DEVELOPMENT OF SPECIES-SPECIFIC POLYMERASE CHAIN REACTION (PCR), REAL-TIME PCR AND LOOP MEDIATED ISOTHERMAL AMPLIFICATION (LAMP) ASSAYS FOR DETECTION OF *ANAPLASMA MARGINALE* STRAINS IN SOUTH AFRICA

By

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DECLARATION

I the undersigned hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree. I further cede copyright of the dissertation in favour of the University of the Free State

Signature 

Date 31/05/2013

DEDICATION

To my parents and siblings who understood the value of studying. To my fiancé for his sustained support at all times. To my unborn baby who kept his calm spirit and accepted that mom is a hard worker.

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LIST OF ABBREVIATIONS

°C	Degree(s) Celsius
µL	microlitres(s)
µM	micromolar(s)
A	Adenine
ABI	Applied Biosystems Inc.
BLAST	Basic local alignment search tool
BLASTn	Basic Local Alignment search tool for nucleotide
Bp	base pair
BSA	Bovine Serum Albumin
Bst	*Bacillus stearothermophilus*
C	Cytosine
cELISA	Competitive Enzyme-linked immunosorbent assay
C_T	Cycle threshold
DDW	double distilled water
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribose Nucleic Acid
DNTP's	Deoxy-nucleotide Triphosphate
EDTA	Ethylenediamine tetra-acetic acid
FRET	Forester Resonance Energy
G	Guanine
HPLC	High Performance liquid Chromatograph
IDT	Integrated DNA Technologies
IFA	Indirect immunoflourescent antibody
LAMP	Loop-mediated Isothermal Amplification
MGB	Minor Groove Binder
min	minute(s)
mM	millimolar(s)
MSP	Major Surface Protein
NCBI	National Center for Biotechnology Information
ng	nanograms(s)
ng/µl	nanogram(s) per microlitre

nm	nanometer(s)
PCR	Polymerase Chain Reaction
pg	picogram
pmol	picamole(s)
qPCR	real-time Polymerase Chain Reaction
Rn	normalized reporter
RNA	Ribonucleic Acid
sec	second(s)
T	Thymine
Taq	*Thermus aquaticus*
UV	Ultraviolet

ABSTRACT

Anaplasma marginale is a virulent intra-erythrocytic pathogen that causes bovine anaplasmosis, its closely related species, *Anaplasma centrale* causes mild sickness. The pathogen is transmitted biologically by tick vectors and mechanically through blood contaminated fomites. It has a worldwide distribution extending from tropical to subtropical regions in correlation with the vector distribution. Bovine anaplasmosis is often characterised by progressive anaemia, jaundice, decreased milk production, abortion and a sudden death. The commonly used method for the diagnosis of *A. marginale* of infected cattle in South Africa is microscopic examination of Giemsa stained blood smears and detection of antibodies from serum using cELISA. However the diagnostic methods have limitations in cases of low parasitemia and in carrier cattle (microscopy) and they fail to differentiate closely related *Anaplasma spp* due to antigenic similarity (serology), the detection limitations of the diagnostic methods influenced the aim of this study which is to develop molecular species -specific assays for the detection of *A. marginale* strains in South Africa, specifically Including conventional polymerase chains reaction, real-time polymerase chain reaction and loop-mediated isothermal amplification assay.

Chapter one of this study discusses bovine anaplasmosis and its causative agent *A. marginale*, the diversity of the strains transmission, distribution, clinical signs, treatment and economic importance of the disease.

The first objective of this study was to develop a species specific conventional PCR for detection of *A. marginale* in cattle in South African regions based on *msp1b* gene. The conventional PCR primers were designed through visual inspection and were named F3 and B3 primers. In the specificity test, the primers were specific whereby the amplified only *A. marginale* DNA and did not amplify control DNA's: *A. centrale*, *Babesia bovis*, *B. bigemina* and *Ehrlihia rumunantium.* The sensitivity of the conventional PCR primers was examined using a 10 ng/ul DNA and the detection limit of the assay was 0.01 ng/ul, The assay was validated on field samples to confirm the infection of the cattle with *A. marginale,* out of 144 samples, (60%) infection rate was obtained with the newly developed conventional PCR, the homogeneity of the sequences were confirmed with the GenBank, the maximum similarity varied from 94 - 100%.

The second objective of this study was to develop a species-specific real-time PCR for detection of *A. marginale* in cattle in South African regions based on *msp1b* gene. The real-time PCR primers and probes were designed using Genescript program, one set of primer (Prf 2, PrR2, and PrB2) was chosen to carry out the study as it showed high sensitivity with the detection limit of 0.001 ng/ul .The specific and sensitive TaqMan based real-time PCR was successfully developed for the of *A. marginale* infections in South Africa. Validation of the assay on field samples showed that the rate of infection was 74% in different sampled provinces of South Africa.

The third objective of this study was to develop loop-mediated isothermal amplification for the detection of *A. marginale* in South African regions based on *msp1b* gene. The LAMP primers were designed using primer Explorer version 4, the LAMP primers were named LA-F3, LA-B3, LA-FIP, LA-BIP,LA-LF and LA-LB. The LAMP assay showed positive results with specific amplification, but as far as the validation of the assay false positive results were obtained, troubleshooting involved the addition of additives, changing of primer purification and manufacturers, however the results were not consistent, false positive results were obtained, speculations were that it could be possible contamination of the laboratory resulting in the amplification of control DNA and distilled water.

The first three objectives of this study were achieved. The newly developed assays were further compared for specificity, sensitivity and detection performance on field derived samples. The developed assays are specific and sensitive; they form a good tool of diagnosis of bovine anaplasmosis, with each assay having its own unique characteristic over the other, they are sensitive giving a correct determination of the infection status, aiding in compiling of epidemiological information. These assays will aid in understanding the major constraint to develop control measures due to the genetic diversity of *A. marginale,* and will also help in constructing of phylogenetic tree between strains from South Africa and other countries.

RESEARCH OUTPUTS

The work written here has been presented by the author in the following international conference and local symposium.

- Khumalo Z.T.H, Mtshali M.S and Thekisoe O.M.M. Development of a species-specific conventional PCR, real-time PCR and LAMP for detection of *A. marginale* infections in South Africa. The 41st PARSA conference. University of the Free State, Bloemfontein Campus, October 2012.

- Khumalo Z.T.H, Mtshali M.S and Thekisoe O.M.M. Development of a species-specific conventional PCR, real-time PCR and LAMP for detection of *A. marginale* infections in South Africa. University of the Free State, Bloemfontein Campus. The Zoology Department Seminar, Faculty of Zoology and Entomology, November 2012.

- Khumalo Z.T.H, Mtshali M.SS and Thekisoe O.M.M. Development of species-specific conventional PCR, real-time PCR and LAMP for detection of *A. marginale* infections in South Africa. The 3rd research symposium of the National Zoological Gardens of South Africa, Pretoria, November 2012.

CHAPTER 1

1. INTRODUCTION AND LITERATURE REVIEW

1.1.1 Preamble

Bovine anaplasmosis also known as gall sickness is an infectious, non-contagious tick-borne disease caused by the intraerythrocytic rickettsial pathogen *Anaplasma marginale* (Theiler 1910). It is of economic importance as it is a major constraint to agricultural development (Kocan *et al.*, 2003). Ticks are generally the biological vectors of *A. marginale*, and five tick species are known as vectors of *A. marginale* in South Africa namely, *Rhiphecephalus decoloratus*, *R. microplus*, *R. evertsi evertsi*, *R. simus* and *Hyalomma marginatum rufipes* (Kocan et al., 2004: Mtshali *et al.,* 2007), however the pathogen is often transmitted mechanically to susceptible vertebrate hosts by blood contaminated mouthparts of biting flies or fomites (Kocan *et al.*, 2003; Kocan *et al.*, 2004). *Anaplasma centrale* is also known to infect cattle but causes mild clinical disease and is used as a live vaccine against the virulent *A. marginale* (Theiler 1911, Kocan *et al.*, 2003; Shkap *et al.*, 2002; Mtshali *et al.*, 2007). Six major surface proteins (MSPs) of *A. marginale* are recognized to date namely; MSP1a, MSP1b, MSP2, MSP3, MSP4 and MSP5 (Kocan *et al.*, 2001; de la Fuente *et al.*, 2007). Previous studies have focused mainly on MSP1 as it is primarily involved in adhesion to bovine erythrocytes and tick cells, and as a result it has been used widely as a stable genetic marker for identification of *A. marginale* geographical isolates (Kocan *et al.*, 2001; de la Fuente *et al.*, 2007; Rodriguez *et al.*, 2009)

1.1.2 Classification

Anaplasma belongs to the Order Rickettsiales which has recently been re-organized into two families: Anaplasmataceae and Rickettsiaceae based on 16S rRNA, groELS and major surface protein on genetic analyses (Sergio *et al.*, 2009). *A. marginale* falls within the Anaplasmataceae family which is characterized by obligate intracellular organisms found only within membrane bound vacuoles in the host cell cytoplasm (Kocan *et al.*, 2003).

1.1.3 *Anaplasma marginale* strains

Anaplasma marginale geographic strains have been identified worldwide, they differ in morphology, rate of infection, antigenic characteristics and protein sequence (de la Fuente *et al.*, 2007; Kocan *et al.*, 2010; Aubry *et al.*, 2010). The increasing number of *A. marginale* genotypes identified in geographic regions resulted from intensive cattle movement and high level of transmission events (de la Fuente *et al.,* 2007; Molad *et al.*, 2009). The major surface proteins play a major role in interaction of *A. marginale* with host cells, major surface proteins, MSP1ba, MSP4 and MSP5 are from single gene and do not vary antigenically, while MSP1b, MSP2 and MSP3 are from multigene families and they vary antigenically, mostly with persistently infected cattle (Kocan *et al.*, 2003; de la Fuente *et al.*, 2007; Mtshali *et al.*, 2007; Corona *et al.*, 2009; Aubry *et al.*, 2010;). The MSP1a of *A. marginale* geographic strains differs in molecular weight because of variable number of tandem repeats, and is used for identification of geographic strains (de la Fuente *et al.*, 2007; de la Fuente *et al.*, 2009).

A study by Molad *et al* (2009) showed the existence of a co-infection in a herd with two different *A. marginale* strains that carried two distinct genotypes. Rodrigues *et al.* (2009) explained that this phenomenon result from cases where there is a presence of different MSP2 pseudogenes in two field strains of *A. marginale*. In South Africa only one study has been conducted to genetically characterize the geographical strains of *A. marginale* (Mtshali *et al.,* 2007). The results presented by the latter authors indicated the presence of common genotypes between South African, American and European strains of *A. marginale.* However the study focused only on blood samples of cattle collected from Free State Province, so there is a need of further studying the prevalence and genetic diversity of *A. marginale* strains in cattle in other South African regions in order to design epidemiological and control strategies for *A. marginale.*

1.1.4 Distribution

De Waal (2000) pointed that anaplasmosis distribution is widespread in South Africa with more than 99% of cattle population at risk; hence, the disease has worldwide distribution (Mtshali *et al.*, 2007; Ndou *et al.*, 2010) in tropical and subtropical areas. It is reported that the current distribution will continue to change due to global warming trends (Kocan *et al.*, 2004; Kocan *et al.*, 2010). Anaplasmosis is endemic in almost every part of the world and is

transmitted by two transmission modes (biological and mechanical). In contrast, areas where tick vectors have been eradicated, mechanical transmission has become an alternative form of transmission and broadened distribution is effected by movement of cattle (Kocan *et al.,* 2003; Kocan *et al.*, 2010). In South Africa few studies have been undertaken for serological evidence of anaplasmosis endemicity, in the Free State, Limpopo and North West Provinces (Dreyer *et al.*, 1998, Rikhotso *et al.*, 2005 and Ndou *et al.*, 2010).

1.1.5 Transmission and epidemiology

Anaplasma marginale is transmitted mechanically through blood contaminated objects and by blood sucking dipterans and different mosquito species (Kocan *et al.*, 2003, Kocan *et al.*, 2004; Kocan *et al.*, 2010). Biological transmission is effected by a tick vector, it can also be transmitted from cow to calf during gestation period (Kocan *et al.*, 2003), and the transmission can occur from stage to stage (interstadial) or within a stage (intrastadial). Successful infection does not vary with age, but severity of anaplasmosis increases with age (Aubry *et al.*, 2010). Clinical signs are prominent in cattle than other ruminants. Red blood cells were thought to be the only known site of development of *A. marginale* but recently, Rodriguez *et al.*, (2009) reported that *A. marginale* also replicates in endothelial cells, although more information is lacking about this phenomenon.

Developmental cycle of *Anaplasma* is complex and associated with the tick feeding cycle, whereby ticks ingest infected erythrocytes during a blood meal from the vertebrate host, providing a source *of A. marginale* infection for tick gut cells, when feeding for the second time many tissues become infected and the final developmental stage takes place in the salivary glands, where the rickettsia is transmitted to the vertebrate host and is infective (Kocan *et al.,* 2010), for each site of infection in ticks, *A. marginale* develops within membrane-bound colonies, the form in a colony is reticulated form, which divides by binary fission forming large colonies that may contain hundreds of organisms, the reticulated form changes into dense form, which is infective and can survive outside the host cells. Cattle are infected with *A. marginale* when the dense form is transmitted during tick feeding through the salivary glands (Kocan *et al.*, 2003, Kocan *et al.*, 2004).

Calves are much more resistant to disease infection than older cattle. This resistance is not due to colostral antibody from immune dams. In endemic areas where cattle are infected with *A. marginale* early in life, losses due to anaplasmosis are minimal. After recovery from the acute phase of infection, cattle remain chronically infected carriers but are generally immune to further clinical disease. However, these chronically infected cattle may relapse to anaplasmosis when immunosuppressed, when infected with other pathogens, or after splenectomy. Carrier cattle serve as a reservoir for further transmission. Serious losses occur when mature cattle with no previous exposure are moved into endemic areas or when under endemically unstable situations when transmission rates are insufficient to ensure all cattle are infected before reaching the more susceptible adult age (Kocan *et al.*, 2003, Kocan *et al.*, 2004).

1.1.6 Clinical signs

The first clinical sign of anaplasmosis is pyrexia which occurs after infection of 1% of red blood cells, fever persists as the level of parasitemia increases and the most remarkable sign is anaemia through removal of infected red blood cells perpetuating to clinical signs due to lack of red blood cells, further signs include, weakness, low milk production, development of infertility and as a result death may occur (De Waal, 2000; Kocan *et al.*, 2010). Cattle that survive the infection remain persistently infected and they serve as reservoirs for continuous transmission of *A. marginale* (Kocan *et al.*, 2003). *Bos indicus* breeds of cattle appear to possess a greater resistance to *A. marginale* infection than *B. taurus* breeds, but variation of resistance of individuals within breeds of both species occurs. Difference in virulence between *Anaplasma* strains and the level and duration of the rickettsemia also play a role in the severity of clinical manifestations (De Waal, 2000).

1.1.7 Diagnosis

Diagnosis of anaplasmosis is usually performed at a time when clinical signs are most pronounced. The most commonly used method for diagnosis of cattle infected with *Anaplasma* is microscopic examination. However it is problematic and require careful examination as the level of parasitemia may be very low due to removal of most infected erythrocytes from circulation (De Waal, 2000), which is a serious problem in case of carrier cattle (Fyumagwa *et al.,* 2009, Vahid *et al.,* 2009). Serological tests, such as competitive

enzyme-linked immunosorbent assay and indirect immunoflourescent antibody test are advantageous in large scale epidemiological surveys, but their ability to detect antibodies of pathogen both during infection and after the pathogen has been cleared from the cattle host, makes it difficult to draw a conclusion on current state of infection. The cELISA has three main limitations in detection of the *Anaplasma* infection, low sensitivity for the detection of early infection. Cross reactivity with other *Anaplasma* species and insufficient specificity for identifying true negative cattle at time of chemosterilization.

Polymerase chain reaction is the most commonly used method for diagnosis of *A. marginale* infections (Lew *et al.*, 2002, Shkap *et al.,* 2002). Most of the PCR assays have been targeting the *msp4* and *msp1α* genes for differentiating strains of *A. marginale*, which is a useful method for tracking the origin of the outbreak (lew *et al.*, 2002, Mtshali *et al.*, 2007, de la Fuente *et al.*, 2007). PCR has been shown to reliably detect *Anaplasma* at the lowest levels of persistent rickettsemia (Lew *et al.*, 2002, Shkap *et al.,* 2002), however other studies have shown that PCR fails to detect the infection of *A. marginale* at low levels or during the early stages of infection (Molad *et al.*, 2009).

Real-time PCR based on *msp1b* gene was successfully developed for detection and quantification of *A. marginale* DNA in blood of naturally infected cattle (Carelli *et al.*, 2007), The assay was shown to be distinctively sensitive and specific as there were no cross-reactions with other haemoparasites of ruminants (*A. centrale*, *A. bovis*, *A. phagocytophilum*, *Babesia bigemina* and *Theleria buffeli*).

1.1.8 Treatment, prevention and control

Control measures for anaplasmosis vary with different geographic location, including maintenance of anaplasmosis free herds, control of tick vectors, administration of antibiotics and vaccination (De Waal, 2000; Aubry *et al.*, 2010). Dairy and beef cattle farmers have relied on dipping for control of tick infestation, however in areas where tick vectors are well established, the exposure to ticks gives high degree of immunity against anaplasmosis (De Waal, 2000). Prophylaxis has been through administration of antibiotics. Chemotherapy is intended for prevention of clinical anaplasmosis but it does not prevent cattle from becoming persistently infected with *A. marginale*, as a result cattle receiving antibiotics therapy may not be cleared of infection. Tetracycline administration is accompanied by

disadvantages of expenses, and the demand of continuous feeding and also the risk of development of resistant *Anaplasma* organisms, although the resistance of *A. marginale* to antibiotics has not been reported. Live vaccine was introduced by Sir Arnold Theiler has been used widely in South Africa but the vaccination renders partial protection against *A. marginale* and the success vary according to genotypes of *A. marginale* (De Waal, 2000).

