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Occurrence and genetic diversity of *Anaplasma marginale* in cattle from two diptanks in Zambezia Province, Mozambique

By

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Table of Contents

LIST OF FIGURES iii

LIST OF TABLES iv

DEDICATION v

ACKNOWLEDGEMENTS vi

DECLARATION vii

ABSTRACT ix

ABBREVIATIONS x

1 INTRODUCTION 1

2 LITERATURE REVIEW 3

2.1 The organism 4

2.2 The disease 5

2.3 The tick vectors 6

2.4 Control of Anaplasmosis 7

2.5 Detection of A. marginale and A. marginale subsp. centrale 8

2.6 Strain differentiation using *msp1α* gene sequence analysis 10

2.7 Benefits arising from the project 11

2.8 Hypothesis 11

2.9 Aims/Objectives 11

2.9.1 Specific Aims: 11

3 MATERIALS AND METHODS 12

3.1 Sample collection 12

3.2 Quantitative real-time PCR (qPCR) for detection of *A. marginale* and *A. marginale* subsp. *centrale* 14

3.3 Strain differentiation using *msp1α* gene sequence analysis. 14

3.3.1 Amplification of the *msp1α* gene 15

3.3.2 Purification of *msp1α* PCR products 15

3.3.3 Cloning of the *msp1α* PCR products 15

3.3.4 Sequence Analysis 18

3.4 Statistical analysis 19

4 RESULTS 20

4.1 Detection of *A. marginale* and *A. marginale* subsp. *centrale* using duplex quantitative real-time PCR (qPCR) 20

4.1.1 Detection of *A. marginale* 20

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4.1.2 Detection of *A. marginale* susbsp. *centrale* 21
4.2 Amplification of the *msp1α* gene 26
4.2.1 Selection of samples and amplification of the *msp1α* gene 26
4.2.2 Purification of *msp1α* PCR products 27
4.3 Cloning 28
4.3.1 Colony PCR 29
4.4 Sequence assembly and analysis 31
5 DISCUSSION 38
6 CONCLUSION 42
7 REFERENCES 43
APPENDIX 1: Animal Ethics Certificate 49
APPENDIX 2: Letter of permission to use DNA samples from Mozambique 50
APPENDIX 3: Protocol for blood sample collection in Mozambique 51
APPENDIX 4: Veterinary Import Permit for DNA samples from Mozambique 52
APPENDIX 5: Determination of DNA concentration of *msp1α* PCR products 53
APPENDIX 6: Reverse Line Blot Results 55

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LIST OF FIGURES

Figure 1: Map indicating the location of Botao and Namitangurine diptanks in the Zambezia Province in Mozambique. 13

Figure 2: An example of LC-530 fluorescence generated in Anaplasma marginale positive samples, measured at 530 nm. 20

Figure 3: An example of LC-610 fluorescence generated in Anaplasma marginale subsp. centrale positive samples, measured at 610 nm. 21

Figure 4: Amplification of the msp1α gene from samples 90, 91, 92, 58, 64 (Panel A) and samples 49, 84, 86, 51, 33, 96, 97, 4, 46, 45 (Panel B). PCR products(750 - 1000 bp) were separated on a 1% agarose gel stained with ethidium bromide. The size of the PCR products was determined by comparison with a 1 kb molecular weight marker (Fermentas) (1kb MWM). Positive (DNA from Anaplasma marginale positive sample 208291) and negative (ddH_20) controls were included in all amplification reactions. 27

Figure 5: Purification of msp1α PCR products using the QIAquick® PCR purification kit (Qiagen) from samples 90, 91, 92, 58, 64 (Panel A) and samples 49, 84, 86, 51, 33, 96, 97, 4, 46, 45 (Panel B). PCR products were separated on a 1% agarose gel stained with ethidium bromide. The size of the PCR products was determined by comparison with a 1 kb molecular weight marker (Fermentas) (1kb MWM). 28

Figure 6: Cloning of msp1α PCR products. Plates labelled A show clones from 50 µl of the transformation mix and plates labelled B show results of the rest of the transformation mix (205 µl) after incubation overnight at 37°C. Only E. coli cells containing recombinant plasmids can grow on the agar plates containing ampicillin, as the plasmid confers ampicillin resistance to the E. coli, and the DNA insert disrupts the lethal gene. 29

Figure 7 : Colony PCR for samples 49, 84, 86 (A), 51, 33, 96 ,97 (B), 90, 91, 92 (C), and 58 and 64 (D) using primers pJet 1.2F and pJet 1.2R, resulting in amplification of a 1000 bp insert from colonies containing recombinant plasmids..... 30

Figure 8: ClustalX alignment of novel MSP1a repeats (UP37-UP50) identified in this study with the first MSP1a repeat identified, the A repeat (Allred et al., 1990). Identical amino acid residues in the alignment are shown by black text on a white background; residues that differ from the A repeat are indicated by white text on a black background. 31

Figure 9: Number of msp1α genotypes per animal at Botao diptank (A), Namitangurine diptank (B) and overall (C). 35

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LIST OF TABLES

Table 1: Real-time duplex qPCR results for the 50 samples from Botao diptank. Samples selected for further analysis are highlighted in yellow. 22

Table 2: Real-time duplex qPCR results for the 50 samples from Namitangurine diptank. Samples selected for further analysis are highlighted in yellow. 24

Table 3: MSP1a repeat structure (msp1α genotype) of strains found per sample from each of the diptanks. Novel MSP1a repeats identified in this study are given in red font. 32

Table 4: Analysis of MSP1a repeats of Anaplasma marginale strains from different countries around the world, accessed from the database generated in RepeatAnalyzer (Catanese *et al*., 2016). 36

Table 5: Analysis of msp1α genotypes of *Anaplasma marginale* strains from different countries around the world accessed from the database generated in RepeatAnalyzer (Catanese *et al*., 2016)... 37

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DEDICATION

To my children Maxwell Bukhosi and Shekinah Nobukhosi Phili – you are my greatest inspiration in life. I will always love you.

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DECLARATION

I, Sizanobuhle L. Nyoni-Phili (u15393713) hereby declare that this thesis has been the result of my original efforts and investigations and such work has not been presented elsewhere for any degree. All additional sources of information have been acknowledged by means of references.

……………………………………

Sizanobuhle L. Nyoni-Phili

Date……………………... 10/07/2017

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Occurrence and genetic diversity of *Anaplasma marginale* in cattle from two diptanks in Zambezia Province, Mozambique

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ABSTRACT

Anaplasmosis is a tick-borne disease that can result in serious loss of production and even loss of livestock. It is caused by the most globally prevalent vector-borne pathogen of livestock, *Anaplasma marginale*, with endemic regions in all six permanently inhabited continents. This study examined the occurrence and diversity of *A. marginale* in cattle (n= 100) opportunistically sampled at two diptanks (Botao, n=50, and Namitangurine, n=50) in Zambezia Province, Mozambique. A duplex quantitative real-time PCR was used to detect *A. marginale* and *A. marginale* subsp. *centrale* in cattle samples from the two diptanks. The overall prevalence of *A. marginale* was 87% (95% CI: 80, 93%). There was no significant difference (p>0.05) between the prevalence of *A. marginale* in cattle at Namitangurine diptank (88.0%, n=42) and Botao diptank (86.0%, n=43). The overall prevalence of *A. marginale* subsp. *centrale* was 6.0% (95% CI: 2.0, 11%). Only 2.0% (n=1) of cattle sampled from Botao diptank were positive for *A. marginale* subsp. *centrale*, while 10% (n=5) were positive for *A. marginale* subsp. *centrale* at Namitangurime diptank. There was no significant association (p>0.05) between *A. marginale* subsp. *centrale* presence in cattle and diptank. In order to assess the genetic variability in *A. marginale*, *msp1α* amplicons were sequenced from 27 samples from the two diptanks. Fourteen novel MSP1a repeat sequences were identified. Most samples had mixed infections with one to eight *msp1α* genotypes identified in individual animals. A total of 47 different *msp1α* genotypes were found from 76 *msp1α* sequences generated from the 27 samples. This considerable genetic diversity contributes to the understanding of the regional diversity of *A. marginale* and will be important for the development of appropriate and effective vaccines in the future. In future research, the gene sequences of eight highly promising vaccine candidates will be examined from these samples. In addition, the results will be compared to equivalent results from cattle samples from an area in the Limpopo National Park where cattle are grazed alongside wildlife, to see if the presence of wildlife affects the diversity of the A. marginale population in cattle.

Key words: *Anaplasma marginale,* real-time PCR, *msp1α,* genetic diversity

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ABBREVIATIONS

Cp	crossing Point
DNA	deoxyribonucleic acid
hrs.	hours
min	minutes
MSP1a	major surface protein 1a
msp1α	the gene encoding MSP1a
PCR	polymerase chain reaction
qPCR	quantitative real-time polymerase chain reaction
sec	seconds

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1 INTRODUCTION

Anaplasma marginale is the most globally prevalent vector-borne pathogen of livestock, with endemic regions in all of the six permanently inhabited continents (Brayton *et al.*, 2009). Bovine anaplasmosis results from *Anaplasma marginale* infection (OIE, 2012). *Anaplasma marginale* subsp. *centrale* causes a milder form of anaplasmosis (Potgieter and Stoltsz, 2004), and is used as a vaccine in many countries. *Anaplasma marginale* is responsible for almost all outbreaks of clinical disease. Anaplasmosis is characterised by anaemia and jaundice but the clinical disease can only be confirmed by identifying the organism (OIE, 2012). Once infected, cattle may remain carriers for life, and identification of these animals depends on the detection of specific antibodies using serological tests, or rickettsial DNA using molecular techniques.

The distribution and prevalence of tick-borne diseases in Mozambique is still not clearly understood (Alfredo *et al.*, 2005). A large number of cattle mortalities in southern Africa, including Mozambique, are caused by tick-borne diseases such as anaplasmosis, babesiosis, heartwater and theileriosis. Tick-borne diseases can be controlled through a combination of the strategic use of acaricides and application of vaccines. A live blood vaccine containing *A. marginale* subsp. *centrale* is available, but it has not been widely used in Mozambique. After the end of the civil war in Mozambique in 1992, cattle were imported from Zimbabwe and South Africa, and dipping services were reactivated (Tembue *et al.*, 2011). However, mortalities of up to 50% were observed in imported cattle and these mortalities were largely attributed to inadequate control of tick-borne diseases.

A lot of research has been done in recent years towards the development of a recombinant vaccine. It is known that the outer membrane preparations of *A. marginale* induce protection from disease in nearly all animals that have been tested (Palmer *et al*., 1986; 1988; 1989; 1999). These studies demonstrate that it would potentially be possible to design a subunit vaccine based on the components of the outer membrane that induce immunity. Eight highly promising vaccine candidates have been identified primarily from strains of *A. marginale* from the United States of America (USA) (Lopez *et al.,* 2005;

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Noh *et al.,* 2010; Agnes *et al.,* 2011). While it is known that these vaccine candidate genes are conserved amongst *A. marginale* strains from the USA (Dark *et al.,* 2009), it is not known if these candidates are sufficiently conserved to be broadly useful in other parts of the world or if vaccine development based on regional pathogen strains is necessary. In order to study the vaccine candidate genes in *A. marginale* from other regions, *A. marginale* positive samples must first be identified from cattle in the field, and genetically diverse *A. marginale* field strains should be selected from the positive samples. Genotyping can be done using *msp1α* gene analysis, since, within a herd situation, variation in MSP1a repeat structure has been shown to be indicative of sequence variation in antigenically important genes (Rodriguez *et al*., 2005). In a first step towards examining the diversity of *Anaplasma* strains in Mozambique, bovine field samples from Zambezia Province that are positive for *A. marginale* and *A. marginale* subsp. *centrale* were identified using a duplex qPCR assay (Carelli *et al*., 2007; Decaro *et al*., 2008), and the genetic diversity amongst *A. marginale* positive samples was determined using *msp1α* gene sequencing. Future studies will compare the results to equivalent results from cattle samples from an area in the Limpopo National Park where cattle are grazed alongside wildlife to see if the presence of wildlife affects the diversity of the *A. marginale* population in cattle. In addition, the gene sequences of the vaccine candidates from these samples will be examined.

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2 LITERATURE REVIEW

Tick-borne diseases are spread between animals by the bite of an infected tick. Ticks become infected when they feed on the blood of animals that are either sick from the disease or carriers, and infected ticks transmit the parasites through their saliva when feeding on uninfected animals (Turton, 1994). Tick-borne diseases can cause heavy losses of animals and can prevent the introduction of high-producing animals to upgrade or replace local stock. Tick-borne diseases have common occurrences in both the medical and veterinary clinical settings. It is often very difficult to control and prevent tick-borne diseases because it requires the disruption of a complex transmission chain, involving both vertebrate hosts and ticks, which interact in a constantly changing environment (Torres *et al.*, 2014). Tick-borne diseases are caused by infection with a variety of pathogens that include rickettsia, bacteria, viruses and protozoa. Tick-borne diseases that are commonly associated with cattle in southern Africa include anaplasmosis, babesiosis, heartwater and theileriosis.

Bovine anaplasmosis, caused by *Anaplasma marginale,* is the most globally prevalent of the virulent haemoparasites of ruminant livestock, and it is endemic in most of the cattle-farming areas in southern Africa (Potgieter and Stoltsz, 2004; Mtshali *et al.*, 2004; 2007; Stevens, 2007; Marufu *et al.*, 2010). Five tick species have been implicated in the transmission of *A. marginale* in South Africa, *Rhipicephalus decoloratus*, *Rhipicephalus microplus*, *Rhipicephalus evertsi evertsi*, *Hyalomma marginatum rufipes* and *Rhipicephalus simus* (Potgieter *et al.*, 1981; Potgieter and Stoltsz, 2004). In southern Africa, the mechanical transmission of *Anaplasma* spp. has not been studied in detail. In Zimbabwe it is thought that, after the introduction of anaplasmosis by infected ticks, mechanical transmission may play a significant role in the epidemiology of the disease (Tembue *et al.*, 2011). In related research done in South Africa it was shown that close social association of cattle, in dairy herds and feedlots, promotes mechanical transmission by flies after a tick-transmitted outbreak has occurred (Tembue *et al.*, 2011).

