Molecular characterisation of potential vaccine candidates from *Anaplasma marginale* strains in South Africa

By

PAIDASHE HOVE

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Supervisor

DR. NICOLA E. COLLINS

Co-Supervisors

PROF. MARINDA C. OOSTHUIZEN

DR. KELLY A. BRAYTON

DR. MOSES S. MTSHALI

May, 2018

DECLARATION

I declare that this thesis, which I hereby submit for the degree of **Philosophiae Doctor** at the University of Pretoria, is my own work and has not been previously submitted by me for a degree at any other tertiary institution.

_____

Paidashe Hove

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DEDICATION

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LIST OF ABBREVIATIONS AND ACRONYMS

2D	two-dimensional
ARC-OVR	Agricultural Research Council-Onderstepoort Veterinary Research Institute
BCA	bicinchoninic Acid
cELISA	competitive enzyme-linked immunosorbent assay
Cq	quantification cycle
DNA	deoxyribonucleic acid
E	assay efficiency
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
gDNA	genomic DNA
HRP	horse radish peroxidase
HVR	hyper variable region
IDE8	*Ixodes scapularis* deer-collected embryos isolated on day 8 after onset of oviposition
IFN-γ	interferon gamma
Ig	immunoglobulin
INDEL	insertion/deletion
IPTG	isopropylthio-β-galactoside
iRBCs	infected red blood cells
ISE6	*Ixodes scapularis* southern embryos isolated on day 6 after onset of oviposition
kDa	kiloDalton
LB	Luria-Bertani
LC-MS/MS	liquid chromatography-tandem mass spectrometry
MB	megabytes
MHC	major histocompatibility complex
ml	millilitre
Msp	major surface protein
N	neutralization sensitive
NGS	next-generation sequencing

nPCR	nested PCR
NZG	National Zoological Gardens
OBP	Onderstepoort Biological Products
OMPs	outer membrane proteins
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PCV	packed cell volume
PPE	percentage parasitized erythrocytes
PVDF	polyvinylidene difluoride
qPCR	quantitative real-time PCR
R^2	regression co-efficient
RLB	reverse line blot hybridization
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecylsulphate
SNPs	single nucleotide polymorphisms
SUMO	small ubiquitin-like modifier
Taq	*Thermus aquaticus*
TMB	tetramethylbenzidine
TPB	tryptose phosphate broth
tRNA	transfer ribonucleic acid
USA	United States of America
UTR	untranslated region
µl	microlitre

THESIS SUMMARY

Bovine anaplasmosis, a tick-borne disease caused by the rickettsia *Anaplasma marginale,* has a significant economic impact for cattle farmers in South Africa. We have estimated the economic cost due to mortality arising from bovine anaplasmosis in the country to be R115 million ($US9.6 million) per year. Further costs are attributable to chemotherapeutic treatment and tick control using acaricides. *Anaplasma centrale* is a species that is closely related to *A. marginale,* and in most cases causes a milder, less virulent form of anaplasmosis. It provides cross protection against field strains of *A. marginale* infection and is therefore employed as a blood vaccine against bovine anaplasmosis in some countries, including South Africa. Despite the economic impact of this disease, there are few studies on the prevalence and control of bovine anaplasmosis in South Africa. This study was therefore carried out in order to evaluate the presence and genetic diversity of *A. marginale* in the country using quantitative real-time PCR (qPCR) and repeat variation of MSP1a, respectively. In a comprehensive examination of 517 bovine samples from all provinces of South Africa, using the *A. marginale* and *A. centrale* qPCR, *A. marginale* was detected in 57% of our test samples. The rickettsial pathogen was present in all provinces of South Africa with the exception of the Northern Cape province where the tick vector is absent. However, due to recently reported range extension of the important anaplasmosis tick vector *Rhiphicephalus microplus*, it is believed that this situation may change, and needs close monitoring. *Anaplasma centrale* was also detected in 17% of the samples, with 15% of the samples being co-infected. An analysis of *A. marginale* strains present in the samples revealed high genetic diversity, as reflected by the 190 genotypes derived from 99 Msp1a amino acid repeats. This genetic diversity is attributable to a high rate of evolution. Our data also reveal that 22% of the 99 amino acid repeats and, interestingly, only 2 genotypes we found in South Africa, were shared with other countries around the world. Because this study is centred on contributing to the development of a recombinant subunit vaccine, this strain variation should be taken into account in such an undertaking.

The current *A. centrale* blood vaccine has some drawbacks, the two main problems being that it does not protect against heterologous challenge with field strains of *A. marginale* and it may contribute to transmitting other emerging diseases resulting from a contaminated blood vaccine. Outer membrane protein (OMP) preparations are known to induce immune protection in nearly all animals tested, thus demonstrating the potential

efficacy of a subunit vaccine. Five potential OMP vaccine candidates Am779, Am854, Omp7, Omp8 and Omp9 were identified from North American *A. marginale* strains and have been well-characterised in *A. marginale* strains from United States of America (USA), but their levels of conservation in other countries were not known. This information would be needed to show that they could be used in a vaccine formulation for a broad application to control bovine anaplasmosis worldwide or in making specific vaccine formulations well-suited for geographic regional strains. In this study, we demonstrated the amino acid variation in these five vaccine candidate OMPs in South African *A. marginale* strains. We also assessed the immunogenic relationships between South African recombinant versions of these OMPs, and the extensively studied St. Maries and Florida *A. marginale* strains, from USA. OMPs Am854 and Am779 were found to be highly conserved, with 99–100% amino acid identity. Omp7, Omp8 and Omp9 were also found to be conserved with 79–100% identity with St. Maries and Florida strains. We also found, as has been shown previously, that the latter OMPs possess conserved N- and C-terminals, along with a pronounced, central hypervariable region. A previously identified, highly conserved T-cell epitope, FLLVDDAI/V, was also found in the conserved N-terminus of these three OMPs. Through recognition of South African recombinant OMPs by anti-*A. marginale* and *A. centrale* bovine sera from South Africa and USA, we were able to demonstrate immunological cross-reactivity between the *A. centrale* and *A. marginale* organisms. This suggests that there are significant antigenic and immunological relationships between South Africa and USA strains of *A. marginale,* and provides evidence for the continued use of the *A. centrale* blood vaccine for immunisation against *A. marginale* infections. Our study also provides evidence to suggest that the *A. marginale* OMPs are good vaccine candidates for use in a global vaccine cocktail, although further work on the best formulation and delivery methods is necessary.

For the purposes of creating a biobank of *A. marginale* strains for downstream '-omic' studies and to provide challenge material for future vaccine trials, we attempted to culture field strains of *A. marginale* from 17 bovine blood samples in ISE6 and IDE8 tick cell lines. Blood from three persistently infected and 12 clinically sick animals was used to attempt direct infection of tick cell lines, but yielded negative cultures after approximately 160 days in culture. We therefore attempted to initiate cultures using blood from two splenectomised calves that were infected with blood from *A. marginale*-carrier animals. These have yielded promising results as small colonies could be observed after about 60

days of culture, and DNA extracts of these cultures were qPCR-positive. We will continue to monitor these cultures by Giemsa staining, light microscopy and qPCR, for progression of the infection.

CHAPTER 1

Literature Review

1.0 Introduction

Anaplasmosis (or Gall-sickness as it was formerly known) is a tick-borne disease of ruminants caused by microbial pathogens of the genus *Anaplasma* which are obligate, intra-erythrocytic bacteria of the order Rickettsiales and family *Anaplasmataceae* (Theiler, 1910a, b; Dumler et al., 2001; Potgieter & Stoltsz, 2004; Merck, 2017). *Anaplasma marginale* is the most virulent and most prevalent vector-borne, bovine pathogen on a global scale, as it is found on all six inhabited continents (Kocan et al., 2004; de la Fuente et al., 2007; Brayton et al., 2009; Merck, 2017).

Bovine anaplasmosis was first characterized by Sir Arnold Theiler between 1907 and 1910 in South Africa, when he observed, in cattle imported from England, the intra-erythrocytic, membrane-bound coccus-like bodies of *A. marginale*, which he described as 'marginal points' (Theiler, 1910a; b; 1912; de Waal, 2000; Palmer, 2009). Theiler, through a combination of experiments and epidemiological observations, identified *A. marginale* as the causative agent of bovine anaplasmosis, which had been earlier mistaken as a lifecycle stage of the causative agent of redwater (*Babesia bigemina*). Theiler also identified an organism which he called '*Anaplasma marginale* variety *centrale*' (referred to as *A. centrale* hereafter) which generally causes a milder, less virulent form of the disease (Theiler, 1911). Infection with *A. centrale* confers some cross-protection against *A. marginale*, and it has therefore been employed as a live vaccine from the time it was developed by Theiler in 1910 (Potgieter & Stoltsz, 2004; Palmer, 2009; Theiler, 1912).

Anaplasmosis is one of the most economically important diseases of cattle in South Africa (Potgieter & Stoltsz, 2004; Mtshali et al., 2007), and results in significant productivity losses and in some cases mortality (de Waal, 2000; Aubry & Geale, 2011). In South Africa like the world over, the effects of tick-borne diseases on animals are often synergistic, where animals are infected with more than one pathogen at a time (de Waal, 2000). As a consequence, studies to quantify losses specifically attributable to anaplasmosis are yet to be carried out in South Africa (de Waal, 2000; Potgieter & Stoltsz, 2004). However, based on the 2015 figure of 13.7 million cattle in South Africa, (Directorate: Statistics and Economic Analysis, 2016),

and a yearly average mortality rate of 3% of cattle (Scholtz & Bester, 2010), we estimate the cost due to mortalities from anaplasmosis to be approximately R115 million ($US9.6 million) per year. In other parts of the world, costs arising from anaplasmosis have been estimated from $US300 to $US800 million (Kocan et al., 2003). Furthermore, economic costs attributable to the disease burden and control for babesiosis and anaplasmosis have been approximated at $US875 million in South America (Brown, 1997) and $US30.5 million in Australia (Bock & de Vos, 2001). Due to the high economic impact, vaccination with *A. centrale* has been deemed cost effective for many countries despite the risk of transmitting emerging pathogens along with the blood borne vaccine (Theiler, 1912; Potgieter & Stoltsz, 2004).

