pos	pos	neg	neg	neg	neg	neg	neg	neg
NW 12	F	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
NW 13	M	neg	neg	pos	pos	neg	neg	neg	neg	neg	neg	neg
NW 14	F	neg	neg	pos	neg	neg	neg	neg	neg	neg	pos	neg
NW 15	M	neg	neg	neg	pos	neg	neg	neg	neg	neg	neg	neg
NW 16	M	neg	neg	pos	pos	neg	neg	neg	neg	neg	pos	neg
NW 17	M	neg	neg	neg	neg	neg	neg	neg	pos	pos	neg	neg
NW 18	M	neg	neg	neg	pos	neg	neg	neg	neg	neg	pos	neg
NW 19	M	neg	neg	pos	pos	neg	neg	neg	neg	neg	neg	neg
NW 20	M	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg
NW 21	F	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
NW 22	M	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
NW 23	F	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg
NW 24	F	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
NW 25	M	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg

NW 26	M	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
NW 27	M	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
NW 28	F	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
NW 29	F	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
NW 30	F	neg	neg	neg	neg	neg	pos	pos	neg	neg	neg	neg
NW 31	F	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
NW 32	M	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
NW 33	F	neg	neg	neg	neg	neg	pos	pos	neg	neg	pos	neg
NW 34	F	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
NW 35	M	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
NW 36	M	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
NW 37	F	neg	neg	neg	neg	neg	pos	pos	neg	neg	neg	neg
NW 38	M	neg	neg	neg	neg	neg	pos	pos	neg	neg	neg	neg
NW 39	M	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
NW 40	M	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
NW 41	F	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
NW 42	F	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
NW 43	F	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
NW 44	F	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
NW 45	F	pos	neg	neg	neg	pos	neg	neg	pos	pos	neg	neg
NW 46	F	pos	neg	neg	neg	pos	neg	neg	neg	neg	neg	neg
NW 47	M	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
NW 48	M	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
NW1(D)	M	pos	neg	neg	pos	pos	neg	neg	neg	neg	neg	neg
NW2 (D)	M	pos	neg	neg	neg	pos	neg	neg	neg	neg	neg	neg
NW3 (D)	F	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
NW4 (D)	M	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
NW5 (D)	M	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
NW6 (D)	M	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
NW7 (D)	F	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg

NW8 (D)	F	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
NW9 (D)	F	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
NW10 (D)	F	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
NW11 (D)	F	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
NW12 (D)	F	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
NW13 (D)	F	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
NW14 (D)	F	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg
NW15 (D)	F	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg
NW16 (D)	F	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg
NW17 (D)	F	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg
NW18 (D)	F	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg
NW19 (D)	M	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg
NW20 (D)	M	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg
Total pos		8	0	11	15	7	4	4	4	4	14	0

Mpumalanga Province

			Trypanosoma equiperdum					*T. equi*		*B. caballi*		*A. phagocytophilum*	*N. risticii*
Sample name	**Age**	**Sex**	**PCR**	**LAMP**	**ICT**	**caELISA**	**rELISA**	***T. equi* PCR**	***T. equi* LAMP**	***B. caballi* PCR**	***B. caballi* LAMP**	**PCR**	**PCR**
MP H1	12	F	neg	neg	neg	neg	neg	pos	pos	neg	neg	neg	pos
MP H2	15	F	neg	neg	neg	pos	neg	neg	neg	neg	neg	pos	neg
MP H3	15	F	neg	neg	neg	pos	pos	neg	neg	neg	neg	neg	neg
MP H4	2	F	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg
MP H5	8	F	neg	neg	neg	pos	neg	neg	neg	neg	neg	pos	neg
MP H6	8	M	neg	neg	neg	neg	neg	pos	pos	neg	neg	neg	neg
MP H7	0.5	M	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
MP H8	8	F	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
MP H9	6	F	neg	pos	neg	pos	pos	pos	pos	neg	neg	pos	pos
MP H10	8	M	neg	pos	neg	pos	pos	pos	neg	pos	pos	neg	neg
MP H11	7	F	neg	neg	neg	pos	pos	neg	neg	pos	pos	pos	neg
MP H12	3	F	neg	neg	neg	pos	pos	neg	neg	neg	pos	neg	neg
MP H13	8	F	neg	neg	neg	pos	pos	neg	neg	neg	pos	pos	neg
MP H14	10	F	pos	pos	neg	neg	pos	pos	neg	neg	neg	pos	neg
MP H15	10	F	neg	pos	neg	pos	pos	neg	neg	neg	neg	pos	neg
MP H16	12	M	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
MP H17	8	M	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
MP H18	8	M	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
MP H19	10	M	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
MP H20	5	F	neg	neg	neg	neg	neg	neg	pos	neg	neg	neg	neg
MP H21	4	F	neg	neg	neg	neg	neg	pos	neg	pos	pos	neg	neg
MP H22	4	M	neg	neg	neg	neg	neg	neg	neg	pos	pos	neg	neg
MP H23	2	F	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
MP H24	2	F	neg	neg	neg	neg	neg	pos	neg	neg	neg	neg	neg
MP H25	5	F	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg