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In Mozambique livestock production grew significantly in the mid-1990s (Tembue *et al.*, 2011) due to importation of cattle from Zimbabwe and South Africa, and also due to reactivation of dipping services after the end of the civil war in 1992. However, a mortality rate of 50% was observed in imported cattle and this has been largely attributed to inadequate control of tick-borne diseases, namely anaplasmosis, babesiosis and cowdriosis. The distribution and prevalence of these tick-borne diseases at the moment is still not clearly understood (Alfredo *et al.*, 2005).

2.1 The organism

Anaplasma marginale occurs in most tropical and subtropical countries, and in some more temperate regions. The organism was first identified as a distinct pathogen by Sir Arnold Theiler in South Africa (Theiler, 1910a). Theiler (1910b) differentiated this parasite from the *Piroplasma bigeminum* by the differences in their incubation period, anaplasmosis has a longer incubation period than babesiosis; the parasite present at the periphery of the red blood cells coincided with onset of the fever and disappeared when the animal recovered; in anaplasmosis there was no haemoglobinuria; also the absence of marginal points in blood samples from cattle from England, and the animals infected with *Anaplasma* produced a fever reaction. He later discovered another variety of *Anaplasma* which he named *Anaplasma marginale* variety *centrale* which appeared at a more central point of the red blood cell and was less virulent than *A. marginale.* Theiler (1912) noticed that animals injected with this subspecies did not suffer as severely as those injected with *A. marginale*, and therefore *A. marginale* subsp. *centrale* can be used as a vaccine against anaplasmosis. The organism has since been imported by other countries, including Australia and some countries in South America, South-East Asia and the Middle East, for use as a vaccine against *A. marginale* (Uilenberg, 1995)

Anaplasma species were originally regarded as protozoan parasites, but later research showed they had no significant attributes to justify this description. Since the last major accepted revision of the taxonomy in 2001 (Dumler *et al.*, 2001), the Family *Anaplasmataceae* (Order *Rickettsiales*) is now composed of four genera, *Anaplasma*, *Ehrlichia, Neorickettsia,* and *Wolbachia*. The genus

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Aegyptianella is retained within the Family *Anaplasmataceae* as genus *incertae sedis*. The revised genus *Anaplasma* now contains *A. marginale* as the type species, *A. phagocytophilum* (formerly known as *Ehrlichia phagocytophila*, *E. equi* and the unclassified agent of human granulocytic ehrlichiosis), *A. platys,* and *A. bovis*. *Haemobartonella* and *Eperythrozoon* are now considered most closely related to the mycoplasmas (Dumler *et al.*, 2001).

Taxonomic classification of *Anaplasma* spp. (Dumler *et al.*, 2001; Brayton *et al.*, 2009)

Superkingdom	Bacteria
Phylum	Proteobacteria
Class	Alpha-proteobacteria
Order	Rickettsiales
Family	Anaplasmataceae
Genus	*Anaplasma*
Species	*A. marginale* (type species)
	A. marginale subsp. *centrale*
	A. bovis
	A. ovis
	A. phagocytophilum
	A. platys

2.2 The disease

Anaplasma marginale invades and destroys the red blood corpuscles causing primarily an acute oligocythaemia accompanied by high fever, progressive anaemia and degeneration of all parenchymatous organs (Theiler, 1910a; Potgieter and Stoltsz, 2004). In anaplasmosis there is no haemoglobinuria and haemoglobinaemia and this helps in the differential diagnosis of anaplasmosis from babesiosis which is normally endemic in the same region. Recovery from the disease gives resistance to subsequent infections (Theiler, 1910a). The immune animal acts as a reservoir for the

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organism and the blue tick *(Rhipicephamlus decoloratus*) acts as host or transmitter of the parasite. Outbreaks of bovine anaplasmosis are due to *A. marginale*. *Anaplasma marginale* subsp. *centrale* produces mild anaemia but clinical outbreaks in the field are extremely rare. *A. phagocytophilum*, with a reservoir in rodents, rarely infects cattle (Dreher *et al.*, 2005). Anaplasmosis is widely distributed in the world and is endemic in most cattle-farming areas in southern Africa.

2.3 The tick vectors

Minjauw (2001) states that "*A. marginale* stands out among the major vector-borne livestock pathogens (*Anaplasma*, *Babesia*, *Ehrlichia, Theileria*, and *Trypanosoma*) due to its transmissibility by multiple vectors". *Anaplasma* species are transmitted either mechanically or biologically by arthropod vectors. Reviews based on careful study of reported transmission experiments list up to 19 different ticks as capable of transmitting *A. marginale* experimentally (Kocan *et al.*, 2004). These include *Argas persicus, Ornithodoros lahorensis, Rhipicephalus annulatus, B. calcaratus, R. decoloratus, R. (B. microplus, Dermacentor albipictus, D. andersoni, D. hunteri, D. occidentalis, D. variabilis, Hyalomma excavatum, H. rufipes, Ixodes ricinus, I. scapularis, R. bursa*. Intrastadial or transstadial transmission is the usual mode, even in the one-host *Rhipicephalus (Boophilus)* species. Male ticks (of one-host ticks) are particularly important as vectors because they can become persistently infected as they move between hosts to find mates and serve as a reservoir for infection (Aguirre *et al*., 1994). Experimental demonstration of vector competence does not necessarily imply a role in transmission in the field. However, *Rhipicephalus* species are clearly important vectors of anaplasmosis in countries such as Australia and countries in Africa, and some species of *Dermacentor* are efficient vectors in the USA (Kocan *et al.*, 2004).

Various other biting arthropods have been implicated as mechanical vectors, particularly in the USA. Experimental transmission has been demonstrated with a number of species of *Tabanus* (horseflies), and with mosquitoes of the genus *Psorophora* (Kocan *et al.*, 2004). The importance of biting insects in the natural transmission of anaplasmosis appears to vary greatly from region to region. *Anaplasma*

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marginale also can be readily transmitted during vaccination against other diseases unless a fresh DNA-deoxyribonucleic acid or sterilised needle is used for injecting each animal. Similar transmission by means of unsterilized surgical instruments has been described (Kocan *et al.*, 2004).

The main biological vectors of *A. marginale* subsp. *centrale* appear to be multihost ticks peculiar to Africa, including *R. simus.* The common cattle tick, *R. microplus*, has not been shown to be a vector. This is of relevance where *A. marginale* subsp. *centrale* is used as a vaccine in *R. microplus*-infested regions.

2.4 Control of Anaplasmosis

The control of anaplasmosis has not really changed in the past 50 years (Kocan *et al*., 2000). The control measures that can be used to control anaplasmosis include chemoprophylaxis, vaccination, vector control and being able to maintain an *Anaplasma* free herd. Palmer (1989) explains that these control measures are greatly influenced by their cost, their feasibility and, most importantly, their availability.

2.4.1 Chemoprophylaxis

Chemotherapy does not prevent cattle getting infected with *A. marginale.* It is rather directed towards prevention of clinical anaplasmosis. Antibiotic therapy includes use of tetracycline drugs, imidocarb and gloxazone (Richey, 1981). The antibiotic therapy can be administered as feed supplements, although it is difficult to ensure equal doses per animal, or it can be given as injectable (Kocan *et al*., 2000).

2.4.2 Vaccination

Long-term development of immunity following vaccination has been extensively used to control anaplasmosis in most parts of the world (Palmer, 1989). The vaccine could be killed or live. The vaccines are normally targeted at preventing of morbidity and mortality, but they do not prevent cattle from infection upon challenge exposure. Immunized cattle may serve as a reservoir of *A. marginale* for

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mechanical or biological transmission (Kocan *et al*., 2000). Live and killed vaccines for anaplasmosis depend on the use of infected blood as a source of infection antigen, therefore further extensive purification is required in order to remove contaminating bovine cell material from the antigen.

2.4.3 Vector control

The control of anaplasmosis through controlling ticks and biting flies is both labour intensive and expensive. It can cause environmental pollution and repeated control by arcaricides can result in ticks becoming resistant (Kocan *et al*., 2000). In areas of endemic stability the interruption of the transmission cycle by arcaricides may risk the development of a susceptible cattle population, leading to massive outbreaks of the disease (Norval *et al*., 1992). Tick control is widely used in Africa but rarely used in the USA.

2.4.4 Maintaining an *A. marginale*-free herd

In non-endemic areas, anaplasmosis can be controlled by maintenance of *A. marginale*-free herds through avoiding importation of *Anaplasma* carriers that could serve as a source of infection (Kocan *et al*., 2000). This can be achieved by testing newly introduced cattle with a serological test such as the competitive enzyme linked immunoassay (cELISA). The cELISA makes use of recombinant major surface protein 5 (rMSP5) which has been proven to be a very good diagnostic antigen, making the cELISA a very sensitive serological test for anaplasmosis (French *et al*., 1998).

2.5 Detection of A. marginale and A. marginale subsp. centrale

There are different techniques that are used to diagnose anaplasmosis and identify the causative pathogen, namely microscopy, serology and molecular techniques such as the polymerase chain reaction.

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2.5.1 Microscopy

A. marginale and *A. marginale* subsp. *centrale* organisms can be identified in blood and organ smears stained in Giemsa stain. Blood smears are observed under the microscope at a magnification of x1000. *A. marginale* appear dense, rounded and are located on or near the margin of the erythrocyte. *A. marginale* subsp. *centrale* have a more central location in erythrocytes. The problem with this technique of diagnosing is that microscopic examination of blood smears from suspected cases of anaplasmosis is done when clinical signs are most pronounced, by this time the parasitaemia may be very low because of the removal of infected cells in the circulation. Also subclinical carriers are difficult to identify because of low levels of parasitaemia (Shkap *et al*., 2002).

2.5.2 Serological tests

Several serological tests have also been developed for the detection of *A. marginale*. Serological tests, such as the immunofluorescence antibody test (IFA) and enzyme linked immunosorbent assay (ELISA) (Visser *et al*., 1992) were developed principally to identify carrier cattle for regulatory reasons, chemotherapeutic eradication, disease control, epidemiological studies, screening of experimental animals and potential live vaccines donors for susceptibility to *Anaplasma* infections. Serological tests have non-specific cross-reactivity and they lack sensitivity (Potgieter and Soltsz, 2004).

2.5.3 Polymerase chain reaction (PCR)

Nucleic acid-based tests have been developed in order to detect *A. marginale* infection in carrier cattle even though they have not been fully validated (OIE, 2012). The analytical sensitivity of PCR-based methods has been estimated at 0.0001% infected erythrocytes, but at this level only a proportion of carrier cattle would be detected (OIE, 2015). Identification of *A. marginale* carrier cattle in blood samples has been done using a nested PCR. However, nested PCR poses significant quality control and specificity problems for routine use (Torioni De Echaide *et al.*, 1998).

Quantitative real-time PCR (qPCR) tests have also been described for identification of *A. marginale* (Carelli *et al.*, 2007; Decaro *et al.*, 2008; Reinbold *et al.*, 2010), and should be considered in place of

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the nested PCR. The two advantages of using real-time PCR for amplification and analysis, are that there are reduced chances for amplicon contamination and the test yields a semi quantitative assay result. However, the equipment needed for real-time PCR is expensive and requires preventive maintenance, and may be beyond the capabilities of some laboratories. Real-time PCR assays may target one of several genes, including *msp1b*, *groEL* (Carelli *et al.*, 2007; Decaro *et al.*, 2008), or 16S rRNA (Reinbold *et al.*, 2010), and are reported to achieve a level of analytical sensitivity equivalent to nested conventional PCR (Carelli *et al.*, 2007; Decaro *et al.*, 2008; Reinbold *et al.*, 2010).

2.6 Strain differentiation using *msp1α* gene sequence analysis

Different classes of major surface proteins (MSP) such as MSP1, MSP2, MSP4, and MSP5 are responsible for the genetic and antigenic diversity of *A. marginale*. Geographic isolates of *A. marginale* differ in their biology, protein sequence of MSPs and antigenicity. The MSPs are involved in host-pathogen and tick-pathogen interactions and have been used as markers for the genetic characterization of *A. marginale* strains and in phylogenetic studies. The *msp1α* gene is very diverse and has therefore been used as a marker in different geographic locations to determine the distribution of *A. marginale* genotypes and strains. The MSP1 protein is involved in the adhesion and transmission of *A. marginale* by ticks. The MSP1a peptide sequence varies among geographic strains in the number and sequence of amino-terminal tandem repeats (de la Fuente *et al.*, 2007). Using this technique many different geographic isolates of *A. marginale* have been identified and they differ in biology, morphology, protein sequence, antigenic characteristics and transmissibility by ticks (Mtshali *et al.*, 2007). The MSP1a varies in sequence and molecular weight because of different numbers of tandem 28–31 amino acid repeats. The MSP1a tandem repeats are located after a conserved decapeptide in the amino terminal region of the protein and are exposed extracellularly for interaction with host cell receptors (de la Fuente *et al*., 2003). The frequency of variable amino acid positions within geographic isolates is higher in this region than in the rest of the protein (de la Fuente *et al*., 2001). Determining the diversity of *msp1α* gene allows us to characterise the different strains *of A. marginale* present in infected animals.

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2.7 Benefits arising from the project

This project will identify and characterise regional *A. marginale* strains from Mozambique based on *msp1α* gene sequences, which can be used in future studies to help determine whether vaccine candidates of *A. marginale* identified in North American strains are conserved in *A. marginale* from Mozambique, and if they can be broadly used in a subunit vaccine.

2.8 Hypothesis

A diverse range of *A. marginale* and *A. marginale* subsp. *centrale* strains are present in cattle in Mozambique.

2.9 Aims/Objectives

The main objective of this study is to examine and characterise *Anaplasma* spp. from blood samples from cattle from Mozambique.

2.9.1 Specific Aims:

1. Determine the occurrence of *A. marginale* and *A. marginale* subsp. *centrale* in cattle from Mozambique, using a duplex quantitative real-time polymerase chain reaction (qPCR) assay (Carelli *et al*., 2007; Decaro *et al*., 2008).
2. Determine the genetic diversity of *A. marginale* in positive samples using *msp1α* sequence analyses.