1.1.9 Economic impact

Anaplasmosis is of economic importance, as it is associated with significant losses related to impaired production, mortalities and control measures (Ndou *et al.*, 2010; Mbati *et al.*, 2002). The losses due to anaplasmosis are measured through several parameters such as loss of weight, low milk production, abortion and mortality (Kocan *et al.*, 2003).

1.10 OBJECTIVES

1.1.10 Statement of the problem

Anaplasmosis is endemic in South Africa, there are high numbers of carrier cattle which serve as reservoirs for continuous infection. Various control measures have been applied to control anaplasmosis but each seems to have its own complications. Genetic diversity of *A. marginale* strains presents a major barrier in the development of a vaccine that provides full protection against *A. marginale*. The currently used vaccine (of live *A. centrale*) induces protective immunity that only mutes or prevent clinical disease and does not provide cross protection in case of co-infected host with different *A. marginale* strains (Eriks *et al.*,1994; Shkap *et al.*, 2002; Molad *et al.*, 2009).

South Africa is a developing country that depends largely on farming, thus failure to develop successful control methods to prevent persistent infection contributes to the further spread of *A. marginale* and these causes constraints in economic development of the livestock farming sector.

Different PCR assays have been developed for detection of *A. marginale* PCR-RFLP, Nested PCR, Duplex Real time-PCR reverse transcriptase and conventional PCR (Vahid *et al.*, 2010; Molad *et al.*, 2006, Reinbold *et al.*, 2010; Picoloto *et al.*, 2010). Carelli *et al* (2007) developed a real-time PCR assay that was successful in detection and quantification of *A. marginale* in blood of naturally infected cattle, which was highly specific, however Carelli *et al* (2007) utilized samples collected from limited geographic region, not on global scale, which does not give a representative number of *A. marginale* strains. Lew *et al* (2002) used conventional PCR for detection of *A. marginale* strains, however standard PCR has limitations such as lower sensitivity for the detection of early infection and during part of chemosterilization treatment and it fails to quantify *A. marginale* in blood of infected host. It is important therefore to develop more PCR assays with different target genes to alternately end up with desired characters.

Therefore development of new assays to differentiate and quantify DNA of the strains of *A. marginale* is required. Loop-mediated isothermal amplification (LAMP) assay has been recently developed (Mori *et al.*, 2008; Pandey *et al.*,2008; Nakao *et al.*, 2010; Liu *et al.*, 2008) and is characterized by it rapidity, accuracy and cost effectiveness. To date, there

are no reports of LAMP assays that have been developed for detection of *Anaplasma* parasite infections.

The aim of the study is to develop species-specific PCR real-time PCR and LAMP assays for the detection of *A. marginale* strains in South Africa that can detect *A. marginale* strains from different Provinces of South Africa.

1.1.11 Objectives

1.1.11.1 General objective

To develop molecular diagnostic assays (PCR, real-time PCR and LAMP) for detection of *A. marginale* strains infections in South Africa.

1.1.11.2 Specific objectives

- To develop a conventional PCR assay for detection of *A. marginale* infections in South Africa
- To develop a real-time PCR assay for detection of *A. marginale* infections in South Africa
- To develop a LAMP assay for detection of *A. marginale* infections in South Africa
- To validate the newly developed assays (PCR, real-time PCR and LAMP) using field derived samples from all Provinces of South African.

CHAPTER 2

2. DEVELOPMENT OF A SPECIES-SPECIFIC CONVENTIONAL PCR FOR THE DETECTION OF *ANAPLASMA MARGINALE* IN SOUTH AFRICA

2.1.1 Introduction

Polymerase Chain Reaction is a nucleic acid amplification technique that is widely used in molecular biology because of its simplicity. The technique amplifies specific DNA fragments from minute quantities of template DNA material, even when the template DNA is of poor quality (Erlich, 1989). It is used widely in many fields of science and it enables the study of DNA sequences, mutation and the structure. PCR uses two synthetic DNA primers (oligonucleotides) to amplify a unique DNA sequence through the use of thermostable DNA polymerase isolated from organisms such as *Thermus aquaticus* (*Taq*). PCR-based methods have been developed to identify *Anaplasma* infections, however at present serological methods based on major surface proteins remains the most practical means of routine screening large numbers of cattle for evidence of infection (Kocan, 2010). No adequate PCR-based methods have been developed that are capable of detecting low levels of infection in carrier cattle (Aubry *et al.*, 2010).

In order to carry out PCR, different components are required, which include (Erlich, 1989, Burke, 1996);

Template DNA: DNA must be extracted from the specimen of interest, and adequate amounts of template DNA range between 0.1 and 1 µg of genomic DNA.

Primers: PCR primers should be 10 - 24 nucleotides in length and must be specific for gene or DNA sequence to be amplified GC content should range from 40% - 60%, melting temperatures of primer pairs should not differ by more than 5°C.

$MgCl_2$ concentration: The recommended range of $MgCl_2$ concentration is 1 mM, under the standard reaction conditions specified, but can be increased depending on various factors such as template structure and type of enzyme used.

***Taq* DNA polymerase**: Enzyme that withstands high temperatures needed for DNA-strand separation and is responsible for replicating DNA.

dNTPs: The concentration of each dNTP (dATP, dCTP, dGTP, dTTP) in the reaction mixture is usually 200 µM.

The PCR amplification is based on three phases (Burke, 1996).

Denaturation phase: Separation of two strands of DNA by temperatures of around 94 - 98°C, DNA melts and opens into two pieces of single stranded DNA.

Annealing phase: Binding of DNA primer to the separated strands, this occurs at 50 - 65 °C

Extension phase: Elongation of the strands using the DNA primer with heat–stable DNA polymerase e.g. (*Taq*) occurs at 70°C and upward. Primers hybridize to complementary sequence and these fragments are amplified by the DNA polymerase.

The annealing step is repeated over and over again, stimulating the primers to bind to the original sequences and newly synthesized sequences. The enzyme will again extend primer sequences. Cycling of phases result in an exponential increase in the number of copies of the specific target sequence.

2.1.2 Analysis of PCR products

Gel electrophoresis is a widely used technique for the analysis of nucleic acids and proteins. Agarose gel electrophoresis is routinely used for the preparation and analysis of PCR products. Gel electrophoresis separates molecules on the basis of their rate of movement through a gel under the influence of an electrical field. DNA is negatively charged and when placed in an electrical field, DNA will migrate towards the positive pole (anode). An agarose gel is used to show the movement of DNA and separate by size. Within an agarose gel, linear DNA migrates inversely proportional to the log10 of their molecular weight. The PCR product is then visualized by staining of the gel and then subjected under UV light. (Burke, 1996)

2.1.3 Objective of this study

To develop a conventional PCR assay for detection of *A. marginale* strains infections in South Africa

2.2 MATERIALS AND METHODS

2.2.1 PCR primer design

2.2.1.1 Identification of target amplification region on the gene

Primers which were previously designed by Molad *et al.*, 2006, (5'-CCATCCTCGGCCGTATTCCAGCGCA-3') and (5'-CTGCCTTCGCGTCGATTGCTGTGC-3') were used for amplifying a target region within the *msp1b* gene. PCR was performed using DNA samples in (Figure 1) which were previous determined as positive of *A. marginale,* the following conditions were used to perform the reaction, The initial denaturation was at 95°C for 3 minute, amplification cycles following an initial denaturation which consisted of 30 cycles of denaturation at 95°C for 10 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 3o seconds. Final extension was at 72°C for 10 minutes and the final hold at 4 °C as described by Molad *et al* (2006) and the PCR products were sent to Inqaba Biotechnical Industries (Pty) Ltd. (Pretoria, RSA) for DNA sequencing. The sequencing reactions were performed using the BigDye kit, ver3.1, (Applied Biosystems, Johannesburg, South Africa) according to manufacturer's instructions. The labelled fragments were purified using Zymo sequencing clean-up kit (Zymo Research) and subsequently analysed on a 3500xl Genetic Analyser (Applied Biosystems, Johannesburg, South Africa).

The nucleotide sequences were then analysed using BioEdit program (www.mbio.ncsu.edu/bioedit/bioedit.html). A total of 6 sequences (appendix II) were aligned by BioEdit program to find the conserved region within the sequences (Figure 1) sequences. The conserved region was identified and primers were designed (Table 2). The primers were designed based on visual inspection using the IDT (Essential advanced PCR short course, chapter 8); the parameters are shown in (Table 1).The primer orientation is shown in the consensus sequence (Figure 2).

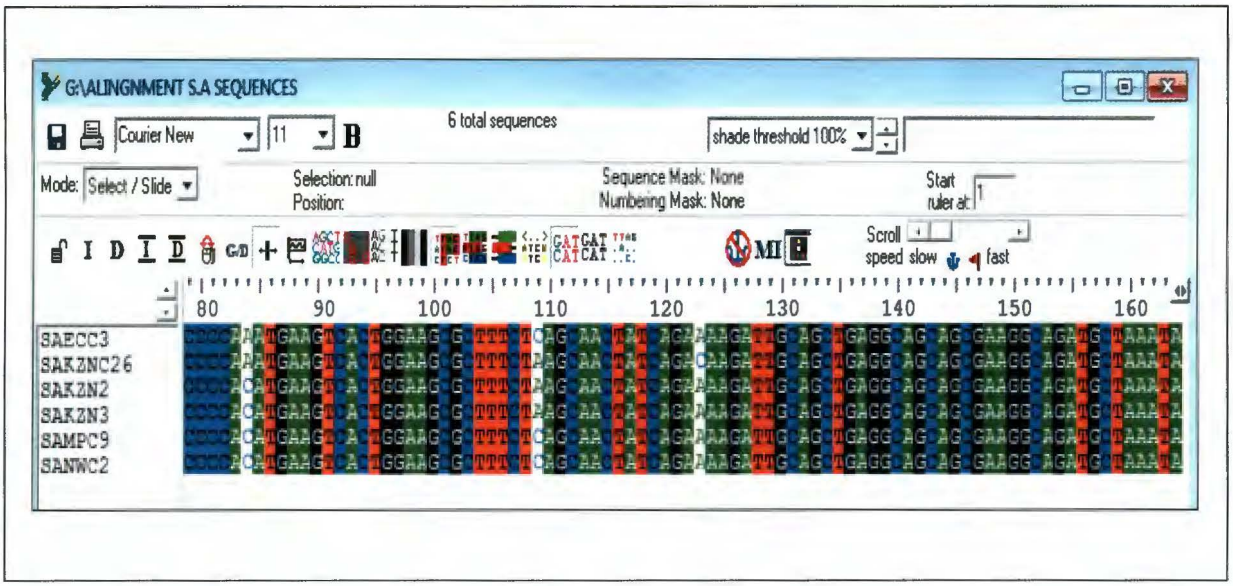


Figure 1: Alignment of six South African sequences using Bioedit program to obtain the consensus sequence for specific primer design.

CATGACTTCATAAAAGGCGCTGATGGTACACTCAAGAACGTCCATCCCCACATGAAGTCACTGGAAGCGCTTTCTAAG

CAACTATCAGAAAAGATTGCAGCTGAGGCAGCAGCGAAGGCAGATGCTAAATACGAGAGCGTGGGACTACGTGCTAAA

GCAGCTGCAGCATTAGGTAATCTCGGGCGGCTTGTCGCTCGTGGTAAACTCACAAGCTCAGATGCACCCAAGAA**CCTT**

GACCAGAGCATTGACAACATACCGTTCATGGATGAAGCACCTGACACTGGTGAGCGGGTTGTTGTGCAGTATGGTGAG

GAGAGAGAATTTGGCAAGGCAGCGGCTTGGGGTCTAGCAGGCTTCAAGCGTACAGTGGATGAAAGCCTGGAGATGTTA

GGCCGAGGCATGAACATGCTCGCGGAAGGCCAGGCACAGAT**ATCACAGGGGATTGCAGACA**AGAGTACTGCACTAGTT

AGGGAAGGTCTGGAAACATCTAGACTTGGTGCAGGGTTATGTCGCAATGGCTTGGTAGAGGCCTCCTACGGCGTTGGT

TATGCCAACAAGACCATGGGCAAGTATGCCGGCAAGGGTCTAGACAAGTGTAAAAACAAACTCGACGATGCATGCCAC

AAGTGGAGCAAGGCTCTCGATGAGATTGAAAGCCTGCGCCACAGCA

Figure 2: The consensus sequence showing primers designed for conventional PCR. The primers were designed based on the *msp1b* gene, forward primer LA-F3 and reverse primer LA-B3 primers are underlined with blue colour and in bold.

Table 1: Primer design checklist of critical parameters (www.DNAbiotec.com)

Parameters	**Details**
Primer length	18 - 25 bases
Melting temperature	55 - 65°C Less than 5°C difference between forward and reverse primer
GC content	45 - 60%
Hairpin formation	3' end hairpin more than -2 kcal.mol-2 Internal hairpin more than -3 kcal.mol-2 Less than four bases annealing
Self-dimer	3' end hairpin more than -5 kcal.mol-2 Internal hairpin more than -6 kcal.mol-2
Cross dimer	3' end hairpin more than -5 kcal.mol-2 Internal hairpin more than -6 kcal.mol-2
3' end sequence	3' end terminates with G or C More than 3 G's or C's should be avoided in the last 5 bases at the 3'end of the primer

Table 2: Specific conventional PCR primers for detection of *A. marginale* infections.

Primer name	**Specific**	**Orientation**	**Sequences (5'- 3')**
LA-F3	*A. Marginale*	Forward primer	CCTTGACCAGAGCATTGACA
LA-B3	*A. Marginale*	Reverse primer	TGTCTGCAATCCCCTGTGAT

2.2.2 Optimization of conventional PCR conditions

Primers: The primers were reconstituted to 100 µMol stock solution as specified by the Inqaba Biotechnical Industries (Pty) Ltd. (Pretoria, SA). Primers were prepared to 100 pmol working concentration.

Annealing temperature: Both primers had different melting temperatures; both primers melting temperature were added and divided by two to give the average annealing temperature which was at 55°C. The assay involved the testing of optimal annealing temperature using gradient from 55 - 65°C.

Buffer: DreamTaq Green PCR Master mix (2x) Inqaba Biotechnical Industries (Pty) Ltd. (Pretoria, RSA), was used for the reaction, supplied with DreamTaq DNA polymerase 2x DreamTaq Green buffer, 0.4 mM of dATP, dCTP, dTTP and 4 mM $MgCl_2$. The master mix is supplemented with two tracking dyes and a density reagent that allows for direct loading of the PCR product on a gel.

Conventional PCR cycles: The PCR conditions included initial denaturation at 95°C for 1 minute, denaturation at 95°C for 30 seconds, annealing at 55-65°C for 1 minute and extension at 72°C for 1 minute. Final extension was at 72°C for 7 minutes and the final hold at 4°C. The cycles of the denaturation and annealing steps were finalized as 40 cycles. The optimal annealing temperature was finalized to be at 55°C.

2.2.3 Conventional PCR assay

2.2.3.1 Optimized conditions for conventional PCR

The LA-F3 and LA-B3 (Table 2) primers were used as forward and reverse primers for conventional PCR. The PCR was performed using DreamTaq Green Master mix (Thermoscientific, Europe). The PCR reaction mixture contained 12.5 µl of DreamTaq Green Master mix, 2 µl primer mix of each 10 µM primers, 5 µl of DNA template and double distilled water was used for adjusting to a final volume of 25 µl. The following conditions were optimal for the PCR assay. PCR mixtures were cycled under the following thermal conditions: the initial denaturation was at 95°C for 1 minute, amplification cycles following an initial denaturation which consisted of 40 cycles of denaturation at 95°C for 30 seconds,

annealing at 55ºC for 1 minute and extension at 72ºC for 1 minute. Final extension was at 72ºC for 7 minutes and the final hold at 4 ºC.

2.2.3.2 Specificity of conventional PCR assay

To determine specificity of the PCR assay 5 µl of DNA template of *A. marginale, A. centrale, B. bigemina, B. bovis*, *Ehrlichia ruminantium* and uninfected bovine blood was added separately to each reaction. PCR was performed using the Thermo cycler (Bio-Rad T100™, Germany), with initial denaturation at 95ºC for 1 minute, amplification cycles followed the initial denaturation which consisted of 40 cycles of denaturation at 95ºC for 30 seconds, annealing at 55ºC for 1 minute and extension at 72ºC for 1 minute. Final extension was at 72ºC for 7 minutes and the final hold at 4 ºC.

2.2.3.3 Sensitivity of conventional PCR assay using genomic DNA

To determine the detection limit or (lowest sensitivity) of the PCR assay, genomic DNA (10 ng/ul) was serially diluted in 6 tubes with the lowest concentration 0.01 pg/ µl. The PCR mixture contained 12.5 µl of DreamTaq Green Master mix, 2 µl primer mix of each 10 µM primers, 5 µl of each serially diluted genomic DNA and double distilled water was used for adjusting the total volume. The PCR reaction mixture was performed using thermo cycler (Bio-Rad T100™, Germany), with initial denaturation at 95ºC for 10 minutes, amplification cycles followed the initial denaturation which consisted of 40 cycles of denaturation at 95ºC for 30 seconds, annealing at 55ºC for 1 minute and extension at 72ºC for 1 minute. Final extension was at 72ºC for 7 minutes and the final hold at 4ºC.

