COMPARISON OF REAL-TIME POLYMERASE CHAIN REACTION WITH THE CONVENTIONAL PCR ASSAY FOR THE DIAGNOSIS OF *THEILERIA PARVA* IN SOUTH AFRICA

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30 November 2007

DECLARATION

I, Natasha Ektha Papli, declare that the thesis hereby submitted by me for the MSc degree at the University of Free Sate is my own independent work and has not previously been submitted by me at another university/faculty.
I furthermore, cede copyright of the thesis in favour of the University of the Free State.

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ACKNOWLEDGEMENTS

I would like to express my deepest gratitude and appreciation to the following people and authority:

Dr. Abdalla Latif and Professor Peter Mbati, my supervisors for their guidance, encouragement, support and belief in me during the study.

Ms Thembi Sepeng, Dr Erich Zweygarth, Mr Christo Troskie, Ms Ronel Pienaar, Dr Otto Koekomoer, Dr Fred Potgieter, Dr Juanita Liebenberg and Ms Antoinette Josemans for their help and support.

NRF for providing the financial assistance to complete the study.

The management and staff of OVI and ARC for allowing me to complete this study.

Anna Kenyon, Austin Cohen and Nico Gunter from Roche Diagnostics, for their help with the operation of equipment and analysis of results.

Dr Marinda Oosthuizen and Ms Kgomotso Sibeko for their scientific input with the BioPAD Project consortium.

The OVI Library staff for their assistance with literature searches.

My husband, parents and siblings for their undying love, encouragement, support, sacrifices and faith in me. I dedicate this to you all.

God, who gave me the insight, strength and endurance to complete this study.

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SUMMARY

Comparison of Real-time Polymerase Chain Reaction with the conventional PCR assay for the diagnosis of *Theileria parva* in South Africa

by

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Theileria parva (*T. parva)* is transmitted from carrier buffalo to cattle causing Corridor disease in cattle. The 989/990 conventional Polymerase Chain Reaction (PCR) assay used for the detection of *T. parva* is labour-intensive and has the potential for contamination due to the need for post-amplification handling. Real-time PCR offers a way of addressing these limitations. This thesis describes the development of a TaqMan assay for the detection of *T. parva* and a comparison between this real-time assay with the real-time Hybridization probe assay and the conventional PCR assay for the diagnosis of *T. parva.*

Theileria general forward and reverse primers and a *T. parva* TaqMan probe specific for the recognition of a conservative region of the *T. parva* 18S rRNA gene was designed. The TaqMan PCR assay could detect *T. parva* DNA at a $2x10^{-5}$% parasitaemia with a 93% certainty. The primer pairs and probe only cross-reacted with *Theileria* sp. (buffalo) and no amplification with other *Theileria* species, bacteria or related haemoparasites was observed. *Theileria* sp. (buffalo) is genetically closely related to *T. parva*. However, its biology and

disease relations are not known. The TaqMan probe assay detected 87% of all positive samples for evidence of the diagnostic sensitivity and 100% of all negative samples tested negative for the diagnostic specificity assay.

These results were compared with those obtained from 989/990 conventional PCR and BioPAD Hybridization probe PCR which targeted the same gene. The Hybridization probe PCR appeared to be more sensitive than the TaqMan probe PCR or conventional PCR assay. With the specificity test, the Hybridization probe PCR proved to be more specific than the other two assays. All three tests gave similar results for the diagnostic specificity. The TaqMan probe assay with its high sensitivity, wide range of detection ability and simplicity is particularly useful in the detection of *T. parva*. However, further studies are required to improve the specificity of the TaqMan PCR assay in order to eliminate the detection of *Theileria* sp. (buffalo).

Keywords: *Theileria parva*, *Theileria*, Corridor disease, real-time PCR, TaqMan probe, Hybridization probe, 18s rRNA, amplification, sensitivity, specificity

OPSOMMING

‘n Vergelykking tussen die Werklike-tyd polimerase kettingreaksie en die konvensionele polimerase kettingreaksie vir die diagnose van *Theileria parva* in Suid Afrika

deur

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Wanneer *Theileria parva* (*T. parva*) oorgedra word vanaf draer buffels na beeste, veroorsaak dit ’n siekte toestand genaamd ‘Korridor Siekte’. Die 989/990 konvensionele polimerase kettingreaksie (PKR) toets wat gebruik was vir die diagnose van *T. parva,* is arbeidsintensief en dra ’n risiko van moontlike kontaminasie as gevolg van die hantering van materiaal na amplifikasie. Werklike-tyd PKR bied moontlikhede om hierdie beperkings aan te spreek. Hierdie werk beskryf die ontwikkeling van ’n TaqMan toets vir die diagnose van *T. parva* en ’n vergelyking tussen hierdie werklike-tyd toets met die werklike-tyd Hibridisering peiler toets en die konvensionele PKR toets vir die diagnose van *T. parva*.

Theileria algemene sens en antisens voorvoerders en ’n *T. parva* TaqMan peiler spesifiek vir die uitkenning van ’n konserwatiewe area van die *T. parva* 18S rRNA geen is ontwerp. Die TaqMan PKR toets kon *T. parva* DNA teen $2x10^{-5}$% parasitemie met 93% akkuraatheid vasstel**.** Die voorvoerderpare en peiler het slegs met *Theileria* sp. (buffalo) gekruisreageer. Geen amplifikasie met ander *Theileria* spesies, bakterieë of verwante bloedparasiete is waargeneem nie. *Theileria* sp.(buffalo) is geneties naverwant aan *T. parva* alhoewel die biologie en siekte verwantskappe nie bekend is nie. Die TaqMan peiler het 87% van alle positiewe monsters korrek geidentifiseer as

bewys vir die diagnostiese sensitiwiteit en 100% van alle negatiewe monsters was negatief vir die diagnostiese spesifisiteit van die toets.

Hierdie resultate is vergelyk met die resultate wat verkry is met die 989/990 konventionele PKR toets en die BioPAD Hibridisering peiler toets wat dieselfde geen teiken. Die Hibridisering peiler PKR toets bleik om meer sensitief as die TaqMan peiler PKR toets en die konvensionele PKR analise te wees. Met die spesifisiteit bepaling was die Hibridisering peiler PKR toets meer spesifiek as die ander twee toetse. Al drie toetse het dieselfde resultate getoon vir diagnostiese spesifisiteit. Die TaqMan peiler analise met sy hoë sensitiwiteit, wye veld van deteksie en eenvoud om te gebruik is besonders nuttig vir die diagnose van *T. parva*. Nietemin is verdere studies nodig om die spesifisiteit van die TaqMan peiler PKR toets te verbeter om die kruisreaksie met *Theileria* sp. (buffalo) te elimineer.

ABBREVIATIONS USED IN TEXT:

bp	base pairs
BioPAD	Biotechnology Partnership and Development
°C	degrees Celsius
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
EDTA	ethylene diamine tetra-acetic acid
FAM	carboxyfluorescein
FN	false negatives
FP	false positives
g	gram
IFA	Indirect fluorescent antibody
MAbs	Monoclonal Antibodies
M	Molar
mg	milligram
µg	microgram
µl	microlitre
µM	micromolar
min	minutes
mm	milliliter
mM	millimolar
mmol	millimoles
$MgCl_2$	magnesium chloride
NaOH	sodium hydroxide
OVI	Onderstepoort Veterinary Institute
PCR	polymerase chain reaction
PCV	packed cell volume
^{32}P	radioactive Phosphorous 32
rRNA	ribosomal ribonucleic acid
sec	seconds
ssu	small subunit
SDS	sodium dodecyl sulphate

TAMRA	*N,N,N',N'*-tetramethyl-6-carboxyrhodamine
TBE	Tris borate EDTA
TE	Tris EDTA
T. annulata	*Theileria annulata*
T. buffeli	*Theileria buffeli*
T_m	Melting temperature
T. mutans	*Theileria mutans*
TN	True negatives
T. orientalis	*Theileria orientalis*
T. parva	*Theileria parva*
T. p. bovis	*Theileria parva bovis*
T. p. lawrencei	*Theileria parva lawrencei*
T. p. parva	*Theileria parva parva*
TP	True positives
T. sergenti	*Theileria sergenti*
T. sp. (buffalo)	*Theileria* species (buffalo)
T. taurotragi	*Theileria taurotragi*
T. velifera	*Theileria velifera*
UDG	uracil deoxyglycosylase

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Preamble

The introduction of cattle into southern Africa occurred around 1000 AD with the Khoi and Bantu tribes (Norval *et al*., 1992). These cattle were a source of milk, draught power for cultivation and a symbol of wealth for the people (Norval *et al*., 1992). The European settlers used the cattle for transport and cultivation as well as a source of meat and milk. Several serious cattle diseases occurred during this time and in 1896 an outbreak of rinderpest resulted in severe losses of cattle estimated to be more than 2.5 million cattle (Norval *et al*., 1992). In 1902 a highly pathogenic strain of *Theileria parva* caused an epidemic of East Coast fever in Southern Africa which resulted in the deaths of 5.5 million cattle (Potgieter *et al.*, 1988). It was finally eradicated from South Africa in 1955 through tick control, quarantine and slaughter of diseased cattle.

Since the mid-1970s the demand for cattle meat and milk products increased dramatically (Norval *et al*., 1992). However, livestock disease was and still is a major health impediment, contributing to the shortfall in supply (Norval *et al*., 1992).

The African buffalo being one of the famous Big Five is considered a very valuable asset on game farming and conservation areas in South Africa. The presence of buffalo on a property increases the value tremendously in terms of eco-tourism and hunting. The popularity of commercial game farms and nature reserves has led to a greater demand for buffalo. The buffalo serves as the natural reservoir hosts of several controlled diseases including Brucellosis, Trypanosomosis, Tubercullosis, Foot and Mouth Disease, Heartwater and Corridor disease (Coetzer and Tustin, 2004), some of which are transmitted to cattle by ticks.

1.2 History of Corridor disease

In 1953 a severe outbreak of a disease resulting in the deaths of 300 cattle occurred in the corridor between Hluhluwe and Umfolozi Game Reserves in Natal (Norval *et al*., 1992). This disease was named Corridor disease. Corridor disease is an acute, usually fatal disease of cattle. It is caused by infection with *Theileria parva lawrencei*, which is transmitted by ticks from African buffalo to cattle (Uilenberg, 1981). It was first found in Zimbabwe in 1934 and then discovered in South Africa twenty years later. It was then that the causal organism was identified as a new species, *Theileria lawrencei* which was subsequently found to be a subspecies of *T. parva* and renamed *T. parva lawrencei.*

1.3 Tick vectors of Corridor disease

Theileria parva lawrencei is mainly transmitted by *Rhipicephalus appendiculatus* and by *Rhipicephalus zambeziensis* ticks in more arid areas (Walker *et al.*, 1981) from carrier buffalo to cattle causing Corridor disease in cattle. *Rhipicephalus appendiculatus,* more commonly known as the Brown ear tick, is a three-host tick which exhibits a seasonal life cycle and is usually found in wetter areas of South Africa (Norval, 1994). The adults usually attach to the ears of their hosts.

1.4 Classification and life cycle of *Theileria parva*

The genus *Theileria* belongs to the phylum Apicomplexa, class Sporozoea, subclass Piroplasmia, order Piroplasmida and family Theileriidae (Norval *et al*., 1992). *Theileria* is a haemoprotozoan parasite which is transmitted by ticks and affects various wild and domestic animals. The different species, which infect cattle and buffalo, are *T. parva*, *T. annulata*, *T. taurotragi*, *T. mutans*, *T. buffeli*, *T. velifera*, *T. orientalis* and *T. sergenti. Theileria parva* is infective to the Cape buffalo (*Syncerus caffer*), cattle and the Asiatic buffalo (Norval *et al.*, 1992). It occurs predominantly in eastern, central and southern Africa (Norval *et al.*, 1992). *Theileria parva* was previously classified into three subspecies *T. p. parva* (causes classical East Coast Fever in cattle*)*, *T. p. lawrencei* (causes

Corridor disease in cattle) and *T. p. bovis* (cause of Zimbabwean/ January disease). However, this trinomial system was dropped since studies showed no conclusive genetic evidence that the sub-species were distinguishable (Norval *et al*., 1992). The classification is now based on their host species of origin for convenience, as buffalo associated or cattle associated (Norval *et a*l., 1992).

The life cycle of *T. parva* alternates between the host animal and tick vectors (Figure 1). The piroplasms in the erythrocytes of the host are ingested by the tick during its feeding and develop into gametes, which fuse to form a zygote (Norval *et al*., 1992). These invade the epithelial cells of the tick. As the tick moults, a motile kinete forms and migrates to the salivary glands where it invades the epithelial cells and develops into a sporoblast within which tiny sporozoites develop. Sporozoites are inoculated into the host animals with the tick saliva during feeding. They enter the lymphocytes in the nearest lymphatic nodes where they develop into schizonts. The pathogenic effects of *T. parva* infection are normally associated with the schizont stage rather than the piroplasm stage (Norval *et al.*, 1992). Schizonts divide synchronously with the cells and later form merozoites, which invade the host's erythrocytes and develop into piroplasms.