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3 MATERIALS AND METHODS

3.1 Sample collection

Ethical clearance for the study was obtained from the Animal Ethics Committee (AEC) of the Faculty of Veterinary Science, University of Pretoria (project number V120-15) as shown in Appendix 1. A total of 100 blood samples were opportunistically collected from two diptanks, namely Botao and Namitangurine, in the Zambezia Province, Mozambique by Mr. Fernando Mulandane. Zambezia Province is located in north-central Mozambique (Fig. 1) and it is predominantly rural and depends almost entirely on subsistence farming and fishing (Moon *et al*., 2010).

Zambezia Province has a total area of 103,478 km^2, and most of it is drained at the Zambezi River. The greater part of the coast consists of mangrove swamps, and there is considerable forest inland. The average temperature is 24-26ºC with an annual average precipitation of up to 2000 mm (Moon *et al*., 2010).

Blood samples were collected as part of a routine surveillance project to examine trypanosomiasis resistance (Appendix 2 and 3). Prior to the survey, the nature and purpose of the survey/study was explained in detail to the cattle owners. The owners of the cattle participated voluntarily and an oral consent was obtained from each of them. The cattle were professionally handled in a crush pen and blood samples were collected in 10 ml Vacutainer® EDTA tubes from the coccygeal vein using 21 guage needles. Blood samples were transported to Eduardo Molandane University (EMU) in Maputo, Mozambique where DNA was extracted using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. All the vacutainers, pipette tips and Eppendorf tubes were disposed of in the Biotechnology Center – EMU, following the standard procedures for the disposal of hazardous material.

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A veterinary import permit for pathology specimen was obtained by Dr. Nicola Collins from the Directorate of Animal Health, Import-Export Policy Unit (Appendix 4). The import permit granted permission to the laboratory of the Department of Veterinary Tropical Diseases to import 100 DNA samples (100 µl each), extracted from cattle blood.

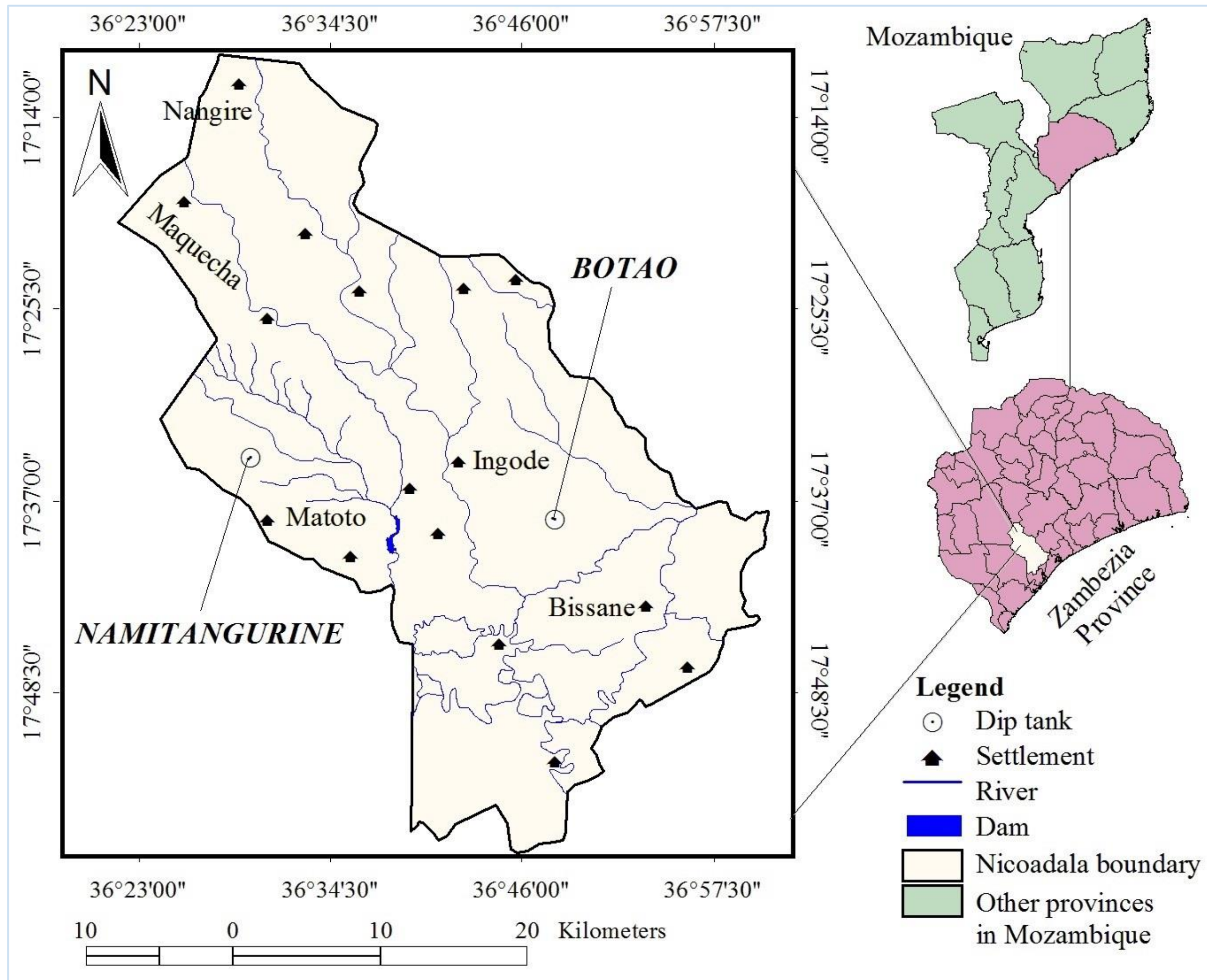

Figure 1*: Map indicating the location of Botao and Namitangurine diptanks in the Zambezia Province in Mozambique.*

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3.2 Quantitative real-time PCR (qPCR) for detection of *A. marginale* and *A. marginale* subsp. *centrale*

The Taqman duplex qPCR assay (Decaro *et al*., 2008) targeting the *msp1β* gene of *A. marginale* (Carelli *et al*., 2007) and the *groEL* gene of *A. marginale* subsp. *centrale*, was used to detect the parasite DNA in the samples. The assay was adapted for use on a LightCycler™ v2 (Roche Diagnostics).

The reaction mixture (20 µl) contained 4 µl of the Fast Start Taqman mix (Roche Diagnostics), 0.5 µl UDG, 0.6 µM each of *A. marginale*-specific primers AM-For (5'-TTG GCA AGG CAG CAG CTT-3') and AM-Rev (5'-TTC CGC GAG CAT GTG CAT-3'), 0.2 µM of probe AM-Pb (5'-6FAM-TCG GTC TAA CAT CTC CAG GCT TTC AT-BHQ1-3'), 0.9 µM each of *A. marginale* subsp. *centrale*-specific primers AC-For (5'-CTA TAC ACG CTT GCA TCT C-3') and AC-Rev (5'-CGC TTT ATG ATG TTG ATG C-3'), 0.2 µM of probe AC-Pb (5'-LC610-ATC ATC ATT CTT CCC CTT TAC CTC GT-BHQ2-3') and 2.5 µl of genomic DNA. Thermal cycling conditions were: 1 cycle of UDG activation at 40°C for 10 min with no analytical mode, 1 cycle of pre-incubation at 95°C for 10 min with no analytical mode, 40 cycles of denaturation at 95°C for 1 min and annealing-extension at 60°C for 1 min set at quantitative analytical mode, and a final cooling step at 40°C for 30 sec. A positive control for *A. marginale* (field strain 208291) and *A. marginale* subsp. *centrale* (field strain 9410) and negative control (water) were included in each PCR run. The data were analysed using the LightCycler Software version 4.0.12. The Cp (crossing point-PCR-cycle) value is the cycle at which fluorescence achieves a defined threshold. It corresponds to the cycle at which a statistically significant increase in fluorescence is first detected. The number of cycles needed for the amplification-associated fluorescence to reach the Cp value is inversely correlated to the amount of the target nucleic acid that was in the original sample (Rodriguez-Lazaro and Hernandez, 2013).

3.3 Strain differentiation using *msp1α* gene sequence analysis.

A. marginale strains in positive samples were characterized by sequence analysis of the *msp1α* gene.

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3.3.1 Amplification of the *msp1α* gene

Twenty-seven samples that were positive for *A. marginale* were selected for cloning based on low crossing point (Cp) values obtained in the qPCR, as these correlated with higher parasite DNA concentration in the samples. The variable region of the *msp1α* gene from *A. marginale* positive samples was amplified using primers 1733F (5'-TGT GCT TAT GGC AGA CAT TTC C-3') and 2957R (5'-AAA CCT TGT AGC CCC AAC TTA TCC-3') (Lew *et al*., 2002). Amplification was performed in a 25 µl volume consisting of 1x Phusion Flash High-Fidelity PCR Master Mix (ThermoFisher Scientific), 0.5 µM primers and 2.5 µl of template DNA (approximately 200 ng). The thermal cycling conditions were 98°C for 0.10 sec, 40 cycles of 98°C for 0.01 sec, 63°C for 0.05 sec and 72 °C for 18 sec, and a final extension at 72°C for 60 sec.

3.3.2 Purification of *msp1α* PCR products

Samples were run on a 1% agarose gel with a 1000 bp molecular weight marker (Fermentas) to determine their size. Purification of PCR products was done using the QIAquick PCR Purification kit (Qiagen). Briefly, five volumes of buffer PB were added to 1 volume of the PCR sample. To bind DNA, the sample was applied to the QIAquick column which was then centrifuged for 30-60 sec, and the flow-through was discarded. Buffer PE (0.75 ml) was added to the QIAquick column to wash away impurities and the column was centrifuge for 30-60 sec. The QIAquick column was placed in a clean 1.5 ml micro centrifuge tube, and to elute DNA, 50 µl of Buffer EB [10 m MTris·Cl, (pH 8.5)] or water (pH 7.0–8.5) was added to the center of the QIAquick membrane and the column was centrifuged for 1 min.

3.3.3 Cloning of the *msp1α* PCR products

The *msp1α* PCR products from 27 samples were selected for cloning. Cloning was done using the Clone JET PCR Cloning Kit (ThermoFisher Scientific).

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3.3.3.1 Ligation reaction mix

A 3:1 insert:vector molar ratio was used in the ligation reactions. The amount of PCR product (insert) to add to the ligation reaction was calculated as follows:

$$\frac{\text{ng of vector x kb size of insert}}{\text{kb size of vector}} \text{ x insert: vector molar ratio = ng of insert}$$

The pJET vector is provided at a concentration of 50 ng/µl, and is 2974 bp (2.974 kb) in length. The PCR products were approximately 900 bp (0.9 kb) in length.

$$\frac{\text{50 ng (pJET) x 0.9 kb}}{\text{2.974 kb}} \text{ x } \frac{3}{1} = 45.39 \text{ ng} \cong 50 \text{ ng}$$

A spectrophotometer reading was taken using a PowerWave HT Microplate spectrophotometer (BioTek) in order to determine the concentration of the PCR products. After determining the concentration of the PCR products, the simple proportion method of calculation was used to determine how much of each PCR product to add to the ligation mix for cloning purposes [X µl = 50 ng x 1 µl / (concentration of PCR product)].

For each *msp1α* PCR product, a 20 µl ligation reaction mix was prepared containing a final concentration of 1x reaction buffer, 50 ng of PCR product, 50 ng pJET vector and 5U T4 DNA ligase. The ligation mix was vortexed for 3-5 sec and incubated at room temperature for 5 min. A blunting reaction was done to prepare the control PCR product provided in the CloneJET PCR Cloning Kit for cloning. The components of the blunting reaction and the volumes used are shown below:

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Component	Volume
2x reaction buffer	10µl
Control PCR product (24 ng/µl)	2 µl
Water	5 µl
DNA blunting enzyme	1 µl
Total volume	18 µl

The blunting reaction mix was vortexed briefly and then centrifuged for 3-5 sec. It was incubated for 4 hrs at 70°C. Then 1 µl of pJET vector (50 ng/µl) and 1 µl of T4 DNA ligase (5 U/ µl) was added to the blunting reaction. The control ligation mix was vortexed and centrifuged briefly for 3-5 sec, and incubated at room temperature for 5 min.

3.3.3.2 Transformation

ImMedia™ Amp Agar (ThermoFisher Scientific) and ImMedia™ liquid medium was used to grow the transformed cells. ImMedia™ growth medium was prepared following the manufacturer's instructions. For transformation, 50 µl of JM109 competent *E. coli* cells (Promega) was added to 5 µl of each ligation reaction. The transformation mix (cells plus ligation mix) was placed on ice for 20 min, after which the transformation mix was subjected to heat shock for 2 min at 42°C in a water bath. The transformation mix was then cooled on ice for 2 min, 200 µl of SOC medium (ThermoFisher Scientific) was added, and the transformed cells were incubated at 37°C for 1.5 hrs with shaking at 150 rpm. Each transformation mix (50 µl) was plated out onto one ImMedia™ Amp agar plate and the rest of the mix was plated out onto a second ImMedia™ Amp agar plate. The plates were incubated overnight (approximately 16 hrs) at 37°C to allow for growth of the colonies.

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3.3.3.3 Colony PCR

To determine which colonies contained insert of the correct size, a colony PCR was done using the DreamTaq Green PCR Master Mix[1] (ThermoFisher Scientific). The colony PCR master mix was prepared as shown below:

	20 µl Reaction volume			
Component	**x 1 (µl)**	**x 55 (µl)**	**x 56 (µl)**	**x 111 (µl)**
DreamTaq Green PCR Master Mix 2x	10	550	560	1110
pJet 1.2F primer (10 µM)	0.4	22	22.4	444
pJet 1.2R primer (10 µM)	0.4	22	22.4	444
Water	9.2	506	515.2	1021.2

A 20 µl aliquot of the master mix was pipetted into each Eppendorf tube for each colony to be tested. A 10 µl tip was used to pick-up a single colony from the Amp agar plates. The tip was used to streak the colony onto duplicate ImMedia™ Amp agar plates, after which it was dipped into the master mix. Ten recombinant colonies were picked per sample. The cycling conditions for the colony PCR were: an initial denaturation at 95°C for 3 min; 25 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 1 min; a final extension at 72°C for 7 min; a final hold at 4°C. The samples were then run on a 1% agarose gel stained with ethidium bromide and visualised on a gel electrophoresis Gel Doc XR+ System (Biorad).