2.2.3.4 Validation of the PCR assay on field samples

Blood samples of cattle were collected from different regions within Provinces of South Africa, namely, KwaZulu-Natal, Free State, Gauteng, Mpumalanga, Limpopo, North West, Eastern Cape, Western Cape and Northern Cape and were used for validation of the developed PCR assay, and the same PCR conditions were used for screening DNA samples for presence of *A. marginale* infections (Table 3).

2.2.3.5 Sequencing of PCR products

To confirm that the amplified PCR products were *A. marginale*, positive PCR products were randomly selected and sent for sequencing at Inqaba Biotechnical Industries (PTY) LTD (Pretoria, RSA). The sequencing reactions were performed using BigDye ver3.1 (Applied biosystems, Johannesburg, S.A) according to manufactures instructions. The labelled fragments were purified using Zymo research sequencing clean-up kit (Zymo Research) and subsequently analysed on a 3500xl Genetic Analyser (ABI, Life Technologies). The obtained nucleotides sequences were then analysed using BioEdit program. The sequences were then analysed using the BLASTn program (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST) to confirm target sequence homogeneity.

2.3 RESULTS

2.3.1 Specificity of the PCR assay

Amplification reaction of *A. marginale*, *A. centrale*, *B. bigemina*, *B. bovis* and *E. ruminantium* were analysed on 1% agarose gel electrophoresis and viewed under the UV light. 100 bp marker was used for examination of the expected amplicon size. Only *A. marginale* DNA was amplified (Figure 3).

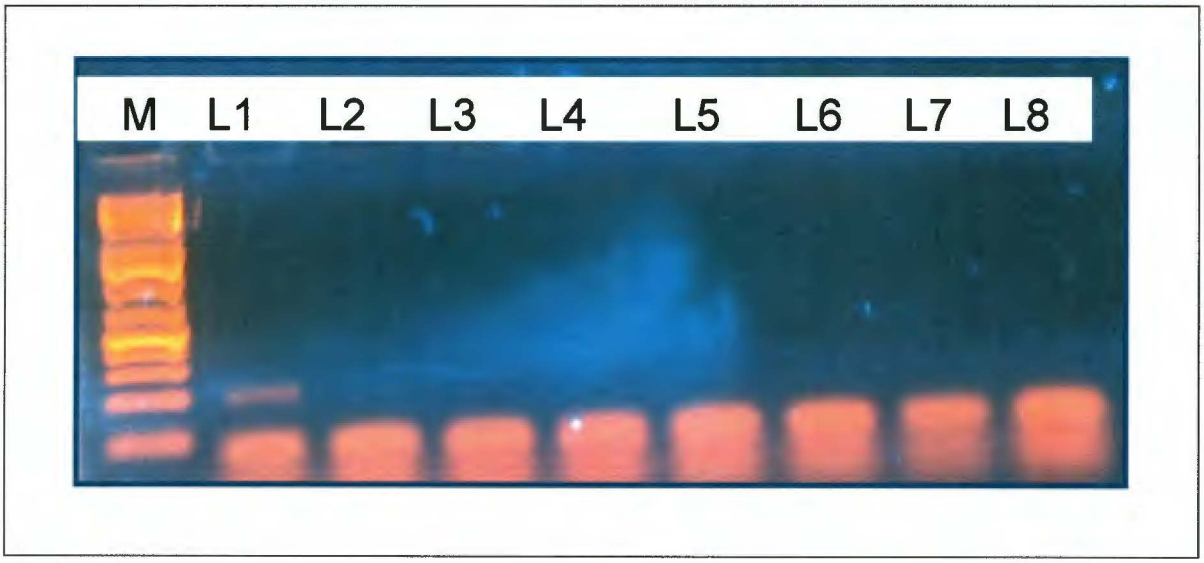


Figure 3: Specificity of a PCR assay using gel electrophoresis. L1:*A. marginale*, L2: *A. centrale*. L3: *B. bigemina*, L4: *B. bovis*, *E. ruminantium*, L5-L8: DDW

2.3.2 Sensitivity of the PCR assay

The PCR products (10-fold serial dilutions) were analysed on 1% agarose gel electrophoresis and viewed under the UV light. The detection limit was at lane 3 (Figure 4).

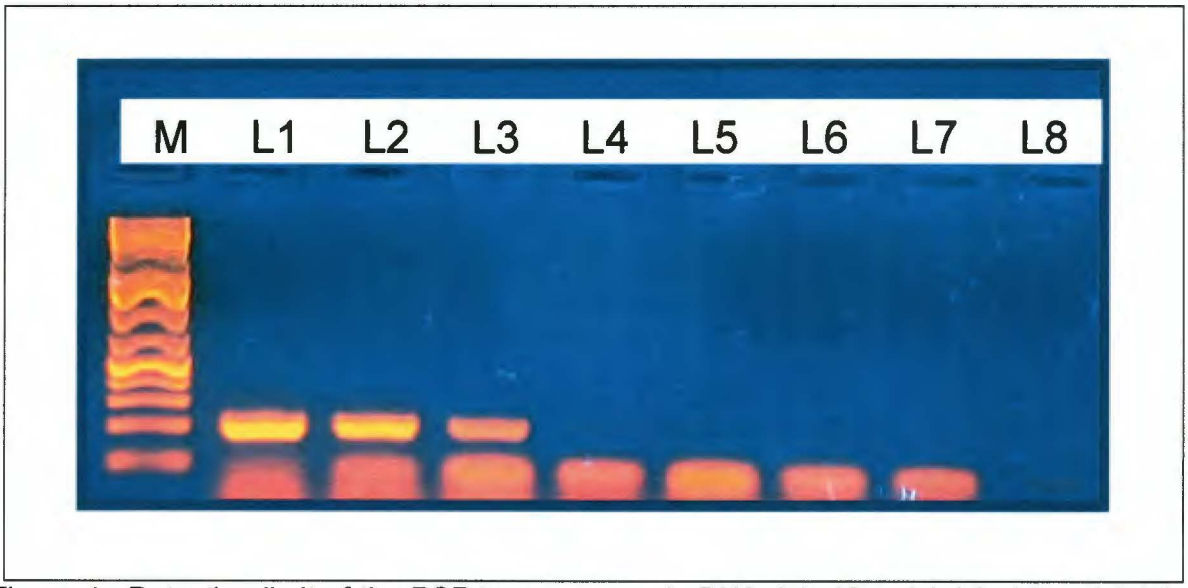


Figure 4: Detection limit of the PCR assay, genomic DNA, L1: 10 ng/µl, L2: 1.0 ng/µl, L3: 0.1 ng/µl, L4: 0.01 ng/µl, L5: 0.001 ng/µl, L6: 0.0001 ng/µl, L7:0.00001 ng/ul and L8: DDW.

2.3.3 Validation of field samples

With exception to samples from N. Cape Province, the newly developed conventional PCR assay amplified DNA samples from 8 provinces with detection performance of 53%, 67%, 69%, 82%, 71%, 83%, 63% and 25% for the EC, FS, GP, KZN, LP, MP, NW and WC respectively as shown in Table 3.

Table 3: Screening of blood samples from different Provinces of South Africa using PCR.

Province	**Number of Samples**	**PCR (Positive)**	**Rate of infection (%)**
Eastern Cape	30	16	53%
Free State	16	12	67%
Gauteng	13	9	69%
KwaZulu-Natal	17	14	82%
Limpopo	14	10	71%
Mpumalanga	18	15	83%
North West	12	8	63%
Northern Cape	16	0	0%
Western Cape	8	2	25%
Total sample	**144**	**86**	**60%**

2.3.4 Sequencing of PCR products

Sequences were analysed using BioEdit software, blasted on NCBI website for confirmation of homogeneity with other *Anaplasma marginale* strains sequences on the GenBank. The maximum similarity of our sequences with accession number AF112480.1 varied from 99%, 96% and 94% with GP & NW, LP and KZN respectively, with AF111196.1 is 99% with both GP and KZN, with AF348137.1 is 98% for LP, with CP1001079 is 100% for MP (Table 4).

Table 4: Homogeneity of South African sequences with those in the GenBank (*msp1b* gene)

GenBank accession number for *A. marginale* strains	South African sequences	Maximum identity
AF112480.1	*GP_C9LA	99%
AF111196.1	*GP_C1_LA	99%
AF112480.1	**KZN_C9LA	94%
AF111196.1	**KZN_C2_LA	99%
AF348137.1	***LP_C9LA	98%
AF112480.1	***LP_10_LA	96%
CP1001079	****MP_C1_LA	100%
AF112480.1	*****NW_C4_LA	99%

*GP- Gauteng sample, **KZN- KwaZulu-Natal sample, ***LP- Limpopo sample, ****MP- Mpumalanga sample, *****NW- North West samples.

2.4 DISCUSSION

Anaplasmosis, caused by *A. marginale*, is one of the most prevalent tick-transmitted rickettsial diseases of cattle worldwide. Lack of sufficient diagnostic assays due to poor sensitivity and specificity, together with the potential for cross-reactivity among closely related *Anaplasma* species has made the accurate determination of infection status problematic. The significance of anaplasmosis is frequently underestimated due to seasonal outbreaks and stability in areas of endemicity.

In this study a PCR assay was developed for routine detection of *A. marginale* DNA in blood of naturally infected cattle. The assay was specific for *A. marginale*, and there was no cross reaction with other haemoparasites namely, *A. centrale*, *B. babesia*, *B. bovis* and *Ehrlichia ruminantium*. Clinical signs such as anaemia and jaundice are often common among cattle infected with the previous stated haemoparasites and the vector ticks *Rhipicephalus* spp commonly transmit the same haemoparasites. Previous studies based on PCR assays for detection of *A. marginale* have shown that assays based on 16S rRNA are invaluable for specific detection of *A. marginale* this is due to the few different sites in the sequence alignment of *A. marginale and A. centrale* (Vahid *et al.,* 2010, Aubry *et al.,* 2011*).* The necessity of differentiating the bovine anaplasmosis, babesiosis and erhlichiosis by correct identification of the causative agent is very important thereof. Successful development of species-specific conventional PCR assay for the detection of *A. marginale* infections based on the *mpsp1b* makes it ideal to understand or study the clinical signs specific for *A. marginale* infections, antigenicity of different strains and to document the epidemiological information of bovine anaplasmosis in South Africa.

The detection limit of the conventional PCR assay developed in this study is 0.1 pg/ul of the serially diluted *A. marginale* DNA. The sensitivity of the assay is fundamental when developing an assay for detection of infections in the field as the infected DNA may contain inhibitors. Conventional PCR has been shown to incapably detect infections of *A. marginale* during early stages of infections and in carrier cattle due to the sensitivity limitations, however the newly developed assay has its own detection limit, but it does not deter its capability to detect *A. marginale* infections in cattle.

Detection performance of the newly developed conventional PCR assay was further evaluated with field- derived samples collected from different regions of South Africa. The DNA samples were chosen randomly with no particular order, infections varied with geographic location. Samples from Mpumalanga, Kwazulu-Natal, Limpopo, Gauteng, North West, Eastern Cape and Free State Provinces showed high infection rates with *A. marginale,* while Western Cape showed low infection rate. The Northern Cape had 0% infection of *A. marginale* which corresponds with the fact that samples were collected from a known anaplasmosis free area within the Kuruman region. The diversity in the rate of infection within Provinces can be explained by the vector tick distribution and the season of sample collection. A study conducted alongside (Mtshali K, personal communication) shows high occurence of the vector tick *R. evertsi evertsi* with samples collected from November 2011 to February 2012, however the conventional PCR has its own detection limit capabilities which explain the discrepancy of the infection status within these South African Provinces where anaplasmosis is endemic.

The PCR products were sequenced and aligned on BLASTn to check the maximum similarity of the sequences against those homogenous sequences, the maximum similarity with different *A. marginale* isolates varied from 94-100%, The sample C1_MP_LA when entered on the BLASTn search tool was shown to be 100% similar to the sequence with accession number CP1001079. The sequencing of PCR products confirms the three factors, specificity of amplified fragment, causative agent *A. marginale* and the homogeneity of *A. marginale* strains. The developed assay was specific and sensitive, and proves to be an assay which can be used for detection of *A. marginale* infections even in samples collected from the field where there is presence of co-infections with other bovine haemoparasites.

CHAPTER 3

3. DEVELOPMENT OF A SPECIEC-SPECIFIC REAL-TIME PCR FOR THE DETECTION OF *ANAPLASMA MARGINALE* IN SOUTH AFRICA

3.1.1 Introduction

Real-time Polymerase Chain Reaction enables the monitoring of the progress of the PCR as it occurs in real time. Data information is collected throughout the PCR process, rather than at the end of the PCR. This completely modernizes the way one approaches PCR-based quantization of DNA and RNA. In real-time PCR, reactions are characterized by the point in time during cycling when amplification of a target is first detected rather than the amount of target accumulated after a fixed number of cycles. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed. In contrast, an endpoint assay measures the amount of accumulated PCR product at the end of the PCR cycle (Ginzinger, 2002; Decaro *et al.*, 2005).

Today, there are different chemistries including: 5' nuclease assays using TaqMan probes, molecular beacons and SYBR Green I intercalating dyes. All these chemistries allow detection of PCR products through the generation of fluorescent signal. Molecular Beacons and TaqMan probes depend on Forester Resonance Energy (FRET) to produce the fluorescence signal through the coupling of a fluorogenic dye molecule and a quencher moiety to the same or different oligonucleotide substrate, while SYBR Green is a fluorogenic dye that exhibits little fluorescence when in solution, but when binding to double stranded DNA it emits a strong fluorescence (Ginziger, 2002).

3.1.2 TaqMan

TaqMan probes depend on the 5' nuclease activity of the DNA polymerase used for PCR to hydrolyze an oligonucleotide that is hybridized to the target amplicon. TaqMan probes are oligonucleotides that have a fluorescent reporter dye attached to the 5' end and a quencher moiety coupled to the 3' end (Figure 5). These probes are designed to hybridize to an internal region of a PCR product. In the hybridized state the closeness of the fluor and the quench molecules prevents the detection of the fluorescent signal from the probe (Ginziger, 2002).

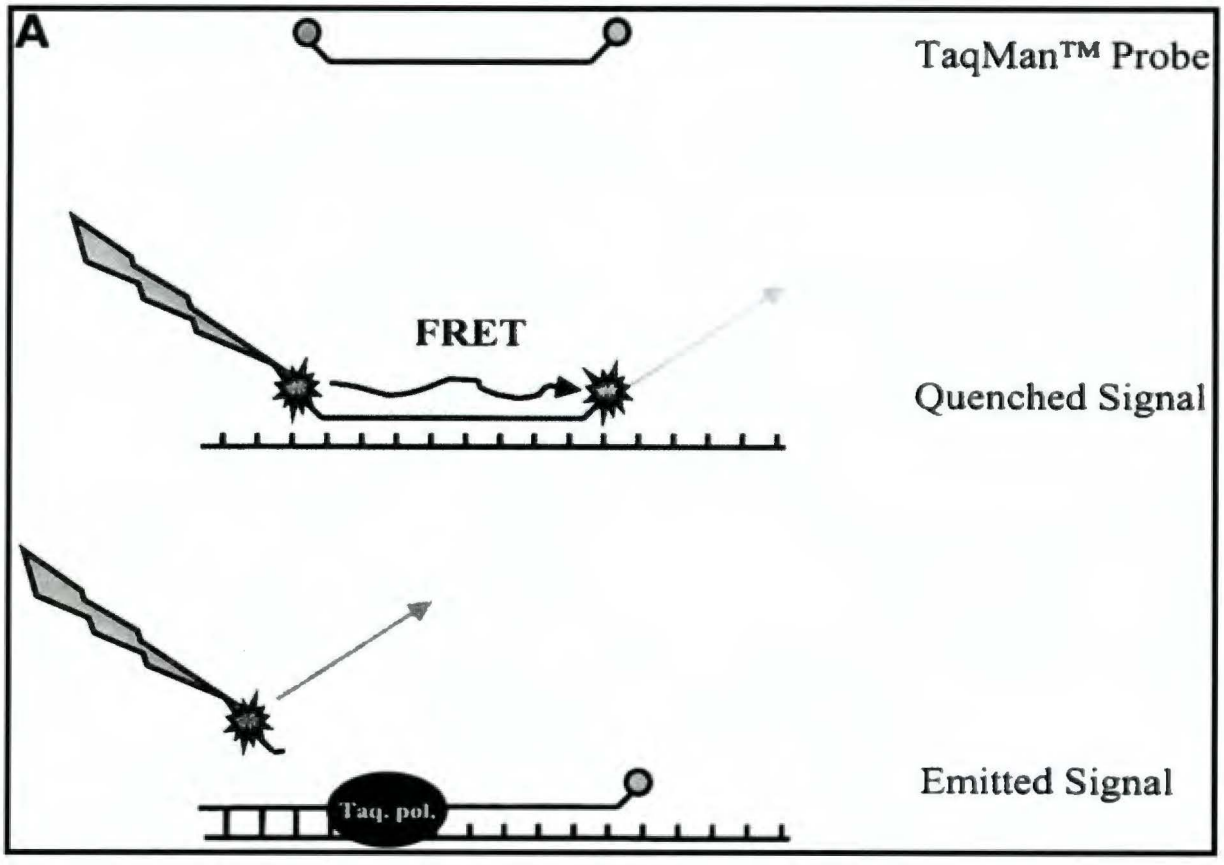


Figure 5: The schematic presentation of TaqMan probe chemistry of a real-time PCR (Ginzinger, 2002).