1.5 Epidemiology

The vectors of *T. parva* are ticks of the genus *Rhipicephalus* and are widespread in South Africa (Grootenhuis, 1988). This has become a serious threat to the cattle farming industry in South Africa wherever cattle come into contact with buffalo (Potgieter *et al.*, 1988). In 1988, an outbreak of Corridor disease in South Africa resulted in more than 90% mortality rate of cattle involved (Potgieter *et al.*, 1988). Corridor disease in the presence of tick vectors is transmitted when infected buffalo and cattle share the same pasture, when cattle are moved into an area where infected buffalo are present or when infected buffalo stray into farming areas.

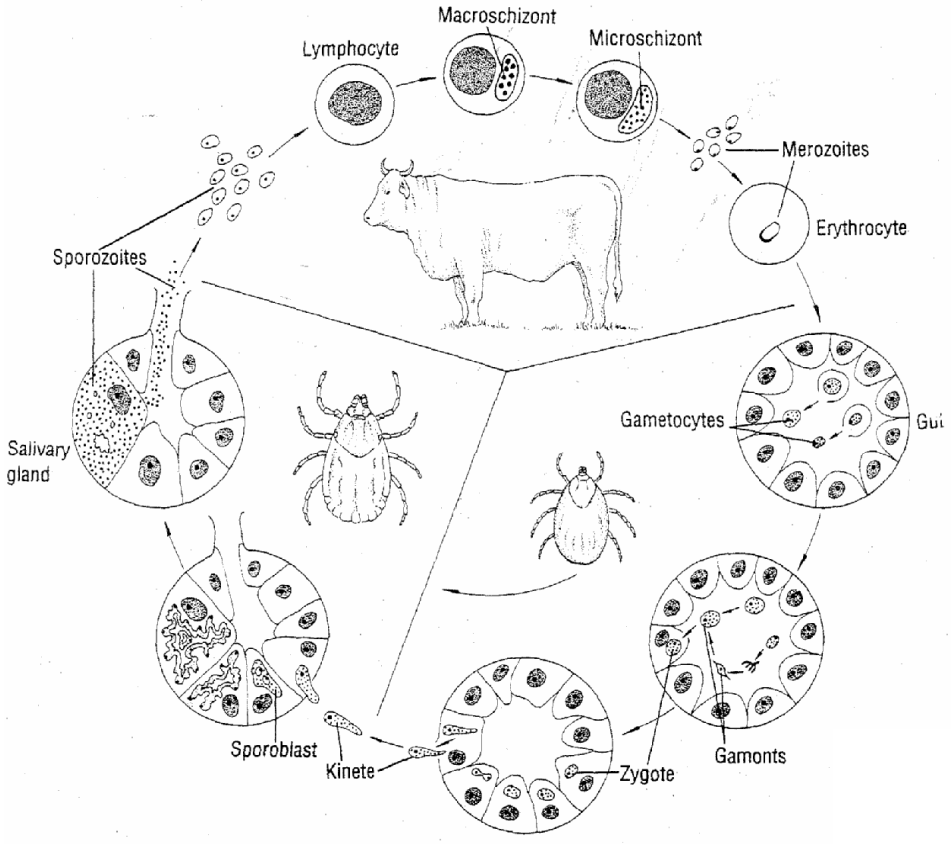


Figure 1: Life cycle of *Theileria parva* (Lawrence *et al*., 1994)

By law (Animal Diseases Act 1984, Act 35) it is necessary that before buffalo are to be translocated to non-endemic areas they have to be certified free from Corridor disease, Foot and Mouth Disease, brucellosis and tuberculosis. Corridor disease is endemic in the Kruger National Park and the Natal parks (Collins *et al*., 2002).

1.6 Economic impact of Corridor disease

Disease free buffalo are few in number and therefore very expensive. In 1998, a disease free buffalo was worth above US$16,500 whilst a *T. parva* infected buffalo was valued between US$4,300 and US$7,200 (Vosloo *et al.*, 2001). Some operators could resort to illegal translocation of buffalo, which could result in Corridor disease outbreaks. Since many areas bordering game reserves serve as cattle grazing land, the cattle industry becomes at risk of facing major losses due to an outbreak of Corridor disease. Hence Corridor disease is a major constraint in cattle production and the expansion of the cattle farming industry.

1.7 Clinical signs

Clinical symptoms of Corridor disease in cattle are fever, enlargement of superficial lymph glands, frothy fluid from nose and mouth, difficult breathing, loss of appetite, lacrimation and loss in milk production. This condition is usually associated with a high rate of fatality (Toye *et al.*, 1995).

1.8 Control and treatment

Corridor disease can be controlled by physical separation of buffalo and cattle, strict quarantine measures and tick control (Potgieter *et al*., 1988). The use of chemotherapeutic drugs is illegal in South Africa due to the risk of the animals developing a carrier state (Maritim *et al*., 1989). A carrier state means that the parasites present in the infected and recovered animal are able to infect ticks, which then transmit the parasites to a susceptible host (Young *et al*., 1986). The carrier state of *T. parva* was found to exist in naturally recovered buffalo and cattle for long periods (Barnett and Brocklesby, 1966).

When an outbreak of Corridor disease does occur, the cattle are dipped, removed to uninfected pastures, all sick and recovered cattle are culled, tick control procedures are carried out, the source of contact is investigated and the camp is kept free of cattle for 18 months. Rapid, sensitive and specific laboratory diagnostic methods are therefore essential in order to prevent an epidemic.

1.9 Laboratory diagnosis

There are several traditional laboratory techniques currently being used for the diagnosis of *T. parva* and each method has its own limitations.

- Giemsa stained blood and lymph node smears are examined for piroplasms or schizonts respectively. However, the level of parasitaemia in recovered or carrier animals may be below the microscopy detection threshold (Skilton *et al*., 2001) and it is difficult to differentiate between the different *Theileria* species using this technique (Ogden *et al.,* 2003).

- The indirect fluorescent antibody test (IFA) is the most widely used serological diagnostic test. Cross-reactions with antibodies produced against other *Theileria* species, particularly *T. taurotragi*, occur (Shayan *et al.*, 1998). It is also subjective in nature (Apfalter *et al*., 2003).

- Monoclonal Antibodies (MAbs) profiles are used to demonstrate antigenic diversity and to characterize *T. parva* stocks. Parasites that have been cultured in vitro are fixed and spotted onto microscope slides and the reactions to the MAbs are determined with an indirect fluorescent antibody test (Bishop *et al.,* 2001).

- Polymerase Chain Reaction (PCR) /DNA probe - A universal set of primers 989/990 is used to amplify the small subunit rRNA gene of the *Theileria* parasite. A *T. parva* specific oligonucleotide is used to probe the amplified DNA to detect *T. parva.* No records of previous investigations into the detection limits of this test could be found.

1.10 Polymerase Chain Reaction

The polymerase chain reaction is a molecular technique that employs the target DNA, a DNA polymerase, primers, dNTP's, salts and buffer to allow a target sequence to be amplified several million-fold in a thermal cycler in a few hours. There are various PCR-based methods available for *T. parva* detection. These methods include conventional PCR with 989/990 primers and nested PCR with p104 primers. The conventional PCR method with the *Theileria* general primers 989 AGT TTC TGA CCT ATC AG and 990 TTG CCT TAA ACT TCC TTG was used for the specific amplification of a section of the *Theileria* parasite 18s rRNA genes in presence of mammalian DNA (Allsopp *et al.*, 1993). The expected size of the amplicon is approximately 1100 base pairs (bp) (Allsopp *et al.*, 1999). It was found that small subunit (SSU) rRNA from different *Theileria species* revealed species-specific regions. This resulted in the generation of synthetic oligonucleotides specific for the various species (Morzaria *et al.*, 1999) including the *T. parva* specific oligonucleotide, 1348 CAA AGC GAA CTC CGT CCG, which is used to probe ribosomal DNA to detect the presence of *T. parva*. This assay was optimized for the Onderstepoort Veterinary Institute (OVI) laboratory conditions to produce a single product for *T. parva* and was used to diagnose *T. parva*.

Other methods, such as the p104 gene PCR assay are based on the conserved sequences of the 104kDa rhoptry antigen (p104) gene. Rhoptries are secretory organelles implicated in host cell invasion (Skilton *et al.*, 1998). It was found to be highly specific for *T. parva* and there were no cross-reactions with other *Theileria* species (Morzaria *et al.*, 1999). Skilton *et al.* (2001) showed that this assay was able to detect parasite DNA in cattle that were experimentally infected with *T. parva* stocks. According to Morzaria *et al.* (1999), the p104 primers (IL 3231 ATT TAA GGA ACC TGA CGT GAC TGC and IL 755 CCG TTT GAT CCA TCA TTC AAG G) generate a secondary product in uninfected animals. However, this does not hybridize with a p104 nested probe hence the product does not originate from *T. parva*. The expected PCR product for p104 primers is 496bp.

For both the above mentioned techniques, PCR of purified DNA is followed by electrophoresis through an agarose gel and transfer of amplified DNA onto Hybond-N-nylon membrane using slotblotting or standard capillary blotting.

One drawback of these assays is that a complex hybridization step has to be performed or a radio labeled probe has to be used to achieve high levels of sensitivity. In the case of the 989/990 primers the membrane bound DNA is then hybridized with the *T. parva* specific oligonucleotide labeled with radioactive ^{32}P. With p104 assay a nested probe is generated by PCR and the probe is labelled with ^{32}P using a Megaprime random priming kit. Following this the membrane bound PCR products are hybridized with the labelled probe and autoradiographed. This takes between 3-4 days to be performed. Also of importance is that this post-amplification handling presents the risk of contamination (Contini *et al*., 2005). Apart from the fact that the continuous use of ^{32}P in a diagnostic laboratory could be a health hazard, these tests are both laborious and time consuming.

Since the advent of real-time PCR for the detection and quantitative analysis of amplicon (Yue *et al*., 2006) using fluorescent dyes, it is inevitable that diagnostic methods will follow the real-time PCR route. It is a technological leap forward for scientists around the world because of its increased sensitivity and ability to monitor the generation of PCR products in real-time (Valasek and Repa, 2005). Real-time PCR for detection of various targets has been reported to be faster, simpler (Alhassan *et al*., 2005, Becker *et al*., 2004) and more sensitive (Roy *et al.,* 2005; Nicolas *et al*., 2002). In recent years a real-time PCR method developed for malaria diagnosis was found to increase the safety and accuracy of such diagnosis (Andrew *et al*., 2005).

Sequence specific templates can be detected in real-time with the introduction of fluorogenic probes and cross contamination is prevented (Gibson *et al*., 1996). There are several assay formats such as the DNA binding assays which include Ethidium Bromide and SYBR Green and the sequence specific probe binding assays which include Hybridization probes, Molecular Beacons, Scorpions and Hydrolysis or TaqMan probes.

The Hybridization probe format has been used for the detection of *Brucella abortus* (Newby *et al*., 2003), *Legionella pneumophila* (Ballard *et al*., 2000), *Entamoeba histolytica* (Blessmann *et al*., 2002), for direct molecular haplotyping (Pont-Kingdon and Lyon, 2005), for rapid genotyping of hemochromatosis gene mutations (Mangasser-Stephan *et al*., 1999) and more recently for the detection of *T. parva* (Sibeko pers. comm., 2007). The Hybridization probe assay uses two specifically designed sequence- specific oligonucleotide probes labeled with different dyes (Roche Applied Science). These probes hybridize to the target sequence on the amplified DNA in a head-to-tail arrangement (Roche Applied Science). When the two fluorophores are in close proximity, the donor fluorophore emits energy which excites the acceptor fluorophore attached to the second probe. The acceptor dye emits fluorescent light at a different wavelength which is proportional to the amount of target DNA generated (Roche Applied Science). More specifically the real-time Hybridization PCR for the detection of *T. parva* (Sibeko pers. comm., 2007) includes two sets of probes, one *Theileria* genus specific and the other *T. parva* specific.

TaqMan probe chemistries (also known as the 5' nuclease assays) are some of the most widely used formats in real-time PCR assays because of its sensitivity (Hardick *et al*., 2004), specificity, accuracy and reproducibility (Tian *et al*., 2004). Some examples in which TaqMan probe formats have been used is in the diagnosis of pestiviruses (McGoldrick *et al*., 1998; Bhudevi and Weinstock, 2003), for the detection of hypodermal and hematopoietic necrosis virus from shrimp (Yue *et al*, 2006), for the detection of classical swine fever (Hoffmann *et al*., 2005), for the detection of hepatitis B viral particles in patients (Pas *et al*., 2000) and to type and subtype influenza viruses (Schweiger *et al*., 2000).