1.1 Classification of *Anaplasma* species

Rickettsiales classification is reviewed in: Dumler et al. (2001), Brayton et al. (2009), and Kocan et al. (2004; 2015). Historically, *Anaplasma* spp. were incorrectly classified as anything from viruses to protozoa (Brayton, et al., 2009). A taxonomic reclassification and reorganization of the genus using genetic analyses (Dumler et al., 2001) provided an invaluable contribution to the systematics of the *Anaplasma* spp., and resulted in the classification shown below [adapted from Brayton et al. (2009)] with seven formally recognised species and two others that have not yet been formally described.

Anaplasma marginale is currently regarded as the type species for the genus *Anaplasma* (Dumler et al., 2001), which was expanded to accommodate three species reclassified from the genus *Ehrlichia* that invade the cells of haemopoietic origin (neutrophils and erythrocytes) in their vertebrate host species. These are *A. phagocytophilum* (formerly known as *Ehrlichia phagocytophila*, *E. equi*, and the agent of human granulocytic ehrlichiosis), *A. bovis* (formerly *E. bovis*) and *A. platys* (formerly *E. platys*). Also included in the genus *Anaplasma* is another species, *A. ovis*, that causes mild to severe disease in sheep, deer and goats. The name *A. caudatum* was given to an organism with appendages that causes bovine anaplasmosis (Potgieter & Stoltsz, 2004). Although this is formally recognised as a separate species, it is believed to simply be a tailed strain of *A. marginale*, but has not been studied in great detail (Merck, 2017).

Superkingdom	Bacteria
Phylum	Proteobacteria
Class	Alpha-proteobacteria
Order	Rickettsiales
Family	*Anaplasmataceae*
Genus	*Anaplasma*
Species	*A. marginale* (type species)
	A. bovis
	A. caudatum
	A. centrale
	A. ovis
	A. phagocytophilum
	A. platys
	Not formally described:
	A. capra
	Anaplasma sp. (Omatjenne)

There are additional species that have been described in the literature that are not formally recognized, including *Anaplasma* sp. (Omatjenne) [formerly *Ehrlichia* sp. (Omatjenne)] (Allsopp et al., 1997) and *A. capra* (Li et al., 2015; Yang et al., 2017). *A. centrale* was erroneously classified as a separate species, an error which is attributable to Ristic (1968) who incorrectly stated: "In 1911, Theiler, who first described *A. centrale*, indicated that it was a separate species and thus distinct from *A. marginale*". While some authors recognised this error and continued to refer to *A. centrale* as a variety or subspecies of *A. marginale*, the organism is listed as a separate species in List No. 15 of new names and new combinations previously effectively published outside the International Journal of Systematic Bacteriology (List Number 15, 1984) and is described in Bergey's Manual of Systematics of Bacteriology (Ristic & Kreier, 1984). It is thus referred to as a separate species in many publications. We have recently shown, through sequence analyses of the 16S rRNA gene, *groEL* and *msp4* from several isolates of *A. marginale* and *A. centrale* from around South Africa, that *A. centrale* consistently forms a separate clade from *A. marginale* (Khumalo, 2017). These results, combined with morphological differences, and differences in Msp1a/Msp1aS gene structure (Khumalo et al., 2016), as well as genome architecture (Brayton et al., 2005;

Herndon et al., 2010) provide evidence to suggest that *A. centrale* is, in fact, a separate species.

1.2 Aetiology and Life Cycle

1.2.1 Aetiology

The causative agent of bovine anaplasmosis is considered to be *A. marginale* (Theiler, 1910a; b; de Waal, 2000), though *A. centrale* is also known to cause sub-clinical to severe infections in Africa (Potgieter & Stoltsz, 2004). In South Africa, five tick species (section 1.3) found on cattle have been experimentally demonstrated to transmit bovine anaplasmosis, although they are probably not all significant as field vectors (Potgieter, 1981; Potgieter & van Rensburg, 1987; de Waal, 2000). Globally, up to 20 tick species are implicated as vectors of bovine anaplasmosis (Merck, 2017). The absence of any known small laboratory animals that can be infected with *A. marginale* and used as laboratory hosts has been a major constraint in *A. marginale* research (Potgieter & Stoltsz, 2004).

1.2.2 Life Cycle

Persistent and life-long infections in wild ruminants, cattle and ticks enable them to serve as reservoir hosts for *A. marginale* (de Waal, 2000; Kocan et al., 2004; Brayton, 2012). *Anaplasma marginale* rickettsiae cycle between the vertebrate and tick hosts (**Fig. 1.1**): ticks ingest *A. marginale* through the consumption of infected red blood cells in a blood meal, leading to infection of the tick gut cells by the bacteria. The rickettsiae infect epithelial cells of the midgut, where they develop within membrane bound vacuoles (Kocan et al., 2004; Aubry & Geale, 2011). At this stage they appear as the reticulated (vegetative) form, which divides by binary fission, leading to membrane-bound colonies containing large numbers of the pathogen (Kocan et al., 2004). Thereafter, the reticulated form matures to become the dense (infective) form, which can survive outside the membrane-bound epithelial cells of the tick midgut. This dense form transfers to various tissues of the tick including salivary glands, where they undergo division, and from where they are transmitted to the bovine host when an infected tick takes on a blood meal; once in the bovine host, the pathogens infect bovine erythrocytes (Kocan et al., 2004; Potgieter & Stoltsz, 2004; Aubry & Geale, 2011). Thereafter, the completion of the developmental cycle of the rickettsiae takes place in mature bovine erythrocytes (Potgieter & Stoltsz, 2004).

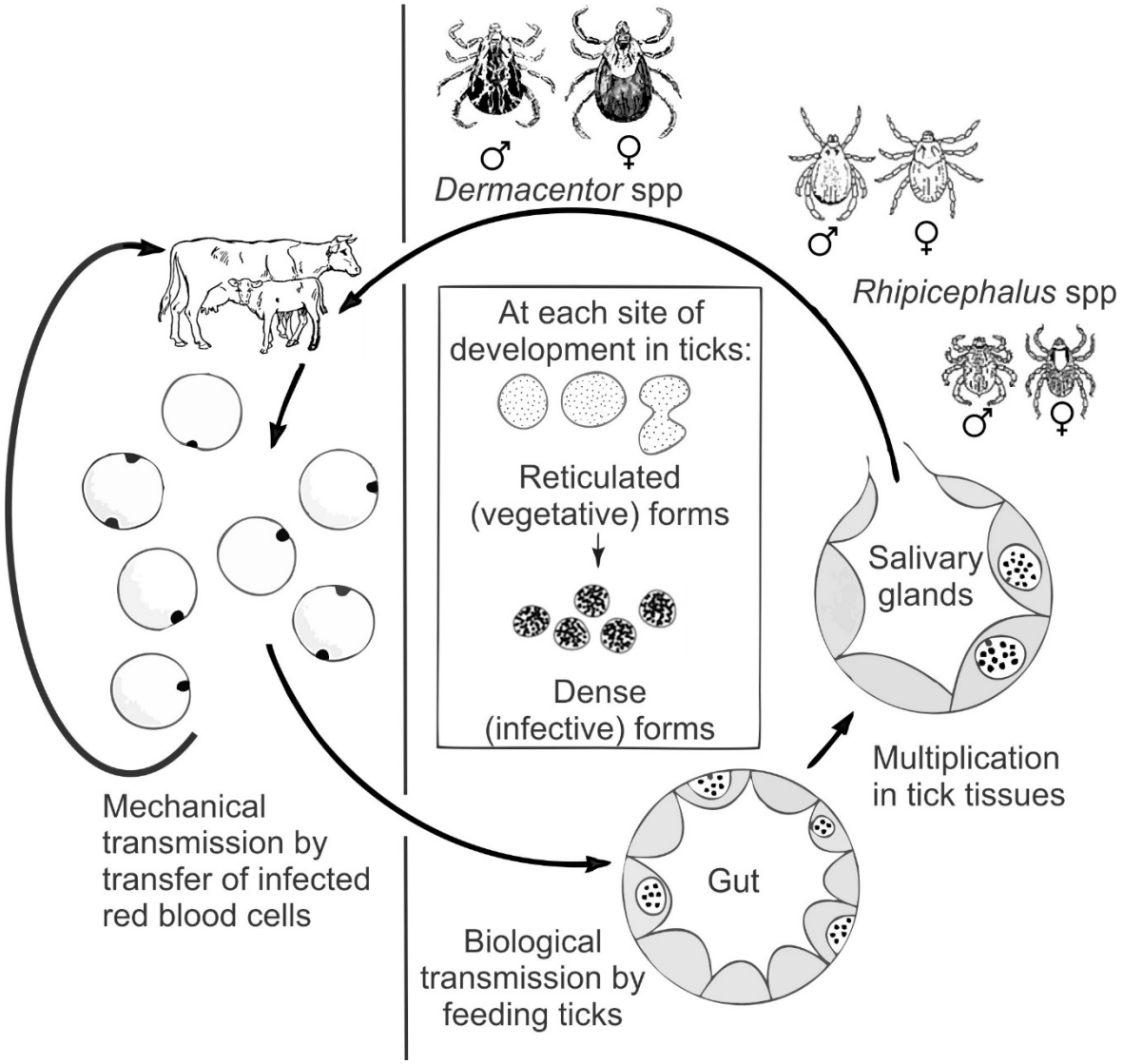

Figure 1.1: **A diagrammatic representation of the life cycle of *Anaplasma marginale*** (modified from Palmer et al., 1999). *Dermacentor* spp. and *Rhipicephalus* (formerly *Boophilus*) spp. are major biological tick vectors.