3.1.3 Molecular beacons

Molecular beacons also uses FRET to detect and quantitate the synthesized PCR product through flour coupled to the 5' end and a quench attached to the 3' end of an oligonucleotide substrate, not like TaqMan probes, molecular beacons are designed to remain intact during the amplification reaction, and must rebind to target in every cycle for signal measurement. Molecular beacons form a stem-loop structure when free in solution (Figure 6) .Therefore the close proximity of the flour and quench molecules prevents the probe from fluorescing. When a molecular beacon hybridizes to a target, the fluorescent dye and quencher becomes separated, FRET does not occur and the fluorescent dye emits light upon irradiation (Ginziger, 2002).

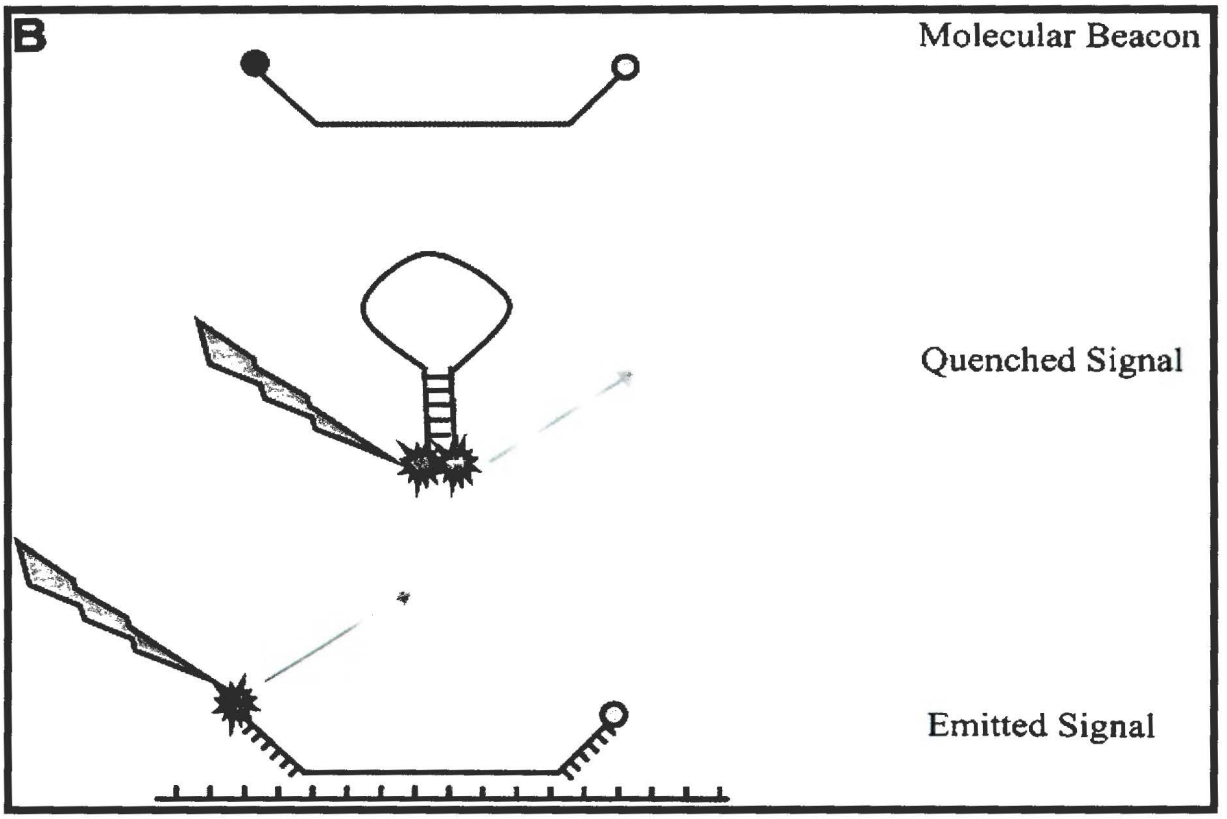


Figure 6: The schematic presentation of molecular beacon chemistry of a real-time PCR (Ginzinger, 2002).

3.1.4 SYBR Green

SYBR Green is the simplest format of detecting and quantitating PCR products in a real-time reactions, it binds double-stranded DNA, and upon excitation emits light, as the PCR products accumulates, fluorescence increases (Figure 7). It is easy to use and inexpensive but will bind to any double-stranded DNA in the reaction and it requires extensive optimization (Ginziger, 2002).

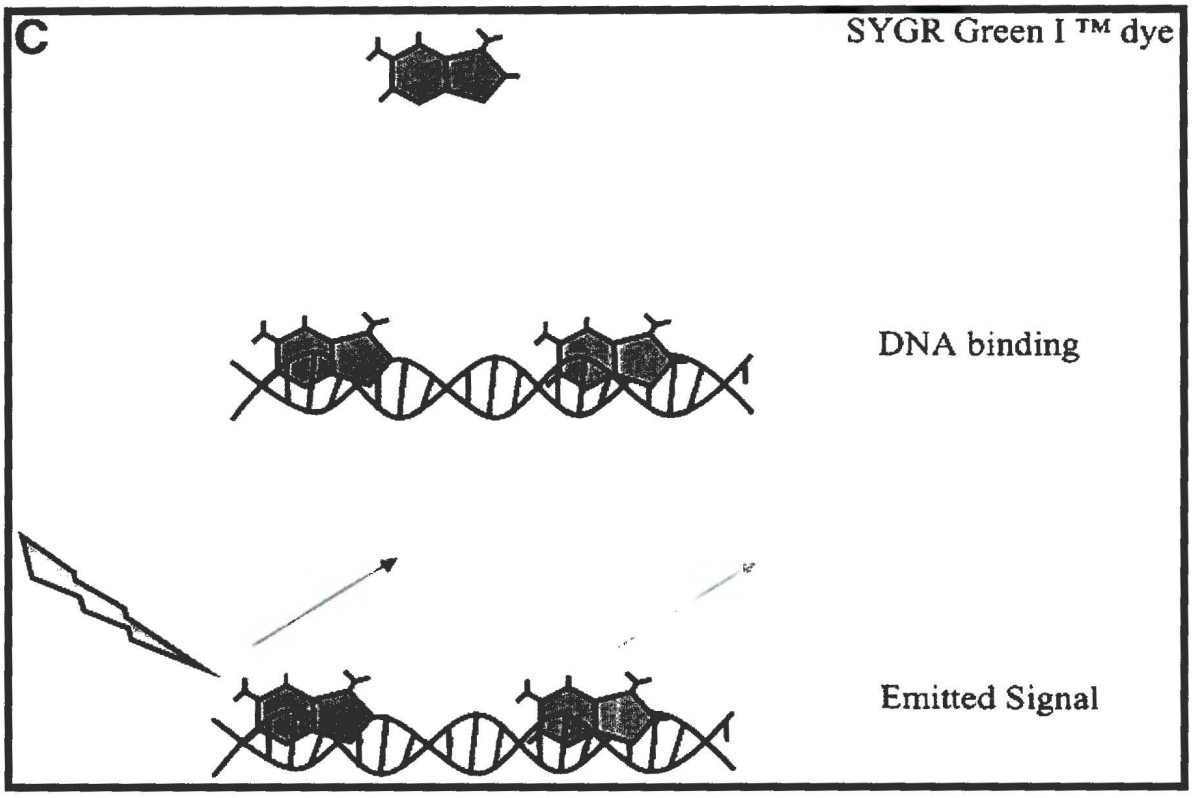


Figure 7: The schematic presentation of SYBR Green I dye chemistry of a real-time PCR (Ginzinger, 2002).

Real-time PCR has outstanding advantage over other previously developed assays. It has an ability to quantify nucleic acids over a wide dynamic range, along with high sensitivity allowing detection of low DNA concentration of a target sequence. It is performed in a closed reaction tube that requires no post PCR analysis, reducing the chances for cross contamination in the laboratory (Valasek *et al.*, 2005).

3.1.5 Objective of this study

To develop a species-specific real-time PCR for the detection of *A. marginale* strains infections in South Africa.

3.2 MATERIALS AND METHODS

3.2.1 Real-Time PCR assay

3.2.1.1 Designing of primers and a probe

Six samples of A. marginale DNA from Eastern Cape, KwaZulu Natal, Mpumalanga and North West were amplified by PCR according to the published method of (Molad, *et al* 2006). The PCR products were sequenced. Sequences were aligned by BioEdit program to find the conserved region within the sequences (Figure 1). The target region was identified. Specific primers and probes were designed using Gene Script program (https://www.genescript.com/ssl-bin/app/primer). The TaqMan probe labelled with fluorescent reporter dye 6-caboxy-fluorosean (6-FAM) at the 5' end with minor groove binder quencher. The primers and probe were synthesized at Applied Biosystems, Johannesburg, South Africa (Table 5). The primers and probe orientation are shown in Figure 8.

Table 5: Real-time PCR Primers and TaqMan probes of *msp1b* gene

Name	Orientation	Sequence (5'-3')	Modification
#PrF2	Forward	AGCGAAGGCAGATGCTAAAT	
#PrR2	Reverse	GAGTTTACCACGAGCGACAA3	
#PBR-2	Reverse	AGCACGTAGTCCCACGCTCTCG	5'Fam-3'MGB
*PrF3	Forward	TTGTCGCTCGTGGTAAACTC	
*PrR3	Reverse	CTCACCAGTGTCAGGTGCTT	
*PBR-3	Forward	TGCACCCAAGAACCTTGACCAA	5'Fam-3'MGB

real-time PCR primers set 2, * real-time PCR primers set 3

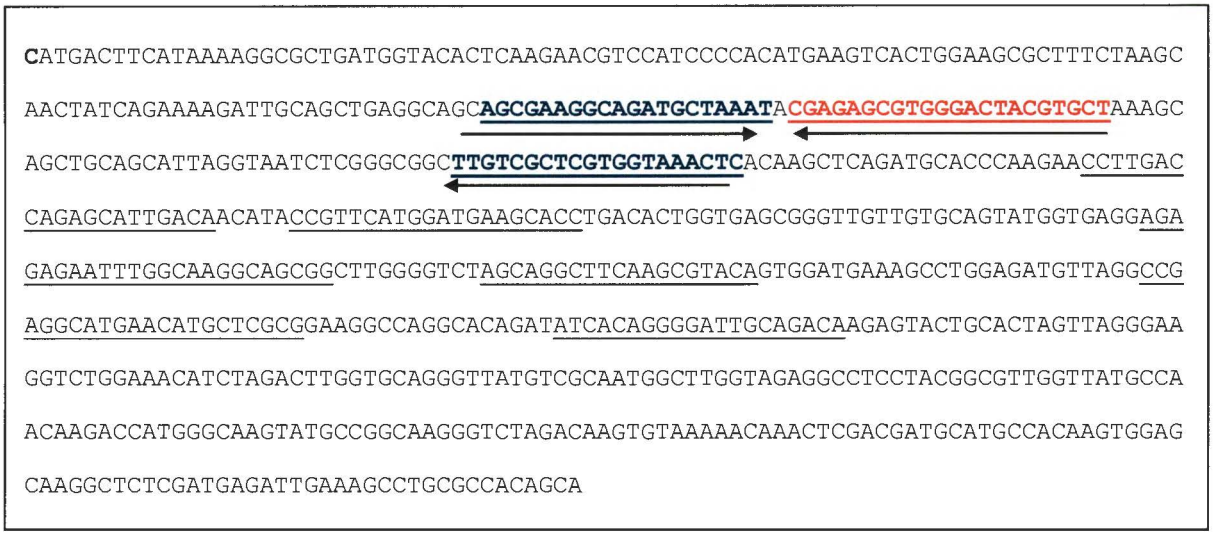


Figure 8: The consensus sequence showing primers designed for real-time PCR. The primers were designed based on the *msp1b* gene PrF2, PrR2 and PBR-2 primers and a probe, underlined and in bold blue colour and in bold red colour (probe).

3.2.1.2 Optimization of Real-Time PCR conditions

Primers: The primers were reconstituted to 100 µMol stock solution: by multiplying the given primer nano moles by 10 and then prepared to 10 pmol working concentration. Primers were then tested using different volumes (1 - 3 ul) within the reaction mixture of 25 ul. The reaction was then carried out with the volume of 2.5 ul of 10 pmol of forward and reverse primers.

Probe(s): The probe was received at a concentration of 100 µMol and was used at the same concentration; volume of probe was taken as 0,125 ul in a 25 ul reaction mixture.

Cycles: The real-time PCR conditions consisted of activation of TaqMan DNA polymerase at 95°C for 10 minutes, denaturation at 95ºC for 15 seconds, and annealing-extension was carried out at different temperatures (55 - 60ºC) for 1 minute. The optimal annealing-extension was 60ºC at 60 cycles.

3.2.2 TaqMan real-time PCR assay

Anaplasma marginale real-time PCR assay was performed on a 7500 real-time PCR system (Applied Biosystems StepOne, USA) with TaqMan master mix. The reaction mixture of 25 µl contained; 12.5 µl of TaqMan master mix, each primer at a concentration of 100 pmol, probe at 100 pmol (both two sets of primers) and 5 µl of template DNA. The thermal cycling consisted of activation of TaqMan DNA polymerase at 95°C for 10 minutes and 60 cycles of denaturation at 95°C for 15 seconds and annealing-extension at 60°C for 1 minute. The TaqMan assay was carried out in duplicate for each unknown and standard sample and double distilled water was included as a negative control. The increase in fluorescent signal was registered during the extension step of reaction and data were analysed with the appropriate sequence detector software (7500 system software V.1.3.1).

The growth of PCR product is proportional to an exponential increase in fluorescence (ΔR_n). The application software produces an amplification curve resulting from a plot of ΔR_n versus cycle number. The threshold cycle number C_T for each analysed sample was regarded as the cycle number at which the amplification curve crossed the threshold which is usually automatically selected from the average of the (ΔR_n) values corresponding to greater amount of initial template and negative result was considered to have a C_T value of 60 or more cycles.

3.2.3 Specificity of TaqMan real–time PCR

The PCR reaction mixture contained 12.5 µl of TaqMan universal PCR Master (Applied biosystems, Johannesburg, S.A) 2.5 µl of each 10 pmol primer, 0.125 ul of TaqMan probe, 5 µl of DNA template which had each of *A. marginale, A. centrale, B. bigemina, B. bovis*, *Ehrlichia ruminantium* and uninfected bovine blood and double distilled water was used for adjusting volume. The thermal cycling consisted of activation of TaqMan DNA polymerase at 95°C for 10 minutes and 60 cycles of denaturation at 95°C for 15 seconds and annealing-extension at 60°C for 1 minute. The TaqMan assay was carried out in duplicate for each sample and double distilled water was included as a negative control. The increase in fluorescent signal was registered during the extension step of reaction and data were analysed with the appropriate sequence detector, software (7500 system software V.1.3.1). The growth of PCR product is proportional to an exponential increase in fluorescence (ΔR_n). The application software produces an amplification curve resulting from a plot of ΔR_n

versus cycle number. The threshold cycle number C_T for each analysed sample was regarded as the cycle number at which the amplification curve crossed the threshold.

3.2.4 Sensitivity of TaqMan real–time PCR

The PCR reaction mixture contained 12.5 µl of TaqMan universal PCR Master (Applied Biosystems, Johannesburg, S.A), 2.5 µl of each 10 pmol primer, 0.125 ul of TaqMan probe, 5 µl of DNA template which was diluted in 10 fold serial dilutions each of (10 ng/ul, 1.0 ng/ul, 0.1 ng/ul, 0.001 ng/ul 0.0001 ng/ul and 0.00001 ng/ul) and double distilled water was used for adjusting volume. The thermal cycling consisted of activation of TaqMan DNA polymerase at 95°C for 10 minutes and 60 cycles of denaturation at 95°C for 15 seconds and annealing-extension at 60°C for 1 minute. The TaqMan assay was carried out in duplicate for standard sample and DDW was included as a negative control. The increase in fluorescent signal was registered during the extension step of reaction and data were analysed with the appropriate sequence detector, software (7500 system software V.1.3.1). The growth of PCR product is proportional to an exponential increase in fluorescence (ΔR_n). The application software produces an amplification curve resulting from a plot of ΔR_n versus cycle number. The threshold cycle number C_T for each analysed sample was regarded as the cycle number at which the amplification curve crossed the threshold.

3.3 RESULTS

3.3.1 Specificity of TaqMan real-time PCR

3.3.1.1 The PrF2, PrR2 and PrB2 primers and probe

The real-time PCR with primers and probe PrF2, PrR2 and PRB2 was performed as explained above. Amplification reaction of *A. marginale*, *A. centrale*, *B. bigemina*, *B. bovis* and *E. ruminantium* was observed as the reaction progresses. Only *A. marginale* DNA was amplified by real-time PCR with PrF2, PrR2 and PrB2 primers and probe. None of the reaction control DNA's were amplified (Figure 9).