The TaqMan assay includes a fluorogenic labeled probe in the PCR which detects the sequence specific templates (Gibson *et al*., 1996). The probe is dual-labeled with a fluorescent reporter dye on the 5′ end and with a quencher dye at the 3′ end (Figure 2). The fluorescence in the intact probe is suppressed due to the close proximity of the quencher to the reporter dye (Roche Applied Science). During the extension phase, the probe hybridizes to the specific

sequence but extension is not possible due to the 3´ phosphorylation. Due to the 5´- 3´nucleolytic activity of the DNA polymerase (*Taq* polymerase), the probe is cleaved during extension. This results in the dyes separating and an increase in fluorescence. The fluorescence is proportional to the amount of PCR product generated and is measured with a LightCycler® instrument.

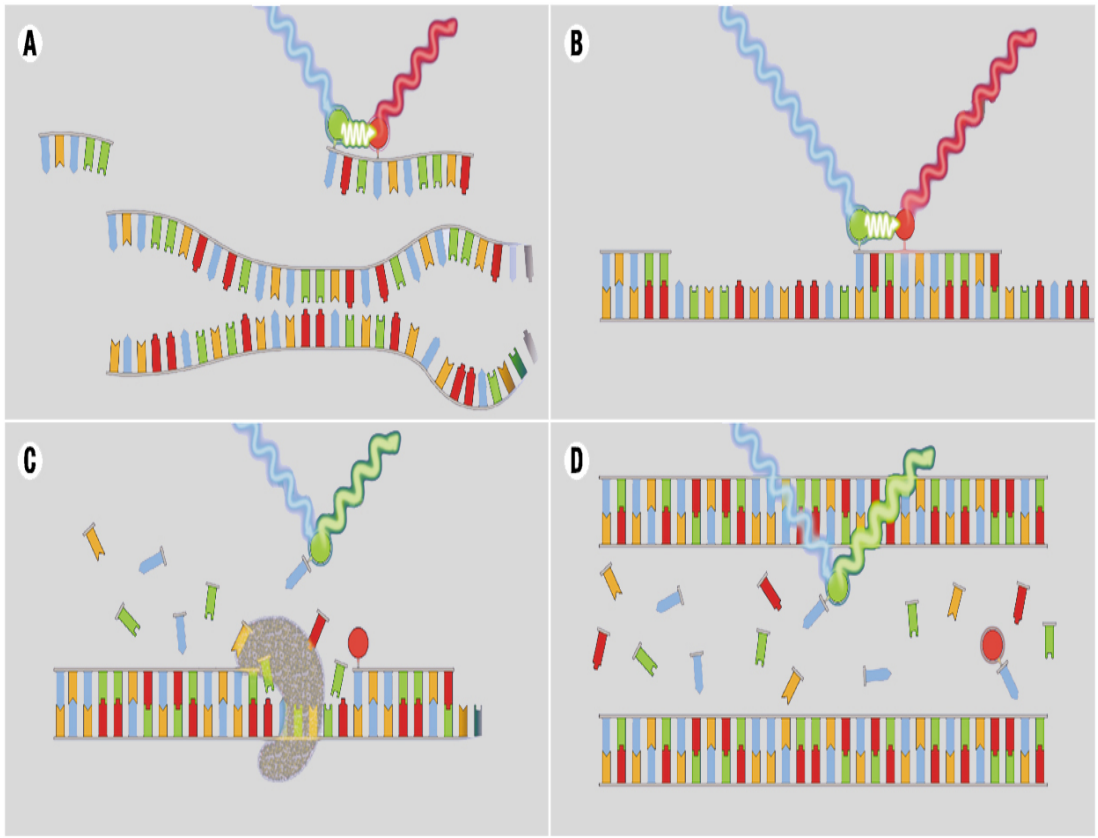


Figure 2: Schematic representation of TaqMan probe PCR (Roche Diagnostics, LightCycler® 2.0 System catalogue, Mannheim Germany)

A-D represents the stages during the cycling reaction

A- Denaturation

B- Annealing

C- Extension

D- product of the PCR

1.11 LightCycler® 2.0 system

The LightCycler® 2.0 Instrument (Figure 3) allows one to simultaneously amplify and analyse results by monitoring fluorescence during amplification (Roche Diagnostics, Mannheim, Germany) in real-time. The software allows for various types of data analyses to be performed for the appropriate chemistry used i.e. TaqMan, molecular beacon, Hybridization probe format or SYBR Green.

Figure 3: LightCycler® 2.0 system developed by Roche Diagnostics, Mannheim, Germany

CHAPTER 2: OBJECTIVES OF STUDY

2.1 Justification of study

Cattle losses due to Corridor disease in South Africa could have a disastrous impact on the cattle farming industry and a high monetary loss to buffalo breeders and game owners. Early and accurate diagnosis of Corridor disease is crucial to control the disease. In infected and recovered cattle and in the buffalo, *T. parva* infection persists and is maintained by two stages of the parasite life cycle (Maritim *et al*., 1989). The schizont-infected lymphocytes (Figure 1) undergo merogony to produce merozoites which infect the erythrocytes (RBC) and produce the piroplasms. The slow intermittent division of the schizonts and the piroplasm keep the infection in cattle and the buffalo. In the absence of re-infection, the piroplasm infected RBCs become very scarce and undetectable by microscopy. It is, therefore, possible that a buffalo or cattle can act as a carrier continuously or intermittently (Latif *et al*., 2001). In order to detect a carrier state i.e. an animal with low piroplasm parasitaemia, a sensitive and specific test is required. In the absence of re-infection, the antibody titres in carrier cattle wane over time and serological tests (IFA) become of no value.

Molecular methods, such as PCR have the advantages of being a species-specific technique and a more sensitive test for the detection of *T. parva* infection (Bishop *et al.*, 1992) than serological methods. However, conventional PCR methods are cumbersome, require the use of radioactive material and rely on post-amplification processing to achieve results, and therefore, could be prone to carryover contamination of PCR products. Real-time PCR makes it possible to amplify and detect a target gene in one step and improves the sensitivity (Hardick *et al*., 2004).

Many different real-time PCR formats can be used to generate the fluorescence signal. A study describing the development of the Hybridization probe real-time PCR assay for the detection of *T. parva* (Sibeko pers. comm., 2007) found that the assay was more sensitive and faster than other molecular tests. This study describes the development and evaluation of a real-time TaqMan probe PCR

assay for the detection of *T. parva* with the Roche LightCycler® in order to replace the conventional PCR assay. The ensuing experiments would be to develop the real-time TaqMan PCR for *T. parva*, to compare the TaqMan PCR assay to the conventional PCR test and the alternate real-time hybridization PCR test and to apply the test to field samples. Collectively this study will demonstrate whether the TaqMan test is a suitable test for the detection of *T. parva* in South Africa.

2.2 Specific objectives

i. To develop and optimize a Real-time PCR test based on TaqMan chemistry for the detection of *T. parva*

ii. To evaluate the diagnostic sensitivity (i.e. the proportion of known infected animals, which give positive test results) and diagnostic specificity (i.e. the proportion of known uninfected animals, which give negative results) of the TaqMan PCR test in comparison to 2 other detection methods:

   a. conventional 989/990 PCR/probe; and

   b. Hybridization probe PCR developed by the BioPAD Consortium

iii. To compare the analytical sensitivity (i.e. the smallest amount of parasite DNA that can be detected in a test sample) and analytical specificity (i.e. the ability of an assay to detect the DNA from a particular causative agent and not DNA that may be from a related or unrelated organism) of the 3 different detection techniques for *T. parva*

iv. To determine the duration and repeatability of the real-time TaqMan PCR assay

v. To apply the real-time TaqMan test to samples from the field

CHAPTER 3: MATERIALS AND METHODS

3.1 Blood samples

3.1.1 Gold standard positive controls

The gold standard positive samples included buffalo 102 which was a naturally infected buffalo, bovine 9288 and bovine 9433 which were experimentally infected cattle in the laboratory. Two buffalo from Welgevonden that were identified as *T. parva* carrier animals were subjected to conventional PCR and found to be positive. They were brought to the OVI stable and their carrier states were confirmed by applying *Rhipicephalus appendiculatus* nymphs from the colony and subsequent adult ticks produced Corridor disease in bovine 9288. The bovine 9288 tested positive for *T. parva* with the Hybridization probe PCR and recovered spontaneously. Unfed nymphs were applied to feed on bovine 9288 and the adult ticks produced classical Corridor disease in a clean bovine 9455 which died on day 19 after infection. Bovine 9433 was a splenectomised animal which received blood from a *T. parva* positive Hluhluwe buffalo. Subsequently ticks fed on bovine 9433 transmitted Corridor disease to bovine 8106. Buffalo 102 was identified as a *T. parva* carrier animal in the Kruger National Park, brought to OVI stables and unfed nymphs were applied to feed on the buffalo. The adult ticks were fed on bovine 9446 which later died of classical Corridor disease.

3.1.2 Collection of blood samples

Blood samples were received in EDTA vacutainer tubes from buffalo and cattle from various parts of South Africa. These were kept in cool boxes and transported to the lab. Twenty known *T. parva* negative blood samples were collected from the herd of cattle kept by the programme for over 30 years to produce tick borne disease blood vaccines. The cattle are kept in a tick and tick borne disease free environment. These samples were used for the diagnostic specificity test and two samples from these negative cattle were also used as gold standard negative controls. The blood from 69 buffalo from different areas in South Africa including Nelspruit, Malelane, Hoedspruit and Kimberley

which tested negative for *T. parva* using the conventional and the Hybridization probe PCR were considered as negative control samples and used for the diagnostic specificity test.

Twenty four blood samples collected from buffalo from Hluhluwe (a Corridor disease endemic area), and 21 other buffalo samples collected from different areas in KwaZulu Natal, Free State and the Limpopo province (which were confirmed to be positive for *T. parva* DNA by Hybridization probe PCR) were used in the diagnostic sensitivity tests.

3.2 Extraction of DNA

The DNA extractions from all blood samples were performed using the automated Roche MagNa Pure LC (Roche Diagnostics, Mannheim, Germany) (Figure 4) and the MagNa Pure Large Volume Kit (Roche Diagnostics, Mannheim, Germany) according to the instructions supplied for the MagNa Pure LC program "DNA Large Volume" protocol. The 200 µl blood samples are lysed during incubation with Proteinase K and Lysis/Binding buffer. The Lysis/Binding buffer contains chaotropic salts which increase the ionic strength of the mixture. The DNA binds to the Magnetic Glass Particles (MagNa Pure Large Volume Kit). Following several washing steps which remove the proteins, cell membranes, PCR inhibitors, cellular debri and chaotropic salts, the purified DNA is eluted in 100 µl of elution buffer. The extracted DNA samples were stored at -20^{o}C until further use. DNA from these samples was then amplified using the three PCR techniques. All PCR runs included at least one negative DNA control consisting of either PCR-grade water or a known negative animal and a DNA sample from a gold standard positive animal.

Figure 4: Roche MagNa Pure LC system developed by Roche Diagnostics, Mannheim, Germany

3.3 Polymerase chain reaction

3.3.1 Primer and Probe design

The primers *Theileria* S and *Theileria* R designs (TIB MOLBIOL, Berlin, Germany) were based on the *Theileria* 18S rRNA gene (Genbank L02366) since parts of the nucleic acid sequence of these genes are highly conserved (Collins *et al.*, 2002, McGoldrick *et al*., 1998). The primers generate a 233 bp amplicon. It has been recommended that the TaqMan probe be complementary to a stretch of 20-30 nucleotides for the TaqMan assay (McGoldrick *et al*., 1998). The hypervariable regions show species specific variations of the rRNA genes and allowed for a TaqMan probe (TIB MOLBIOL, Berlin, Germany) to be designed for the detection of a 22 bp region of the 18S rRNA gene amplicon. The selected area was found to differ by atleast two nucleotides from other *Theileria* species such as *T. mutans*, *T. taurotragi* and *T.* sp. (buffalo) (Figure 5). This was thought to be appropriate since the TaqMan PCR had previously been used for allelic discrimination where the target differed by one nucleotide (Lee *et al*., 1993). The sequences of the primers and probe are listed in Table 1. The *T. parva* specific probe was labeled with 6-carboxyfluorescein (FAM) at the 5' end and with *N,N,N',N'*-tetramethyl-6-carboxyrhodamine (TAMRA) at the 3' end (Roche). The cycling conditions for PCR were then optimised.