3.3.4 Sequence Analysis

For each cloned *msp1α* PCR product, ten recombinant colonies were sent to Inqaba Biotechnologies (Pretoria, South Africa) for bidirectional Sanger sequencing on an ABI 3500XL Genetic Analyzer, using primers pJet 1.2F and pJet 1.2R. Sequences were assembled and aligned using CLC Main Workbench version 7.6.4 (https://www.qiagenbioinformatics.com/products/clc-main-workbench). The resulting sequences were analyzed to determine their genotype using the online tandem repeats finding

[1] Thermo Scientific DreamTaq Green PCR Master Mix (2X) is a ready-to-use solution containing DreamTaq™ DNA polymerase, optimized DreamTaq Green buffer, $MgCl_2$ and dNTPs. 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.

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program, Tandem Repeats Finder 4.07b (Benson, 1999) to identify the number of repeats, and RepeatAnalyzer (Catanese *et al.,* 2016) to identify the repeats named according to Allred *et al.* (1990). Both programs were operated on default parameters when submitting sequences to determine the repeats present in the sequence.

3.4 Statistical analysis

The qPCR results were analyzed using SPSS (version 23). The prevalence of *A. marginale* and *A. marginale* subsp. *centrale* was determined with a 95% confidence interval, the confidence interval was calculated by adjusting for cluster within diptanks with 1000 bootstrap replication. The association between the presence of *A. marginale* in cattle and location (diptank) was tested using a chi-squared test (χ^2). Univariate analysis of associations using the Chi-squared test was carried out for the exposure variable (diptank) with the infection status of the animal.

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4 RESULTS

4.1 Detection of *A. marginale* and *A. marginale* subsp. *centrale* using duplex quantitative real-time PCR (qPCR)

The 100 DNA samples, consisting of 50 from Botao diptank (Table 1) and 50 from Namitangurine diptank (Table 2), both in the Zambezia Province, Mozambique, were tested for the presence of *A. marginale* and *A. marginale* susbsp. c*entrale* using the duplex qPCR assay (Decaro *et al.*, 2008).

4.1.1 Detection of *A. marginale*

Signals generated by the FAM fluorophore linked to the *A. marginale*-specific probe, measured at 530 nm, were generated in *A. marginale* positive samples. Fig. 2 shows an example of results obtained using the *A. marginale*-specific primers and probe, viewed on channel 530 of a LightCycler v2 (Roche Diagnostics). No fluorescence was observed in the negative control (water) and in negative samples. Positive results are indicated by the Cp value. A total of 86 samples were found to be positive for *A. marginale* (Table 1 and 2).

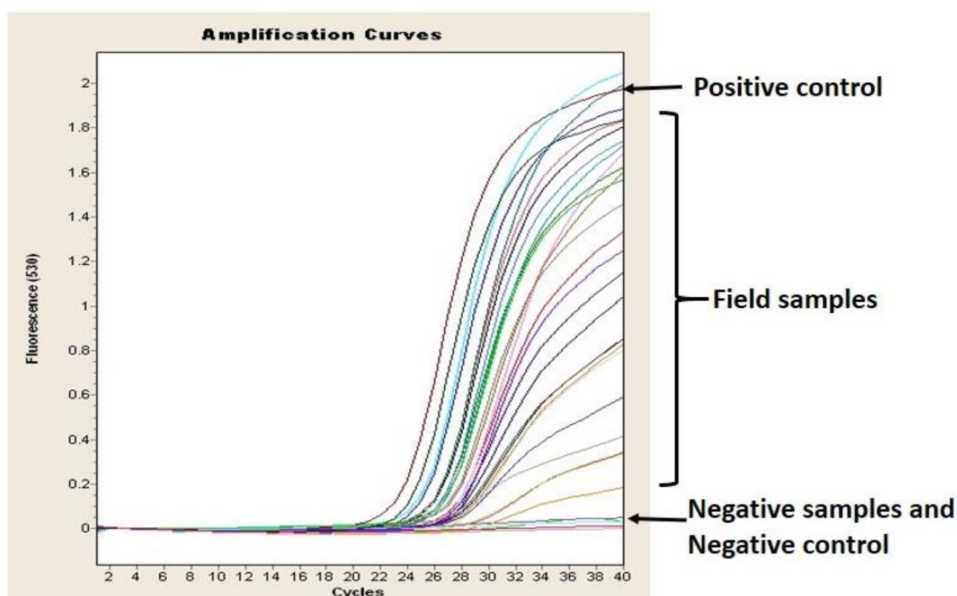

Figure 2: *An example of LC-530 fluorescence generated in Anaplasma marginale positive samples, measured at 530 nm.*

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The overall prevalence of *A. marginale*, determined with a 95% interval calculated by adjusting for cluster within diptanks with 1000 bootstrap replication, was found to be 87% (95% CI: 80, 93%). There was no significant association (p>0.05) between the prevalence of *A. marginale* in cattle at Namitangurine diptank (88.0%, n=42) and Botao diptank (86.0%, n=43).

4.1.2 Detection of *A. marginale* susbsp. *centrale*

Signals generated by the LC-610 fluorophore, linked to the *A. marginale* subsp. *centrale*-specific probe, measured at 610 nm, were generated in *A. marginale* subsp. *centrale* positive samples. Fig. 3 shows an example of results obtained using the *A. marginale* subsp. *centrale*-specific primers and probe, viewed on channel 610/530 of a LightCycler v2 (Roche Diagnostics, Germany). A total of six samples were positive for *A. marginale* subsp. *centrale* (Table 1 and 2)

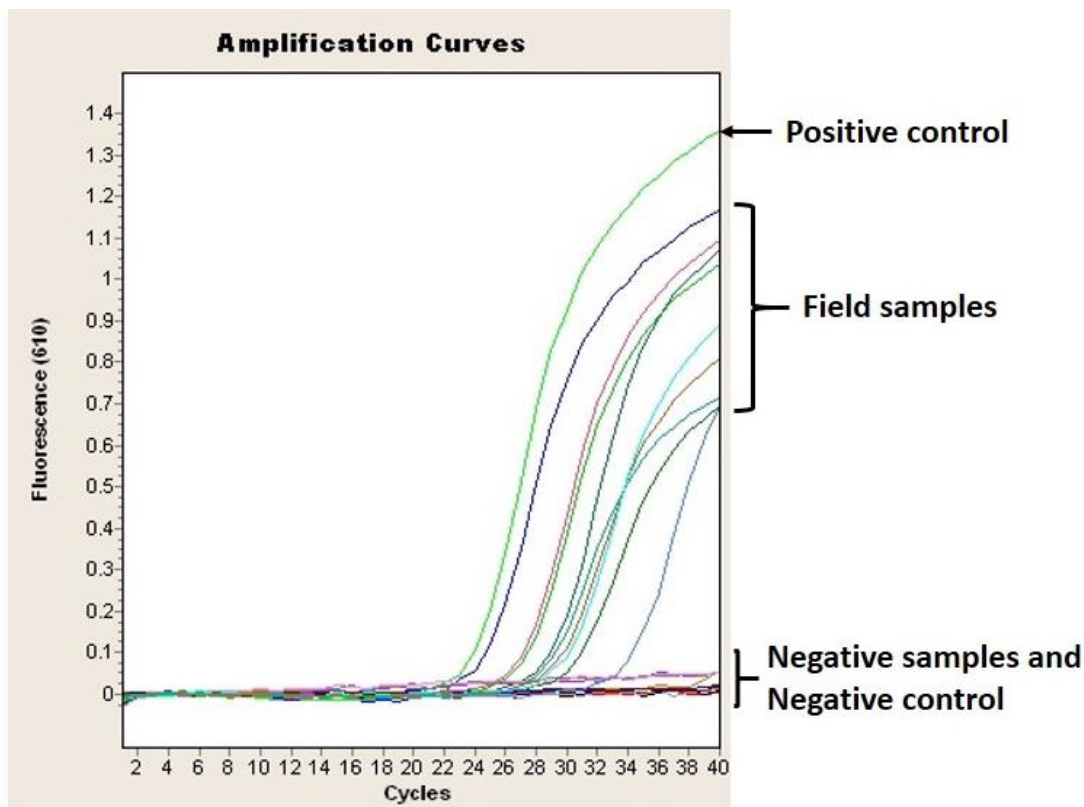

Figure 3*: An example of LC-610 fluorescence generated in Anaplasma marginale subsp. centrale positive samples, measured at 610 nm.*

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The overall prevalence of *A. marginale* subsp. *centrale* was 6.0% (95% CI: 2.0, 11%). Only 2.0% (n=1) of cattle sampled from Botao diptank were positive for *A. marginale* subsp. *centrale* while 10% (n=5) were positive for *A. marginale* subsp. *centrale* in Namitangurime diptank. There was no significant association ($p>0.05$) between *A. marginale* subsp. *centrale* presence in cattle and diptank.

***Table 1**: Real-time duplex qPCR results for the 50 samples from Botao diptank. Samples selected for further analysis are highlighted in yellow.*

Botao (17.6354S/36.7998E)						
Sample No.	***A. marginale* (Channel 530)**			***A. marginale* subsp. *Centrale* (Channel 610/530)**		
	Target	**Cp[c]**	**Score**	**Target**	**Cp**	**Score**
***Am* +ve (sample 208291)**	P[a]	22.71	5	N		
***Ac +ve* (sample 9410)**	N[b]			P	16.98	5
1	P	24.74	5	N		
2	P	24.74	5	N		
3	P	25.23	5	N		
4	P	23.45	5	N		
5	P	25.57	5	N		
6	P	25.12	5	N		
7	P	24.95	5	N		
8	P	24.82	5	N		
9	P	24.03	5	N		
10	N			N		
11	N			N		
12	N			N		
13	N			N		
14	N			N		
15	P	24.66	5	N		

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16	P	25.43	5	N
17	P	24.38	5	N
18	P	26.23	5	N
19	P	25.00	5	N
20	P	24.90	5	N
21	P	25.05	5	N
22	P	26.51	5	N
23	P	25.60	5	N
24	N			N
25	N			N
26	P	26.87	5	N
27	P	26.29	5	N
28	P	26.69	5	N
29	P	25.83	5	N
30	P	26.63	5	N
31	P	25.27	5	N
32	P	26.19	5	N
63	P	23.74	5	N
64	P	23.60	5	N
65	P	25.57	5	N
66	P	24.08	5	N
67	P	24.21	5	N
68	P	24.31	5	N
69	P	27.83	5	N
70	P	26.76	5	N
71	P	26.18	5	N
72	P	27.24	5	N
73	P	27.08	5	N

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74	P	27.00	5	N		
75	P	25.81	5	N		
76	P	25.44	5	N		
77	P	26.82	5	N		
78	P	26.13	5	N		
79	P	25.86	5	N		
80	P	26.72	5	P	29.88	5
Water (-ve)	N			N		

[a] P: positive; [b] N: negative; [c] Cp: Crossing point (the cycle at which fluorescence from amplification at the exponential phase exceeds back ground fluorescence).

Table 2: *Real-time duplex qPCR results for the 50 samples from Namitangurine diptank. Samples selected for further analysis are highlighted in yellow.*

Namitangurine (17.5748S/36.4950E)						
Sample No.	***Anaplasma marginale*** **(Channel 530)**			***Anaplasma centrale*** **(Channel 610/530)**		
	Target	Cp[c]	Score	Target	CP	Score
***Am*+ ve- (sample 208291)**	P[a]	22.71				
***Ac +ve* (sample 9410)**	N[b]				16.98	5
33	P	22.46	5	N		
34	P	25.56	5	N		
35	P	26.13	5	N		
36	P	23.98	5	P	24.52	5
37	P	25.32	5	N		
38	P	27.46	5	N		
39	P	27.95	5	N		
40	P	26.46	5	N		

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41	P	25.64	5	N		
42	P	27.83	5	N		
43	P	26.91	5	N		
44	N			N		
45	P	23.09	5	N		
46	P	23.09	5	N		
47	N			N		
48	P	26.17	5	N		
49	P	19.39	5	N		
50	P	25.85	5	N		
51	P	22.72	5	N		
52	P	24.28	5	P	25.5	5
53	P	25.71	5	N		
54	P	25.63	5	N		
55	P	26.30	5	N		
56	N			N		
57	N			N		
58	P	23.50	5	N		
59	N			N		
60	N			N		
61	P	24.33	5	N		
62	P	24.51	5	N		
81	P	25.27	5	N		
82	N			N		
83	P	27.07	5	N		
84	P	19.31	5	N		
85	P	27.24	5	P	27.01	5
86	P	19.49	5	N		

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87	P	26.14	5	P	25.82	5
88	P	27.52	5	P	27.56	5
89	P	26.70	5	N		
90	P	23.51	5	N		
91	P	23.07	5	N		
92	P	23.49	5	N		
93	N			N		
94	P	26.76	5	N		
95	P	24.78	5	N		
96	P	22.27	5	N		
97	P	22.22	5	N		
98	P	23.84	5	N		
99	P	26.11	5	N		
100	P	26.92	5	N		
Water(-ve)	N			N		

[a] P: positive; [b] N: negative; [c] Cp: Crossing point (the cycle at which fluorescence from amplification at the exponential phase exceeds back ground fluorescence).

4.2 Amplification of the *msp1α* gene

4.2.1 Selection of samples and amplification of the *msp1α* gene

Twenty-seven *A. marginale* positive samples with low crossing point (Cp) values were selected as indicated in Table 1 and 2, since a low Cp correlates with a high starting concentration of target DNA in a sample. The region of the *msp1α* gene that contains the repeats used for genotyping was amplified from all 27 samples. Amplification products between 750 bp and 1000 bp were obtained (Fig. 4).