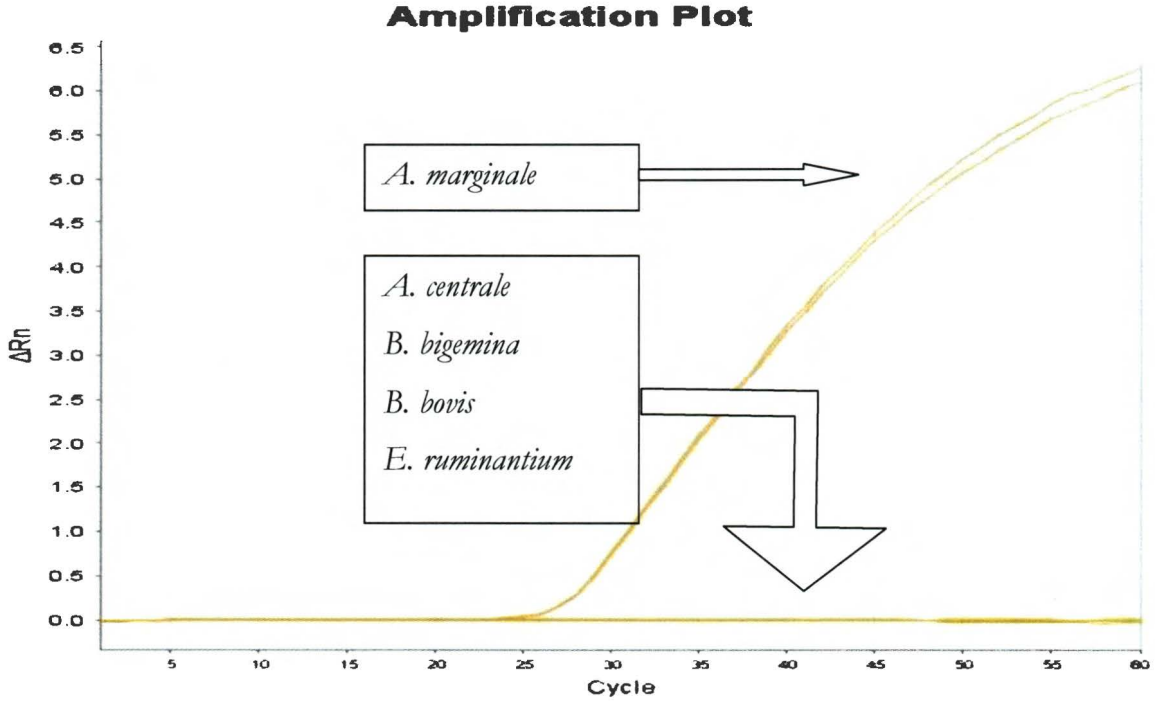


Figure 9: Detection of *A. marginale* genomic DNA using the *A. marginale* TaqMan MGB™ real-time PCR assay, indicated by an increase in the fluorescence signal. No increase in fluorescence was observed from *A. centrale*, *B. bigemina*, *B. bovis*, *E. ruminantium*.

3.3.1.2 The PrF3, PrR3 and PrB3 primers and probe

The real-time PCR with primers (PrF3, PrR3 and PRB3) was performed as explained above. Amplification reaction of *A. marginale*, *A. centrale*, *B. bigemina*, *B. bovis* and *E. ruminantium* was observed as the reaction progresses. Only *A. marginale* DNA was amplified by real-time PCR with PrF2, PrR2 and PrB2 primers and probe. None of the reaction control DNA's were amplified (Figure10).

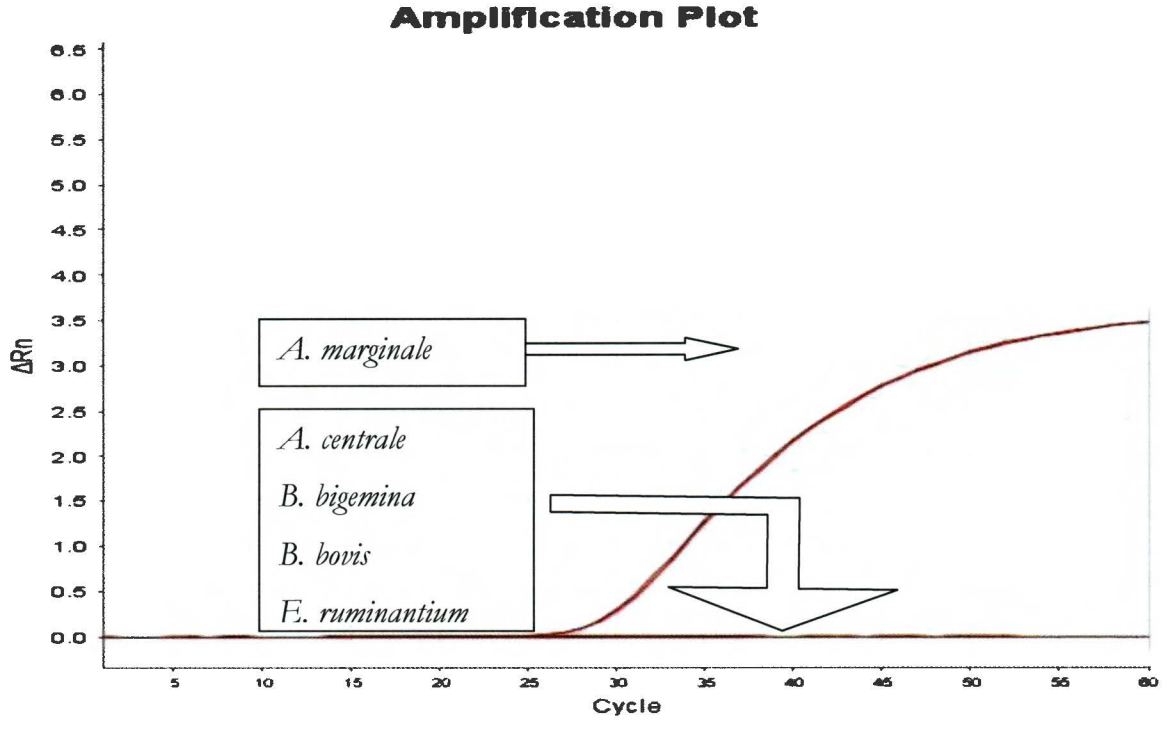


Figure 10: Detection of *A. marginale* genomic DNA using the *A. marginale* TaqMan MGB™ real-time PCR assay, indicated by an increase in the fluorescence signal. No increase in fluorescence was observed from *A. centrale*, *B. bigemina*, *B. bovis*, *and E. ruminantium*.

3.3.2 Sensitivity of TaqMan real-time PCR

Determination of the detection limit with primers (PrF2, PrR2 and PRB2) was analysed on the standard curve; amplification plot was used to construct the standard curve of fluorescence versus quantity of the DNA. As a result the assay amplified serially diluted DNA from 10 ng/ul down to 0.001 ng/ul (Figure 11).

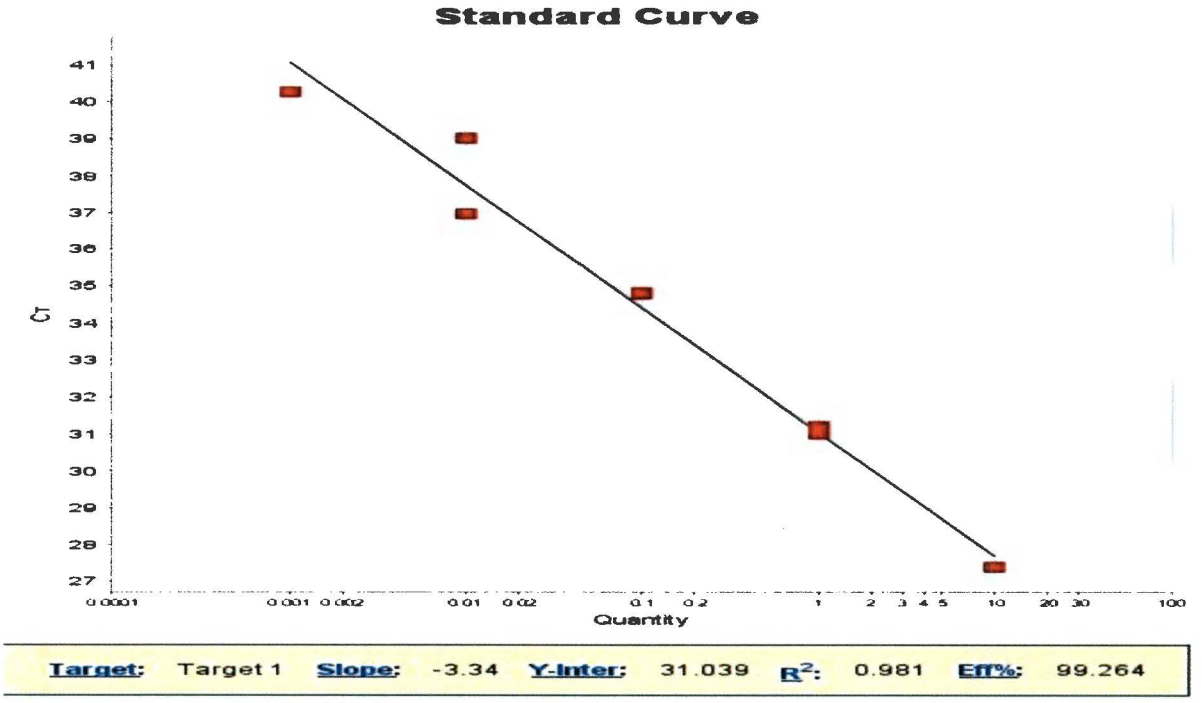


Figure 11: Standard curve for the quantification of *A. marginale* using the *A. marginale* TaqMan MGB™ real-time PCR assay with 10 ng/ul as a starting concentration to 0.001 ng/ul.

3.3.3 Sensitivity of TaqMan real-time PCR

Determination of the detection limit with primers (PrF3, PrR3 and PRB3) was analysed on the standard curve; amplification plot was used to construct the standard curve graph of fluorescence versus quantity of the DNA. As a result the assay amplified serially diluted DNA from 10 ng/ul down to 0.01 ng/ul (Figure 12)

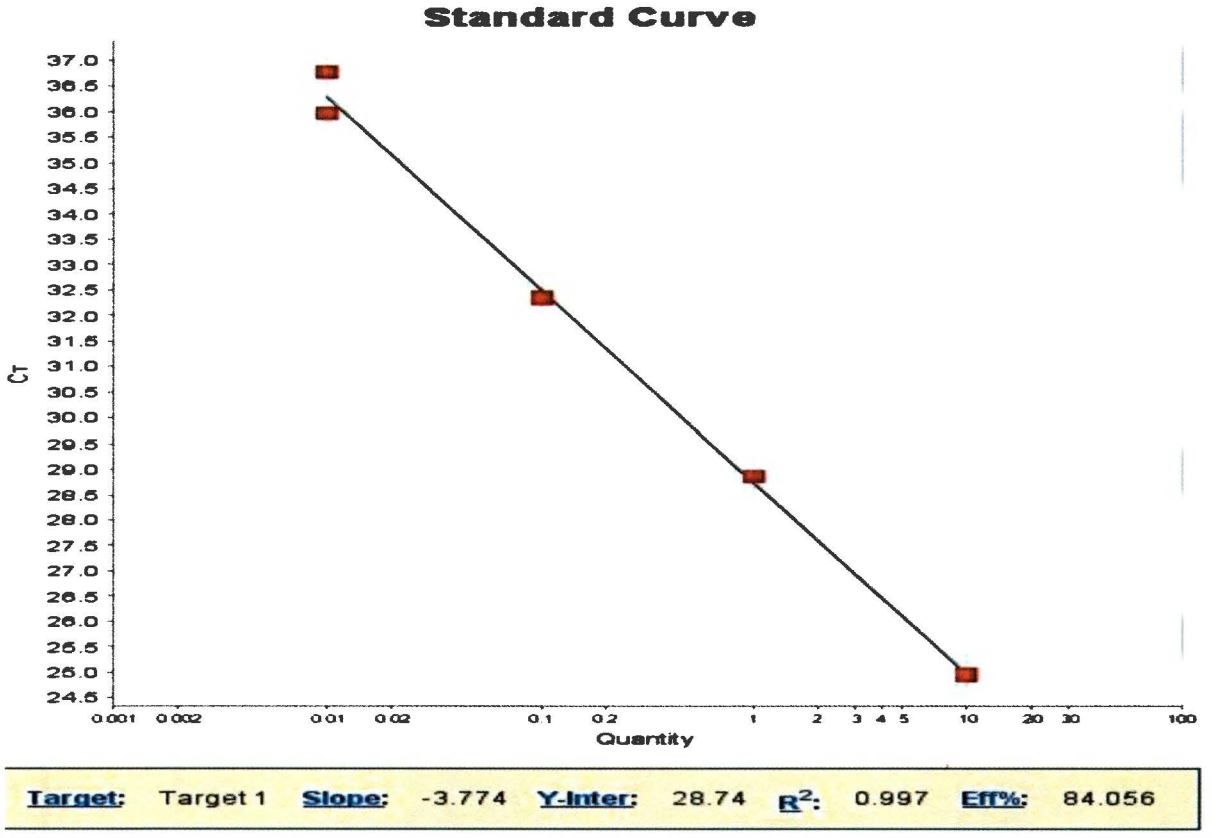


Figure 12: Standard curve for the quantification of *A. marginale* using the *A. marginale* TaqMan MGB™ real-time PCR assay with 10 ng/ul as a starting concentration to 0.01 ng/ul.

3.3.4 Detection performance of real-time PCR assay on field samples

The newly developed real-time PCR assay amplified DNA samples from 9 provinces with detection performance of 100%,100%, 100%, 94%, 71%, 100%, 83%, 19% and 88% for EC, FS, GP, KZN, LP, MP, NW, NC and WC, respectively (Table 6).

Table 6: Screening of blood samples from different Provinces of South Africa

Provinces	Number of samples	Positives	Rate of infection (%)
Eastern Cape	30	30	100%
Free State	16	16	100%
Gauteng	13	13	100%
KwaZulu Natal	17	16	94%
Limpopo	14	10	71%
Mpumalanga	18	18	100%
North West	12	10	83%
Northern Cape	16	3	19%
Western Cape	8	7	88%
Total sample	**144**	**107**	**74%**

3.4 DISCUSSION

Real-time PCR has distinct advantages over previously developed assays, because of its characteristics of detecting and also quantifying target sequence in a single tube in real-time with no post manipulation of the PCR product. Real-time PCR technology has recently been applied for the diagnosis of many organisms of veterinary and medical importance (Carelli *et al.*, 2007, Bhoora *et al.*, 2010); this technology has shown high sensitivity and rapidity of the TaqMan MGB^{TM} real time PCR.

In this study TaqMan-based chemistry was chosen for the development of the real-time assay, because of that the fluorescent signal is generated only when there is specific hybridization of the probe to the target sequence. No fluorescent signal is generated from any non-specific amplification products that may arise as the reaction progresses (Valasek *et al.*, 2005). Two sets of primers and probe based on *msp1b* gene (Table 5) were tested using the validated conditions for real time PCR, the primers both showed specificity, the test was proven to be highly specific: there were no cross reactions with other haemoparasites of bovines including *A. centrale*, *B. bigemina*, *B. bovis* and *E. ruminantium*.. However primers set two showed early fluorescence signal at 25 cycles with the highest peak than of primer set three which signalled at 27 cycles with the shortest peak.

The two sets of primers (Table 5) were examined for sensitivity. Ten-fold dilutions of standard DNA were tested and used to construct the standard curve by plotting DNA concentration against the measured C_T values. Primer set two standard points covered a linear range of five orders of magnitude (from 10 - 0.001 ng/ul) of standard DNA, showed linearity with slope = -3.34. The efficiency of the TaqMan MGB^{TM} (primer set two) was determined to be 99.264% for detection of *A. marginale*. Primer set three standard points covered a linear range of four orders of magnitude (from 10 - 0.01 ng/ul) of standard DNA, showed linearity with slope = -3.77. The efficiency of the TaqMan MGB^{TM} (primer set three) was determined to be 84.05% for detection of *A. marginale* infections. Primer set two showed high sensitivity than primer set three, therefore it detection performance was further determined on field derived samples. The sensitivity of the assay is vital when developing a detection assay, the detection limit of the primer set two is efficient enough to detect infections in carrier cattle and also during early infections of anaplasmosis disease.

The real-time PCR assay primer set two was further tested for the ability to detect DNA extracted from blood of the cattle collected from different Provinces of South Africa. DNA samples were chosen randomly with no particular order in order to examine the performance of the newly developed assay on field samples, anaplasmosis infections varied from one Provinces to another, All Provinces showed high rate of infection with *A. marginale* and ranged (71 - 100%), while only Northern Cape showed low anaplasmosis infection (19%). This confirms that samples were collected from anaplasmosis free area within Kuruman region. This further shows that the assay developed based on TaqMan probe is sensitive and efficient to give a correct status of current infection even in cases of low parasitemia.

CHAPTER 4

4. DEVELOPMENT OF A SPECIES-SPECIFIC LOOP-MEDIATED ISOTHERMAL AMPLIFICATION FOR THE DETECTION OF *ANAPLASMA MARGINALE* IN SOUTH AFRICA

4.1.1 Introduction

Loop-mediated isothermal amplification (LAMP) is a relatively recent and novel technique for specific amplification of DNA with high sensitivity under isothermal conditions (Notomi *et al.*, 2000; Mori *et al.*, 2009). It is characterized as a simple, rapid, specific and cost effective amplification method (Notomi *et al.*, 2000; Thekisoe *et al.*, 2005; Mori *et al.*, 2009). Therefore LAMP has the potential for being used in resource-limited veterinary laboratories in developing countries, such as South Africa, where many endemic diseases exist.