The dense forms of the rickettsiae, which infect bovine host erythrocytes, are referred to as initial bodies (Potgieter & Stoltsz, 2004). Initial bodies undergo binary fission in bovine erythrocytes to form structures known as inclusion bodies, containing four to eight initial bodies, which are cyclically transmitted to uninfected erythrocytes (Kocan et al., 2004; Potgieter & Stoltsz, 2004; Brayton et al., 2009). The location of inclusion bodies in host red blood cells observed using light microscopy, was the main characteristic used by Sir Arnold Theiler in naming the species (de Waal, 2000; Palmer, 2009), and the location of inclusion bodies differs for the two *Anaplasma* spp., with *A. marginale* inclusion bodies being more

marginally located, whereas those for *A. centrale* are more centrally located. The initial bodies are released from a disrupted erythrocyte and spread to invade other uninfected erythrocytes by invagination of the erythrocyte membranes, forming membrane-bound vacuoles containing the organism (Potgieter & Stoltsz, 2004), therefore perpetuating the infection to other uninfected host erythrocytes.

1.3 Epidemiology

Anaplasmosis, found in virtually all human-inhabited geographical locations of the world (de la Fuente et al., 2007; Brayton et al., 2009), is endemic in most cattle-rearing areas of southern Africa (de Waal, 2000). Outbreaks of anaplasmosis, though rare in countries like Canada and France, are also well documented (de Waal, 2000; Potgieter & Stoltsz, 2004; Howden et al., 2010; Aubry & Geale, 2011). This points not only to the global presence of the bacterium, but economic importance also.

As shown in **Fig. 1.2**, most of the South African cattle production areas are considered as anaplasmosis endemic and epidemic areas (de Waal, 2000). Anaplasmosis endemic areas are present in all provinces of the country except the Northern Cape, where the tick vectors are mostly absent. Of all cattle mortalities in South Africa, 18% originate from the tick-borne diseases babesiosis, heartwater and anaplasmosis, with anaplasmosis contributing approximately 3% of the total cattle mortalities. It is however, suspected that there is gross under-reporting of disease incidences (de Waal, 2000). Mtshali et al. (2007) also described the anaplasmosis incidence in communal and commercial cattle herds in the Free State province of South Africa. This study measured disease incidence using seroprevalence to *Anaplasma* spp. by competitive enzyme-linked immunosorbent assay (cELISA), which ranged between 44% and 98%. Additionally, Mtshali et al. (2007) reported *A. marginale* infection in 129 out of 215 samples tested, using an *A. marginale*-specific *msp1α* polymerase chain reaction (PCR) assay.

In South Africa, the role played by tick species in anaplasmosis transmission is poorly studied, and it has long been assumed that the one-host tick, *Rhipicephalus decoloratus* is the main disease vector. This is due to the co-occurrence of this tick with the disease in endemic areas of the country (Potgieter & Stoltsz, 2004). *Rhipicephalus microplus* is spreading in South Africa and is therefore probably increasing in importance as a vector (Nyangiwe et al.,

2013; 2017). Five tick species have been shown to experimentally transmit anaplasmosis in South Africa and have therefore been implicated in the epidemiology of the disease (de Waal, 2000; Potgieter & Stoltsz, 2004) [A review of some of the tick species in South Africa is given in Spickett et al. (2011)]. These five tick species are *R. decoloratus* and *R. microplus* (these were formally members of the genus *Boophilus*, but have now been reclassified into a subgenus of *Rhipicephalus* (Barker & Murrell, 2004; Spickett et al., 2011)), *R. evertsi evertsi*, *R. simus* and *Hyalomma marginatum rufipes* (de Waal, 2000; Potgieter & Stoltsz, 2004). More recent data from an unpublished South African study conducted between 2014 and 2017 (Khumalo, 2017), in which ticks were collected and analysed for *A. centrale* infection, suggests that *A. centrale* is also transmitted by the tick vector *R. appendiculatus*. However, this is yet to be confirmed by performing transmission studies.

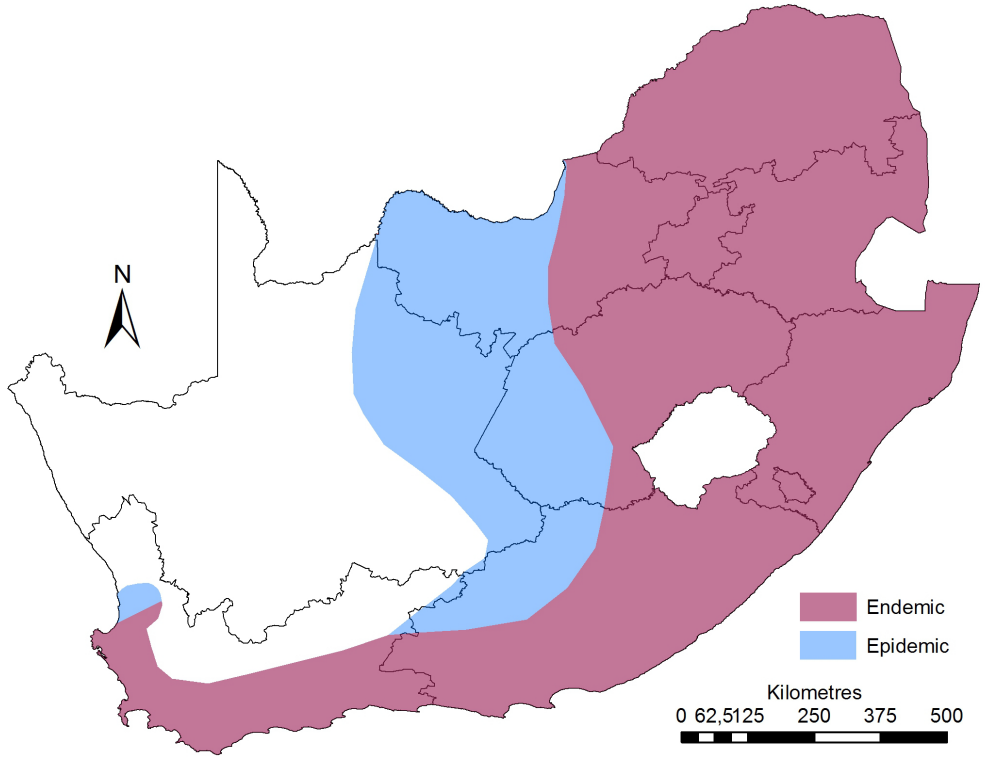

Figure 1.2: Endemic (red) and epidemic (blue) areas of anaplasmosis disease coverage in South Africa (from de Waal, 2000).

Rhipicephalid ticks are the most important anaplasmosis vectors in Africa and Australia. The adult ticks of the five important South African tick species mentioned above, all transmit *A. marginale* intrastadially, and while *R. decoloratus* and *R. microplus* transmit the rickettsia

transstadially. Additionally, *R. simus* has the ability to transmit both *A. centrale* and *A. marginale* transstadially, showing that infection dynamics vary between different tick species (de Waal, 2000; Potgieter & Stoltsz, 2004). Other genera which are important vectors for anaplasmosis in other countries or continents are *Dermacentor* [United States of America (USA)], and *Ornithodoros* (Kocan et al., 1985; Potgieter & Stoltsz, 2004; Merck, 2017).

Additionally, biting flies, louse flies, face flies, stable flies, mosquitoes, eye gnats, and filarial worms, are thought to play a minor role in the transmission of *A. marginale* in livestock. While these species have been reported to transmit *A. marginale* by mechanical means, this is not as efficient as biological transmission. Mechanical transfer of *A. marginale* by veterinary surgical tools has been implicated in the spread of the disease (Kocan et al., 2004; Aubry & Geale, 2011). This method of transmission has not been studied in southern Africa, though incidences have been reported for needle transfer in East Africa (Potgieter & Stoltsz, 2004). Transplacental (*in utero*) transmission of *Anaplasma* spp. from infected cows to calves has also been reported in South Africa (Potgieter & van Rensburg, 1987), Brazil (Grau et. al., 2013) and USA (Swift & Paumer, 1976), and is now believed to play a more important role in *A. marginale* and *A. centrale* than has been previously reported (de Waal, 2000).

Many antelope and other game species are abundant both in game reserves and farming areas in South Africa, and they are likely to play a role in the epidemiology of anaplasmosis. However, the role of wildlife as reservoir hosts of *Anaplasma* spp. has not been extensively studied. Blesbok (*Damaliscus pygargus phillipsi*), common duiker (*Sylvicapra grimmia*) and black wildebeest (*Connochaetes gnou*) have been experimentally infected with *A. marginale* and *A. centrale*, although the infections were subclinical (Neitz, 1935; Potgieter & Stoltsz, 2004). It has also been shown that blesbok are susceptible to *A. centrale* infection (Potgieter & Stoltsz, 2004). *Anaplasma* spp. have also been recorded in giraffe (*Giraffa camelopardalis*), sable antelope (*Hippotragus niger*), buffalo (*Syncerus caffer*) and black wildebeest (*Connochaetes gnou*) (Potgieter, 1979; Potgieter & Stoltsz, 2004). A more complete understanding of the epidemiology of anaplasmosis is important for both domestic and wild animal health.