MP H26	10	M	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
MP H27	8	M	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
MP H28	8	M	pos	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg
MP H29	1	M	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
MP H30	8	F	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg
MP H31	8	F	pos	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg
MP H32	5	F	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
MP H33	8	M	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
MP H34	5	F	neg	neg	neg	pos	neg	pos	pos	pos	pos	pos	pos
MP H35	2	M	neg	neg	neg	neg	neg	pos	neg	neg	neg	neg	neg
MP H36	8	M	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg
MP H37	10	F	neg	neg	neg	neg	neg	pos	neg	neg	neg	neg	neg
MP H38	10	M	neg	neg	neg	neg	neg	pos	neg	neg	neg	neg	neg
MP H39	5	F	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
MP H40	4	M	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
MP H41	2	F	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
MP H42	6	M	neg	neg	neg	neg	neg	pos	neg	neg	neg	neg	neg
MP H43	6	F	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
MP H44	6	F	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
MP H45	3	M	neg	neg	neg	neg	neg	pos	neg	neg	neg	neg	neg
MP H46	7	M	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
MP H47	10	M	pos	neg	neg	neg	neg	pos	pos	neg	neg	neg	neg
MP H48	5	F	neg	neg	neg	pos	neg	neg	neg	neg	neg	neg	neg
MP H49	8	F	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
MP H50	10	F	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
MP H51	10	F	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
MP H52	5	F	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
MP H53	8	M	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
MP H54	8	M	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
MP H55	6	M	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg

MP H56	5	M	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
MP H57	4	M	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
MP H58	5	M	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
MP H59	7	F	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg
MP H60	2	F	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
MP H61	0.5	F	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
MP H62	1	F	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
MP H63	8	F	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
MP H64	3	F	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
MP H65	3	F	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
MP H66	2	M	neg	neg	neg	pos	pos	neg	neg	neg	neg	neg	neg
MP H67	7	M	neg	neg	neg	neg	pos	neg	neg	neg	neg	neg	neg
MP H68	3	F	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
MP H69	8	F	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
MP H70	9	M	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
MP H71	6	F	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
MP H72	3	M	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
MP H73	3	M	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
MP H74	10	F	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
MP H75	10	M	neg	neg	neg	pos	neg	neg	neg	neg	neg	neg	neg
MP H76	4	M	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
MP H77	6	M	pos	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg
MP H78	6	F	neg	neg	neg	pos	neg	neg	neg	neg	neg	neg	neg
MP H79	10	M	neg	neg	neg	pos	neg	neg	neg	neg	neg	neg	neg
MP H80	5	M	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
MP H81	4	F	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg
MP H82	3	M	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg
MP H83	5	M	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
MP H84	5	F	neg	neg	neg	pos	pos	neg	neg	neg	neg	neg	neg
MP H85	3	M	neg	neg	neg	pos	neg	neg	neg	neg	neg	neg	neg

MP H86	7	M	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
MP H87	7	M	pos	neg	neg	pos	pos	neg	neg	neg	neg	neg	neg
MP H88	9	M	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
MP H89	6	M	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
MP H90	3	F	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
MP H91	9	F	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
MP H92	7	M	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
MP H93	8	M	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
MP H94	2	F	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
Total pos			16	6	0	19	12	14	7	5	7	15	3