Various LAMP assays have been developed for rapid detection of ruminant's haemoparasites (Liu *et al.*, 2008, Ma *et al.*, 2011). Loop-mediated isothermal amplification assay for rapid diagnosis of ovine theileriosis showed high specificity and sensitivity, the results showed that at 0.1 pg DNA concentration LAMP can detect both ovine parasites including *Theileria luwenshi* and *Theileria uilenbergi*, which is ten times more sensitive than PCR method for detection of the same parasites (Liu *et al.*, 2008). Ma *et al.*, 2011 developed LAMP for rapid detection of *Anaplasma ovis* (Ma *et al.*, 2011). The latter authors demonstrated that the LAMP primers specifically amplified genomic DNA of *A. ovis* isolates but not *A. marginale*, confirming the high specificity of LAMP for the diagnosis of *A. ovis* infection.

Loop-mediated isothermal amplification uses a DNA polymerase with strand-displacement activity *Bacillus stearothermophilus* (*Bst* polymerase), along with two inner primers (FIP, BIP) and outer primers (F3 and B3) which recognize six separate regions within a target DNA (Pandey *et al.*, 2008; Mori *et al.*, 2009). Additional primers called Loop F (LF) and Loop B (LB) can be added in order to make the reaction be much faster. During the initial LAMP reaction all four primers are used but for the cycling step, only the inner primers are used for further amplification of DNA. The inner primers form the stem-loop structures and

initiates strand displacement DNA synthesis by the outer primers (Notomi *et al.*, 2000; Tomita *et al.*, 2008).

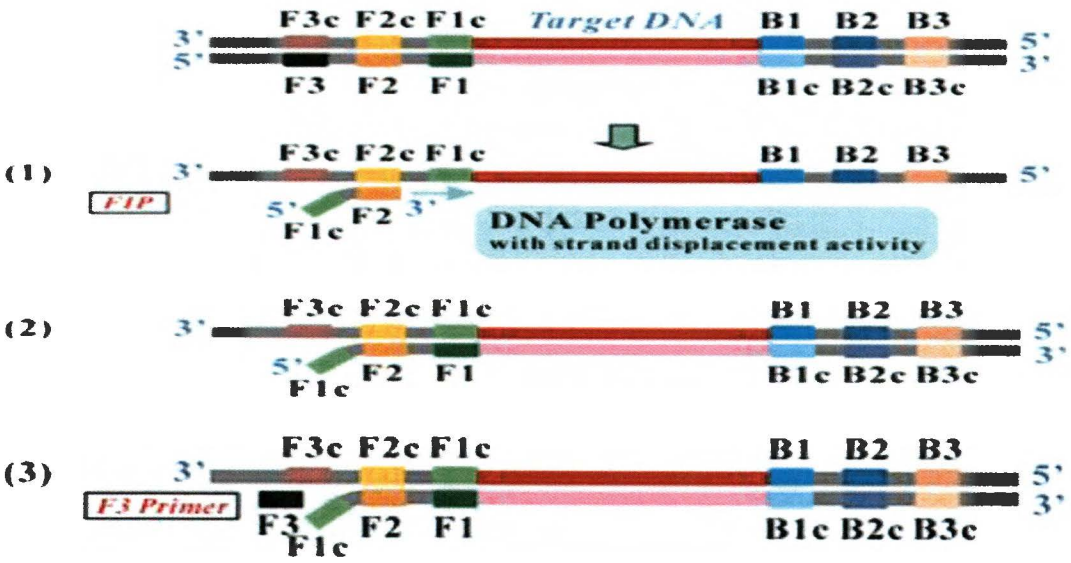
4.1.2 LAMP mechanism

Starting material producing step shown in figure 13 (Notomi *et al.*, 2000)

The LAMP primers anneal to the complimentary sequence of double stranded target DNA, initiating DNA synthesis using the DNA polymerase with strand displacement activity, displacing and releasing a single stranded DNA. In the LAMP mechanism, unlike with PCR, there is no need for heat denaturation of the double stranded DNA into a single strand. The following amplification mechanism explains from when the FIP anneals to such released single stranded template DNA (step1). Through the activity of DNA polymerase with strand displacement activity, a DNA strand complementary to the template DNA is synthesized, starting from the 3' end of the F2 region of the FIP (step 2). The F3 Primer anneals to the F3c region, outside of FIP, on the target DNA and initiates strand displacement DNA synthesis, releasing the FIP-linked complementary strand (step3). A double strand is formed from the DNA strand synthesized from the F3 Primer and the template DNA strand (step 4). The FIP-linked complementary strand is released as a single strand because of the displacement by the DNA strand synthesized from the F3 Primer. Then, the released single strand forms a stem-loop structure at the 5' end because of the complementary F1c and F1 regions (step 5).

This single strand DNA in (step 5) serves as a template for BIP-initiated DNA synthesis and subsequent B3-primed strand displacement DNA synthesis. The BIP anneals to the DNA strand produced in (step 5). Starting from the 3' end of the BIP, synthesis of complementary DNA takes place. Through this process, the DNA reverts from a loop structure into a linear structure. The B3 Primer anneals to the outside of the BIP and then, through the activity of the DNA polymerase and starting at the 3' end, the DNA synthesized from the BIP is displaced and released as a single strand before DNA synthesis from the B3 Primer (step 6). In step 7, double stranded DNA is produced through the processes described in (step 6). The BIP-linked complementary strand displaced in (step 6) forms a structure with stem-loops at each end, which looks like a dumbbell structure. This structure serves as the starting structure for the amplification cycle in the LAMP method, LAMP cycling (step 8).

A dumbbell-like DNA structure is quickly converted into a stem-loop DNA by self-primed DNA synthesis. FIP anneals to the single stranded region in the stem-loop DNA and primes strand displacement DNA synthesis, releasing the previously synthesized strand. This released single strand forms a stem-loop structure at the 3' end because of complementary B1c and B1 regions. Then, starting from the 3' end of the B1 region, DNA synthesis starts using self-structure as a template, and releases FIP-linked complementary strand (step 9). The released single strand then forms a dumbbell-like structure as both ends have complementary F1 - F1c and B1c - B1 regions, respectively (step (11). This structure is the 'turn over' structure of the structure formed in step (8). Similar to the steps from (8) to (11), structure in step (11) leads to self-primed DNA synthesis starting from the 3' end of the B1 region. Furthermore, BIP anneals to the B2c region and primes strand displacement DNA synthesis, releasing the B1-primed DNA strand. Accordingly, similar structures to steps (9) and (10) as well as the same structure as step (8) are produced. With the structure produced in step (10), the BIP anneals to the single strand B2c region, and DNA synthesis continues by displacing double stranded DNA sequence. As a result of this process, various sized structures consisting of alternately inverted repeats of the target sequence on the same strand are formed.



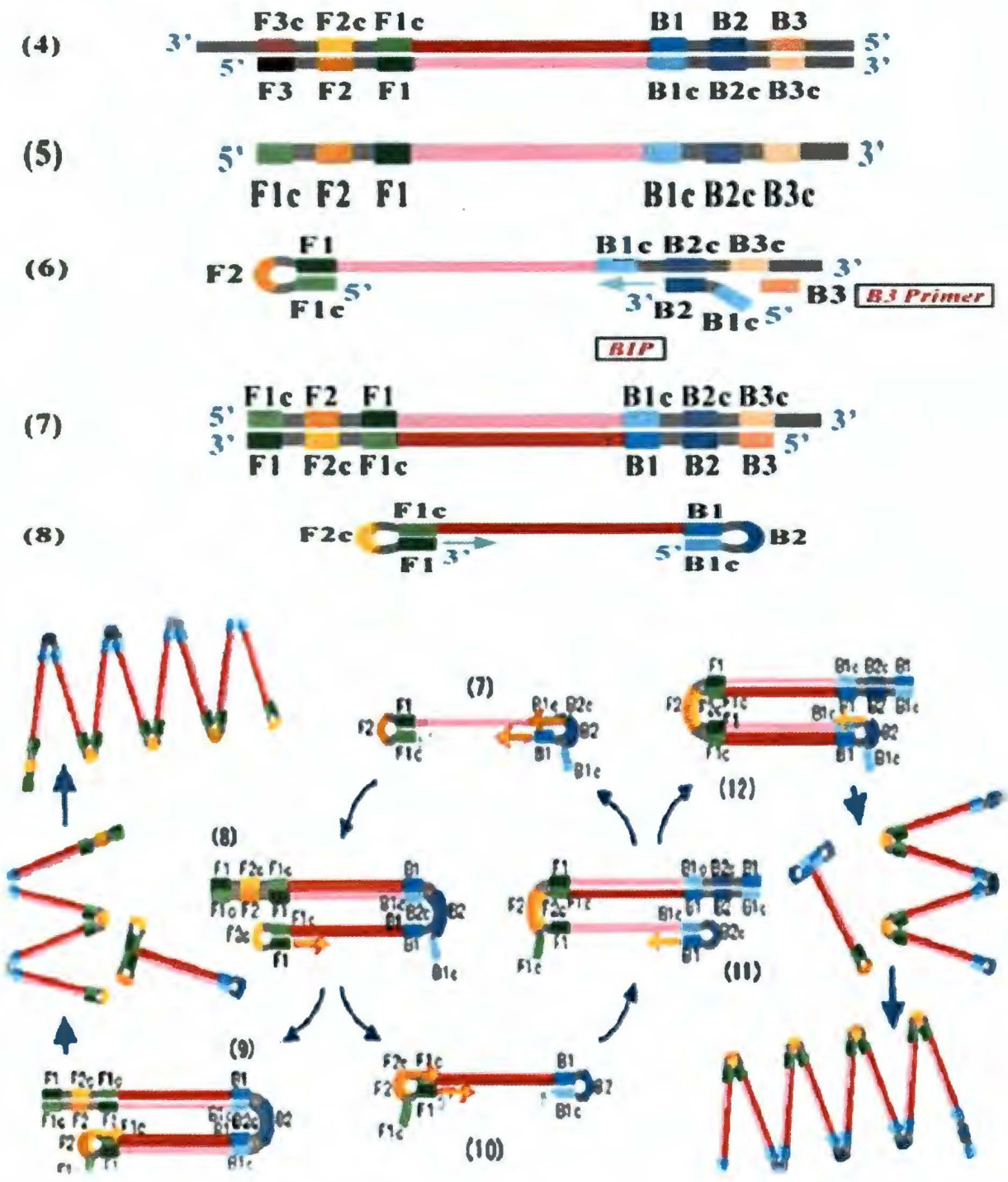


Figure 13: Schematic representation of the LAMP mechanism Step 1-11 (http://loopamp.eiken.co.jp/e/lamp/rt_principle.html)

4.1.3 Analysis of LAMP products

The LAMP product can be easily analysed on the agarose gel electrophoresis and subjected under the UV for examination (Notomi *et al.*, 2000). LAMP reaction produces insoluble magnesium pyrophosphate in the course of the amplification, thus enabling turbidity measurement by photomer, LAMP positive reaction can be detected by visual inspection (Mori *et al.*, 2001). Thekisoe *et al* (2010), showed a typical logarithm amplification curve through utilizing of real-time turbiditimeter developed by Mori *et al* (2009). The graph is plotted with measurements taken from fluorescence relative to the reaction time. Tomita *et al* (2008) developed an endpoint detection using calcein, the fluorescence is quenched by the binding of manganese ions, and therefore calcein produces bright fluorescence in a positive reaction.

4.1.4 Objective of this study

To develop a species-specific LAMP assay for the detection of *A. marginale* strains infections in South Africa.

4.2 MATERIALS AND METHODS

4.2.1 Designing of LAMP primers

The forward outer primer (F3), backward outer primer (B3), forward inner primer (FIP), and backward inner primer (BIP) were designed on the basis of the sequences *msp1b* genes using the Primer Explorer V4. (http://primerexplorer.jp/elamp4.0.0/index.html). The primers were generated and were examined to make sure they are within recommended parameters (Table 7). All primers were synthesized at Inqaba Biotechnical Industries (Pty) Ltd, and are shown in Table 8 and figure 14.

Table 7: Primer design checklist of critical parameters

(http://loopamp.eiken.co.p/e/lamp/primer.html)

Parameters	Details
Distance between primer regions	Distance between 5' end of F2 and B2 is 120-180bp Distance between F2 and F3 as well as B2 and B3 is 0-20bp The distance for loop forming regions is 40-60bp
Melting temperature	60-65°C for GC rich 55-60°C for AT rich
GC content	50-60 % for GC rich 40-50°C for AT rich
Secondary structure	Primers should not form secondary structures. 3' end sequence should not be AT rich or complementary to other primers

Table 8: Sequences of LAMP primers for detection of *A. marginale*

Primer name	Orientation	Sequences (5'-3')
LA-F3	Forward outer primer	CCTTGACCAGAGCATTGACA
LA-B3	Backward outer primer	TGTCTGCAATCCCCTGTGAT
LA-BIP	Backward inner primer (B1c+ B2)	AGCAGGCTTCAAGCGTACAGT-GAGCATGTTCATGCCTCGG
LA-FIP	Forward inner primer (F1c+F2)	CCGCTGCCTTGCCAAATTCTCT-CCGTTCATGGATGAAGCACC
**LA1-LB	Loop backward primer	GATGAAAGCCTGGAGATGTTAGG
**LA1-LF	Loop forward primer	TCACCATACTGCACAACAACCC

** Loop primers

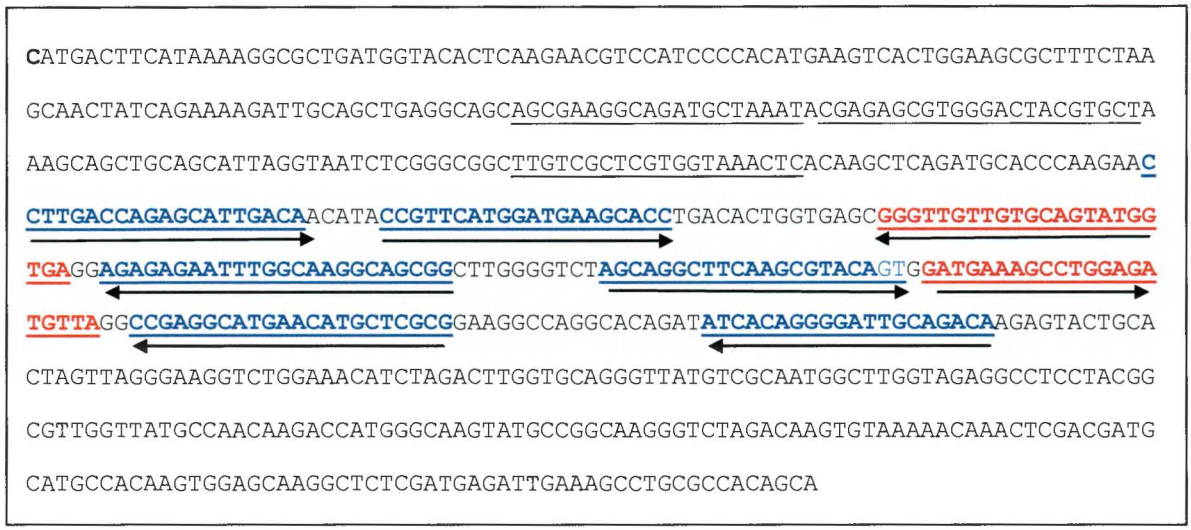


Figure 14: The consensus sequence showing primers designed for LAMP. The primers were designed based on the *msp1b* gene LA-F3, LA-B3, LA-FIP (F1c+F2) and LA-BIP (B1c+B2) primers are underlined and in bold blue colour. Loop primers LA-LF and LA-LB are underlined and in bold red colour.

4.2.2 Optimization of LAMP conditions

Primers: The primers were reconstituted to 100 µMol stock solution as specified by the Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, S. A). The LAMP Primers were prepared accordingly to working concentration: FIP and BIP- 40 pmol each F3 and B3 - 5 pmol LP and LF - 20 pmol each.

Annealing temperature: The optimum incubation temperature for LAMP with the *A. marginale* primer set was established using a range of temperatures from 60, 61.5, 62, 62.9, 63.3, 64.2, 64.7 to 65 ºC. At 62.9 - 65 ºC temperatures gave positive results (Figure 15).

4.2.3 Optimization of LAMP assay

The reaction mixture was prepared using Loopamp DNA Amplification kit (EIKEN CHEMICAL CO., LTD), It contained a final volume of 25 µl; with 12.5 µl of LAMP reaction buffer, 1 µl of *Bst* DNA polymerase, 40 pmol each FIP and BIP primers, 5 pmol of each F3 and B3 primers, 3 µl of target DNA, distilled water was used as negative control and the volume was adjusted with double distilled water. The reaction mixture was incubated at 65°C for 60 minutes using conventional PCR thermal cycler (Bio-Rad T100^{TM}, Germany)

and then heated at 80° C for 2 minutes to terminate the reaction. The LAMP products were subjected to gel electrophoresis under UV light (Figure 15).

4.2.4 Specificity of the LAMP assay using genomic DNA

The LAMP assay was tested for specificity using closely related pathogens which included *A. centrale, Babesia bigemina, B. bovis* and *E. ruminantium*. The LAMP reaction mixture contained; 12.5 µl of LAMP reaction buffer, 1 µl of *Bst* DNA polymerase, 40 pmol each FIP and BIP primers, 5 pmol of each F3 and B3 primers, 3 µl of target DNA, water was used as negative control and the volume was adjusted with double distilled water. The reaction mixture was incubated at 65ºC for 60 minutes using conventional PCR thermal cycler (Bio-Rad T100, Germany) and then heated at 80° C for 2 minutes to terminate the reaction. The LAMP products were subjected to the gel electrophoresis and viewed under the UV light.