1.4 Clinical Signs and Diagnosis

1.4.1 Clinical Signs

Anaplasma marginale causes the most virulent form of anaplasmosis, characterized by fever, progressive anaemia and icterus potentially resulting in mortality and morbidity if not treated (Kocan et al., 2004; Potgieter & Stoltsz, 2004). Up to 70% of erythrocytes are infected in the acute stage, which in the bovine host results in inappetence, weight loss, fever, abortion in pregnant cows and lowered milk production (Kocan et al., 2004; Palmer, 2009; Merck, 2017). In animals under one year of age, the disease is usually subclinical, whereas in yearlings as well as those below two years old it presents with moderate severity. However, in older animals, disease is more likely to be severe and fatal (Merck, 2017). A transient febrile response phase characterized by peak rickettsaemia (where 10–30% of the host's erythrocytes are infected), mucous membranes appearing pale then yellow and a body temperature of 41°C, are experienced by the animal, after which surviving animals recuperate (Merck, 2017).

1.4.2 Diagnosis

The main *Anaplasma* spp. infecting bovines are *A. marginale* and *A. centrale* (Potgieter & Stoltsz, 2004), with *A. marginale* and *A. ovis* being morphologically indistinguishable. In Giemsa-stained thin film blood smears, they appear as dense, deep purple, vacuole-bound, near-circular inclusions, with a diameter ranging from 0.3 to 1 μm. These are located marginally in the erythrocytes of all the above-mentioned species with the exception of *A. centrale*, which as the name implies, has inclusion bodies located centrally (Potgieter & Stoltsz, 2004; Merck, 2017). Necroscopy accompanied by microscopic examination may be utilized to detect *Anaplasma* in thin films of internal organs such as liver and spleen, along with peripheral blood stained with dyes such as toluidine blue, new methylene blue and acridine orange.

Furthermore, genus-specific detection in infected animals may be carried out with a fair degree of accuracy using the following serological tests: major surface protein 5 (Msp5) enzyme-linked immunosorbent assay (ELISA), complement fixation and the card agglutination test (Visser et al., 1992; de Waal, 2000; Potgieter & Stoltsz, 2004; Merck, 2017). Deoxyribonucleic acid (DNA)-based tests such as polymerase chain reaction (PCR) (Lew et al., 2002), nested PCR (nPCR) (Molad et al., 2006; Decaro et al., 2008), duplex

qPCR (Carelli et al., 2007; Decaro et al., 2008) and reverse line blot (RLB) hybridization (Bekker et al., 2002) have been demonstrated to be effective for inter- and intra-species differentiation and for the detection of low levels of rickettsaemia, which cannot be detected in thin blood smears. A recent study by Chaisi et al. (2017) evaluated the performance of three of the nucleic acid-based methods, RLB hybridization, nPCR and duplex qPCR in the detection of *A. centrale* and *A. marginale* in South African samples. Their findings showed that the nPCR assay gives false negative results, due to sequence differences in the internal forward priming region in South African *A. marginale* strains. In addition, the authors concluded that duplex qPCR is the most sensitive of these three methods, as it detected more *A. marginale* and *A. centrale* positive samples.

1.5 Treatment, Prevention and Control

1.5.1 Treatment

No chemoprophylactic drugs are currently available for anaplasmosis, therefore drugs are mostly used for chemotherapy especially when used in conjunction with the live blood vaccine (de Waal, 2000). Chemotherapeutic treatment of cattle with early stage anaplasmosis is the preferred long term treatment strategy, which mainly involves the use of tetracyclines such as oxytetracycline (which is the most used drug in South Africa), tetracycline, minocycline, and chlortetracycline and gloxazole and/or imidocarb formulations such as Imizol® used to treat acute infections (de Waal, 2000; Potgieter & Stoltsz, 2004; Merck, 2017). Various formulations of these drugs have been utilized in prolonged oral, parenteral and/or intramuscular administrations for elimination of the carrier status of animals and to prevent clinical disease in susceptible animals in 5–16 day and 21–28 day treatment schemes, respectively (de Waal, 2000; Potgieter & Stoltsz, 2004; Reinbold et al., 2010). However, this is surrounded by controversy as the doses and duration of treatment for some of the drug formulations required to clear *A. marginale* infections, have been found to be unacceptably toxic to the animal patients (Potgieter & Stoltsz, 2004).

1.5.2 Prevention and Control Strategies

Effective prevention and control strategies for anaplasmosis rely on a sound knowledge of the epidemiology (Potgieter & Stoltsz, 2004) and an integrated use of current and traditional methods (de Waal, 2000). Anaplasmosis can be controlled and prevented by implementing two broad strategies: 1) control of the vectors using chemical treatments and 2) by the use of

vaccines (Theiler, 1912; de Waal, 2000). Potgieter & Stoltsz (2004) also noted that the chemical sterilization or slaughter of infected animals are effective control strategies with concurrent eradication of infected ticks using acaricides. Slaughter of infected animals has also been reported to be an effective control method in Canada (Howden et al., 2010). Dipping and pour-on of, among others, pyrethroids, organophosphates and arsenic-based chemical formulations have been used in South Africa to control tick populations (Stevens et al., 2007), though a complete eradication of ticks is not thought to be helpful because it decreases the natural resistance of animals to anaplasmosis (de Waal, 2000). Notable acaricide resistance has been shown to be evolving, however, leading to the lowered efficacy of acaricides in the control of ticks (Cossio-Bayugar et al., 2004; Rajput et al., 2006), and this, coupled with the high cost of acaricides and their environmental unfriendliness, is making them a less favoured choice. An alternative to chemical tick control has been suggested in the form of anti-tick vaccines for use in the host, however, these are still in the developmental phase (de Waal, 2000; Rajput et al., 2006; Kocan et al., 2009).

A live blood vaccine for anaplasmosis has been used as a control strategy in South Africa and many parts of the world since the early 1900s when it was developed (Theiler, 1912; Palmer & McElwain, 1995; de Waal, 2000; Kocan et al., 2003; Potgieter & Stoltsz, 2004; Palmer, 2009). This single dose live blood vaccine is based on the partial cross-protection offered by the milder and less virulent *A. centrale* (de Waal, 2000; Potgieter & Stoltsz, 2004). However, the dose of *A. centrale* used in the vaccine may result in severe vaccine reactions in a small fraction of immunised cattle (Merck, 2017); this can be averted while still achieving long-term protection with a regime of a long-acting tetracycline which may be administered concurrently with the vaccine (de Waal, 2000; Merck, 2017). The use of live vaccines is, however, not permitted in USA because of the potential for introducing *A. centrale* into the country or spreading emerging pathogens. Therefore, the use of a non-living or killed *A. marginale* purified from bovine red blood cells has been the preferred method for vaccination in USA (Kocan et al., 2003; Merck, 2017), though this does not provide protection against infection by a heterologous strain of *A. marginale* (Palmer et al., 1988; 1989; Lopez et al., 2005; Mtshali et al., 2007), and therefore this vaccine is not currently marketed. More recently in USA, Hammac and co-workers (2013), reported the development and use of a live *A. marginale* genetically modified mutant derived from the St. Maries strain, which expresses the green fluorescent protein, as a vaccine. This was also shown to have a protective effect against *A. marginale* challenge resulting in a comparable reduction in the clinical parameters

associated with *A. marginale* infection, as in animals vaccinated with the *A. centrale* live blood vaccine.

Strong evidence suggests that the solution to the aforementioned drawbacks of current vaccines, which has been the subject of intense investigation over the last 25 years or so, may lie in the use of subunit vaccines consisting of recombinant surface proteins from *A. marginale* (Palmer et al., 1988; 1989; 1999; 2012; Brayton et al., 2002; Brown et al., 2003; Kocan et al., 2003; 2004; Noh et al., 2008; Agnes et al., 2011).

1.6 Immune response to *Anaplasma marginale* infection in the bovine host

Significant work has been carried out on the immune response in animals exposed to infection by *A. marginale*, with Palmer et al. (1999) giving an overview of the molecular biology of the process. Protective immunity has been hypothesized as being not only mediated by the generation of high titres of immunoglobulin G2 (IgG2) antibodies against the surface B-cell epitopes, but by a simultaneous $CD4^+$ T-cell mediated macrophage activation for opsonization and killing of the rickettsiae (Palmer & McElwain, 1995; Brown et al., 1998a; b). Therefore, an effective vaccine needs to induce both high IgG2 titres and possess both $CD4^+$ T- and B-cell epitopes, which produce robust B- and T-cell memory responses in future infections by *A. marginale*. For example, in addition to the antibody-sensitive neutralization epitope, Q(E)ASTSS, first described by Allred et al. (1990), both T-cell (VSSQSDQASTSSQLG) (Brown et al., 2002) and B-cell (SSAGGQQQESS) (Garcia-Garcia et al., 2004) epitopes have been described at the N-terminus of the Msp1a protein, making it a favourable vaccine candidate for further exploration. More recently, Omp7, 8 and 9 have also been reported to possess a shared T-cell epitope FLLVDDAI/VV which is conserved globally between strains of *A. marginale* and *A. centrale* (Deringer et al., 2017).

Macrophage activation and control of acute rickettsaemia and anaemia have been found to be correlated with a corresponding increase in the titre of antibodies directed specifically against major surface proteins (Msps) of *A. marginale* (Palmer et al., 1999). In cattle, $CD4^+$ T-cells expressing interferon γ (IFN-γ) are central to protective immunity, since IFN-γ is responsible for the enhancement of IgG2. Furthermore, $CD4^+$ T-cells have been found to help B-cells in their production of specific IgG2 antibodies. Also, IFN-γ provides the activation needed for macrophages, which control rickettsiae by specific cell-mediated phagocytosis, phagolysis

and by the production of rickettsicidal nitric oxide (Palmer et al., 1999). It needs to be noted that major histocompatibility complex (MHC) class 1 and class 2-restricted cytotoxic lymphocytes cannot directly control the intraerythrocytic pathogens, hence the reliance on the immune mechanism described above (Palmer et al., 1999; Brown et al., 2002).