Table 1: The sequences of *T. parva* primers and TaqMan probe

Primer	Sequence 5'-3'	Location
Theileria S primer	5'-GGT AAT TCC AGC TCC AAT AG -3'	552-571
Theileria R primer	5' –ACC AAC AAA ATA GAA CCA AAG TC- 3'	761-785
TaqMan probe	5'- ACG GAG TTC GCT TTG TCT GGA T- 3'	665-686

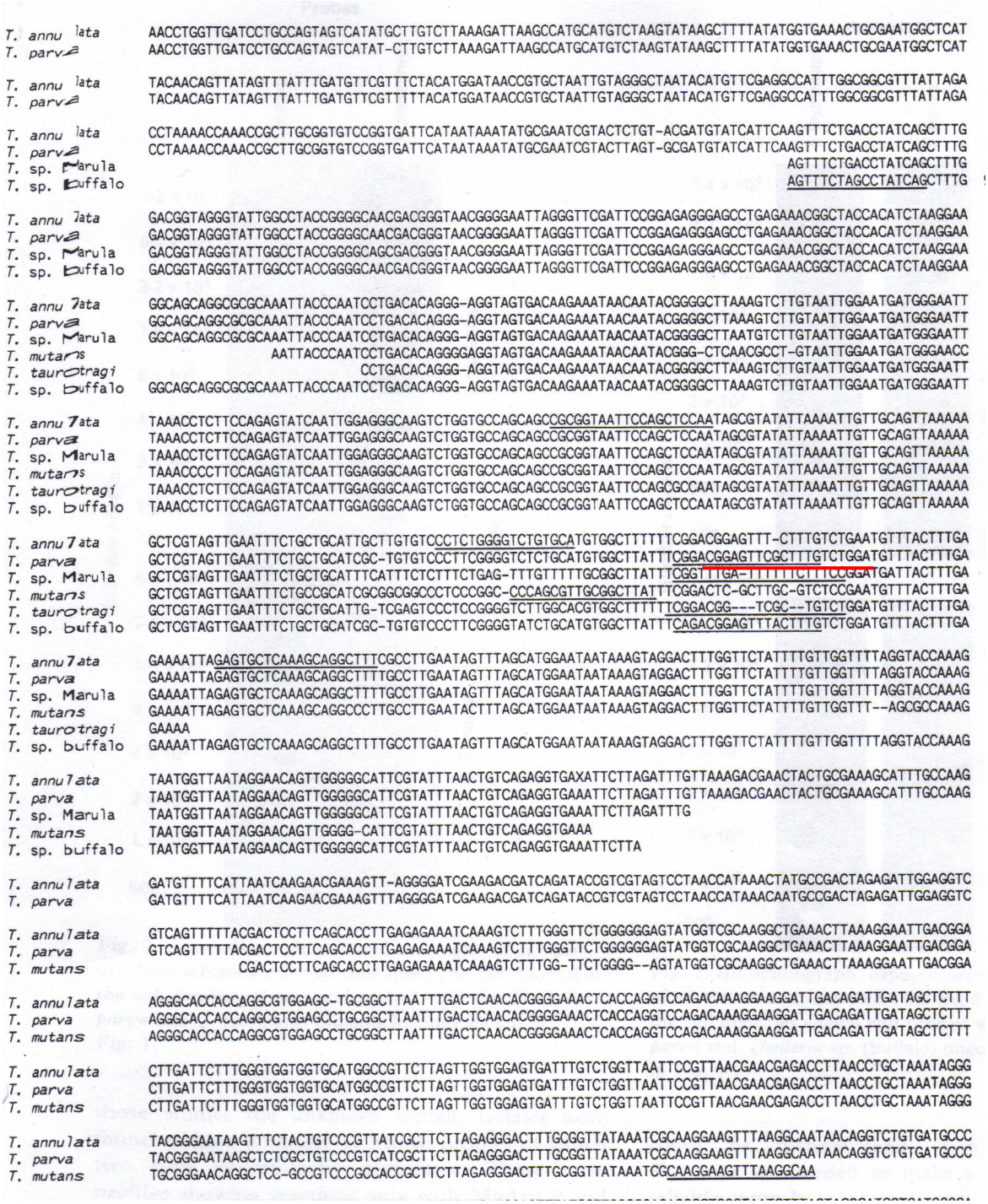


Figure 5: CLUSTAL alignment of complete and partial gene sequences (Allsopp *et al*., 1993)

The red line indicates the position of the *T. parva* TaqMan probe.

The TaqMan probe assay involves a single probe with a dye at each end of the oligonucleotide. Following binding to the target DNA the probe is hydrolysed by the DNA polymerase and the reporter (FAM) is released from the quencher (TAMRA). The reporter dye emits fluorescent light which is measured by the LightCycler® instrument's photometer. The increase in fluorescence directly correlates to the amount of amplicon generated and the results can be analysed in channel 530 of the Absolute Quantification analysis.

3.3.2 Optimisation of the TaqMan PCR

The TaqMan PCR was performed with the LightCycler® 2.0 system (Roche Diagnostics, Mannheim, Germany) using the primers and probe shown in Table 1. Four microlitres of 5x LightCycler® FastStart DNA Master^{Plus} Hybridization Probe reaction mix (including enzyme), 12.1 µl PCR grade water (LC Faststart DNA Master^{Plus} Hybridization Probe Kit), 0.5 µM each of primers *Theileria* S and *Theileria* R and 0.4 µM *T. parva* probe were combined to make up the master mix. 2.5 µl of template DNA was added to make up a 20 µl final reaction volume. Amplification was performed under the following conditions: an initial denaturation step at 95°C for 10 minutes, followed by 40 cycles of 95°C for 10 seconds, 50°C for 5 seconds and 59°C for 20 seconds. The data were collected during extension and was monitored in the F1 (530nm) channel (Hardick *et al*., 2004). A negative template control and a *T. parva* positive control were included in every run.

3.3.3 Conventional 989/990 PCR/probe assay

The conventional PCR using the 989/990 primers (Table 2) as described by Allsopp *et al*. (1993) was used. This test has been optimized, modified and employed by the OVI to screen *T. parva* in cattle and buffalo since 1996. The master mix comprised of reaction buffer without $MgCl_2$ (5 µl), $MgCl_2$ (2.5 mM) and enzyme (0.75 µl) from the High Fidelity Expand PCR kit (Roche Diagnostics), 4 µl dNTP (2.5 mM each, Takara), primers 989 and 990 (1 µM each of 10 µM solution) and water to make 47.5 µl. DNA (2.5 µl) was added to

the master mix and amplified with the Eppendorf Mastercycler® Gradient under the following conditions, 95°C for 2 minutes followed by 35 cycles of 95°C for 30 seconds, 52°C for 40 seconds and 72°C for 40 seconds, and a final elongation step at 72°C for 10 minutes. PCR products (8 µl) and phiX174DNA/Hae III Markers were electrophoresed through a 1% agarose gel and the expected size of the amplicon was 1094 bp.

Table 2: The sequences of 989/990 primers and *T. parva* specific probe (Allsopp *et al*., 1993)

Primers	Sequence 5'-3'	Location
989	5'-AGT TTC TGA CCT ATC AG -3'	278-294
990	5' –TTG CCT TAA ACT TCC TTG- 3'	1363-1380
T. parva specific probe	5'- CAA AGC GAA CTC CGT CCG- 3'	664-681

3.3.4 Slotblotting

Ten microlitres of the PCR products from the 989/990 PCR assay was added to a master mix containing TE, 0.5M EDTA and 6M NaOH. This was allowed to incubate on ice for 10 minutes and then 100 µl of 2M Nh_4OAc was added. The samples were slotblotted (Saiki *et al.,* 1986) onto Hybond N+ membranes (Amersham International). The membranes were rinsed in 6 x SSC solutions, dried, incubated for 10 minutes in 0.4M NaOH, rinsed again in SSC and dried. The membranes were prehybridized in a mixture of 14% SDS and 1M NaOH for 3 hours at 40°C. The membranes were then probed with *T. parva* specific oligonucleotide probes (as in Table 2.) labeled with [α-^{32}P]-dATP. The oligonucleotide probes were 3' end-labeled with [α-^{32}P]-dATP using terminal transferase according to the manufacturer's instructions (Promega). Hybridization was performed overnight and the 4 washing steps were performed before the stringency wash at 60°C for 1 minute the following day. The membranes were placed into plastic bags. Each membrane and a film were

sandwiched between two screens in an X-ray cassette and the film was exposed at -70°C for 4 hours. The films were developed and fixed in the dark room.

3.3.5 BioPAD Hybridization PCR

The BioPAD Hybridization PCR assay (Sibeko pers. comm., 2007) was also performed on the LightCycler® 2.0 system (Roche Diagnostics, Mannheim, Germany). The sequences of the primers and probes used are shown in Table 3. Four microlitres of 5x LightCycler® FastStart DNA Master^{Plus} Hybridization Probe reaction mix including enzyme (Roche Diagnostics, Mannheim, Germany), 0.5 µM each of Parva F and *Theileria* R primers, 0.1 µM of each Hybridization probe, 1U uracil deoxy-glycosylase (UDG) (Roche Diagnostics, Mannheim, Germany) were combined to make up the master mix. A final reaction volume of 20 µl is made up by the addition of 2.5 µl of template DNA.

Table 3: The sequence of primers and probes for the BioPAD Hybridization PCR (Sibeko pers. comm., 2007)

Primer	Sequence 5'-3'	Location
Parva-F	5'-CTG CAT CGC TGT GTC CCT T -3'	619-637
Theileria-R	5' –ACC AAC AAA ATA GAA CCA AAG TC-3'	763-785
TheilPr640	5'- LCRed640- TCG GAC GGA GTT CGC T—PH- 3'	662-677
Theileria Anch. Probe	5'-GGG TCT CTG CAT GTG GCT TAT--FL - 3'	640-660
Theileria positive	5'-AGA AAA TTA GAG TGC TCA AAG CAG GCT TT –FL- 3'	699-727
Posit LC705	5'-LCRed705- GCC TTG AAT AGT TTA GCA TGG AAT--PH - 3'	729-752

Amplification was performed under the following conditions (Sibeko pers. comm., 2007):

Programs

Program Name	Cycles	Analysis Mode
UDG activation	1	None
Pre-incubation	1	None
Amplification	45	Quantification
Melting curve	1	Melting curves
Cooling	1	None

Program	Temp ^{o}C	Hold	Ramp/rate ^{o}C/sec	Acquisition Mode
UDG	40	10 min	20	None
Pre-incubation	95	10 min	20	None
Amplification	95	10 sec	20	None
	58	10 sec	20	Single
	72	15 sec	20	None
Melting curves	95	0	20	None
	40	40 sec	20	None
	95	0	0.2	Continuous
Cooling	95	30 sec	20	None

The PCR product generated was 167 bp and the *T. parva* specific probes targeted a region of 40 bp in length combined.

3.4 Sensitivity

The diagnostic sensitivity of the TaqMan, Hybridization probe and conventional PCR assays were compared using the *T. parva* positive samples indicated in the collection of blood samples. The analytical sensitivity was determined by preparing 10 fold serial dilutions ranging from 2 x 10^{-3} to 2 x 10^{-9}% parasitaemia using the blood from the gold standard positive buffalo 102.

The blood from a negative animal, as described in collection of blood samples, was used to dilute the positive sample. The red blood cell (RBC) count was estimated using a haematocrit centrifuge. The blood sample of buffalo 102 was placed in a capillary and spun in the centrifuge at 12000 rpm for 3 minutes. The packed cell volume (PCV) reading was 35%. The parasitaemia was estimated by 2 experienced personnel in the parasitology lab at OVI by examination of a

Giemsa stained blood smear from the animal on that day using a compound microscope and was estimated to be 0.002% (1 parasite per 50000 RBC). All dilutions were performed in 30 replicates and each sample was processed as previously described. A total of 210 samples were tested using each PCR assay.

3. 5 Specificity

The diagnostic specificity of the TaqMan, Hybridization probe and the conventional PCR tests were compared using the *T. parva* negative samples referred to in the collection of blood samples. The analytical specificity of the three tests targeting the 18S rRNA gene was evaluated by testing DNA preparations from *T. parva* gold standard positive animals, other *Theileria* species, haemoparasites, diverse microorganisms and gold standard negative animals as indicated in Table 6. PCR was performed on duplicates of each sample for each test.

3.6 Repeatability

DNA from two gold standard positive controls, animal 9433 and 9446, and a negative control was extracted daily for 10 days and tested on the TaqMan assay in different runs.

3.7 Comparing field sensitivity of the TaqMan PCR and Conventional 989/990 PCR/probe

Field samples sent to the OVI for the purpose of the *T. parva* test from 443 buffalo and 134 cattle from various areas in South Africa including Bloemfontein, Pietermaritzburg, Vryheid, Ladysmith, Grahamstown, Kimberley, George, Ellisras, Malelane, Hoedspruit, Elsenburg, Stofberg, Kuruman, Randfontein, Potgietersrus, Thabazimbi, Louis Trichardt, Lydenburg and Nkomazi, were tested for *T. parva* using the 989/990 PCR assay and the TaqMan PCR. Positive and negative controls were included in each test for every run. Results were only accepted if the controls performed as expected. Data were analysed using the percent agreement and the kappa coefficient values (Appendix 4).