4.2.5 Sensitivity of the LAMP assay using genomic DNA

The *A. marginale* genomic DNA (10 ng/µl) was diluted serially in 6 tubes down to the lowest concentration $1x10^{-7}$ ng/µl. The reaction mixture was prepared using Loopamp DNA Amplification kit (EIKEN CHEMICAL CO., LTD). It contained a final volume of 25 µl with 12.5 µl of LAMP reaction buffer, 1 µl of *Bst* DNA polymerase, 40 pmol each FIP and BIP primers, 5 pmol of each F3 and B3 primers, 3 µl of each serial diluted genomic DNA, double distilled water was used as negative control and the volume was adjusted with the double distilled water.

4.3 RESULTS

4.3.1 Optimal conditions for LAMP reaction

The LAMP products which were ran on different reaction temperatures (60 - 65°C) at 60 minutes were examined on gel electrophoresis. LAMP amplification produced a ladder-like pattern. The optimal conditions for the *A. marginale* LAMP assay were 65°C for 60 minutes (Figure 15). The real-time turbidimeter LoopAmp (EIKEN, JAPAN) following the same protocol as described in section 4.2.3 showed amplification of *A. marginale* started at 42 minutes (results not shown).

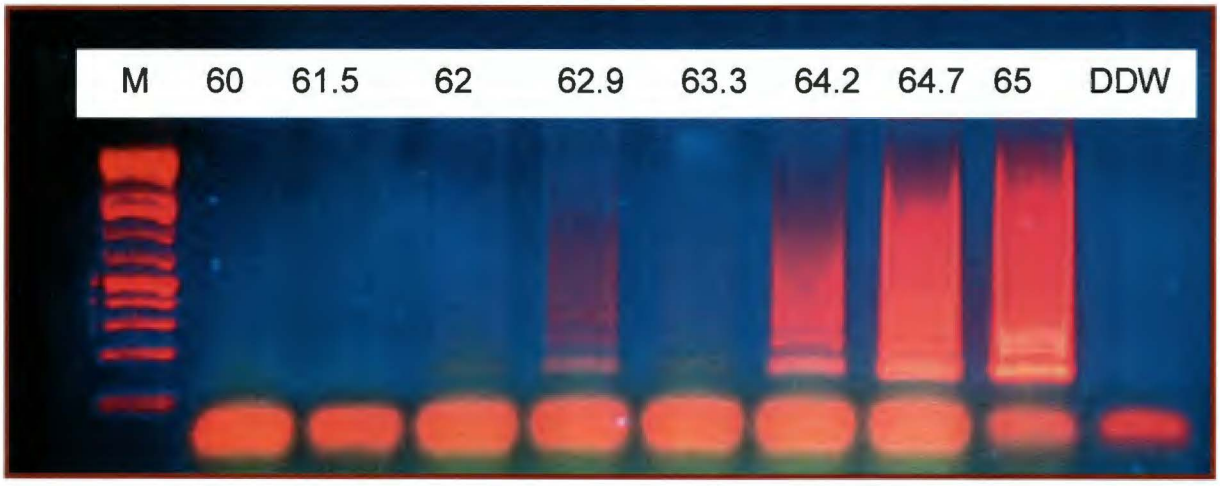


Figure 15: Temperature optimizations for LAMP reaction (60 - 65°C). (M: marker)

4.3.2 Specificity of the LAMP assay

The LAMP assay specificity was examined using other haemoparasites including *A. centrale*, *B. bigemina*, *B. bovis*, *E. ruminantium*. The LAMP products were visualized on 1% gel electrophoresis. Only *A. marginale* DNA was amplified and control DNA from other parasites was not amplified (Figure 16).

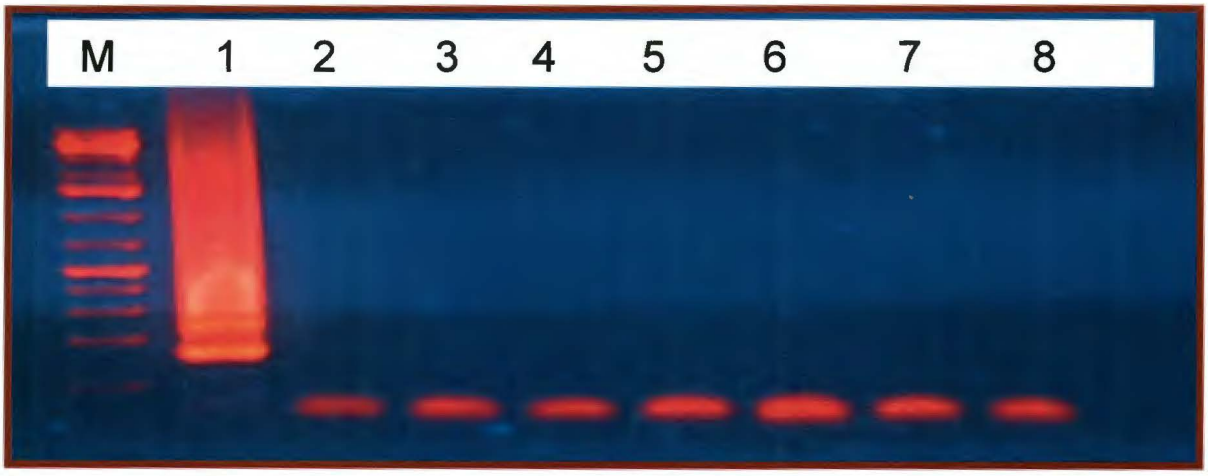


Figure 16: Specificity of the LAMP assay at 65°C for 60 minutes. M: marker, L1: *A. marginale*, L2: *A. centrale*. L3: *B. bigemina*, L4: *B. bovis*, L5: *E. ruminantium*, L6-L8: DDW

The LAMP assay specificity was also examined using real- time turbidimeter. As a result only *A. marginale* DNA was amplified as shown in Figure 17 and other control DNA's were not amplified.

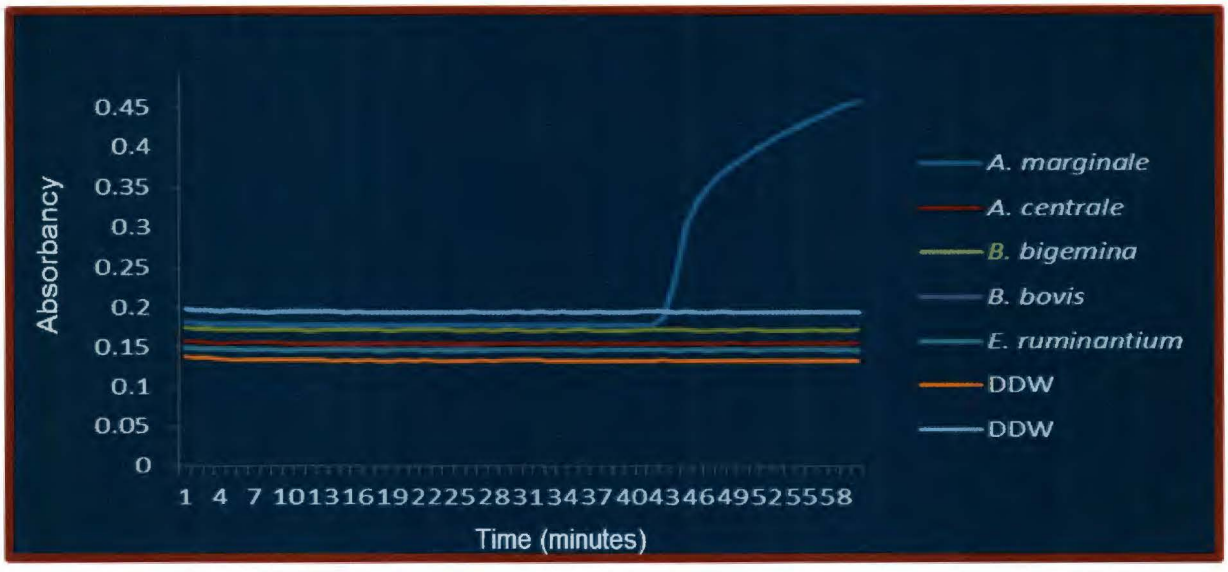


Figure 17: Specificity of LAMP assay at 65°C for 60 minutes using real-time turbidimeter.

4.3.3 Sensitivity of the LAMP assay

Sensitivity of LAMP primers was examined using 10-fold serial dilution of 10 ng/ul DNA. The LAMP products were analysed using 1% gel electrophoresis and visualized under the UV light as shown in Figure 18.

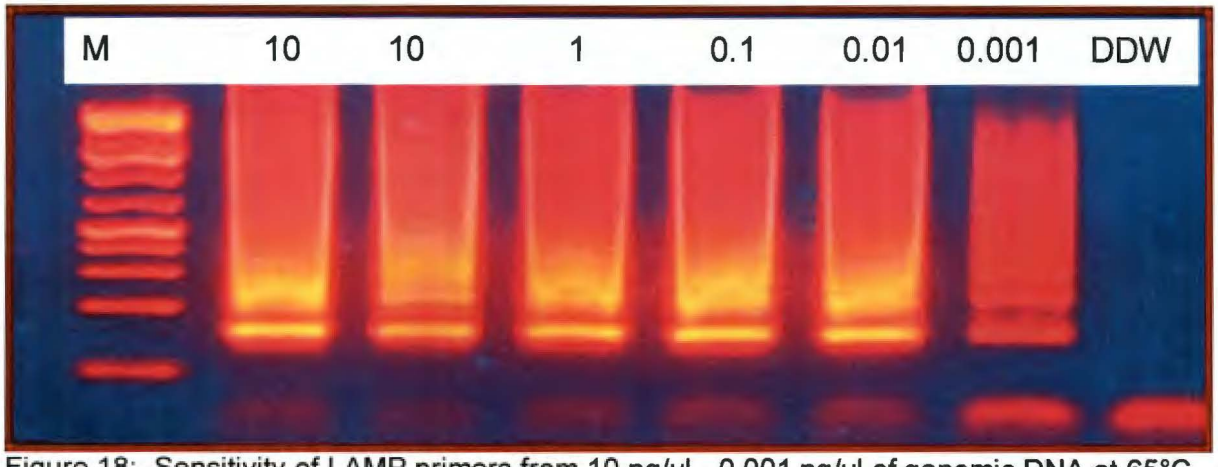


Figure 18: Sensitivity of LAMP primers from 10 ng/ul - 0.001 ng/ul of genomic DNA at 65°C for 60 minutes. Sensitivity of LAMP primers was examined using 10-fold serial dilution of 10 ng/ul DNA.

Sensitivity of LAMP primers was examined using 10-fold serial dilution of 10 ng/ul DNA. The reaction was observed on the real-time turbidimeter and the detection limit was 0.001 ng/ul as shown in Figure 19.

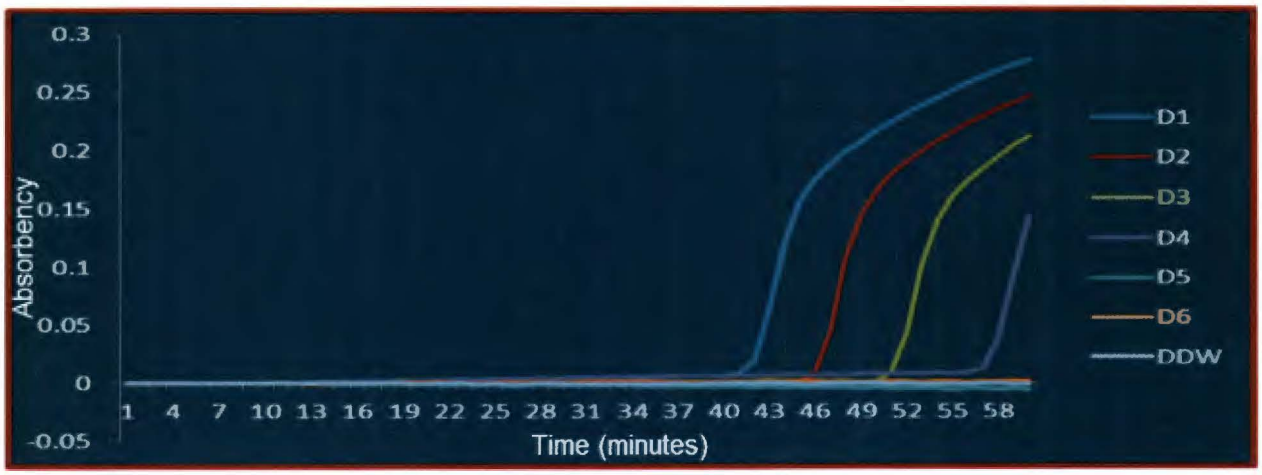


Figure 19: Sensitivity of LAMP primers with serially diluted genomic DNA from 10 ng/ul down to 0.001 ng/ul at 65°C for 60 minutes using real-time turbidimeter.

4.3.4 Challenges of the LAMP assay

The LAMP assay was further modified by addition of Loop primers (LF & LB) to increase the rate of the reaction, conditions explained in section 4.2.3 were adopted for the reaction, however false positive results were obtained, non-targeted DNA controls were amplified together with the negative control water.

4.3.4.1 Troubleshooting of the LAMP assay

a. **Excluding Loop primers**

LAMP assay was performed using only 4 primers in order to tackle the problem of false positive amplification; however the results showed false positive results.

b. **Additives**

The additives such as dimethyl sulfoxide (DMSO), Glycerol, Betaine, bovine serum albumin (BSA) and TE buffer were added to the reaction to increase the yield, specificity and consistency of the LAMP reaction for both sets of primers; 4 primers set and 6 primer set.

c. **Primer purification**

Primers were firstly manufactured at Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, S. A), and purified using cartridge method, the primer manufacturer was changed to Integrated DNA Technologies (IDT), Whitehead Scientific (PTY) Ltd (Johannesburg, S.A), high performance liquid chromatograph (HPLC) purification was used for the primer synthesis . However, false positive results were obtained.

d. **Re-evaluating the target region specificity of the LAMP assay using F3 and B3 primers**

The conventional PCR was performed using outer primers shown in Table 1 under the established conditions described in section 2.2.3, however only *A. marginale* amplified. The results are shown in appendix III.

4.4 DISCUSSION

LAMP is nucleic acid amplification with advantages of being rapid, easy to use, and less expensive than other nucleic acid-based tests for the diagnosis of infectious diseases. It therefore has the potential for use in resource-limited veterinary laboratories in developing countries, such as African countries where many endemic diseases such as anaplasmosis, babesiosis and theileriosis exist.

The LAMP assay protocol was established through applying gradient temperatures and incubation time was set at 60 minutes, the reaction could successfully take place in temperatures ranging from 63 - 65°C. The optimal reaction conditions were then chosen as 65°C for 60 minutes. This reaction temperature is within the reported optimal reaction range of 60 - 65°C for LAMP (Notomi et al., 2000, Thekisoe *et al.*, 2009, Thekisoe *et al.*, 2010)

The LAMP assay under the established optimal conditions was further tested for specificity using bovine haemoparasites *A. centrale*, *B. bigemina*, *B. bovis*, and *E. ruminantium*. The LAMP primers specifical produced positive LAMP amplicons of typically ladder patterns in the gel electrophoresis from genomic DNA of *A. marginale,* while there were no products from DNA of other haemoparasites as well as water control. The amplified product gave a major amplicon of expected size 181bp. When the reaction was performed in a similar manner for primer specificity using the real-time turbidimeter, the specificity of LAMP primers was formally, there was no cross-reaction with other haemoparasites. *Anaplasma marginale* DNA was amplified within 42 minutes at 65°C.

The LAMP primers were examined for sensitivity using 10-fold serially diluted genomic DNA, the reaction was conducted at 65°C for 60 minutes. The assay showed high sensitivity with the detection limit of 0.001 pg/ul. The assay was further conducted using real-time turbidimeter the initial genomic DNA was 1.0 ng/ul. The assay sensitivity was similar with the results ran on the gel electrophoresis giving the same detection limit which is equivalent to 1 pg/ul.

Challenges of the LAMP assay included the amplification of the false positives, *A. centrale*, *B. bigemina, B. bovis*, *E. ruminantium* and water. To solve the problem troubleshooting, involved addition of additives mentioned in section 4.3.4.1; as the additives DMSO and

formamide are known to be helpful when amplifying GC-rich DNA that form secondary structures which can cause DNA polymerase to standstill , therefore they reduce the amount of energy required to separate the double stranded DNA template (Rees *et al.*, 1993) and in poor amplification the additives give rise to high yield of amplified product (Varadaraj and Skinner, 1994) .However in this study the addition of additives did not improve the specificity and consistency of the LAMP reaction whether using four set of primers or including the loop primers, the use of additives either individually or in combination did not yield expected results. In contrast a study by Nagai *et al.*, (1998), showed the effectiveness of BSA and glycerol on PCR when amplifying an enterohemorrgagic *Escherichia coli*, this study demonstrated that the additives individually enhance the amplification efficiency of the target DNA.

Hypotheses was that primers could be possibly not stable, as a results ending up self-priming and giving false positive results, therefore to improve the stability of the primers the primer cartridge purification method offered at Inqaba biotechnical company was altered and primers were synthesized at Whitehead scientific (IDT) and purified using high performance liquid chromatography (HPLC), however false positives results were obtained. This then led into re-evaluating of the outer primers (F3 and B3) by performing the developed conventional PCR assay described in chapter 2. The results obtained showed specificity with primers from both different manufacturer companies (appendix III). The conclusions were that although LAMP assay had shown great performance and specificity with results obtained from the first reactions, the possible problem could be contamination of the working area, although LAMP is tolerant to inhibitory substances (Boehme *et al.*, 2007), its high specificity and production of high LAMP products, can lead to possible contamination of the whole laboratory if special precautions are not considered through the examination of the LAMP product. The second hypothesis is that inner primers of the LAMP assay (FIP, BIP, LF and LB) could be binding to themselves as a result giving false positive results, in contrast when performing PCR with LAMP outer primers the expected specific amplification of *A. marginale* results are obtained (appendix III).