Infection is perpetuated in bovine erythrocytes, which have a lifespan of approximately 160 days, through the generation of escape variants or new clones of *A. marginale* that express the variant Msp2 and Msp3 on the cell surface (Brayton et al., 2001; 2002; Meeus & Barbet, 2001; Meeus et al., 2003; Aubry & Geale, 2011). Multiple *msp2* variants of *A. marginale* are generated by the processes of segmental gene conversion, in which gene segments or whole donor alleles of these genes are inserted into the expression site (Palmer & Brayton, 2007). These new clones have been shown to have a fitness advantage (Palmer et al., 2007) and are able to evade the antibodies that controlled their forerunners, therefore ensuring survival of the rickettsiae in the host, and a persistent infection in the animal, making it a lifelong carrier (French et al., 1998; Brayton et al., 2002; Kocan et al., 2004; Palmer et al., 1999; 2009; Aubry & Geale, 2011).

1.7 Genetic diversity of *A. marginale*

It has been shown that *msp1α* genotype is a surrogate indicator for strain antigenicity, with strains that have different *msp1α* genotypes having different *msp2* genetic diversity; which is important for immune evasion (Rodríguez et al., 2005). Genotyping efforts are well advanced in DNA-based strain differentiation of *A. marginale* strains (Rodríguez et al., 2005; de la Fuente et al., 2007; Mtshali et al., 2007), the basis of which is the *msp1α* gene which codes for major surface protein 1a (Msp1a) (Palmer et al., 1989; Allred et al., 1990). The *msp1α* gene is a single copy gene and strain differences are characterized by variations in the number and sequence of tandem repeats at the 5′ end of the gene (Allred et al., 1990; Palmer et al., 2001) (**Fig. 1.3**). A complex system has been used to name the repeats alphanumerically, in order to distinguish sequence variants, leading to genotypes being described as, for example, J/B/B (the St. Maries strain) or A/B/B/B/B/B/B (Florida strain) (Rodríguez et al., 2005). The current, most widely used PCR-based *msp1α* genotyping protocol is based on the PCR methodology described by Lew et al. (2002) and de la Fuente et al. (2002). This has led to elucidation of the genotypic variation found in *A. marginale* strains in virtually all major regions of the world, including Africa (Mtshali et al., 2007;

Mutshembele et al., 2014; Hove et al., 2018); Asia (Ybañez et al., 2013; 2014; Yang et al., 2017), Australia (Lew et al., 2002); Europe (de la Fuente et al., 2007; Estrada-Peña et al., 2009); South America (Ruybal et al., 2009; Baêta et al., 2015; Machado et al., 2015; da Silva et al., 2016) and North America (Blouin et al., 2000; Palmer et al., 2001; 2004; Ocampo Espinoza et al., 2006; Alamzán et al., 2008).

Estrada-Peña et al. (2009) also described a parallel genotyping system, based on applying a formula to the number of the microsatellite repeats found between the Shine-Dalgarno sequence (GTAGG) and the initiation codon (ATG) sequence upstream of the *msp1α* coding sequence. However, this genotyping scheme is used less frequently, and the significance of the genotypes remains unclear.

The Msp1a protein has also been shown to have features that make it an important immunogen (**Fig. 1.3**). Msp1a has been found to contain an antibody neutralization-sensitive epitope, along with T- and B- cell epitopes (Allred et al., 1990; Brown et al., 2002; Garcia-Garcia et al., 2004).

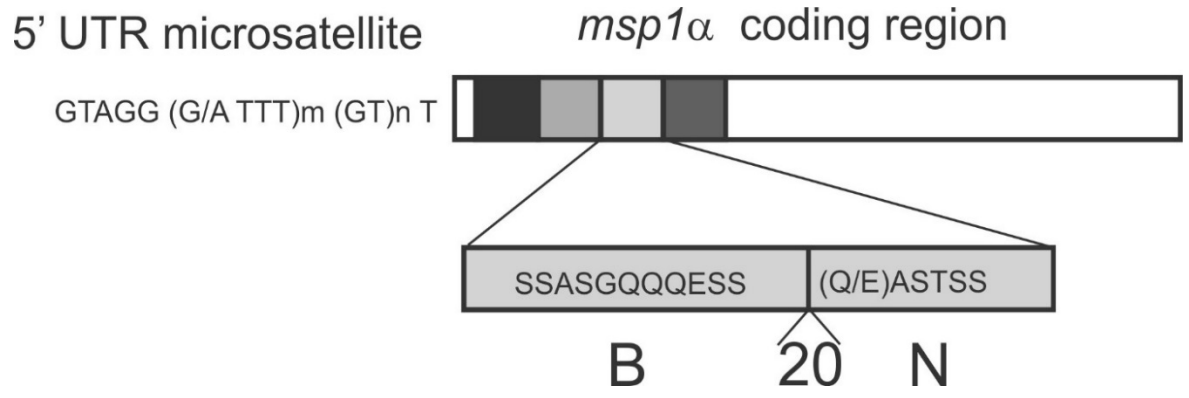

Figure 1.3: A schematic diagram of the *msp1α* gene. The tandem repeats are shown as grey boxes, with each shade representing a different repeat. The microsatellites in the 5' untranslated region (UTR) used in a second genotyping system are shown. The B-cell (B) and neutralization sensitive (N) epitopes, and amino acid 20, found to be important for binding to tick cell extracts, are shown on an enlarged protein repeat.

In the South African context, Mtshali et al. (2007) demonstrated *msp1α*-based genetic diversity (with Msp1a repeats similar to repeats from USA strains as well as repeats unique to South Africa) in the *A. marginale* strains of the Free State province. The *msp1α* genotypes found were also shown to reveal a considerable genetic diversity of *A. marginale* strains in South Africa. Furthermore, 42% of the Msp1a repeats were shown to be shared between the South African strains and those from South America, North American and European strains. Work has also been carried out by Mutshembele et al. (2014) to identify *msp1α* diversity in *A. marginale* strains from other parts of South Africa, with Hove et al. (2018) conducting the most recent diversity study in South Africa; which included comparison with other geographical regions of the world. Furthermore, de la Fuente et al. (2007), Cabezas-Cruz et al. (2013) and Quiroz-Castañeda et al. (2016) give comprehensive reports on global variation in world strains of *A. marginale*, revealing a highly genetically diverse organism. However, caution needs to be taken in interpreting this genetic variation as assessment of genetic diversity using *msp1α* genotypes is based on a single genetic locus, and the inference that this locus is a surrogate reporter for more widespread genomic variation is based on a single study (Rodriguez et al. 2005).

Strains present in different herds show variation in *msp1α* repeat structure and it is thought that this can be indicative of sequence variation of antigenically significant genes (Rodríguez et al., 2005; Brayton et al., 2009; 2012; Palmer et al., 2012).

1.8 Genome sequencing

The *A. marginale* and *A. centrale* genomes have been sequenced and reveal a single, circular chromosome, approximately 1 200 000 bp in length, without any plasmids (Brayton et al., 2005; Herndon et al., 2010). The *A. marginale* St Maries strain genome encodes 1003 predicted genes, of which 62 code for outer membrane proteins (OMPs), 14 functional pseudogenes, 37 transfer ribonucleic acid (tRNA) genes, and three ribosomal ribonucleic acid (rRNA) genes (Brayton et al., 2005). Later studies have increased the number of predicted genes coding for OMPs from 62 to 76 (Brayton et al., 2006). A multi-strain genome comparison of *A. marginale senso stricto* genome sequences indicates that *A. marginale* has a ‘closed-core’ genome (Dark et al., 2009; 2011). The term ‘closed-core’ refers to the fact that no new strain-specific genes are found in the genome, when a new strain is sequenced and a multi-strain genomic comparative analysis is carried out (Tettelin et al., 2005). On the other

hand, the *A. centrale* genome was found to have 984 predicted coding sequences, 19 pseudogenes, 37 tRNA genes, and two rRNA genes (Herndon et al., 2010). *Anaplasma centrale* contains 10 putative genes not found in *A. marginale senso stricto* strains. Also, the *A. marginale* St. Maries strain genome sequence had 18 unique genes that were absent in *A. centrale* (Herndon et al., 2010).

Whole genome sequencing has led to the elucidation of the genomes of several North American strains of *A. marginale,* revealing a significant degree of conservation in synteny and primary structure (Brayton et al., 2005; Dark et al., 2009; 2011). The only strains that have been completely sequenced outside North America come from Australia; these are the attenuated Dawn and virulent Gypsy Plains strains (Pierlé et al., 2014). The study revealed that approximately 9 800 single nucleotide polymorphisms (SNPs) are shared between the two Australian strains compared to St. Maries; with subsets of only 97 and 98 SNPs found to be unique to Gypsy Plains and Dawn strains, respectively. When compared to the North American strains, *omp8-9* genes were found to have an insertion/deletion polymorphism (INDEL) in the Australian Gypsy Plains strain and not in the Dawn strain. Furthermore, a total of 14 SNPs unique to the non-tick transmissible Dawn strain were found to segregate with the Florida strain, which is also not tick transmissible. Interestingly, the study also showed the lowest genetic variation described between any two *A. marginale* strains to date. A total of 195 SNPs were shown to segregate with virulence phenotype, thus revealing genetic markers that are specific to the attenuated Dawn and virulent Gypsy Plains strains. The study also identified an INDEL encompassing the *AM415* gene (*i.e.* missing in the Dawn strain and present in the Gypsy Plains strain) and this can be used as a marker to distinguish these two strains of *A. marginale*.