3.8 Data Analysis

Variables measured included the number of true positives (TP), number of true negatives (TN), number of false positives (FP) and the number of false negatives (FN). Diagnostic sensitivity was calculated as TP/(TP + FN), diagnostic specificity was calculated as TN/(TN + FP) as indicated in Appendix 3. The Kappa coefficient was calculated as indicated in Appendix 4.

CHAPTER 4: RESULTS

4.1 The TaqMan PCR

The analysis of results for the TaqMan PCR is performed with the Absolute quantification analysis tool with the Roche LightCycler®. A sigmoid curve shows amplification of the target area indicating that the sample is positive for *T. parva* and a flat line indicates the absence of the parasite (Figure 6). Samples were considered positive if amplification occurred before cycle number 35 or less since it is likely that the probes will degrade at cycles >35 (with Nico Gunter, Roche Applications Specialist, pers. comm., 2005).

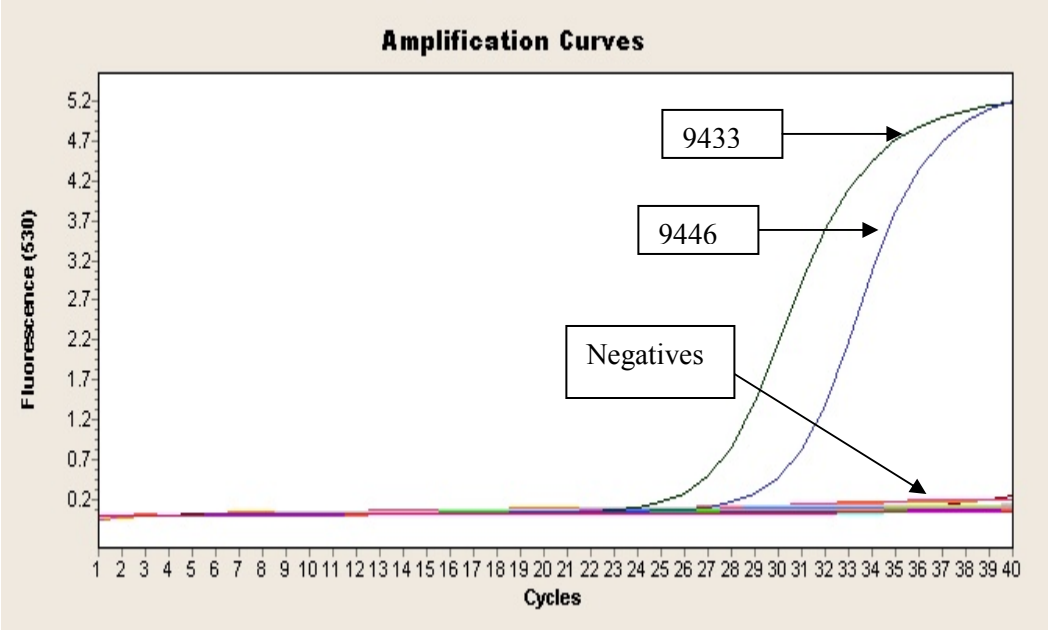


Figure 6: Detection of *T. parva* using the Real-time TaqMan PCR with the *Theileria* general primers and the *T. parva* specific TaqMan probe.
Amplification was only observed in the positive control samples and no amplicons were generated in the negative samples.

4.2 Diagnostic sensitivity

The DNA of 24 positive buffalo from Huhluwe (an endemic area) and 21 positive buffalo as described in the collection of blood samples in Chapter 3 were tested using the three PCR assays. A summary of the results is given in Table 4. Of the 45 positive samples, the Hybridization probe PCR detected 100% of the samples, the TaqMan probe PCR detected 87% and the 989/990 PCR/probe detected 84%.

4.3 Diagnostic specificity

DNA was extracted from the blood of 20 known negative cattle from OVI and 69 negative buffalo as indicated in collection of blood samples in Chapter 3. All three PCR assays did not detect any positive samples and there was 100% agreement between the tests (Table 5).

Table 4: Results for diagnostic sensitivity using the three PCR assays

Sample number	BioPAD Hybridization PCR	Conventional 989/990 PCR/probe	TaqMan PCR
16689	+	+	+
16690	+	+	+
16691	+	+	+
16692	+	+	+
16694	+	+	+
16695	+	+	+
16696	+	+	+
16697	+	+	+
16698	+	+	+
16699	+	+	+
16700	+	+	+
16701	+	+	+
16703	+	+	+
16704	+	+	+
16705	+	+	+
16706	+	+	+
16707	+	+	+
16708	+	+	+
16709	+	+	+
16710	+	+	+
16711	+	+	+
16714	+	-	+
16715	+	+	+
16717	+	+	+
8840	+	-	+
8841	+	-	+
8842	+	-	+
8843	+	-	+
8844	+	-	+
9009	+	+	-
9010	+	+	+
9011	+	+	-
9016	+	+	+
9474	+	+	+
9580	+	+	-
9598	+	+	-
9599	+	+	-
9600	+	+	-
10797	+	+	+
10798	+	+	+
11133	+	+	+
12705	+	-	+
12424	+	+	+
12475	+	+	+
12476	+	+	+
Total positives (%)	45 (100%)	38 (84%)	39 (87%)

Table 5: Results for diagnostic specificity using the three PCR assays

Sample number	BioPAD Hybridization PCR	Conventional 989/990 PCR/probe	TaqMan PCR
9451	-	-	-
9415	-	-	-
9483	-	-	-
9440	-	-	-
9428	-	-	-
9412	-	-	-
9425	-	-	-
9426	-	-	-
9435	-	-	-
9432	-	-	-
9438	-	-	-
9479	-	-	-
9417	-	-	-
9422	-	-	-
9482	-	-	-
9431	-	-	-
9434	-	-	-
9478	-	-	-
9429	-	-	-
9437	-	-	-
12378	-	-	-
12380	-	-	-
12380	-	-	-
12381	-	-	-
12382	-	-	-
12383	-	-	-
12384	-	-	-
12385	-	-	-
12386	-	-	-
12387	-	-	-
12388	-	-	-
12389	-	-	-
12390	-	-	-
12391	-	-	-
12392	-	-	-
12393	-	-	-
12395	-	-	-
12396	-	-	-
12397	-	-	-
12398	-	-	-
12400	-	-	-
12402	-	-	-
12403	-	-	-

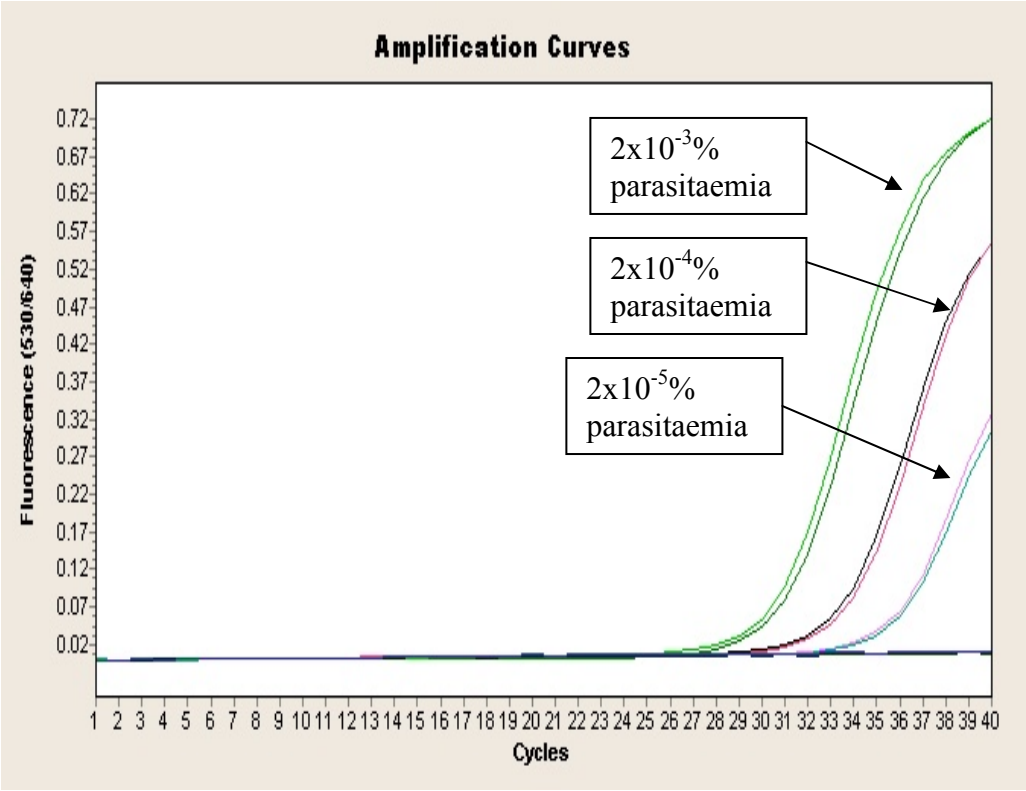
Table 5 continued

Sample number	BioPAD Hybridization PCR	Conventional 989/990 PCR/probe	TaqMan PCR
12404	-	-	-
12405	-	-	-
12407	-	-	-
12408	-	-	-
12410	-	-	-
12411	-	-	-
12412	-	-	-
12413	-	-	-
12414	-	-	-
12415	-	-	-
12416	-	-	-
12417	-	-	-
12418	-	-	-
12419	-	-	-
12420	-	-	-
12421	-	-	-
12422	-	-	-
12423	-	-	-
12425	-	-	-
12426	-	-	-
12427	-	-	-
12428	-	-	-
12429	-	-	-
12430	-	-	-
12431	-	-	-
12432	-	-	-
12433	-	-	-
12434	-	-	-
12435	-	-	-
12436	-	-	-
12437	-	-	-
12438	-	-	-
12439	-	-	-
12441	-	-	-
12443	-	-	-
12444	-	-	-
12445	-	-	-
12446	-	-	-
12447	-	-	-
12450	-	-	-
12451	-	-	-
12454	-	-	-
12456	-	-	-
12457	-	-	-
12458	-	-	-
12459	-	-	-
Total negatives (%)	89 (100%)	89 (100%)	89 (100%)

4.4 Analytical sensitivity of the 3 detection techniques

Ten-fold serial dilutions of blood from a gold standard positive buffalo 102 were made to determine the analytical sensitivity of the 989/990 PCR, TaqMan PCR and the BioPAD Hybridization PCR assays. The samples that showed a sigmoid plot with amplification occurring before cycle 35 were interpreted as positive when tested on the TaqMan PCR assay. Amplification of the *T. parva* DNA at different concentrations showed linearity over a range of dilutions for the TaqMan assay (Figure 7). A standard curve with an efficiency of "2" denotes a perfect amplification curve (Roche Applied Science). The efficiency or slope of this curve (Figure 7b) was found to be 2.4.

a)



b)

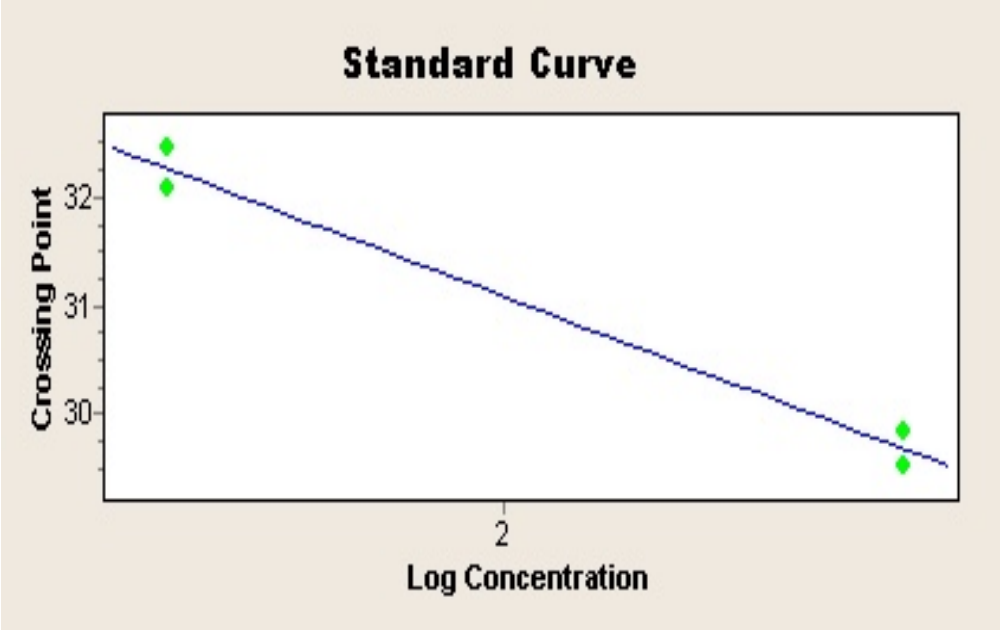


Figure 7: Detection of *T. parva* using the real-time TaqMan probe assay.
a) Absolute quantification analysis showing amplification curves of the 10-fold dilution series ranging from $2x10^{-3}$% parasitaemia to $2x10^{-5}$% parasitaemia and no fluorescence in the negative control. b) Plot of resulting crossing points (cycle numbers) versus logarithm of concentration

For the BioPAD Hybridization probe assay, samples that showed an amplification profile on the Qualitative analysis and then a melting profile at 55ºC and 63ºC in the Tm analysis in channel 640/530 were found to be positive. With the 989/990 PCR assay the appearance of a band on the X-ray film after radiography denotes a positive result.