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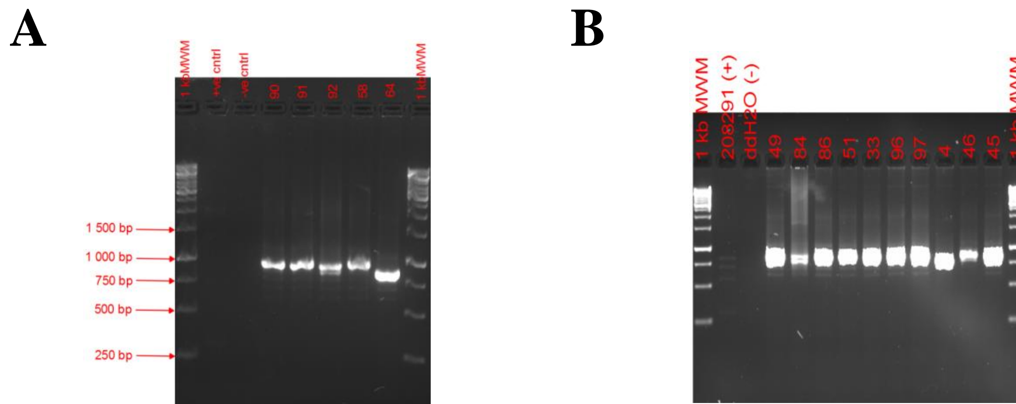

Figure 4*: Amplification of the msp1α gene from samples 90, 91, 92, 58, 64 (Panel A) and samples 49, 84, 86, 51, 33, 96, 97, 4, 46, 45 (Panel B). PCR products(750 - 1000 bp) were separated on a 1% agarose gel stained with ethidium bromide. The size of the PCR products was determined by comparison with a 1 kb molecular weight marker (Fermentas) (1kb MWM). Positive (DNA from Anaplasma marginale positive sample 208291) and negative (ddH$_2$0) controls were included in all amplification reactions.*

4.2.2 Purification of *msp1α* PCR products

The yield of the PCR products was reduced during purification (Fig. 5). Nonetheless, the *msp1α* PCR products were still clearly visible. The resolution between individual bands within PCR products was improved; some samples appeared to have a single band, while others showed thick single bands or clear double or multiple bands (Fig. 5). Samples 84 and 92 showed double bands and sample 46 showed multiple bands. Single bands were observed in the rest of the samples, although some of these were very thick, and could indicate the presence of more than one PCR product of similar size.

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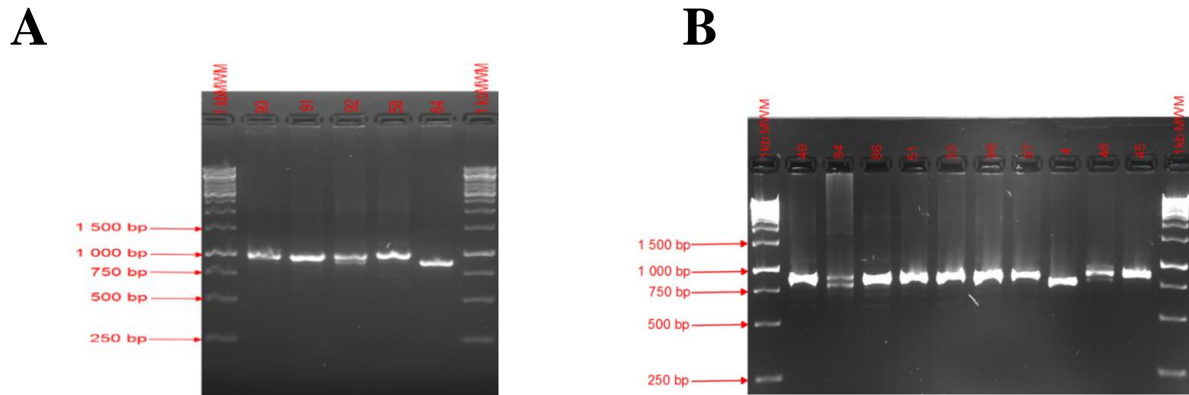

Figure 5: *Purification of* msp1α *PCR products using the QIAquick® PCR purification kit (Qiagen) from samples 90, 91, 92, 58, 64 (Panel A) and samples 49, 84, 86, 51, 33, 96, 97, 4, 46, 45 (Panel B). PCR products were separated on a 1% agarose gel stained with ethidium bromide. The size of the PCR products was determined by comparison with a 1 kb molecular weight marker (Fermentas) (1kb MWM).*

4.3 Cloning

The concentration of each *msp1α* PCR product was determined in order to calculate the optimal amount of each PCR product to include in the ligation reaction (Appendix 5). The *msp1α* PCR products were cloned into the pJET cloning vector, which contains a lethal gene at the cloning site and an ampicillin resistance gene. The lethal gene is disrupted by ligation of a DNA insert into the cloning site, allowing only recombinant clones to survive. The ligation mix was used to transform competent *E. coli* cells, and the transformation mix was plated out onto Amp agar. After overnight incubation (approximately 16 hrs) at 37°C, only *E. coli* cells containing recombinant plasmids were able to propagate on the agar plates containing ampicillin, since the presence of the ampicillin resistance gene in the recombinant plasmid confers ampicillin resistance to the *E. coli* cells (Fig. 6).

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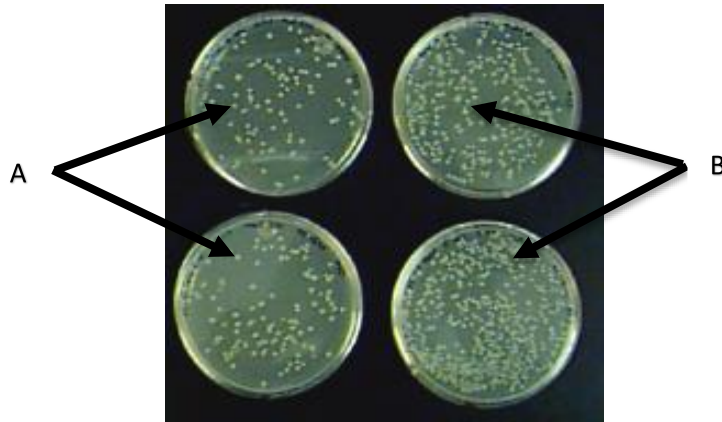

Figure 6: *Cloning of msp1α PCR products. Plates labelled A show clones from 50 µl of the transformation mix and plates labelled B show results of the rest of the transformation mix (205 µl) after incubation overnight at 37°C. Only E. coli cells containing recombinant plasmids can grow on the agar plates containing ampicillin, as the plasmid confers ampicillin resistance to the E. coli, and the DNA insert disrupts the lethal gene.*

4.3.1 Colony PCR

For each PCR product, ten colonies were picked and a colony PCR was done. The results of the colony PCR for selected samples are shown in Fig. 7. Clones with the correct size insert were sent to Inqaba Biotech for sequencing.

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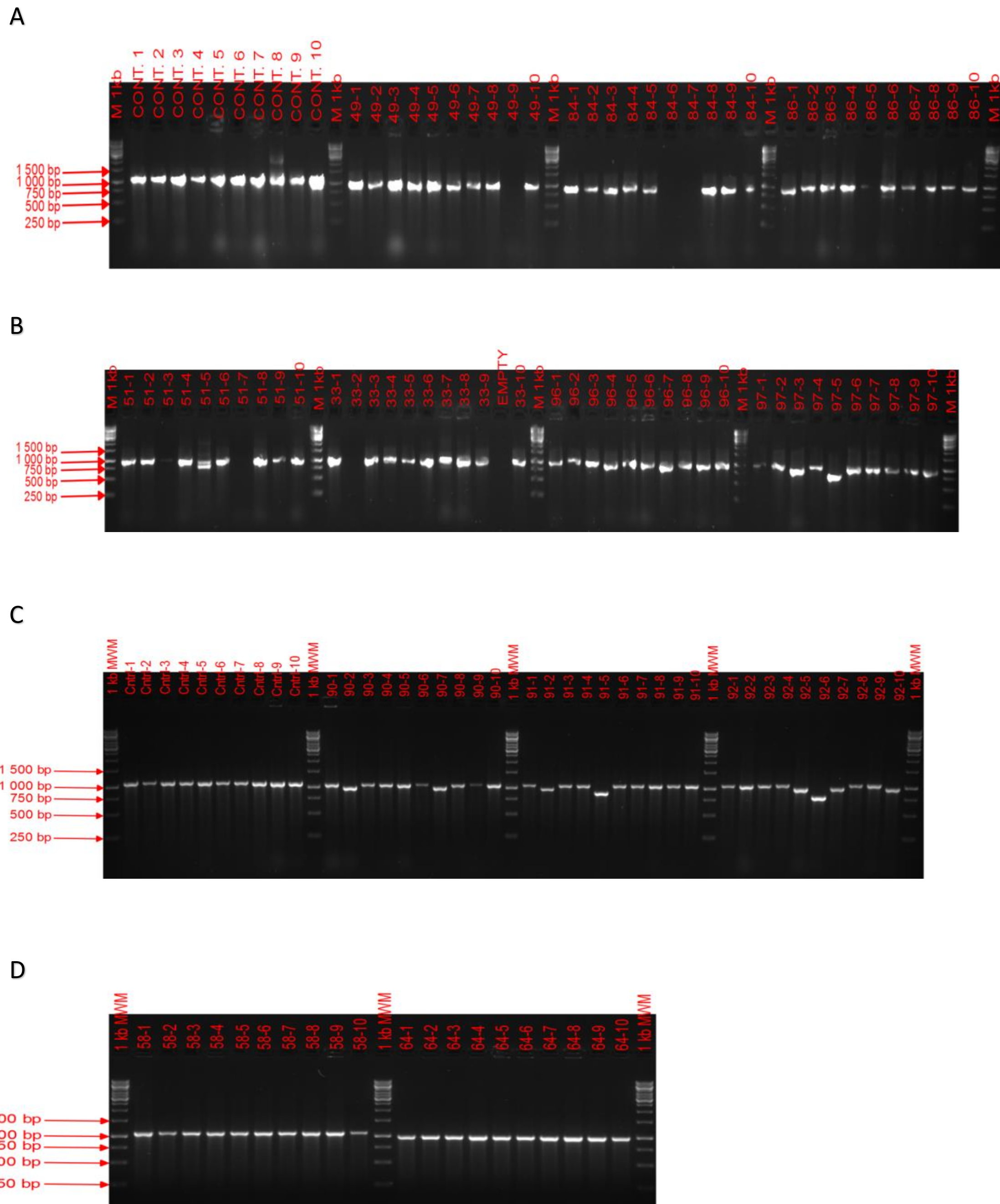

Figure 7 : Colony PCR for samples 49, 84, 86 (A), 51, 33, 96 ,97 (B), 90, 91, 92 (C), and 58 and 64 (D) using primers pJet 1.2F and pJet 1.2R, resulting in amplification of a 1000 bp insert from colonies containing recombinant plasmids

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4.4 Sequence assembly and analysis

Sequences were assembled and aligned using CLC workbench 7.6.4. The repeats were identified using an in-house Java script supplied by Dr. Kelly Brayton (Washington State University). The *msp1α* gene sequences were translated to peptide sequences and MSP1a repeats were identified. Fourteen novel repeats not previously identified were found (Fig. 8, Table 3). The MSP1a repeat structure (*msp1α* genotype) was used to identify *A. marginale* strains. A total of 47 different *msp1α* genotypes were found from 76 *msp1α* sequences generated from the 27 samples. The *msp1α* genotypes (MSP1a repeat structures) identified in each sample are indicated in Table 3.

```
A_repeat_msp1a    DDSSSASGQQQESSVSSQS-EASTSSQLG
UP45              TDSSSAGDQQQESSVLSQSG-ASTSSKLG
UP46              TDSSSAGDQQQESSVLSQSG-ASTSSQLG
UP47              TDSSSAGDQQQESSVSSQSGQASTSSKLG
UP49              ADSSSAGNQQQESSVSSQSSQASTSSQLG
UP50              ADSSSAGDQQQESSVLSQSDQASTSSQLG
UP38              ADSSSAGNQQQESSVLSQSDQASTSSQLG
UP48              TDSSSASGQQQESSVSSQSG-ASTSSQLG
UP37              TDSSSASGQQQESSVSSQSDQASTSSQSG
UP43              AGSSSASGQQQESGVLSQSGQASTSSQLG
UP44              ADSSSASGQQQESGVLSXSGQASTSSQLG
UP41              ADSSSAGDQQQGSGVSSQSGQASTSSQSG
UP42              ADSSSAGDQQQGSGVSSQSGQASTSSQLG
UP39              DNSSSASCRQQESSVLSQSDQASTSSQLG
UP40              DNSSSASCRQQESSVLSQSG-ASTSSQLG
```

Figure 8: *ClustalX alignment of novel MSP1a repeats (UP37-UP50) identified in this study with the first MSP1a repeat identified, the A repeat (Allred et al., 1990). Identical amino acid residues in the alignment are shown by black text on a white background; residues that differ from the A repeat are indicated by white text on a black background.*

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***Table 3**: MSP1a repeat structure (msp1α genotype) of strains found per sample from each of the diptanks. Novel MSP1a repeats identified in this study are given in red font.*

Diptank	***Sample No.***	***No. of clones per sample***	***Genotypes found (MSP1a repeat structure)***
Botao	1	7	100 15 UP43 15 15 100 15 15 15 15 100 15 UP43 15 UP44
	2	8	84 UP3 UP4 UP45 UP46 UP18 81 13 13 18 M M M M UP47 155 13 UP48 UP4 84 UP3 UP4 UP4 UP4 18 13 13 13 18 155 13 13 18 84 UP3 UP3 UP4
	4	10	74 27 27 H M 27 UP37 M 27
	7	6	Is9;78 UP49 43 25 31 25 31
	8	6	Is9;78 UP49 43 25 31 τ 10 10 15 [Strain: Brazil/Marajó Island/E – (τ, 10^2, 15)] τ 22-2 10 15
	9	8	Is9;78 31 Is9;78 UP49 43 25 31 τ 10 15 [Strain: Parana 3;Chaco 5;Brazil/Parana/ - (τ, 10, 15)] τ 10 10 15 [Strain: Brazil/Marajó Island/E – (τ, 10^2, 15)]
	15	6	84 UP3 UP4 τ 15
	17	3	18 13 13 13 18

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			UP18 UP4 UP4 UP4 UP4 UP50 13 UP4 UP4 UP4
	20	8	84 UP3 UP3 UP4
	63	5	100 15 15 15 15
	64	10	84.1 UP3 UP3 UP19
	66	6	18 18 18 13 18 18 18 13 18 18 18 18 84 UP3 UP3 UP4
	67	6	84 UP3 UP3 UP4
	68	3	84 UP3 UP3 UP4 84 84 UP3 UP3 UP4 84 UP3 UP3 UP3 UP4
Namitangurine	33	9	18 18 13 18 18 18 18
	45	7	18 18 13 18
	46	8	18 18 13 18 18 4 18 13 37
	49	9	18 18 18 18 18 18 13 18
	51	9	18 18 13 18 13 18
	58	8	100 15 15 15 15
	84	8	UP38 13 37 UP39 13 37 UP40 62 38 84 UP3 UP3 UP19 84 Γ UP41
	86	10	18 13 18

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			18 18 13 18
			18 18 18 18
	90	10	α β β β 15
			18 18 13 18
			UP42 β β 15
	91	9	α β β β 15
			α β β 15
			α β 15
	92	10	α β β β 15
			18 β β β 15
			18 18 18 18
			α 18
			18 18 13 13
			α β β 15
	96	10	18 18 13 18
			18 18 18
			18 13 18
	97	10	18 18 13 18
			18 13 18
			18

Most of the samples (22 out of 27, 81.5%) contained one, two or three *msp1α* genotypes per animal (Fig. 9). Many samples (21 out of 27, 77.8%) contained more than one genotype, indicating that multiple strains of *A. marginale* were common in the cattle sampled. A few samples contained four to eight genotypes per sample.