1.9 Genomic and proteomic approaches to identification of vaccine candidates

As far back as the mid to late eighties and the early nineties, it has been demonstrated that preparations of *A. marginale* OMPs induce protection in the majority of experimental animals (Palmer et al., 1986; 1988; 1989; Tebele et al., 1991). Furthermore, challenge with the homologous strain of *A. marginale* has shown a corresponding binding of IgG2 to the surface of the pathogen and elicits T- and B-cell responses (Brown et al., 1998; 2002; 2003; Palmer et al., 1999; Han et al., 2010). A high efficacy *A. marginale* vaccine should therefore mimic these important characteristics, and with the advancements in genomic and proteomic tools in

recent years, significant strides have been made in identifying several candidate vaccine OMPs (Brayton, 2012; Palmer et al., 2012).

The OMPs in the *A. marginale* and *A. centrale* genomes are dominated by genes that cluster into the protein superfamilies, Msp1 and Msp2 (Pfam01617). The Msp1 and Msp2 superfamilies consist of OMPs of immunogenic significance and have been elucidated by recent genome sequencing efforts (Brayton et al., 2005; Herndon et al., 2010). Msp1 is a surface exposed heteromeric protein consisting of non-covalently linked Msp1a and Msp1b polypeptide subunits, which are approximately 70–105 kiloDalton (kDa) (Oberle et al., 1988; Allred et al., 1990; Barbet & Allred, 1991). The Msp1b protein is encoded by the *msp1β* multigene family which has been shown to exhibit variation between strains of *A. marginale* of 0.9–1.4% (Barbet & Allred, 1991; Bowie et al., 2002). In the latter study it was shown to be stable during the bovine and tick stages of the lifecycle of *A. marginale* (Bowie et al., 2002). The *msp1β* family is composed of two full length genes and three partial genes. Although *msp1α* and *msp1β* do not share sequence identity, they are considered to be part of the Msp1 superfamily as they compose the Msp1 protein. The Msp2 superfamily consists of Msp2, Msp3, Msp4 and Omp1-15 proteins which fall into pfam01617. Msp2 and 3 have been reported to be highly variable and important in the evasion of the host immune response (Vidotto et al., 1994; French et al., 1998; 1999; Brayton et al., 2003; Brown et al., 2003; Meeus et al., 2003).

Several experiments using advanced genomic and proteomic technologies have led to the identification of potential OMP vaccine candidates. Lopez et al. (2005) used a proteomic approach to identify proteins that induced an immunoprotective response when cattle were challenged with OMP preparations. In this work Lopez et al. (2005) separated OMPs using two-dimensional (2D) electrophoresis and proteins that reacted with serum IgG from OMP-vaccinated animals were identified using immunoblottinng. Thereafter, proteins from immunoreactive spots were excised from the gel and their amino acid sequences determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS). These amino acid sequences were searched against a database of the annotated *A. marginale* genome, thus revealing 24 genes coding for immunogenic proteins. Noh et al. (2008) further narrowed down the vaccine candidates by identifying OMPs associated with the surface proteome of *A. marginale* using cross-linking of adjacent surface proteins. Using a preparation of a cross-linked protein complex as a vaccine, immune protection similar to that achieved using a

whole outer membrane preparation was achieved (Noh et al., 2013). The following subset of proteins was found in the cross-linked protein preparation: Msp1a, Msp2, Msp3, Msp4, OpAG2, Omp1, Omp7, Omp8, Omp9, Am779, Am854.

The OMPs Am202, Am368, Am854, Am936, Am1041 and Am1096, which have been shown to have between 97% and 100% amino acid identity in strains and isolates from different geographical locations; have recently been assessed as vaccine candidates (Ducken et al., 2015). This study revealed that, though the four most conserved of these OMPs were consistently recognised by sera from animals vaccinated with outer membrane complexes, OMPs Am854 and Am936 were recognised most consistently. Notably, animals vaccinated with recombinant Am854 and Am936 showed similar IgG and IgG2 titres to those vaccinated with the outer membrane protein complex of *A. marginale*, demonstrating that Am854 and Am936 are viable vaccine candidates that may be considered in a cocktail vaccine. Interestingly, when animals were immunized with recombinant versions of these proteins, vaccinates developed higher bacteraemia after challenge than adjuvant immunized animals despite robust IgG and IgG2 responses (Ducken et al., 2015). This highlights the need for OMP formulations containing these and other promising vaccine candidates to be extensively tested (with different delivery platforms) before they can have full efficacy.

Herndon et al. (2010), also in an effort to define specific vaccine candidate OMPs, used prior knowledge of immunological cross-protection offered by *A. centrale* for *A. marginale* infections, to extrapolate a broad degree of conservation of epitopes between the two organisms using a comparative genomics approach. This work showed poor conservation in the Msp1 superfamily between *A. centrale* and *A. marginale*, suggesting that members of this protein superfamily are poor vaccine candidates. Also, from comparative analysis of genomic sequence data, six vaccine candidate genes (Msp2 superfamily genes: *msp4*, *Omp1*, *Omp7*, and *OpAG2*; and two non-superfamily members: *Am779/ACIS557* and *Am854/ACIS486*) were found through exclusion of sequences that did not have homologues in the vaccine strain and the highly variable *msp2* and *msp3*. Sequence similarity between these candidate genes between the vaccine strain and *A. marginale* was found to range from 63% to 88%.

Using 2D electrophoresis and immunoblotting, coupled with LC-MS/MS, Agnes et al. (2011) identified *A. marginale* antigens recognized by IgG2 in sera from calves across multiple MHC haplotypes immunised by inoculation with *A. centrale*. A total of 15 proteins were

identified, five housekeeping genes and ten OMPs, including *Omp7, Omp8, Omp9, Omp11, Omp13, Omp14, Am779, Am854, Am1144, Am1063*. Dark et al. (2011), using comparative genome sequence analysis, found that the vaccine candidate genes were conserved amongst *A. marginale senso stricto* strains from USA, corroborating the validity of the OMPs found in the aforementioned studies. The above-mentioned list was narrowed down to five vaccine candidates: *Am779, Am854, Omp7, 8* and *9* (Palmer et al., 2012; Ducken et al., 2015; Deringer et al., 2017), which we test in this study.

It must however be noted, that these OMP vaccine candidates have been identified in North American *A. marginale* strains, and the level of immune protection they offer for strains from other geographical locations is yet to be established. This study was therefore aimed at elucidating how the primary structure and antigenicity of these OMPs vary and compare to one another in South African and North American endemic strains, with the overall aim of showing the most significant OMPs for future vaccine trials.

1.10 Overall study aim

Vaccine candidate OMPs of *A. marginale* can be used in a recombinant vaccine to protect cattle against bovine anaplasmosis. It is unknown whether these OMPs are conserved enough between the North American and the South African strains to be used in a broad-spectrum vaccine, or if region and strain-based vaccine development is a necessity. Therefore, to address these important questions, this study proposes to assess the level of conservation between North American (St. Maries and Florida) and South African strains, through a comparative analysis of primary structure and antigenicity of the vaccine candidates in *A. marginale* from both regions.

1.11 Objectives

The primary objective of this work is to determine the degree of structural and antigenic variation between five OMPs shown to be promising vaccine candidates in North American strains of *A. marginale*, as compared to South African strains, all in an effort to develop a recombinant subunit vaccine against anaplasmosis. To accomplish this goal, we must establish the genotypic variation of South African *A. marginale* strains to ensure that we are capturing a broad selection of diverse strains, therefore secondary objectives include the establishment of a South African collection of *msp1α* genotyped *A. marginale* infected blood

samples, and a cell culture system of a subset of *A. marginale* strains, which will contribute to a better understanding of epidemiology of *A. marginale* and strain dynamics in the country. Therefore, the objectives of this work were as follows:

i. Identification of *A. marginale* positive blood samples and *msp1α* genotyping of collected *A. marginale* strains

Collection of *A. marginale* positive samples from cattle of at least 1 year of age in anaplasmosis-endemic areas in South Africa; and the number of strains involved in each infection determined using *msp1α* genotyping and sequence analysis.

ii. Determination of OMP variation between *A. marginale* strains of South Africa and North America

Establishment of the degree of variation between *A. marginale* strains endemic to South Africa and North America using sequence analysis of the genes coding for the five OMP vaccine candidates.

iii. Determination of immunological cross-reactivity between South African and North American strains

Determination of the level of immunological cross-reactivity between currently endemic strains of *A. marginale* in South Africa and North America, based on the ability of OMPs to bind serum Immunoglobin G2 (IgG2) from animals immunised using OMPs derived from the prototypical North American *A. marginale* (St. Maries) strain.

iv. Establishment of *in-vitro* tick cell culture of *A. marginale* from positive field samples

Generation of *in vitro* cultures of South African *A. marginale* strains from positive bovine blood samples derived from the field, using *in vitro* cell culture in *Ixodes scapularis*-derived embryonic tick cell lines, ISE6 and IDE8.

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CHAPTER 2

Co-infections with multiple genotypes of *Anaplasma marginale* in cattle indicate pathogen diversity [1]

2.1 Abstract

Only a few studies have examined the presence of *Anaplasma marginale* and *Anaplasma centrale* in South Africa, and no studies have comprehensively examined these species across the whole country. To undertake this countrywide study, we adapted a duplex quantitative PCR assay for use in South Africa, but found that one of the targeted genes of the assay was variable. Therefore, we sequenced many field samples and tested the assay on the variants detected. We used the assay to screen 517 cattle samples sourced from all nine provinces of South Africa, and subsequently examined *A. marginale* positive samples for *msp1α* genotype to gauge strain diversity. Despite the variation in *A. marginale msp1β* sequences, the qPCR still functions at an acceptable efficiency. The *A. centrale groEL* gene was not variable within the qPCR assay region. Of the cattle samples screened using the assay, 57% and 17% were found to be positive for *A. marginale* and *A. centrale*, respectively. Approximately 15% of the cattle were co-infected. *Msp1α* genotyping revealed 36 novel repeat sequences. Together with data from previous studies, we analysed the Msp1a amino acid repeats from South Africa where a total of 99 amino acid repeats have been described that can be attributed to 190 *msp1α* genotypes. While 22% of these repeats are also found in other countries, only two South African genotypes are also found in other countries; otherwise the genotypes are unique to South Africa. Our study suggests that *A. marginale* was prevalent in the Western Cape, KwaZulu-Natal and Mpumalanga, and absent in the Northern Cape. Similarly, *A. centrale* was prevalent in the Western Cape and KwaZulu-Natal and absent in the Northern Cape and Eastern Cape. None of the cattle in the study were known to be vaccinated with *A. centrale*, so finding positive cattle indicates that this organism appears to be naturally circulating in cattle. A diverse population of *A. marginale* strains is found in South Africa, with some *msp1α* genotypes widely distributed across the country, and others

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appearing only once in one province. This diversity should be taken into account in future vaccine development studies.