Figure 7a shows the amplification curves for samples ranging from $2x10^{-3}$% parasitaemia to the $2x10^{-5}$% parasitaemia using the TaqMan PCR probe assay. The radiographed X-ray film of the $2x10^{-4}$% parasitaemia to the $2x10^{-6}$% parasitaemia dilutions for the conventional PCR can be seen in Figure 8. Twenty nine replicates of the $2x10^{-4}$% parasitaemia, 28 replicates of the $2x10^{-5}$% parasitaemia and 2 of $2x10^{-6}$% parasitaemia showed band patterns. The melting profiles of the serial dilutions ranging from $2x10^{-4}$% parasitaemia

to the $2x10^{-6}$% parasitaemia using the Hybridization probe PCR assay is shown in Figure 9, however *T. parva* was not detected in all the samples with a $2x10^{-6}$% parasitaemia. A summary of the results for the range of dilutions tested using all 3 PCR assays for the analytical sensitivity is shown in Figure 10.

The TaqMan and the Hybridization probe PCR assays were 100% sensitive at a $2x10^{-3}$% parasitaemia which was equivalent to 350 parasites/reaction whilst the conventional 989/990 PCR assay was 70.6% sensitive (95 % confidence interval of 54%-86%). The sensitivity of the BioPAD Hybridization PCR assay, the TaqMan PCR assay and the 989/990 PCR assay at a $2x10^{-4}$% parasitaemia which was equivalent to 35 parasites/reaction was 100% , 100% and 96.7% (95% confidence interval of 90%-100%) respectively.

The TaqMan PCR was 93.3% sensitive (95% confidence interval of 84%-100%) at a $2x10^{-5}$% parasitaemia (3.5 parasites/reaction) whilst the Hybridization probe and the conventional PCR assay were 100% and 93.3% (95% confidence interval of 84%-100%) respectively. At a $2x10^{-6}$% parasitaemia which was $3.5x10^{-1}$ parasites, the sensitivity of the Hybridization probe PCR assay and the 989/990 PCR assay were 36.7% (95 % confidence interval of 19.5%-54%) and 6.67% (95% confidence interval of 2%-15%) respectively. The TaqMan probe assay was not able to detect *T. parva* parasites at a $\leq 2x10^{-6}$% parasitaemia. The Hybridization probe PCR was still able to detect *T. parva* DNA at $2x10^{-7}$% parasitaemia and at $2x10^{-9}$% parasitaemia however the percentage sensitivity was very low at less than 10%.

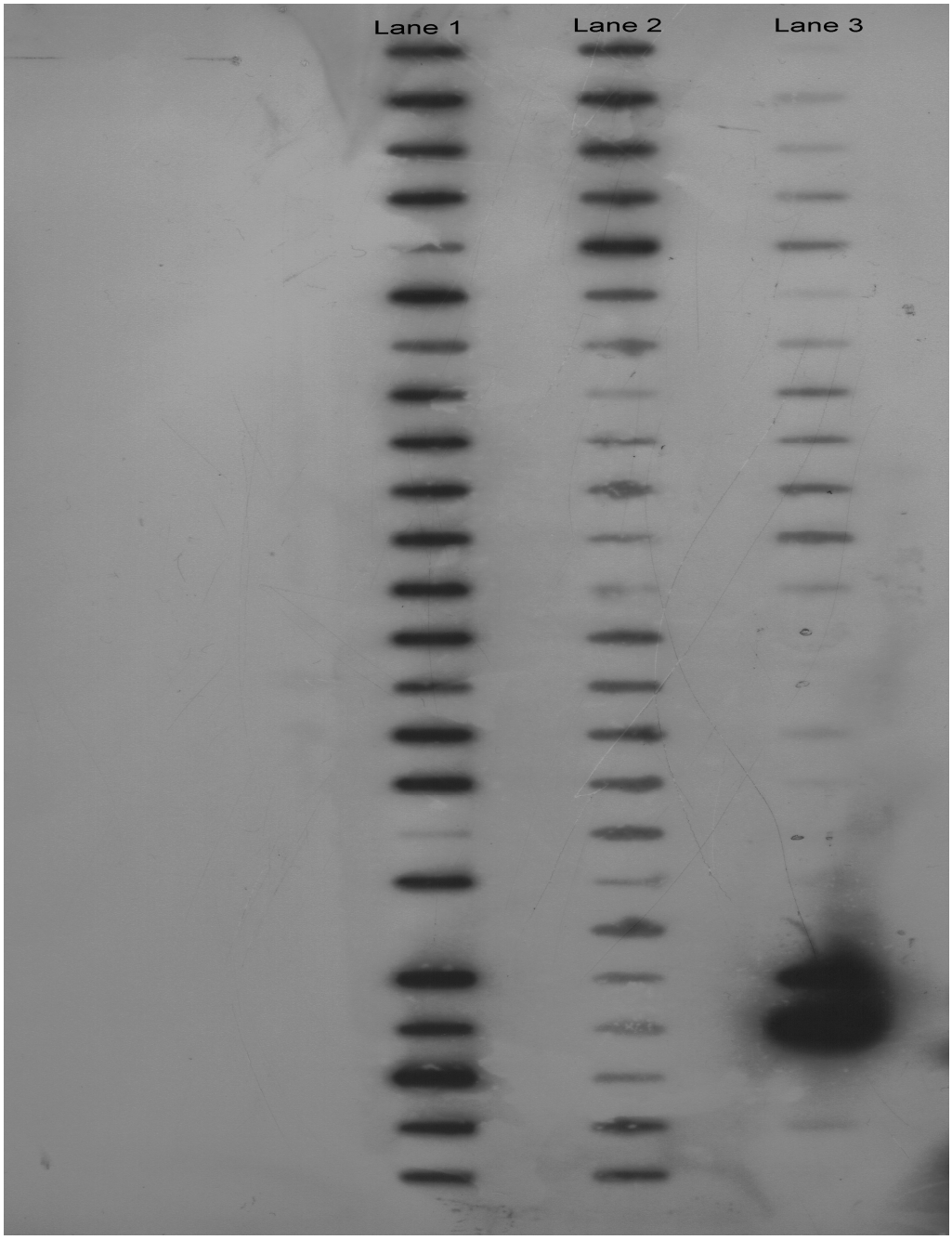


Figure 8: Detection of T. parva using the conventional 989/990 PCR/probe assay.

A radiograph of the 10-fold dilution series ranging from $2x10^{-4}$% parasitaemia to $2x10^{-6}$% parasitaemia was prepared. Lane 1 represents 24 out of the 30 replicates for $2x10^{-4}$% parasitaemia, lane 2 represents 6 out of the 30 replicates of the $2x10^{-4}$% parasitaemia and 18 out of the 30 replicates of $2x10^{-5}$% parasitaemia and lane 3 represents 12 out of the 30 replicates for the $2x10^{-5}$% parasitaemia and 6 out of the 30 replicates of $2x10^{-6}$% parasitaemia and a positive and negative controls.

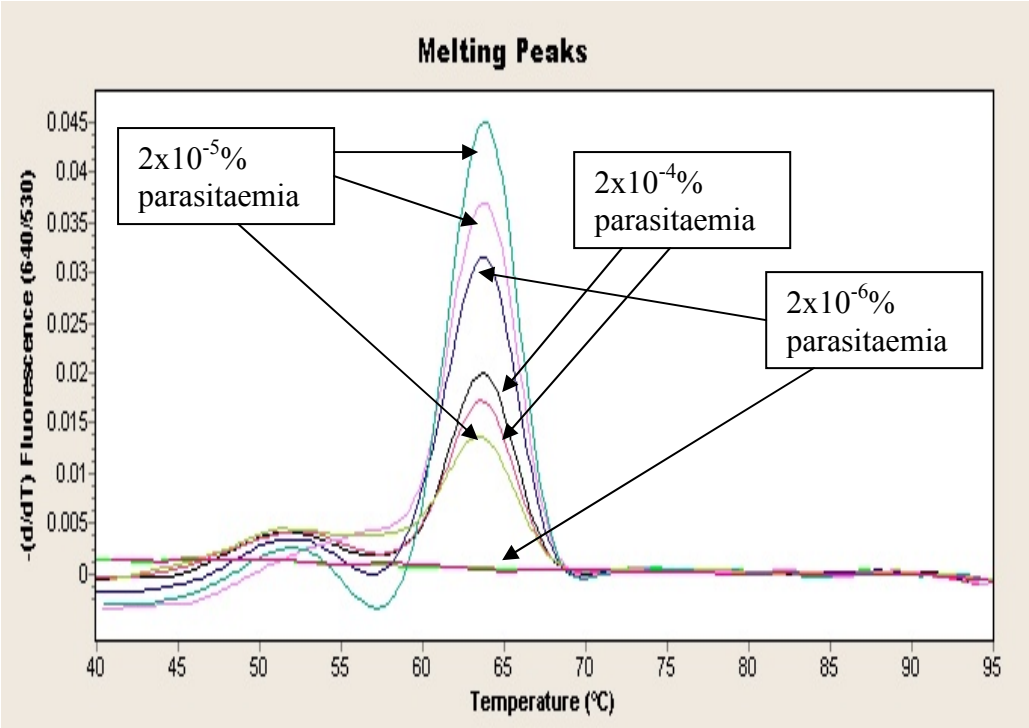


Figure 9: Detection of *T. parva* using the Hybridization Probe PCR assay. Melting profiles of the 10-fold dilution series ranging from $2x10^{-4}$% parasitaemia to $2x10^{-6}$% parasitaemia can be observed.

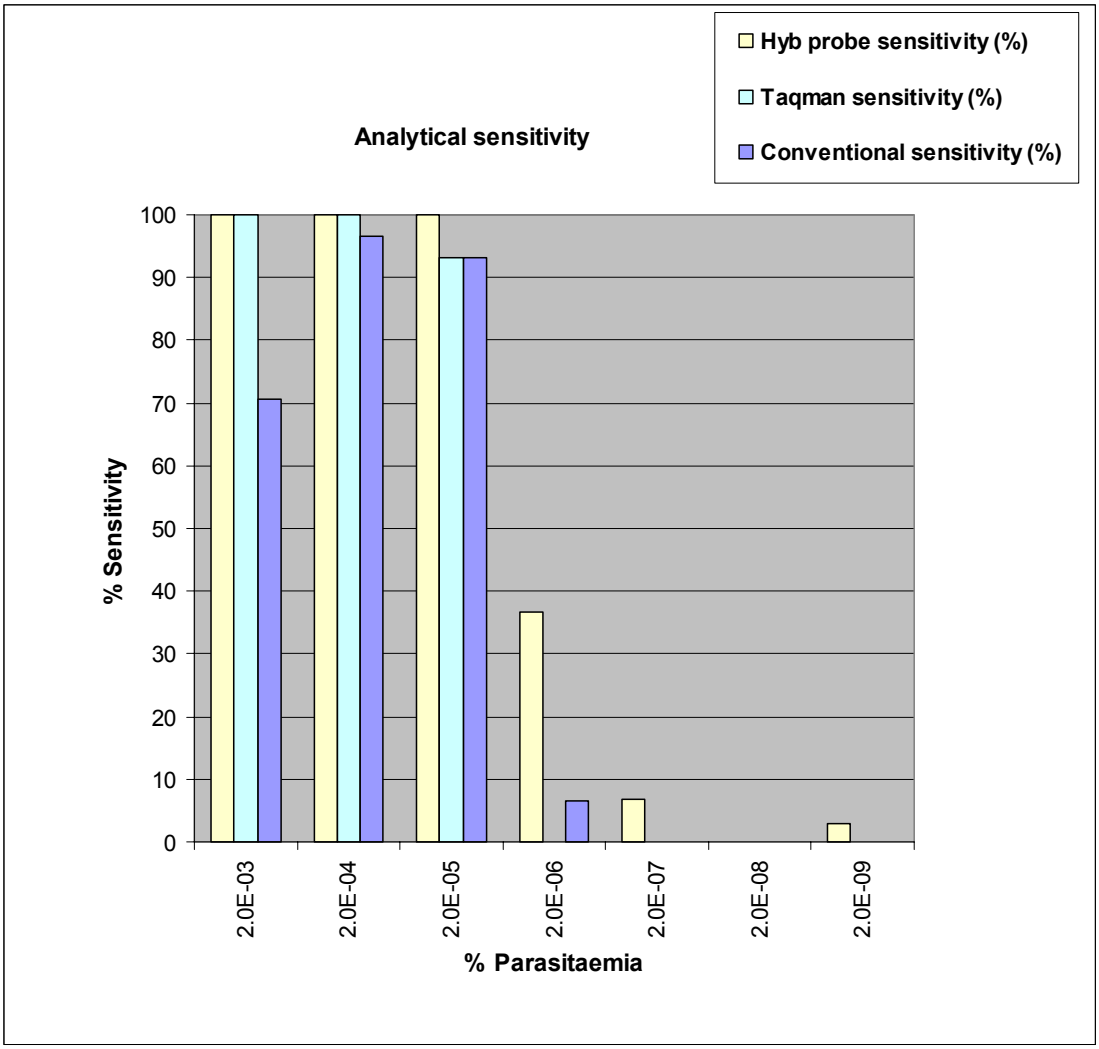


Figure 10: Comparison of the analytical sensitivities of the Hybridization PCR, TaqMan PCR and the 989/990 PCR/probe assay.
The 10-fold serial dilutions range from $2x10^{-3}$% parasitaemia to $2x10^{-9}$% parasitaemia.