Msp1a repeats and *msp1α* genotypes occurring in Mozambique were compared to those identified in five other countries, Brazil, Argentina, Mexico, South Africa and the USA (Mutshembele *et al.*, 2014; da Silva *et al.*, 2015). Only 29.6% of the repeats identified in Mozambique had not previously been

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identified elsewhere (Table 4), yet 95.7% of the *msp1α* genotypes were unique to Mozambique (Table 5). In fact, the percentage of repeats unique to each country (Table 4) was consistently lower than the percentage of genotypes unique to each country (Table 5).

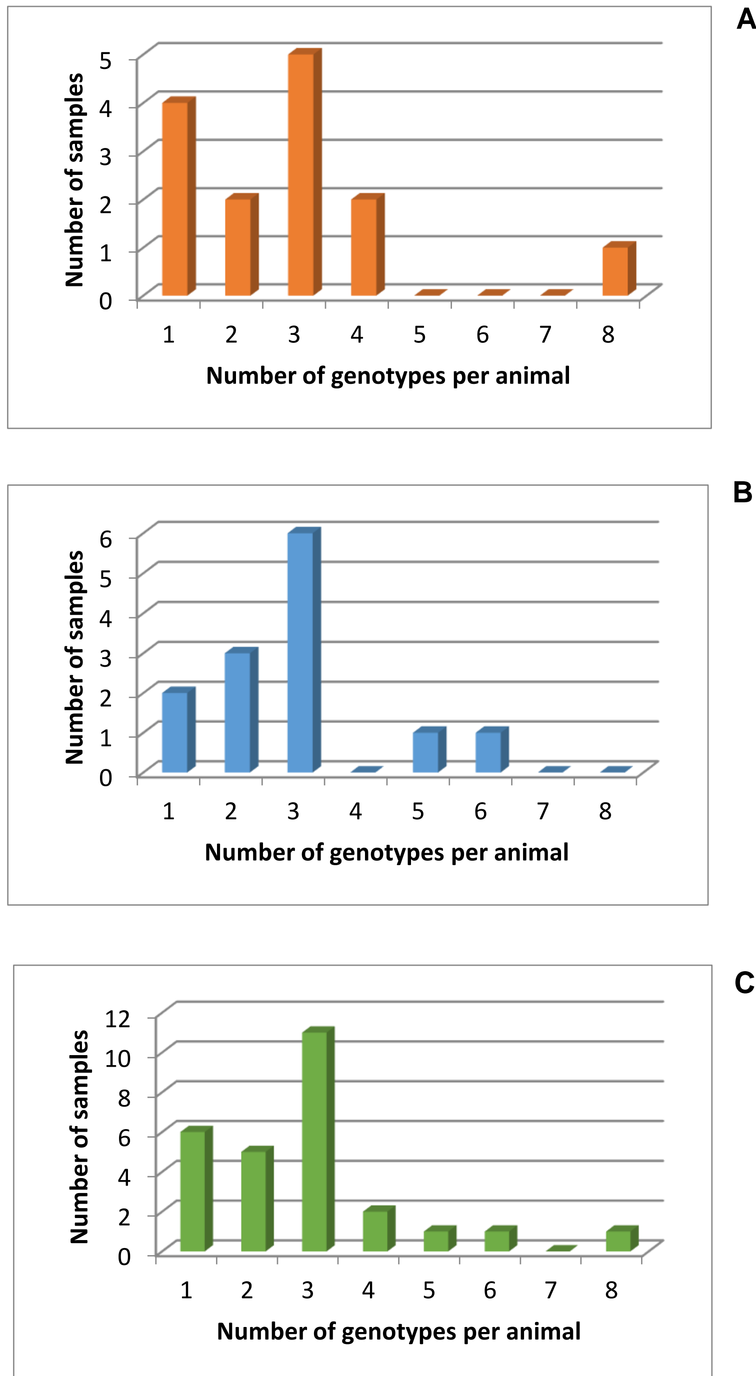

Figure 9: *Number of* msp1α *genotypes per animal at Botao diptank (A), Namitangurine diptank (B) and overall (C).*

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Table 4: *Analysis of MSP1a repeats of Anaplasma marginale strains from different countries around the world, accessed from the database generated in RepeatAnalyzer (Catanese et al., 2016).*

Location	**Brazil**	**USA**	**Argentina**	**Mexico**	**South Africa**	**Mozambique**	**Average %**
# Unique	6	10	12	27	71	13	
# Total	33	22	33	64	99	44	
% Unique	18.2	45.5	36.4	42.2	71.7	29.6	**40.6**
Other locations with repeats in common	Argentina, Mexico, South Africa, USA	Argentina, Brazil, Mexico, South Africa	Brazil, Mexico, South Africa, USA	Argentina, Brazil, South Africa, USA	Argentina, Brazil, Mexico, USA	Argentina, Brazil, Mexico, South Africa, USA	
Common repeats	F	F	F	F	F	–	
	M	M	M	M	M	M	
	13	–	13	13	13	13	
	15	–	15	15	15	15	
	18		18	18	18	18	
	27	–	27	27	27	27	
	B	B	B	B	–	–	
	C	C	C	C	–	–	
	Q	–	Q	Q	Q	–	
	τ	–	τ	τ	τ	τ	
	γ	–	γ	γ	–	γ	
	α	–	α	α	–	α	

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Table 5: *Analysis of msp1α genotypes of Anaplasma marginale strains from different countries around the world accessed from the database generated in RepeatAnalyzer (Catanese et al., 2016).*

Location	Brazil	USA	Argentina	Mexico	South Africa	Mozambique	Average %
# Unique	18	43	15	84	188	45	
# Total	23	43	18	89	190	47	
% Unique	78.7	100.00	83. 3	94.4	99.0	95.7	**91.8**
Other locations with genotypes in common	Mexico, Argentina, South Africa, Philippines, Mozambique	–	Brazil, Mexico, Mozambique	Brazil, Argentina, Philippines, Mozambique	Brazil, Philippines	Brazil, Mexico, Argentina	
Common Genotypes	α β β β β Γ;γ	–	α β β β β Γ;γ	α β β β β Γ;γ	–	–	
	–	–	α β β β Γ;γ	α β β β Γ;γ	–	–	
	α β β Γ;γ	–	–	α β β Γ;γ	–	–	
	τ 57 13 18	–	–	–	τ 57 13 18		
	τ 10 15		τ 10 15	τ 10 15	–	τ 10 15	
	13 27 27*	–	–	–	13 27 27*	–	
	–	–	–	13 13*	–	–	
	τ 10 10 15	–	–	–	–	τ 10 10 15	

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5 DISCUSSION

In this study, 100 samples from two diptanks in the Zambezia province of Mozambique were examined for *A. marginale* and *A. marginale* subsp. *centrale* infections using a duplex qPCR assay (Decaro *et al.*, 2008). *Anaplasma marginale* subsp. *centrale* is considered to be less pathogenic than *A. marginale* and is used in a live blood vaccine. Nevertheless, the vaccine strain can sometimes cause anaplasmosis in susceptible animals (Bigalke 1980; Pipano *et al*., 1985; Carelli *et al.*, 2008). Also it has recently been reported that *Anaplasma marginale* subsp. *centrale* caused clinical cases of bovine anaplasmosis in Italy (Carelli *et al.*, 2008). Therefore, when screening cattle samples, it is important to use an assay that is sensitive and specific to both *A. marginale* and *A. marginale* subsp. *centrale.* A recent study showed that the duplex qPCR assay is a better method of detecting *A. marginale* and *A. marginale* subsp. *centrale* than the reverse line blot (RLB) or nested PCR (Chaisi *et al.*, 2017).

The duplex qPCR assay targets the *msp1b* gene of *A. marginale* (Carelli *et al.*, 2007) and a highly conserved region of the *A. marginale* subsp. *centrale groEL* gene (Decaro *et al*., 2008), which contains enough nucleotide differences from the *A. marginale groEL* gene to prevent binding of the probe to this rickettsia. The assay is therefore a powerful tool for the detection and differentiation of these two closely related infectious agents. This assay can be used to improve diagnosis of bovine anaplasmosis and is very useful as a quantitative assay for studying the pathogen load of *A. marginale* infections in cattle.

Screening of 100 cattle samples from two diptanks in the Zambezia province of Mozambique showed a simultaneous detection of *A. marginale* and *A. marginale* subsp. *centrale* with a total of 86 samples being positive for *A. marginale* and six samples being positive for *A. marginale* subsp. *centrale*. *Anaplasma marginale* was present in large proportions of the samples indicating that many animals are carriers and the parasite is probably endemic in the area. Endemic stability normally occurs if the force of infection is high enough that the attainment of efficient immunity occurs in the majority of the population at a relatively young age (Hay, 2001). A generic mathematical model developed for endemic stability (Coleman *et al.,* 2001) suggested that the probability or severity of clinical disease resulting

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from infection increases with age and that after the first infection, the chance that subsequent infections result in the clinical disease is reduced. The hypothesis put forward by Coleman *et al.* (2001) suggests that when these two criteria are met then endemic stability is reached regardless of vector control measures or vaccination that might be done to prevent the disease. Some researchers investigated the effect of dipping on endemic stability (Rikhotso *et al.*, 2005; Stevens *et al.,* 2007). Their results showed that there was a reduction in tick load on animals and vegetation which led to a reduction in transmission of tick-borne parasites, thereby lowering sero-prevalence in cattle. In another study to investigate the seroprevalence of IgG antibodies to *A. marginale* using the indirect enzyme-linked immunosorbent assay of animals from southern Mozambique in the Provinces of Gaza, Inhambane and Maputo (Tembue *et al.*, 2011), the results indicated that these are areas of enzootic stability to *A. marginale*. This is also an indication that *A. marginale* might also be endemic in the Zambezia Province, although sero-prevalence has not been assessed in this province.

Anaplasma marginale subsp. *centrale* was present in very few of the samples. This could be due to vaccinations that took place when Mozambique imported cattle from Cuba (Anold and Asselbergs, 2004). According to the Anold and Asselbergs (2004), the *Anaplasma* vaccine that was used was the *A. centrale* strain produced at the Veterinary Research Institute, Onderstepoort (Abdala *et al.*, 1990; Tebele *et al.*, 1991). Vaccinated cattle can develop a persistent infection that can induce a lifetime protection and the animals will not need to be vaccinated again. Alternatively, the presence of *A. marginale* subsp. *centrale* might be due to natural infections due to the presence of wild ruminants. Khumalo *et al.* (2016) demonstrated that a wildlife reservoir exists in South Africa, as a diversity of *A. marginale* subsp. *centrale* strains from cattle and wildlife were discovered. These authors developed a test based on *msp1aS*, a homolog of *msp1α* of *A. marginale*, that could differentiate the vaccine strain from natural *A. marginale* subsp. *centrale* infections. The Zambezia Province in Mozambique is also close to a wildlife area where cattle might come in contact with wildlife and ticks contaminated with *A. marginale* subsp. *centrale*.

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Whole-genome sequence (WGS) data can provide information about the gene repertoire and sequence variation which can be used as an approach to associate genotype with phenotype (Bentley and Parkhill, 2015). *Anaplasma marginale* strains can be distinguished by *msp1α* genotyping, which is done by determining variations in the number and sequence of the tandem repeats at the amino-terminal end of the MSP1a sequence (Allred *et al*., 1990; de la Fuente *et al*., 2007; Silva *et al.*, 2015). In this study *A. marginale msp1α* amplicons from 27 cattle samples from Mozambique revealed considerable genetic diversity, providing 14 new MSP1a repeat sequences, and a total of 47 different *msp1α* genotypes. In South Africa, *A. marginale msp1α* genotying has also revealed a considerable genetic diversity (Mtshali *et al.,* 2007; Mutshembele *et al*., 2014), showing that there is a lot of natural diversity within populations of *A. marginale* in Southern Africa. Rodríguez *et al*. (2005) showed that *msp1α* genotype can indicate antigenic diversity, with strains with different *msp1α* genotypes having different *msp2* repertoires. The *msp1α* genotyping can therefore be used to select an antigenically diverse sample set for future studies of vaccine candidate antigens.