2.2 Introduction

Bovine anaplasmosis is one of the most economically important tick-borne diseases of ruminants the world over (de Waal, 2000; Potgieter & Stoltsz, 2004; Aubry & Geale, 2011). The causative agent of the disease is the rickettsia *A. marginale,* a gram-negative, obligate intra-erythrocytic bacteria of the order Rickettsiales and family *Anaplasmataceae* (Theiler, 1910b; Dumler et al., 2001; Potgieter & Stoltsz, 2004; Merck, 2017). *Anaplasma marginale* is the most prevalent vector-borne pathogen and is found on all six inhabited continents (Kocan et al., 2004; de la Fuente et al., 2007; Brayton et al., 2009; Merck, 2017). Approximately 20 tick species worldwide are implicated as biological vectors of the pathogen, although mechanical and transplacental transmission have also been reported (Theiler, 1910a; Swift & Paumer, 1976; Potgieter, 1981; Potgieter & van Rensburg, 1987; de Waal, 2000; Kocan et al., 2003; Potgieter & Stoltsz, 2004; Grau et al., 2013). *Anaplasma centrale*, considered by some authors to be a subspecies of *A. marginale*, generally causes a milder, less virulent form of the disease, with occasional clinical cases (Carelli et al., 2008). Infection with *A. centrale* confers immunity to *A. marginale*. *Anaplasma centrale* has therefore been employed as a live vaccine (Potgieter, 1979; Potgieter & Stoltsz, 2004). In South Africa, bovine anaplasmosis is found in most of the cattle farming regions and is an economically important tick-borne disease (Potgieter, 1979; de Waal, 2000; Potgieter & Stoltsz, 2004). It is endemic in eight of the nine provinces of the country (de Waal, 2000), with the exception of the Northern Cape where the tick vectors are absent. Five tick species, namely *Rhipicephalus decoloratus*, *R. microplus*, *R. evertsi evertsi*, *R. simus* and *Hyalomma marginatum rufipes,* have been shown experimentally to be capable of transmitting *A. marginale* in South Africa (Potgieter, 1981).

Recently we compared three nucleic acid-based tests for detecting *A. marginale* and *A. centrale* (Chaisi et al., 2017). The nested polymerase chain reaction (nPCR) assay (which targets the *msp1β* gene of *A. marginale* and *msp2* of *A. centrale* (Molad et al., 2006; Decaro et al., 2008)) detected fewer *A. marginale* positive samples than the duplex quantitative real-time PCR (qPCR) (which detects *msp1β* of *A. marginale* and *groEL* of *A. centrale* (Carelli et al., 2007; Decaro et al., 2008)). This discrepancy was found to be due to sequence variation in the *msp1β* gene in the target region of one of the internal PCR primers. The reverse line blot (RLB) hybridization assay (Bekker et al., 2002), in which species-specific sequences in the 16S rRNA gene of *Anaplasma* and *Ehrlichia* species are detected, was found to be less

sensitive than the qPCR and nPCR assays. The qPCR assay was thus shown to be the most appropriate assay for detection of *A. marginale* in blood samples from cattle (Chaisi et al., 2017). However, the identification of *msp1β* gene sequence variants indicates the need to assess sequence variation in the target regions of the qPCR assays, to ensure that all *A. marginale* and *A. centrale* genetic variants are detected.

A genotyping method based on the *msp1α* gene (Rodríguez et al., 2005; Mtshali et al., 2007; Mutshembele et al., 2014; da Silva et al., 2015), which encodes major surface protein 1a (Msp1a) (Palmer et al., 1986; Allred et al., 1990), has been developed for characterizing *A. marginale* strains in positive samples and is applied throughout the world. *Anaplasma marginale msp1α* genotyping is not only useful for understanding genetic diversity of the pathogen but is also used to elucidate host-pathogen interactions and co-evolution (de la Fuente et al., 2005; 2007; 2010; Estrada-Peña et al., 2009; Cabezas-Cruz et al., 2013; Mutshembele et al., 2014). *Msp1α* genotyping relies on variation in tandem repeats at the 5' end of the gene that vary both in number and sequence. Msp1a repeats are identified in the deduced amino acid sequence and are given alphanumeric names to distinguish between sequence variants; the Msp1a repeat structure determines the *msp1α* genotype of a strain. Over 250 Msp1a repeats are described, making it a useful marker for discriminating *A. marginale* strains (Allred et al., 1990; Palmer et al., 2001; Bowie et al., 2002; Mtshali et al., 2007; Cabezas-Cruz et al., 2013; Mutshembele et al., 2014; da Silva et al., 2015). In the South African context, *msp1α*-based genotyping has revealed diversity in *A. marginale* strains across the country, and novel repeats have been identified, although other repeats are identical to those detected in Europe and USA (Mtshali et al., 2007; Mutshembele et al., 2014). Earlier studies indicated that infection exclusion resulted in only one *A. marginale* genotype in individual cattle and ticks (de la Fuente et al., 2002), but more recently, infections with multiple distinct *msp1α* and *msp2* genotypes were identified in herds in endemic areas with high infection rates (Palmer et al., 2004; Ueti et al., 2012; Esquerra et al., 2014; Ybañez et al., 2014; Castañeda-Ortiz et al., 2015).

In this study, we used next-generation amplicon sequencing to assess the level of variation in the qPCR target regions of the *msp1β* (*A. marginale*) and *groEL* (*A. centrale*) genes from field samples in order to ensure that the duplex qPCR assay (Carelli et al., 2007; Decaro et al., 2008) can detect all *A. centrale* and *A. marginale* genetic variants in South Africa. The

assay was then used to screen cattle samples from all nine provinces of the country for the presence of these organisms, followed by *msp1α* genotyping from selected positive samples. We cloned *msp1α* PCR amplicons and sequenced multiple clones in order to maximize the diversity of *A. marginale* genotypes detected from individual animals.

2.3 Materials and Methods

2.3.1 Blood sample collection and genomic DNA extraction

A total of 517 EDTA blood samples were obtained from mixed breeds of cattle from all nine provinces of South Africa (**Table 2.1**).

Table 2.1: Number and origin of cattle field samples used in the study.

Province	No. of samples
Limpopo (LP)	30
Mpumalanga (MP)	115
Gauteng (GP)	183
North West (NW)	30
Free State (FS)	30
KwaZulu-Natal (KZN)	30
Northern Cape (NC)	30
Eastern Cape (EC)	43
Western Cape (WC)	26
TOTAL	**517**

These consisted of fresh blood samples collected from cattle in the Mnisi communal area (79) and a private farm near Lydenburg (17), Mpumalanga Province, and 148 samples collected from cattle at the University of Pretoria Experimental Farm (Proefplaas, Gauteng Province), as well as 284 frozen cattle blood samples, collected from different parts of South Africa, obtained from the National Zoological Gardens (NZG), Pretoria, South Africa. Blood samples from cattle were collected in accordance with the animal ethics code of the University of Pretoria in 9 ml Vacuette® EDTA tubes (Greiner Bio-One, Kremsmünster, Austria), from the coccygeal vein of cattle that were at least one-year old. *A. centrale* blood vaccine was obtained from Onderstepoort Biological Products (Pretoria, South Africa).

Genomic DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and DNA was eluted in 100 µl elution buffer and stored at -20°C.

2.3.2 Next-generation amplicon sequencing of *msp1β* and *groEL* genes

Next-generation sequencing (NGS) was used to determine the extent of variation in amplicons of a part of the *msp1β* and *groEL* genes of *A. marginale* and *A. centrale* in, respectively, 40 and 25 known positive field samples from across South Africa. Twenty *A. marginale msp1β* gene sequences from GenBank (accession numbers: M59845, AF110808–AF110810, AF112479, AF112480, AF111195, AF111197, AF221692, AF348137, AF348138, AY841153, KU647713–KU647720) were aligned using CLC Genomics Workbench 7.5.1 (https://www.qiagenbioinformatics.com) and used to design primers Msp1β_F (5′-GAT GAA GCA CCT GAC ACT GGT GAG-3′) and Msp1β_R (5′-CGC GTC GAT TGC TGT GC-3′) in areas conserved in all of these sequences. The primers amplify a 419 bp fragment of the *msp1β* gene spanning the qPCR primer and probe area. The primer pair groEL-ACF and groEL-ACR (Decaro et al., 2008) was used to amplify a 522 bp fragment of the *groEL* gene from both *A. marginale* and *A. centrale*. The primers were modified by adding Illumina-specific adaptor sequences to allow for barcoding of each amplicon and were synthesized at Inqaba Biotechnical Industries (Pretoria, South Africa). The PCRs were performed in a total volume of 25 µl containing 1X Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific, Waltham, USA), 1.5 µM of each primer and 2.5 µl genomic DNA (approximately 200 ng). For amplification of the *msp1β* amplicon, the PCR thermal cycling conditions were 98°C for 10 s, 40 cycles of 98°C for 5 s, 67°C for 15 s, 72°C for 15 s, and a final extension at 72°C for 1 min. The same cycling conditions were used for amplification of the *groEL* amplicon, except that the annealing temperature was 66°C. The amplicons were purified using the QIAquick gel extraction kit (Qiagen, Hilden, Germany) according to the manufacturers' instructions.