4.5 Analytical specificity of the 3 PCR assays

The species specificity of the 3 PCR assays was determined with DNA from several different *Theileria* species, other haemoparasites and microorganisms (Table 6). The TaqMan assay detected the *T. parva* and the *T.* sp. (buffalo) positive samples but did not amplify other *Theileria* species, haemoparasites or microorganisms (Figure 11). However, the shape of the amplification curves for the *T. parva* and the *T.* sp. (buffalo) positive samples were not similar. The *T.* sp. (buffalo) amplification curve did not look sigmoid but was of a more flat nature.

An amplification curve in the 640/530 channel and a melting profile with melts at 51°C and 63ºC were obtained for all the *T. parva* positive samples with the BioPAD Hybridization probe PCR (Figure 12). This PCR assay also amplified *T.* sp. (buffalo) (Figure 13a) but no melting profile was observed (Figure 13b) in the Tm calling analysis. The Hybridization probe PCR did not detect any other organisms.

The conventional 989/990 PCR assay performed detected the *T. parva* positive sample and the other *Theileria* samples containing *T. mutans* but did not detect any of the other organisms.

Table 6: Specificity of the three PCR assays

Species	TaqMan probe	BioPAD Hybridization probe	Conventional PCR
Trypanosoma (8a)	-	-	-
Babesia. caballi 502 (16368)	-	-	-
Theileria equi 20 (16369)	-	-	-
Erlichia ruminantium (Ball vaccine strain)	-	-	-
Babesia bigemina and *Anaplasma centrale* 9456.1 (16932)	-	-	-
Babesia bovis (16823)	-	-	-
Babesia bigemina (16834)	-	-	-
T. tauro + *T. mutans* (14047)	-	-	+
T. mutans (14043)	-	-	+
T. buffeli + *T. mutans* (14044)	-	-	+
T. taurotragi + *T. mutans* and *T. parva* (14048)	+	+	+
T. parva	+	+	+
Arcanobacterium pyogenes (6964)	-	-	-
Bacillus lactosporus (9879)	-	-	-
Staphyloccocus aureus (9351)	-	-	-
Escherichia coli (097)	-	-	-
Enterococcus facium (9351)	-	-	-
T. sp. (buffalo)	+	-	-

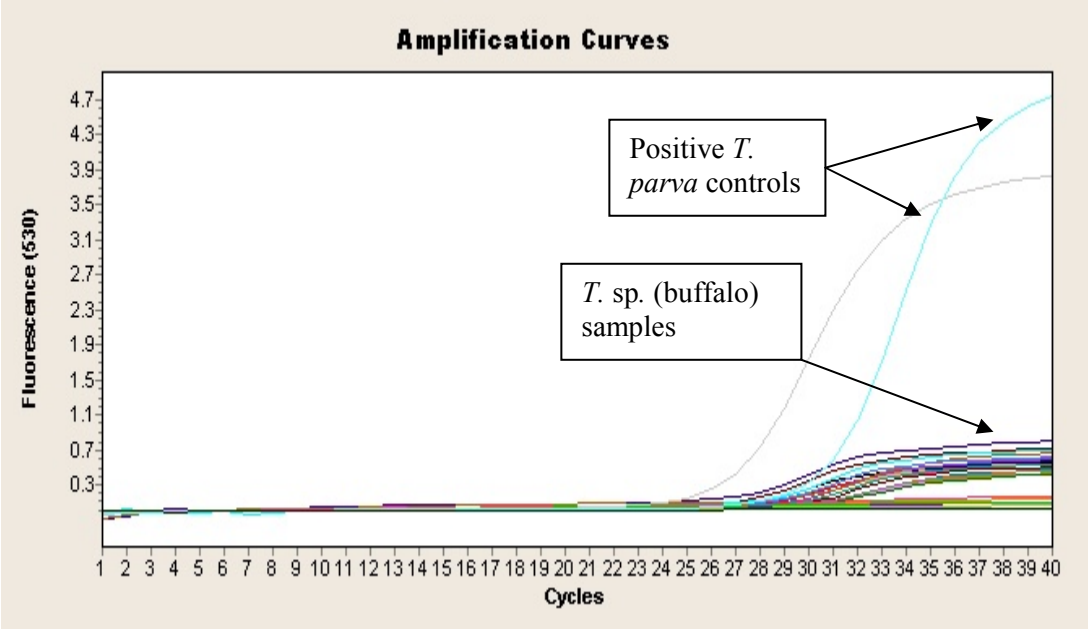


Figure 11: Specificity of TaqMan PCR
Amplification can be observed for the positive *T. parva* control samples and the *T.* sp. (buffalo) samples.

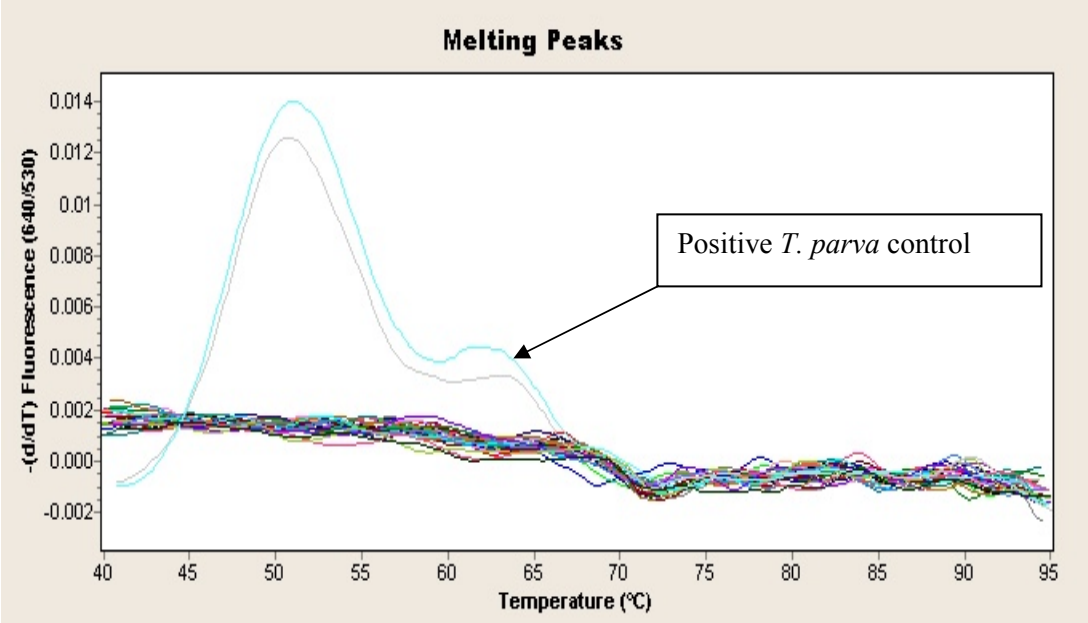
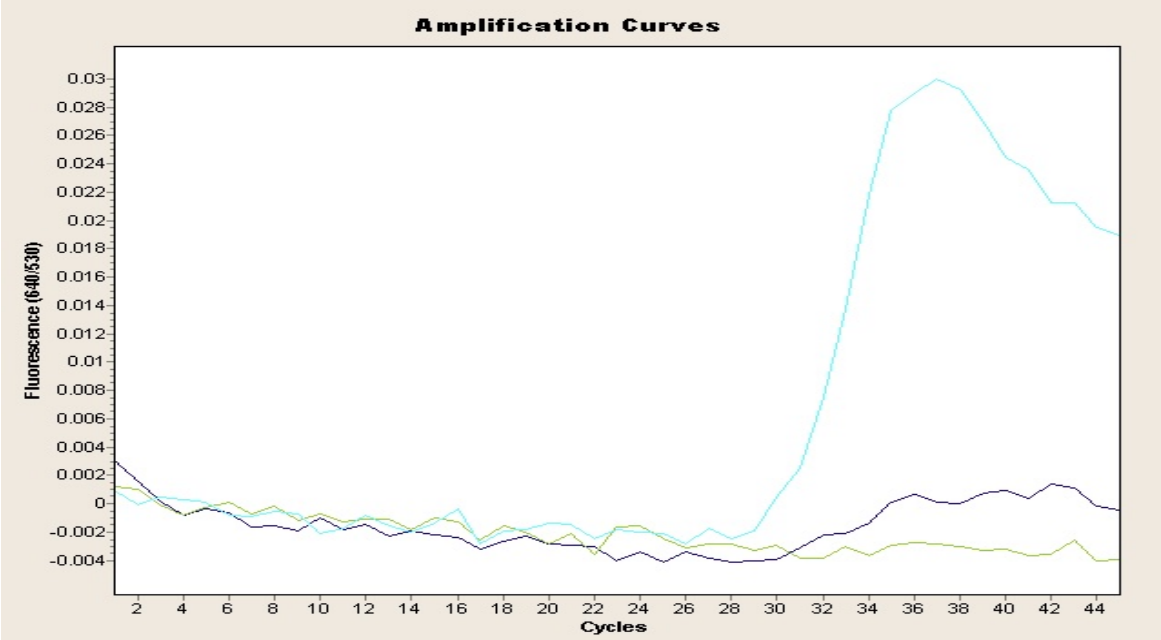


Figure 12: Specificity of BioPAD Hybridization probe PCR.
Melting profiles were observed for the *T. parva* control samples (blue and grey lines).

a)



b)

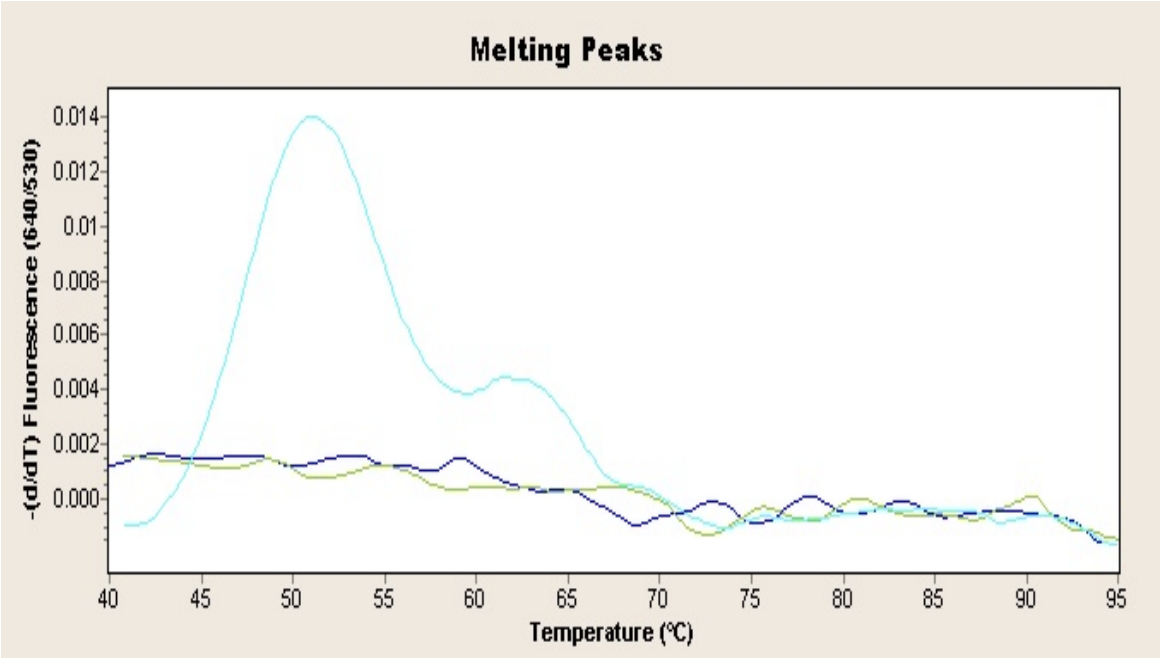


Figure 13: Specificity results using the BioPAD Hybridization probe PCR.
a) *T. parva* (light blue line) and *T.* sp. (buffalo) (dark blue line) experimental DNA samples were amplified using this PCR method. b) Melting curve analysis showed only the *T. parva* melting profile.