Anaplasmosis is compounded by the large antigenic and genetic diversity found in strains from one region to another, within the same herd and even within the same animal. Most samples examined in this study had more than one *msp1α* genotype, indicating that most cattle are infected with multiple strains of *A. marginale*. In fact, eight different strains were identified in one animal. Many of the *A. marginale* MSP1a repeats that were identified in this study have already been identified in other parts of the world (de la Fuente *et al.,* 2001; Quiroz-Castañeda *et al.*, 2016). However, only two (τ 10 15 and τ 10 10 15) of the 47 strains identified were not unique to Mozambique. These results might indicate that some parasites have been imported along with cattle from Brazil, although it is also possible that similar repeats have arisen independently in different countries.

A. marginale subsp. *centrale* is a less virulent strain that induces cross protection to virulent strains (Theiler, 1911) and has been used as a live blood vaccine for over 100 years. It has been used in countries like Zimbabwe, Paraguay, and Argentina (Bell-Sakyi *et al.*, 2015), but some studies have shown that the *A. marginale* subsp. *centrale* vaccine provides little to no protection, meaning the

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rickettsia does not provide full protection against *A. marginale*, perhaps due to different endemic strains found in the different countries and regions (Abdala *et al.*, 1990; Brizuela *et al.*, 1998; Turton *et al.*, 1998; Hammac *et al.*, 2013; Bell-Sakyi *et al.*, 2015). Although the *A. marginale* subsp. *centrale* vaccine has benefits, it also presents the risk of transmitting blood-borne pathogens which may result in diseases like bovine leukemia (de la Fuente *et al*., 2001). It may therefore be better to use naturally avirulent strains of *A. marginale* as vaccines against heterologous *A. marginale* isolates. The wide genetic diversity of *A. marginale* strains identified in Mozambique from just 27 samples from two diptanks (Botao and Namitangurine) may support the suggestion that regional vaccines will be needed to control anaplasmosis, and therefore local avirulent *A. marginale* isolates would need to be identified.

In this study, we identified a high proportion of cattle infected with *A. marginale* at two diptanks in Mozambique, which could be a result of endemic stability as was also suggested by Tembue *et al.*, (2008) in a study of seroprevalence to *A. marginale* done at Maputo, Inhambane and Gaza, in Mozambique. The ability of the animals to achieve endemic stability to *Anaplasma marginale* infection would be advantageous to farmers in Botao and in Namitangurine, however the high level of diversity found in the few samples analysed in this study might cause an outbreak if a more virulent strain infects the animals. Therefore the strains discovered in the Zambezia Province, Mozambique could be used to identify avirulent strains that could also be used as vaccines in Mozambique and other regions of Southern Africa (de la Fuente *et al.*, 2001; Coetzee *et al.*, 2006; Rodr´ıguez Camarillo *et al.*, 2008). This study therefore provides base-line data for future vaccine discovery programs.

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6 CONCLUSION

This study showed that *A. marginale* is endemic in Mozambique and identified 47 different *msp1α* genotypes from only 27 samples, of which only two genotypes had already been identified in other countries. The remaining 45 genotypes were novel to Mozambique. From this diversity of strains, it might be possible to identify avirulent strains that could be used as vaccines in Mozambique. This great diversity of different *A. marginale* strains in Mozambique will be an important factor to consider in the formulation of recombinant vaccines in the future. The study also indicated a very low percentage of *A. marginale* subsp. *centrale* infections which could be attributed to the fact that wildlife, which are probably the reservoir of *A. marginale* subsp. *centrale*, might not be in constant contact with the cattle from these two diptanks.

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APPENDIX 1: Animal Ethics Certificate

Animal Ethics Committee

Extension No. 1

PROJECT TITLE	**Screening for Anaplasma using quantitative real-time PCR to understand the occurrence and genetic diversity of Anaplasma marginale from cattle samples from Mozambique.**
PROJECT NUMBER	**V120-15**
RESEARCHER/PRINCIPAL INVESTIGATOR	**S.L. Phili**

STUDENT NUMBER (where applicable)	**U_15393713**
DISSERTATION/THESIS SUBMITTED FOR	**MSc**

ANIMAL SAMPLES	**Cattle – Blood**	
NUMBER OF ANIMALS	**100**	
Approval period to use animals for research/testing purposes		**January 2017- January 2018**
SUPERVISOR	**Dr. N. Collins**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date 10 March 2017
CHAIRMAN: UP Animal Ethics Committee	Signature

S4285-15

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APPENDIX 2: Letter of permission to use DNA samples from Mozambique

Centro de Biotecnologia

To whom it may concern

We hereby authorise the use of DNA samples collected from cattle in the Zambezia Province, Mozambique by Dr Nicola Collins and her group at the University of Pretoria. The blood samples were collected by Mr Fernando Mulandane, and DNA was extracted by Mr Mulandane at the Eduardo Mondlane University, Maputo. Dr Collins will be responsible for obtaining the necessary permits to allow the DNA samples to enter South Africa.

We request that the results will be shared with Mr Mulandane and he will be acknowledged in any publications emanating from the data. We trust that this research project will lead to further collaborative work and strengthen the ties between the Eduardo Mondlane University and the University of Pretoria.

The Director

Joaquim Saide, PhD

Av. de Moçambique, Km 1,5, C. Postal 257, Tel.: (+258) 21 477227, Fax.: (+258) 21 477227, www.cb.uem.mz
Maputo – Moçambique

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APPENDIX 3: Protocol for blood sample collection in Mozambique

Centro de Biotecnologia

To whom it may concern

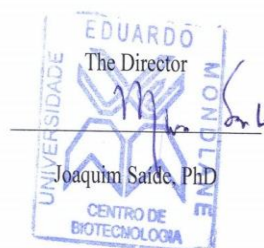
I hereby confirm that Mr. Fernando Mulandane is collecting blood samples from cattle in Zambezia Province, Mozambique as part of a routine surveillance project to examine trypanosome resistance.

Furthermore, he kindly agreed to provide an aliquot of DNA from one hundred samples that will be sent soon to the University of Pretoria.

Prior to the survey the nature and the purpose of it was explained in detail to the cattle owners. The participation of the owners was voluntary and an oral consent was obtained from each of them.

To obtain the blood samples, the cattle were professionally handled in a crush-pen and the blood was drawn, from the coccygeal vein, using vacutainer EDTA tubes and 21G needles.

The blood samples were transported to the Eduardo Mondlane University (EMU) in Maputo where DNA was extracted. All vacutainers, pipette tips and eppendorf tubes were disposed of in the Biotechnology Center –EMU, following the standard procedures for the disposal of hazardous material.



The Director

Joaquim Saíde, PhD

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Maputo – Moçambique

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APPENDIX 4: Veterinary Import Permit for DNA samples from Mozambique

agriculture, forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate of Animal Health
Import-Export Policy Unit
Private Bag X138
Pretoria, 0001
Republic of South Africa
Tel: (27)-012-319 7514
Fax: (27)-012-329 8292
PERMIT NO: 13/1/1/30/2/0-2015/09/003158
Valid from: 2015-09-22
Expiry date: 2015-12-22

IMPORTER:
DR NICOLA E. COLLINS
DEPARTMENT OF VETERINARY TROPICAL DISEASES FACULTY OF VETERINARY SCIENCE
UNIVERSITY OF PRETORIA
ONDERSTEPOORT

VETERINARY IMPORT PERMIT FOR PATHOLOGY SPECIMENS
[Issued in terms of the Animal Diseases Act, 1984 (Act No. 35 of 1984)]

Authority is hereby granted for you to import 100 X 1 ML EPPENDORF TUBES EACH CONTAINING 250 UL OF DNA EXTRACTED FROM 100 CATTLE BLOOD SAMPLES into Republic of South Africa:

From: MOZAMBIQUE
subject to the following conditions:

1. The consignment must be accompanied by this original permit and an original veterinary health certificate, complying with the conditions stipulated overleaf (IMP.PATH.CE.10/2013), duly completed and signed by an official veterinarian, authorised thereto by the Veterinary Authority of MOZAMBIQUE.
2. The specimens are to be securely packed and transported in leakproof containers, sealed by an authorised official of the Veterinary Authority of the exporting country;
3. The specimens must be kept and used for purposes of testing/research at the laboratories of DEPARTMENT OF VETRINARY TROPICAL DISEASES under the personal supervision of DR NICOLA COLLINS;
4. On completion of tests/research the specimens, including all contaminated/infectious things or animal products (as defined by the Animal Diseases Act, 1984 [Act No. 35 of 1984]) derived/produced from or that came into contact with the above-mentioned specimens, must be destroyed by incineration. Records of the incinerations must be maintained for a period of 5 years, and made available for auditing to the Veterinary Authority upon request.
5. The consignment must be airfreighted through port of entry O R TAMBO INTERNATIONAL AIRPORT. **Samples may only be imported as manifest cargo under an airwaybill number and may not be imported as personal luggage.**
6. The consignment must be accompanied by this permit and its arrival reported immediately to the inspecting veterinary official: KEMPTON PARK Tel: 011 973 2827, and may not be released without his/her written permission.
7. Upon arrival the inspecting veterinary official will inspect the consignment and release it to the importer only after he/she is satisfied that all the import conditions have been complied with in full.
8. **This permit does not absolve the importer from compliance with the provisions of any other legislation relating to this import.**
9. This permit is subject to amendment or cancellation by the Director Animal Health at any time and without prior notice being given.
10. This permit is valid for three (3) months from date of issue and FOR ONE CONSIGNMENT ONLY.

SPECIAL CONDITIONS:

pp aufraham
DIRECTOR: ANIMAL HEALTH

NOTE:

- All imports for research purposes require Section 20 permission in compliance with the Animal Diseases Act.
- Any consignment imported into South Africa packed with either wood packaging material or dunnage, will require treatment to remove any pests present (by heat or methyl bromide fumigation). Treatment must be indicated as per IPPC prescript on wood packaging material. [Directorate: Inspection Services Tel: 012 309 8754 or Fax 086 732 4768 or www.daff.gov.za]

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(IMP.PATH.CE.10/2013)

APPENDIX 5: Determination of DNA concentration of *msp1α* PCR products

The *msp1α* PCR products were run on a PowerWave HT Microplate spectrophotometer (BioTek) in order to determine the DNA concentration of each PCR product. For cloning of the PCR products, 50 ng of PCR product is required in each ligation reaction mix. The spectrophotometer reading (DNA concentration) was used to determine the volume of each PCR product that would contain 50 ng of the insert (PCR product) that is required for a 3:1insert: vector molar ratio. The spectrophotometer results are presented in Table 3. An example of how to determine how much of the sample was added to the ligation mix is shown in the calculation below.

Example

The equation in the box below shows an example of sample 49 which is at a concentration of 66.402ng/µl. Simple proportion was used to determine the volume that contains 50 ng.

$$1\ \mu l = 66.402\ ng$$

$$X\ \mu l = 50\ ng$$

$$X\ \mu l = 50\ ng \times 1\ \mu l\ /\ (66.402\ ng)$$

$$X\ \mu l = 0.75\ \mu l$$

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Appendix 5, Supplementary Table 1: *DNA concentration of 15 selected samples. The spectrophotometer results were used to determine what volume of the sample to use for cloning purposes.*

Sample read no.	Location	260 RAW	280 RAW	320 RAW	260	280	260/280	ng/µL	Volume (µL)
49	B2	0.118	0.087	0.05	0.066	0.037	1.805	66.402	0.75
84	B3	0.147	0.118	0.08	0.065	0.037	1.755	65.109	0.768
86	C2	0.111	0.08	0.042	0.067	0.038	1.78	66.76	0.75
51	C3	0.111	0.081	0.045	0.064	0.035	1.823	63.556	0.79
33	D2	0.107	0.078	0.042	0.063	0.035	1.782	62.702	0.8
96	D3	0.115	0.083	0.047	0.067	0.036	1.855	66.754	0.75
97	E2	0.089	0.069	0.043	0.042	0.024	1.725	42.255	1.2
4	E3	0.103	0.079	0.046	0.054	0.032	1.681	53.901	0.93
46	F2	0.104	0.076	0.041	0.059	0.034	1.768	59.498	0.84
45	F3	0.1	0.074	0.043	0.054	0.03	1.783	54.15	0.92
90	B2	0.096	0.072	0.041	0.056	0.03	1.837	55.567	0.92
91	B3	0.102	0.075	0.041	0.062	0.034	1.808	62.189	0.8
92	C2	0.097	0.072	0.04	0.057	0.031	1.829	57.222	0.87
58	C3	0.103	0.075	0.041	0.064	0.034	1.849	63.664	0.79
64	D2	0.088	0.066	0.039	0.048	0.026	1.836	48.349	1.03
BLANK	D3	0.083	0.08	0.076	0.003	0.001	3.625	3.153	

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APPENDIX 6: Reverse Line Blot Results

TEST REPORT
REVERSE LINE BLOT
Enquiries: Mrs Milana Troskie
Ms Ilse Vorster

Tel : +27 12 529 8441 / 8363 Fax: +27 12 529 8312

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

MOLECULAR DIAGNOSTIC SERVICES
Department of Veterinary Tropical Diseases
Faculty of Veterinary Science
Private Bag X04
Onderstepoort, 0110
South Africa

Case Number: **RS15/022**

Date Opened: 23 November 2015
Report Date: 23 March 2016

Total Cost **R 21 200.00**

This document does not serve as an invoice.
An official invoice will be sent by the University
with a reference number to be used.

Sender information: Collins, N. Dr.

Faculty of Veterinary Science
DVTD

Tel: (012) 529-8022
Fax: (012) 529-8312
Mobile:
Email: nicola.collins@up.ac.za

Referring Vet / Contact Person:
Collins, N. Dr.

Owner:
Collins, N. Dr.