Nevertheless, sequencing of the inner regions of LAMP primers using outer primers (F3 and B3) for examining the homogeneity. The sequences obtained in this study were homologous to those in NCBI database, therefore confirming that the LAMP primers target

region for *A. marginale* is specific. However, LAMP assays developed in this study need further troubleshooting in order to solve the problem of false positives.

CHAPTER 5

5. GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 General discussion

Anaplasma marginale is endemic in South Africa, consequently meaning that there is high number of cattle which serve as reservoirs for continuous infection with *A. marginale* which can spell an outbreak in cases of cattle movement or trading (Mtshali *et al.*, 2007; Kocan *et al.*, 2010). The fact that *A. marginale* can persistently infect the recovered cattle function as risk for health management in livestock industries. Anaplasmosis presents many problems to the cattle industry due to complications with the disease control, eradication and treatment. When vaccinating cattle of unknown disease status, hygienic animal husbandry techniques are highly recommended (Reinbold *et al.*, 2010). Therefore it is very imperative to know the correct disease status of cattle.

Genetic diversity of *A. marginale* strains presents a major barrier in the development of control measures that confer full protection. This is due to increasing number of *A. marginale* genotypes caused by cattle movement within different regions (de la Fuente *et al.*, 2007 and 2009). Therefore development of diagnostic methods that are highly sensitive and specific are of greater necessity for correct diagnosis of *A. marginale* infected cattle.

In this study, the development of three assays, that is PCR, real-time PCR and LAMP for detection of *A. marginale* infections in cattle in South Africa was successful. The developed PCR, real time PCR and LAMP assays were specific, only *A. marginale* was amplified and there was no cross reaction with other haemoparasites including *A. centrale*, *B. bigemina*, *B. bovis* and *E. ruminantium*. The assays showed high sensitivity PCR: 0.01 ng/ul, real-time PCR: 0.01 and 0.001 ng/ul primer set two was than chosen with 0.001 ng/ul detection limit, LAMP: 0.001 ng/ul which is equivalent to 1 pg/ul, the sensitivity of the assays were taken as the detection limit of the assays. Although PCR was specific and sensitive, it detection limit was lower than of real-time PCR and LAMP assay as they could detect up to 1 pg/ul of serially diluted *A. marginale* DNA.

Validation of PCR assay on samples collected from the field confirmed the less sensitivity of PCR assay (low infection rates within Provinces). Polymerase chain reaction detected

86/144 (60%) of *A. marginale* infections in DNA samples collected from different regions of South African Provinces, when comparing the detected infection rate of *A. marginale* to that detected using the real-time PCR 107/144 (74%) it shows that conventional PCR assay has limitations in detection of *A. marginale* infections during cases of low parasitemia in red blood cells usually in early infection and also in carrier cattle (Stich *et al.*, 1993; Decaro *et al.*, 2008; Vahid *et al.*, 2010). Aubry *et al* (2011) stated that although PCR assay are sensitive, but they can still misdiagnose a proportion of carrier cattle that would test negative. The PCR assay described in this study has high specificity. When compared to the previously developed PCR assays which mainly targeted the conserved *Anaplasma* regions (Stich *et al.*, 1993) as *A. centrale* is genetically similar to *A. marginale*, therefore not differentiating between *Anaplasma* species.

The real-time PCR assay developed showed high specificity and sensitivity with the detection limit of 1 pg/ul. Real-time PCR has a good reputation in terms of high sensitivity and specificity (Carelli *et al.*, 2007, Decaro *et al.*, 2008 Bhoora *et al.*, 2010). When comparing the sensitivity to that of LAMP assay, both methods had similar detection limit, this is the first study showing similar sensitivity between real-time PCR and LAMP assay. However, real-time PCR showed good efficiency (99.26%) which fall in between the optimal 90 -100% with the slope gradient of Y= -3.34. The real-time PCR assay proved to be of high sensitivity by detecting more positive samples of *A. marginale* infections 107/144 (74%) when compared to those of PCR assay. The DNA samples which were negative with PCR, real-time PCR assay detected them as *A. marginale* positively infected. Real-time PCR assay positively detected few samples few samples from Northern Cape Province with *A. marginale* infections, although it was speculated that it an anaplasmosis free area. This shows outstanding ability to detect cases of low parasitemia or carrier cattle.

The LAMP assay developed in this study showed high specificity and sensitivity. The specificity was confirmed by sequencing the region within the F3 and B3 (which are also forward and reverse primer for PCR). The detection limit of LAMP assay was equivalent to that of real-time PCR, the amplification of genomic DNA was achieved at 43 minutes when using the real-time turbidimeter which is a rapid type of detection with no post manipulation of results, although the LAMP assay is said to be time efficient, cost effective real-time turbidimeter is expensive, but it was good in proving the detection initial amplification time of *A. marginale*. However the LAMP assay is easy to perform, rapid sensitive and gives

several option of endpoint detection methods that is gel electrophoresis or real-time turbidimeter. The LAMP assay was further modified by including loop primers (results not shown), the addition of loop primers brought cross reactivity, and other haemoparasites were amplified, speculations of this matter were that LAMP product would have possibly contaminated the laboratory of the inner primers are self-priming, more work needs to be done in order to achieve a reliable LAMP assay that gives ideal results at all times.

5.2 CONCLUSIONS

Following the objectives of this study which seeked development of molecular diagnostic assays, namely, conventional PCR, real-time PCR and LAMP for detection of *A. marginale* which causes bovine anaplasmosis in cattle, this study has successfully developed the 3 assays targeting the *msp1b* gene. The three assays specifically amplify *A. marginale* DNA only and have reasonable detection limits. Furthermore, the assays are capable of detecting *A. marginale* infections from DNA extracted from blood samples collected from different Provinces of South Africa.

Similar to previous reports, real-time PCR and LAMP showed higher sensitivity than conventional PCR. However, most importantly all 3 assays have showed potential to detect *A. marginale* from samples collected from cattle. The conventional PCR requires further improvements, whilst LAMP also needs improvements to prevent false positive amplifications. As for the real-time PCR assay developed in this study seems to be perfect and ready for use in diagnosis.

With observation of clinical signs as the first line of disease diagnosis and microscopy being a widely applied diagnostic technique, followed by serological assays such as IFAT and ELISA which are also widely applied in epidemiological studies. The molecular diagnostic assays developed in the current study can be used to supplement the above-mentioned traditional diagnostic methods. Furthermore, the targeted fragment of the gene *msp1b* where the primers of the 3 assays have been designed is conserved amongst South African strains. This is an advantage ensuring that assays developed in this study will not be affected by the widely reported genetic diversity of *A. marginale*.

5.3 RECOMMENDATIONS

- Sensitivity of conventional PCR needs to be improved, possibly with the use of different PCR kits modifying the assay into a nested PCR assay.
- The source of LAMP false positives needs to be determined and sorted out.
- All the assays developed in this study need to be further evaluated with more field samples in order to validate their potential for use in large scale of epidemiological studies.
- It will also be interesting to determine if molecular assays developed in this study can amplify DNA of blood samples collected from other countries.

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APPENDICES

Appendix I: *Anaplasma marginale* sequences of South African strains.

>GP_C9_LA _*Anaplasma marginale* major surface protein 1 beta 1 (*msp1b1*) gene
ACACCTGACACTGGTGAGCGGGTTGTTGTGCAGTATGGTGAGGAGAGAGAATTTGGC
AAGGCAGCGGCTTGGGGTCTAGCAGGCTTCAAGCGTACAGTGGATGAAAGCCTGGAG
ATGTTAGGCCGAGGCATGAACATGCTCGCGGAAGGCCAGGCACAGATATCACAGGGG
ATTGCAGACAAACTTATTTCTCCCTGTTTAATTCCCCGTCATAGTGACCAAGAAAATGC
CCAAACTTCTACTTGAACTTAATTCGATTCTGACTATCTCTCTTGTCACATTCATCTCCT
TCTAAAACCAAAAAATGATAGGATTACCTTTTC

>KZN_C9_LA_*Anaplasma marginale* major surface protein 1 beta 1 (*msp1b1*) gene
TATCGCACAACTGACCTGGTGAGCGGTTTGTTGTGCAGCATGGTGAGGACAAAGAATT
TGGCAAGGCACCAGCCTGGGGTCTAGCAGCTTTCAAGCGTACAGTGGATGAAGCCTG
GAGATGTTAGACCGAGGCATGCACATGCTCGCGGAAGGCCAGGCACAGATATCACAG
GGGATTGCAGACAA

>LP_C9_LA_*Anaplasma marginale* major surface protein 1 beta 1 (*msp1b1*) gene
GCCTGGGCCTAACATCTCCAGGCTTTCATCCACTGTACGCTTGAAGCCTGCTAGACCC
CAGGCTGCTGCCTTGCCAAATTCTCCCTCCTCACCATATTGCACAACAACCCGCTCAC
CAGTGTCAGGTGCTTCATCCATGAACGGTATGTTGTCAATGCTCTGGTCAAGGA

>C1_MP_LA_F3_ *Anaplasma marginale* major surface protein 1 beta 1 (*msp1b1*) gene
GCYTGGGGTCTAGCAGGCTTCAAGCGTACAGTGGATGAAAGCCTGGAGATGTTAGAC
CGAGGCATGCACATGCTCGCGGAAGGCCAGGCACAGATATCACAGGGGATTGCAGAC
A

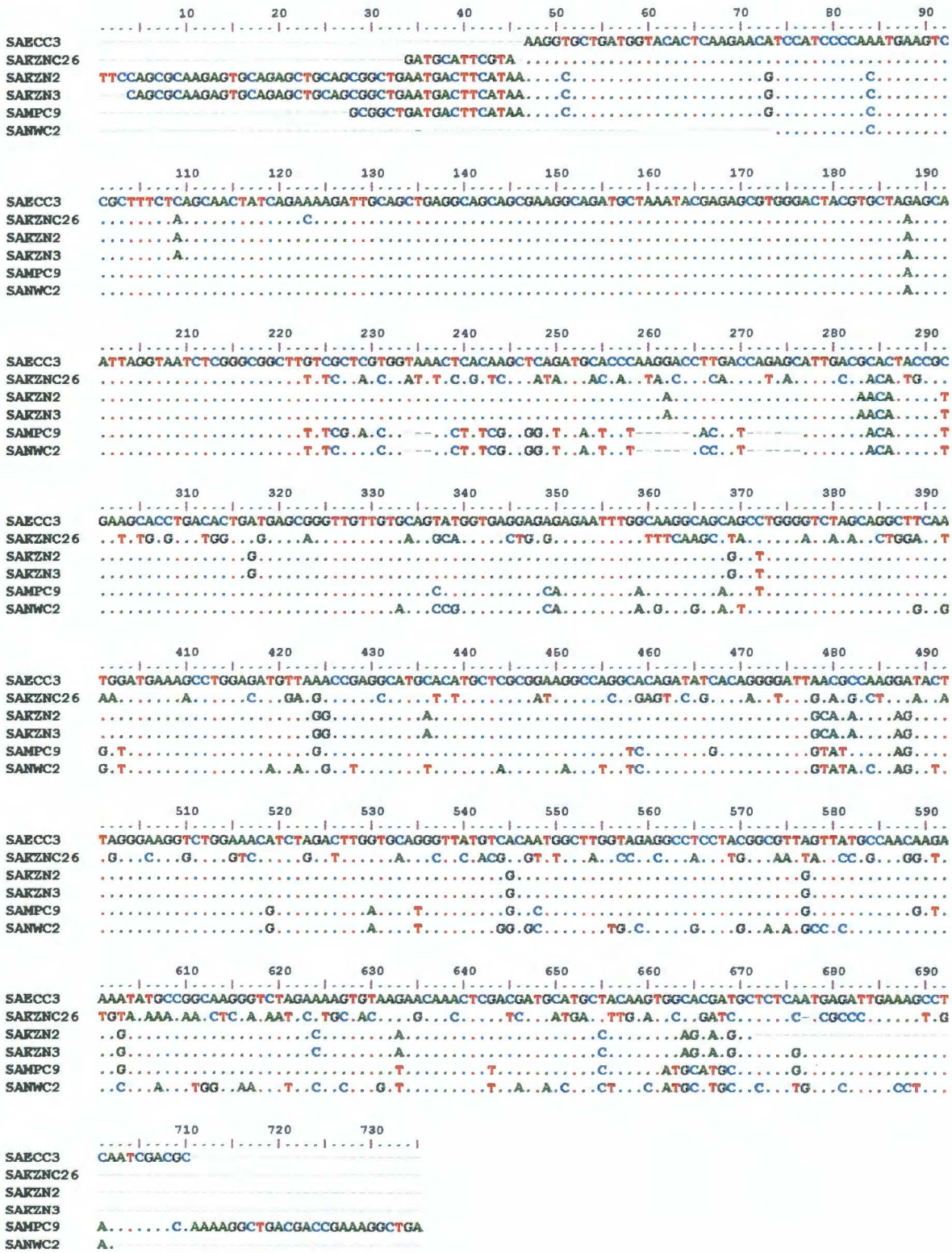
>GP_C1_LA_ F3_ *Anaplasma marginale* major surface protein 1 beta 1 (*msp1b1*) gene
AGCACTGACACTGGTGAGCGGGTTGTTGTGCAGTATGGTGAGGAGAGAGAATTTGGC
AAGGCAGCCGCtTGGGGTCTAGCAGGCTTCAAGCGTACAGTGGATGAAAGCCTGGAG
ATGTTAGGCCGAGGCATGAACATGCTCGCGGAAGGCCAGGCACAGATATCACAGGGG
ATTGCAGACA

>KZN_C2_LA_ F3_ *Anaplasma marginale* major surface protein 1 beta 1 (*msp1b1*) gene
CGCAGCACTGACACTGGTGAGCGGGTTGTTGTGCAGTATGGTGAGGAGAGAGAATTT
GGCAAGGCAGCGGCTTGGGGTCTAGCAGGCTTCAAGCGTACAGTGGATGAAAGCCTG
GAGATGTTAGGCCGAGGCATGAACATGCTCGCGGAAGGCCAGGCACAGATATCACAG
GGGATTGCAGACA

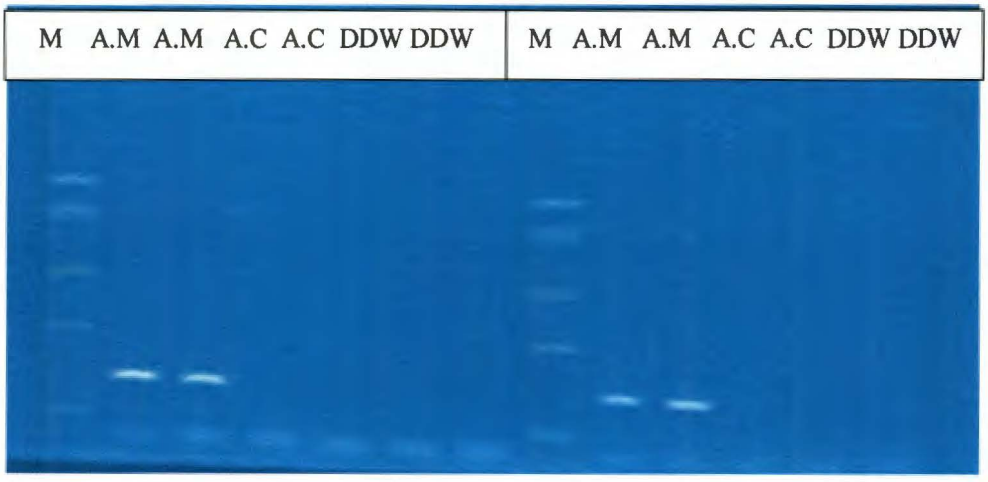
>LP_10_LA_ F3_ *Anaplasma marginale* major surface protein 1 beta 1 (*msp1b1*) gene
AKCAGCACCTGACACTGGTGAGCAGGTTGTTGTAGCAGTMKGGTGAGGAGMGAGAAT
TTGGCAAGGCAGCRGCCTGGGGTCTAGCAGCTTCAAGCGTACAGTGGATGAAAGCCT
GGAGATGTTAGGCCGAGGCATGAACATGCTCGCGGAAGGCCAGGCACAGATATCACA
GGGGATTGCAGACAA

>NW_C4_LA_ F3_ *Anaplasma marginale* major surface protein 1 beta 1 (*msp1b1*) gene
CSAGCACTGACACTGGTGAGCGGGTTGTTGTGCAGTATGGTGAGGAGAGAGAATTTG
GCAAGGCAGCAGCCTGGGGTCTAGCAGGCTTCAAGCGTACAGTGGATGAAAGCCTGG
AGATGTTAGGCCGAGGCATGAACATGCTCGCGGAAGGCCAGGCACAGATATCACAGG
GGATTGCAGACA

Appedix II: Alignment of sequences using BioEdit software.


SAECC3
SARZNC26
SARZN2
SARZN3
SAMPC9
SANWC2

Appendix III: PCR amplification of *A. marginale* using F3 and B3 primers from Inqababiotehnical and IDT.



M- Marker

A.M- *A. marginale*

A.C- *A. centrale*

DDW- double distilled water