4.6 Duration and repeatability of the TaqMan assay

This assay is rapid, taking slightly more than an hour for completion of PCR and detection of target DNA.Only the two gold standard positive controls extracted daily and tested over a period of ten days were detected on all occasions (Table 7) whilst the negative control constantly tested negatively.

Table 7: Test results of 3 animals (2 positive and one negative) over ten days using the TaqMan PCR

Runs	9433	9446	9441	Total
1	+	+	-	2
2	+	+	-	2
3	+	+	-	2
4	+	+	-	2
5	+	+	-	2
6	+	+	-	2
7	+	+	-	2
8	+	+	-	2
9	+	+	-	2
10	+	+	-	2
Total	10	10	0	20

4.7 Comparison of TaqMan PCR to Conventional 989/990 PCR/probe

Since the TaqMan PCR assay was specifically developed to replace the conventional PCR/probe assay, a total of 577 buffalo and cattle samples from various areas in South Africa as described in comparing field sensitivity of the TaqMan PCR and the conventional 989/990 PCR/Probe in Chapter 3 were subjected to both the TaqMan and 989/990 conventional PCR. The 989/990 conventional PCR detected *T. parva* in 4 of the 577 samples (sensitivity in relation to TaqMan PCR was 44%). The 4 samples detected as positive for *T. parva* by the conventional PCR were also detected positive by the TaqMan PCR however, a further 5 samples were also detected as positive by the TaqMan PCR. The observed agreement between the tests was 0.99 and the kappa value which is of importance was 0.55, which indicates that there was a moderate agreement between the two tests.

CHAPTER 5: DISCUSSION

The buffalo industry in South Africa is a highly lucrative business and the demand for disease free buffalo by commercial game farms and nature reserves is growing rapidly. The continuous sale and movement of buffalo across the country and the increasing number of game farms neighbouring cattle farms presents many problems. Corridor disease is a major problem for buffalo breeders and cattle farmers in South Africa and becomes very costly when outbreaks occur therefore control of buffalo movement is necessary. Because the disease is fatal in cattle, control relies upon rapid and sensitive diagnostic tests that can detect the parasite early. Current molecular diagnostic methods based on conventional 989/990 PCR/probe are laborious, time consuming and could pose as a health hazard as it necessitates the use of radioactive probes in order to improve sensitivity.

This work describes the development and evaluation of a real-time TaqMan PCR assay for the detection of *T. parva* in buffalo and cattle. No other TaqMan probe based real-time PCR assay for the detection of *T. parva* has been reported previously. The procedure does not require any post-amplification handling such as lengthy electrophoresis and hybridization to visualize the results thereby reducing significantly the risk of contamination of samples by PCR products, reducing the turn around time of the test and eliminates the need for radioactive probes.

The primers and TaqMan probe were designed and the TaqMan PCR cycling conditions were optimised. There is a clear distinction between a positive and a negative result in terms of fluorescence with a positive sample showing a sigmoid curve and a negative sample showing a flat line. PCR and detection occurs simultaneously and is rapid taking less than two hours to test thirty two samples. In order to evaluate the TaqMan test, the diagnostic and analytical aspects of the test were determined and the results were compared to those of conventional PCR and Hybridization probe PCR.

Early detection of *T. parva* in field animals is essential in order to prevent the spread of Corridor disease and an epidemic. The diagnostic sensitivity of the 3

PCR assays was determined using 45 positive samples. The TaqMan PCR was able to detect 87% of the positive samples whilst the Hybridization probe PCR and the conventional PCR assays were able to detect 100% and 84% of the positive samples respectively. The Hybridization probe PCR appears to be diagnostically more sensitive than the TaqMan probe PCR.

A common concern of using PCR as a diagnostic method is the possibility of a false positive result (Andrew *et al*., 2005). A total of 89 negative samples were tested using the 3 PCR tests and the diagnostic specificity of all 3 assays was found to be 100%.

At a $2x10^{-3}$% parasitaemia both the TaqMan PCR and the Hybridization probe PCR were able to detect *T. parva* DNA with a 100% certainty. The TaqMan PCR could detect *T. parva* DNA at a $2x10^{-4}$% parasitaemia with 100% certainty and at a $2x10^{-5}$% parasitaemia with 93% certainty which was equivalent to 3.5 parasites per reaction. This was comparable to the Hybridization probe PCR and the conventional PCR which could detect $2x10^{-5}$% parasitaemia with 100% and 93.3 certainty respectively. At $2x10^{-6}$% parasitaemia and lower, no amplification was observed for the TaqMan PCR assay however the Hybridization probe PCR and the conventional PCR were still able to detect some *T. parva* DNA. This cannot be regarded as the detection limits of these tests as the accuracy is compromised at these low levels. Parasite DNA detection at $2x10^{-7}$% and $2x10^{-9}$% was still possible to a much lesser degree with the Hybridization probe PCR. The TaqMan PCR results were consistent but the conventional PCR results appear to be inconsistent in this study. This inconsistency could be due to the need for post-amplification processing and the use of smaller volumes of amplicon for hybridization to the specific probe. The Hybridization probe PCR is analytically more sensitive for the detection of *T. parva* DNA than the TaqMan or the conventional PCR.

In the analytical specificity test, the TaqMan PCR assay for *T. parva* could also detect the *T.* sp. (buffalo) positive sample. This was unforeseen since there is a two base pair difference between *T. parva* and *T.* sp. (buffalo) in the region where the probe hybridizes to the target DNA. Previously TaqMan probes were

found to be able to distinguish between one base pair differences. It is possible that since *T. parva* is so closely related to *T.* sp. (buffalo), the strength of the signal obtained for *T. parva* and *T.* sp. (buffalo) in this assay could be correlated with the degree of match between the probe and the target, and with the position of the mismatches (McGoldrick *et al*., 1998) hence the *T* sp. (buffalo) samples showed amplifications of lower strength than the *T. parva* samples.

In this work the conventional PCR assay for detection of *T. parva* was found to detect parasite DNA in samples containing mixed infections of *T. taurotragi*, *T. mutans* and *T. buffeli,* but did not cross react with the other unrelated organisms tested. It is possible that this phenomenon is due to the presence of *T. mutans* in these particular samples with which the *T. parva* specific probe is cross reacting. It is not understood as to why the probe was able to detect *T. mutans* as well instead of *T. parva* only. The conventional PCR did not detect the *T.* sp. (buffalo) positive sample. Allsopp *et al*. (1993) found slight cross hybridization between the *Theileria* sp. (buffalo) probe and both *T. parva* and *T. taurotragi* but no cross hybridization between the *T. parva* probe and the other *Theileria* species. However in this study pure *T. taurotragi* and *T. buffeli* DNA samples were not used but the samples were mixed infections of *T. taurotragi* and *T. mutans* or *T. buffeli* and *T. mutans*.

The Hybridization probe PCR showed amplification for *T.* sp (buffalo). Upon closer observation it appears that the amplification curves for *T.* sp. (buffalo) are similar on both the TaqMan probe PCR and the Hybridization probe PCR. However, no melting curve is observed for *T.* sp. (buffalo) in the Hybridization probe PCR and this is used to differentiate between *T. parva* and *T.* sp. (buffalo) positive samples (Sibeko pers. comm., 2007). A previous study comparing SYBR Green, hydrolysis probe and Hybridization probe assays for real-time detection of *Brucella abortus* also found that the greatest specificity was achieved with the Hybridization probe assay (Newby *et al*., 2003).

The TaqMan test was applied to field samples received from the various areas in South Africa as described earlier in Chapter 3 and the results were compared with those from the conventional PCR. The TaqMan PCR was able to detect 5

more positive samples than the conventional PCR. The kappa coefficient of 0.55 suggests that there is a moderate agreement between the TaqMan test and the conventional PCR test.

The Hybridization probe PCR appears to be a more suitable test for the detection of *T. parva* in South Africa with its increased sensitivity and specificity especially when the accurate determination of the carrier animals is essential. Since the Hybridization probe assay still amplifies *T*. sp. (buffalo) but is able to differentiate between *T*. sp. (buffalo) and *T. parva* by melting curve analysis (Sibeko pers. comm., 2007), it is possible that a false negative result could be obtained should *T. parva* be greatly underrepresented in the presence of double infection with *T. parva* and *T*. sp. (buffalo).

In conclusion this work has demonstrated that the overall sensitivity and specificity of the TaqMan PCR assay appears to be better than the conventional PCR method and is comparable to the other real-time PCR chemistries such as the Hybridization probe PCR. This assay shows good repeatability and provides minimal sample manipulation hence the possibility of contamination is prevented. The TaqMan assay is rapid taking less than 2 hours from the extracted DNA to analysis of PCR results and is useful for the detection of *T. parva* with a detection limit as low as $2x10^{-5}$% parasitaemia. The real-time TaqMan PCR in comparison with the conventional PCR assay has a shorter turnaround time, allows for high throughput as there are approximately 4000 samples per year being sent to the OVI for Corridor disease testing and the use of radioactivity is avoided. The TaqMan PCR assay is simpler to interpret and more inexpensive for routine diagnostics than the Hybridization probe PCR assay. Thus future work is required to increase the specificity of the TaqMan PCR assay by designing more specific primers and probe to eliminate the amplification of *T.* sp. (buffalo) and to increase the sensitivity of the test.

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APPENDICES

APPENDIX 1

1. Calculation of parasitaemia

1 parasite in 50 000
= 0.002% parasitaemia

2. Preparation of buffers and chemical reagents

10 X TBE

Tris base	216 g
Boric Acid	110 g
$Na_2EDTA..2H20$	18.6 g

Make up to 2 litres with water.

1 M NaH_2PO_4

NaH_2PO_4	120 g

Make up to 1 litre with water.

1 M Na_2HPO_4

Na_2HPO_4	142 g

Make up to 1 litre with water.

1 M NaPi (pH 7.5)

1M NaH_2PO_4	16 ml
1M Na_2HPO_4	84 ml

Make up to 1 litre with water.

20X SSC

NaCl	175 g
Sodium citrate	88.2 g

Make up to 1 litre with water.

6X SSC

20X SSC	300 ml

Make up to 1 litre with water.

4X SSC

20X SSC	200 ml

Make up to 1 litre with water.

10 M NH_4OAc

NH_4OAc	77.08 g

Make up to 100 ml with water.

0.5 M EDTA

EDTA	93.06 g

Make up to 500 ml with water.

Gel loading buffer

Bromophenol Blue	0.25 g
Xylene cyanol FF	0.25 g
Ficoll (type 400)	15 g

Make up to 100ml with deionised water.

Gel

Agarose	1.8 g

Make up to in 180 ml with TBE (1X)

TE

1M Tris (pH 8.0, 7.6 or 7.4)	10 ml
0.5M Na2EDTA (pH 8)	2 ml

Make up to 1 litre with water.

14% SDS

SDS	14 g

Make up to 100 ml with water

6 M NaOH

NaOH	240 g

Make up to 1 litre with water.

Ethidium bromide

EtBr	1 g

Make up to 100 ml with water.

APPENDIX 3: Calculation of diagnostic sensitivity and specificity of the three tests using the 2 x 2 contingency table

1. TaqMan PCR

	True status		
Test status	**Diseased**	**Not diseased**	**Total**
Diseased	39	0	39
Not Diseased	6	89	95
Total	45	89	134

Sensitivity = 39/ (39+6) x 100
= 87%
Specificity = 89/ (89+0) x100
= 100%

2. 989/990 PCR/probe

	True status		
Test status	**Diseased**	**Not diseased**	**Total**
Diseased	38	0	38
Not Diseased	7	89	96
Total	45	89	134

Sensitivity = 38/ (38+7) x 100
= 84%
Specificity = 89/ (89+0) x100
= 100%

3. Hybridization PCR

	True status		
Test status	**Diseased**	**Not diseased**	**Total**
Diseased	45	0	45
Not Diseased	0	89	89
Total	45	89	134

Sensitivity = 45/ (45+0) x 100
= 100%
Specificity = 89/ (89+0) x100
= 100%

APPENDIX 4: Calculation of Kappa coefficient between the TaqMan and the Conventional (Conv.) PCR

		TaqMan PCR		
989/990		**Positive**	**Negative**	**Total**
Conventional	**Positive**	4	0	4
PCR	**Negative**	5	568	573
	Total	9	568	577

Sensitivity of 989/990 Conv. PCR in relation to the TaqMan PCR= 44
Specificity = 100
Observed agreement = 0.99
Expected agreement = 0.978
Kappa = (Observed agreement - Expected agreement)
(1- Expected agreement)
= 0.55

Kappa	Interpretation
> 0.81	Almost Perfect Agreement
0.61 - 0.80	Substantial Agreement
0.41 - 0.60	Moderate Agreement
0.21 - 0.40	Fair Agreement
0.01 - 0.20	Slight Agreement
0	Poor Agreement