Number of samples tested **100**

Lab number and Type	*Sample ID and Species*	*Sample condition upon receipt*	*Test*	*Result*
1 RE15/446 DNA	**1** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, A. sp. omatjenne* Procedure Date: 03 November 2015
2 RE15/447 DNA	**2** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, T/B catch all, T. catch all, B. catch all 1, B. bovis, T. mutans* Procedure Date: 03 November 2015
3 RE15/448 DNA	**3** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, A. sp. omatjenne, T/B catch all, T. catch all, T. sp. sable, T. mutans, T. velifera* Procedure Date: 03 November 2015
4 RE15/449 DNA	**4** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, A. sp. omatjenne, T/B catch all, T. catch all, T. sp. sable, T. mutans, T. velifera* Procedure Date: 03 November 2015

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5 RE15/450 DNA	**5** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, A. sp. omatjenne, T/B catch all, T. catch all, T. sp. sable, T. mutans, T. velifera* Procedure Date: 03 November 2015

6 RE15/451 DNA	**6** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, A. sp. omatjenne, T/B catch all, T. catch all, T. sp. sable, T. mutans, T. velifera* Procedure Date: 03 November 2015

7 RE15/452 DNA	**7** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, A. sp. omatjenne, T/B catch all, B. catch all 1, B. bovis (f)* Procedure Date: 03 November 2015

8 RE15/453 DNA	**8** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, A. sp. omatjenne, T/B catch all, T. catch all, B. catch all 1, B. catch all 2, B. bovis, T. sp. sable, T. mutans, T. velifera* Procedure Date: 03 November 2015

9 RE15/454 DNA	**9** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, A. sp. omatjenne, T/B catch all, T. catch all, B. catch all 1, B. catch all 2, B. bovis, T. sp. sable, T. mutans, T. velifera* Procedure Date: 03 November 2015

10 RE15/455 DNA	**10** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, A. sp. omatjenne, T/B catch all, T. catch all, B. catch all 1, B. catch all 2, B. bovis, T. sp. sable, T. mutans, T. velifera* Procedure Date: 03 November 2015

11 RE15/456 DNA	**11** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. sp. omatjenne, T/B catch all, T. catch all, B. catch all 1, B. catch all 2, B. bovis, T. mutans* Procedure Date: 03 November 2015

12 RE15/457 DNA	**12** *Cattle*	Acceptable	RLB-PCR (Res)	No nucleic acid detected. Procedure Date: 03 November 2015

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13 RE15/458 DNA	**13** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. sp. omatjenne, T/B catch all, T. catch all, B. catch all 1, B. catch all 2 (f), B. bovis, T. mutans* Procedure Date: 03 November 2015
14 RE15/459 DNA	**14** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. sp. omatjenne, T/B catch all, T. catch all, B. catch all 1, B. catch all 2, B. bovis, T. mutans* Procedure Date: 03 November 2015
15 RE15/460 DNA	**15** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. sp. omatjenne, T/B catch all, B. catch all 1 (f), T. mutans* Procedure Date: 03 November 2015
16 RE15/461 DNA	**16** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale (vf), A. sp. omatjenne, T/B catch all, T. catch all, B. catch all 1, T. mutans, T. velifera* Procedure Date: 03 November 2015
17 RE15/462 DNA	**17** *Cattle*	Acceptable	RLB-PCR (Res)	No nucleic acid detected. Procedure Date: 03 November 2015
18 RE15/463 DNA	**18** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, A. sp. omatjenne, T/B catch all, T. catch all, T. sp. sable, T. mutans, T. velifera* Procedure Date: 03 November 2015
19 RE15/464 DNA	**19** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, B. catch all 1, B. catch all 2* Procedure Date: 03 November 2015
20 RE15/465 DNA	**20** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, B. catch all 1, B. catch all 2* Procedure Date: 03 November 2015
21 RE15/466 DNA	**21** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, T. catch all, B. catch all 1, B. catch all 2* Procedure Date: 03 November 2015

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22 RE15/467 DNA	**22** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale (f), A. sp. omatjenne (f), T/B catch all, T. catch all, T. sp. sable, T. mutans, T. velifera* Procedure Date: 03 November 2015
23 RE15/468 DNA	**23** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale (f), A. sp. omatjenne (vf), T/B catch all, T. catch all, T. sp. sable, T. mutans, T. velifera* Procedure Date: 03 November 2015
24 RE15/469 DNA	**24** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *T/B catch all, T. catch all* Procedure Date: 03 November 2015
25 RE15/470 DNA	**25** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *T/B catch all, T. catch all* Procedure Date: 03 November 2015
26 RE15/471 DNA	**26** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, T/B catch all, T. catch all, B. catch all 1, B. catch all 2, T. sp. sable, T. mutans, T. velifera* Procedure Date: 03 November 2015
27 RE15/472 DNA	**27** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, T/B catch all, T. catch all, B. catch all 1 (f), T. sp. sable, T. mutans, T. velifera* Procedure Date: 03 November 2015
28 RE15/473 DNA	**28** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, T/B catch all, T. catch all, B. catch all 1 (f), T. sp. sable, T. mutans, T. velifera* Procedure Date: 03 November 2015
29 RE15/474 DNA	**29** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, A. sp. omatjenne, T/B catch all, T. catch all, B. catch all 1, B. catch all 2, B. bovis, T. mutans* Procedure Date: 03 November 2015

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30 RE15/475 DNA	30 *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, A. sp. omatjenne, T/B catch all, T. catch all, B. catch all 1, B. catch all 2, B. bovis, T. mutans* Procedure Date: 03 November 2015

31 RE15/476 DNA	31 *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, A. sp. omatjenne, T/B catch all, T. catch all, B. catch all 1, B. catch all 2, B. bovis, T. mutans* Procedure Date: 03 November 2015

32 RE15/477 DNA	32 *Cattle*	Acceptable	RLB-PCR (Res)	No nucleic acid detected. Procedure Date: 03 November 2015
33 RE15/478 DNA	33 *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, T. catch all (f), B. catch all 1* Procedure Date: 03 November 2015

34 RE15/479 DNA	34 *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, T/B catch all, T. catch all, B. catch all 1* Procedure Date: 03 November 2015

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39 **RE15/484** DNA	39 *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, T/B catch all, T. catch all, B. catch all 1, B. catch all 2, T. mutans* Procedure Date: 03 November 2015
40 **RE15/485** DNA	40 *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, T/B catch all, T. catch all, T. mutans* Procedure Date: 03 November 2015
41 **RE15/486** DNA	41 *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, T/B catch all, T. catch all, B. catch all 1, B. bovis, T. buffeli (vf)* Procedure Date: 20 November 2015
42 **RE15/487** DNA	42 *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, T/B catch all, T. catch all, B. catch all 1, B. catch all 2, T. mutans* Procedure Date: 20 November 2015
43 **RE15/488** DNA	43 *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale* Procedure Date: 20 November 2015
44 **RE15/489** DNA	44 *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, T/B catch all, T. catch all, T. mutans* Procedure Date: 20 November 2015
45 **RE15/490** DNA	45 *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, T/B catch all, T. catch all, T. annulata (vf)* Procedure Date: 20 November 2015
46 **RE15/491** DNA	46 *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all* Procedure Date: 20 November 2015
47 **RE15/492** DNA	47 *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, T/B catch all, T. catch all, T. mutans* Procedure Date: 20 November 2015

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48 RE15/493 DNA	48 *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, T/B catch all, T. catch all, B. catch all 1 (f), T. mutans* Procedure Date: 20 November 2015

49 RE15/494 DNA	49 *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, T/B catch all, T. catch all* Procedure Date: 20 November 2015

50 RE15/495 DNA	50 *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, T/B catch all, T. catch all (vf)* Procedure Date: 20 November 2015

51 RE15/496 DNA	51 *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, B. catch all 1* Procedure Date: 20 November 2015

52 RE15/497 DNA	52 *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. centrale, A. marginale, T/B catch all, T. catch all* Procedure Date: 20 November 2015

53 RE15/498 DNA	53 *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, T/B catch all, T. catch all* Procedure Date: 20 November 2015

54 RE15/499 DNA	54 *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, B. catch all 1* Procedure Date: 20 November 2015

55 RE15/500 DNA	55 *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, B. catch all 1* Procedure Date: 20 November 2015

56 RE15/501 DNA	56 *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *T/B catch all, T. catch all, B. catch all 1, T. mutans* Procedure Date: 20 November 2015

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57 RE15/502 DNA	**57** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *T. catch all, B. catch all 1* Procedure Date: 20 November 2015
58 RE15/503 DNA	**58** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, T. catch all (vf)* Procedure Date: 20 November 2015
59 RE15/504 DNA	**59** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all (vf), T. catch all (vf)* Procedure Date: 20 November 2015
60 RE15/505 DNA	**60** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, T. catch all (vf), B. catch all 1* Procedure Date: 20 November 2015
61 RE15/506 DNA	**61** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *T/B catch all, T. mutans (vf)* Procedure Date: 20 November 2015
62 RE15/507 DNA	**62** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, T. catch all, B. catch all 1, B. catch all 2* Procedure Date: 20 November 2015
63 RE15/508 DNA	**63** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, B. catch all 1* Procedure Date: 20 November 2015
64 RE15/509 DNA	**64** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, T/B catch all, T. catch all* Procedure Date: 20 November 2015
65 RE15/510 DNA	**65** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, T/B catch all, T. catch all* Procedure Date: 20 November 2015
66 RE15/511 DNA	**66** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, T/B catch all, T. catch all* Procedure Date: 20 November 2015

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67 RE15/512 DNA	**67** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, T/B catch all, T. catch all, T. mutans* Procedure Date: 20 November 2015
68 RE15/513 DNA	**68** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, T/B catch all, T. catch all, B. catch all 1, T. mutans* Procedure Date: 20 November 2015
69 RE15/514 DNA	**69** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale (f)* Procedure Date: 20 November 2015
70 RE15/515 DNA	**70** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, B. catch all 1* Procedure Date: 20 November 2015
71 RE15/516 DNA	**71** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, T/B catch all, T. catch all, T. mutans* Procedure Date: 20 November 2015
72 RE15/517 DNA	**72** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, T/B catch all, T. catch all, T. mutans* Procedure Date: 20 November 2015
73 RE15/518 DNA	**73** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, T/B catch all, T. catch all, T. mutans* Procedure Date: 20 November 2015
74 RE15/519 DNA	**74** *Cattle*	Acceptable	RLB-PCR (Res)	No nucleic acid detected. Procedure Date: 20 November 2015
75 RE15/520 DNA	**75** *Cattle*	Acceptable	RLB-PCR (Res)	No nucleic acid detected. Procedure Date: 20 November 2015

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76 RE15/521 DNA	**76** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, T/B catch all, T. catch all* Procedure Date: 20 November 2015
77 RE15/522 DNA	**77** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, T/B catch all, T. catch all, T. mutans* Procedure Date: 20 November 2015
78 RE15/523 DNA	**78** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, T/B catch all, T. catch all, T. mutans* Procedure Date: 20 November 2015
79 RE15/524 DNA	**79** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, T/B catch all, T. catch all, B. catch all 1, T. mutans* Procedure Date: 20 November 2015
80 RE15/525 DNA	**80** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, T/B catch all, T. catch all, T. sp. sable, T. mutans, T. velifera* Procedure Date: 20 November 2015
81 RE15/526 DNA	**81** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, T/B catch all, B. catch all 1, B. bovis* Procedure Date: 24 November 2015
82 RE15/527 DNA	**82** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *T/B catch all, T. catch all, T. mutans* Procedure Date: 24 November 2015
83 RE15/528 DNA	**83** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, B. catch all 1* Procedure Date: 24 November 2015
84 RE15/529 DNA	**84** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, T/B catch all, T. catch all, T. mutans* Procedure Date: 24 November 2015

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85 RE15/530 DNA	**85** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. centrale (f), A. marginale (f), T/B catch all, T. catch all, T. mutans* Procedure Date: 24 November 2015
86 RE15/531 DNA	**86** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, T/B catch all, T. catch all, T. mutans* Procedure Date: 24 November 2015
87 RE15/532 DNA	**87** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. centrale (f), A. marginale (f), T/B catch all, T. catch all, T. mutans* Procedure Date: 24 November 2015
88 RE15/533 DNA	**88** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. centrale (f), A. marginale (f), T/B catch all, T. catch all, T. mutans* Procedure Date: 24 November 2015
89 RE15/534 DNA	**89** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, T/B catch all, T. catch all, T. mutans* Procedure Date: 24 November 2015
90 RE15/535 DNA	**90** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, T/B catch all, T. catch all, T. mutans* Procedure Date: 24 November 2015
91 RE15/536 DNA	**91** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, T/B catch all, T. catch all (f), T. mutans* Procedure Date: 24 November 2015
92 RE15/537 DNA	**92** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, T/B catch all, T. catch all, T. mutans* Procedure Date: 24 November 2015
93 RE15/538 DNA	**93** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, T/B catch all, T. catch all, T. mutans* Procedure Date: 24 November 2015

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94 RE15/539 DNA	**94** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, T/B catch all, T. catch all, T. mutans* Procedure Date: 24 November 2015
95 RE15/540 DNA	**95** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, T/B catch all, T. catch all, T. mutans* Procedure Date: 24 November 2015
96 RE15/541 DNA	**96** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, T/B catch all, T. catch all, T. mutans* Procedure Date: 24 November 2015
97 RE15/542 DNA	**97** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *T/B catch all, T. catch all, T. mutans* Procedure Date: 24 November 2015
98 RE15/543 DNA	**98** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all* Procedure Date: 24 November 2015
99 RE15/544 DNA	**99** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, T/B catch all, T. catch all, T. mutans* Procedure Date: 24 November 2015
100 RE15/545 DNA	**100** *Cattle*	Acceptable	RLB-PCR (Res)	Nucleic acid detected: *E/A catch-all, A. marginale, T/B catch all, T. catch all, T. mutans* Procedure Date: 24 November 2015

*T - Theileria; B - Babesia; A - Anaplasma; E - Ehrlichia; * Non-pathogenic but unusual finding; (f) - faint reaction; (vf) - very faint reaction.*

T/B catch-all: indicates that a sample tested positive for a Theileria or a Babesia species (non specific). T catch-all: indicates that a sample tested positive for a Theileria species (non specific). E/A catch-all: indicates that a sample tested positive for a Ehrlichia or a Anaplasma species (non specific). B catch-all 1 or 2: indicates that a sample tested positive for a Babesia species (non specific). Faint / very faint: indicates the visibility of the reaction on the blot for that specific species.

RLB-PCR: Test performed for the simultaneous detection of Theileria-, Babesia-, Anaplasma-, and Ehrlichia species.

M Troskie.
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The results contained in this report relate only to the samples tested.
The Reverse Line Blot serves as a screening test and is used for research purposes only.
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