Plasmid controls were included to determine the *Taq* and sequencing error rate, to distinguish sequence artefacts from real sequence variants (Esquerra et al., 2014). Multiple strains of *A. marginale* are known to be present in South African samples (Mtshali et al., 2007; Mutshembele et al., 2014), but the relative incidence of different strains in each sample is unknown, and some strains may be present at very low levels. Amplicons were therefore

generated from plasmid controls F48a (*A. marginale msp1β*), 9410c (*A. centrale groEL*) and C14c (*A. marginale groEL*) to determine the frequency of sequence artefacts (including *Taq* or sequencing errors) expected in the field samples. The positive control plasmids were generated previously from field samples that were positive for *A. marginale* (F48 and C14) and *A. centrale* (9410) (Chaisi et al., 2017).

Resulting amplicons were gel purified, end repaired and Illumina-specific adapter sequences were ligated to each amplicon. Following quantification, the samples were individually indexed, and another purification step was performed. Indexed, adapter-ligated amplicons were then sequenced on Illumina's MiSeq platform, using a MiSeq v3 (600 cycle) kit (San Diego, California, USA). About 20 megabytes (MB) of data (2 x 300 bp long paired end reads) were produced for each sample.

Quality filtering was performed on the MiSeq platform, using standard procedures. Only reads that mapped to *A. centrale groEL* 9410c, *A. marginale groEL* C14c and *A. marginale msp1β* F48a reference sequences (Chaisi et al., 2017) were incorporated into the subsequent analysis. The sequences were analysed by first merging corresponding Illumina R1 and R2 reads, and only merged sequences were analysed further. Again, the *groEL* and *msp1β* amplicon sequences were mapped to their respective *A. marginale* or *A. centrale* reference sequences. For each set of merged reads, a clustering based on sequence identity was performed. For the *groEL* control plasmid clone 9410c, included to determine the frequency of artefacts, the highest proportion of sequences (47.6%) was identical to the 9410c reference sequence. All other sequences (artefacts) were present at an abundance of less than 1.5% each, but collectively made up 52.4% of the sequences. For the *msp1β* plasmid clone F48c, 63.8% of the sequences were identical to the F48c reference sequence and all other sequences were present at an abundance of less than 1.4%, collectively making up 36.2% of the sequences. Therefore, for the field samples, sequences present at less than 1.5% of the total after cluster analysis were disregarded as *Taq* or sequencing errors. In each cluster, sequences that were present at ≥1.5% of the total number of sequences were therefore considered to be true variants, and were aligned with published sequences using CLC Genomics Workbench 7.5.1.

2.3.3 Confirmation of *msp1β* variants by Sanger sequencing

The *msp1β* variants identified by NGS were confirmed by Sanger sequencing in eleven

samples. Primers AM456 and AM1164 (Molad et al., 2006) were used to amplify a 750 bp region of the *msp1β* gene flanking the qPCR target area. The reaction mixture contained 1X Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific, Waltham, USA), 0.5 µM of each primer, 2.5 µl of template DNA (approximately 200 ng) and molecular grade water to a final volume of 25 µl. The PCR thermal cycling conditions were 95°C for 3 min, 40 cycles of 94°C for 10 s, 60°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 7 min. The PCR products were purified, quantified and cloned using the CloneJET PCR Cloning Kit (Thermo Fisher Scientific, Waltham, USA). Recombinant plasmids were screened by colony PCR using vector-specific primers pJET1.2F and pJET1.2R. Plasmid DNA was extracted from recombinants using the High Pure Plasmid Isolation kit (Roche Diagnostics, Mannheim, Germany). Plasmids containing the correct insert were sequenced bi-directionally on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, California, USA) at Inqaba Biotechnical Industries. Sequences were assembled and aligned using CLC Genomics Workbench 7.5.1.

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

A duplex qPCR assay with minor modifications for the LightCycler real-time machine (Roche Diagnostics, Mannheim, Germany) targeting *msp1β* of *A. marginale* (with five described members of the family in one strain) (Viseshakul et al., 2000); and the *groEL* gene of *A. centrale,* was used to detect *Anaplasma* spp. in genomic DNA samples as previously described (Chaisi et al., 2017). DNA extracted from the *A. centrale* vaccine strain (Onderstepoort Biological Products, Pretoria, South Africa) or field sample 9410 (confirmed to be infected with *A. centrale* by amplification and sequence analysis of the *groEL, msp2* and 16S rRNA genes (Chaisi et al., 2017)) were used as positive controls. Field samples C14 or C57 (obtained from cattle in the Mnisi Community area) were used as positive controls for *A. marginale,* and molecular grade water as a negative control. To determine *A. centrale* loads, DNA was extracted from 10-fold serial dilutions of vaccine prepared in uninfected bovine blood. The data were analysed using LightCycler Software version 4.0. (Roche Diagnostics, Mannheim, Germany). The linear range of detection and assay efficiency of selected variants were determined as previously described (Chaisi et al., 2017).

2.3.5 Amplification, cloning and sequencing of the *msp1α* gene

The repeat-containing region of the *msp1α* gene 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). Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific, Waltham, USA) reactions were set up as for *msp1β*. Cycling conditions were 98°C for 10 s, 30 cycles of 98°C for 1 s, 69.1°C for 5 s and 72 °C for 18 s, and a final extension at 72°C for 1 min. If these PCR conditions failed to generate an amplicon for a sample, the PCR was repeated using the Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific, Waltham, USA), and the cycling conditions reported by (Lew et al., 2002) except that a pre-PCR denaturation at 94°C for 3 min and *Taq* activation at 98°C for 10 s were included. Samples were analysed on a 1.5% agarose gel and those displaying a single, strong band were purified using the Qiagen PCR product purification kit (Qiagen, Hilden, Germany) according to the manufacturers' instructions. Samples containing multiple PCR products and PCR products that produced mixed sequences were cloned into pJET 1.2 (Thermo Fisher Scientific, Waltham, USA). Recombinant clones and amplicons were sequenced at Inqaba Biotechnical Industries as stated previously.

2.3.6 Analysis of Msp1a repeats to determine strain type

Sequences were assembled and aligned using CLC Genomics Workbench 7.5.1. RepeatAnalyzer (Catanese et al., 2016) was used to identify, curate, map and analyse Msp1a repeats and *A. marginale* strains. New names (UP1 to UP36) were given to novel repeats that were not recognized by RepeatAnalyzer. All South African Msp1a repeats and *msp1α* genotypes (Mtshali et al., 2007, Mutshembele et al., 2014; this study) were pooled and analysed using RepeatAnalyzer, generating diversity metric scores (Catanese et al., 2016). For comparison, similar analyses on previously published data from Argentina, Brazil, Mexico, the Philippines and USA, were also carried out.

2.4 Results

2.4.1 Next-generation sequencing of the *groEL* and *msp1β* genes

A total of 39 *A. centrale* and 40 *A. marginale* partial *groEL* sequences (approximately 520 bp in length) were obtained from 25 bovine samples. The *A. centrale groEL* sequences were conserved within the qPCR target region. The *A. marginale groEL* sequences were also conserved and differed from the *A. centrale groEL* sequences at six nucleotide positions in the probe area and three nucleotide positions in the reverse primer region (**Fig. 2.1A**).

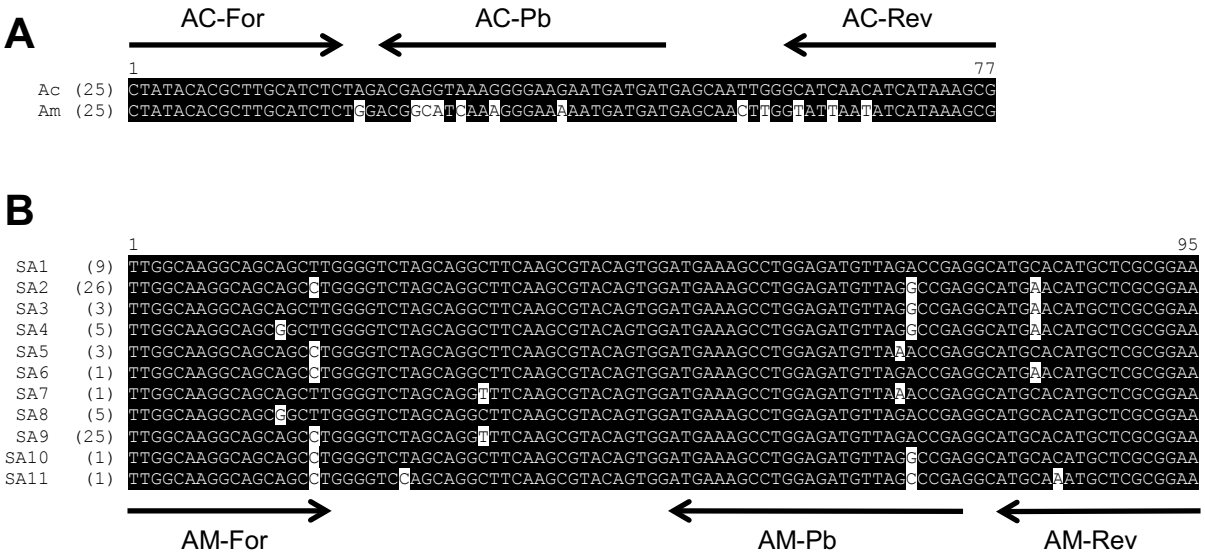

Figure 2.1: Sequence alignment of groEL and *msp1β* sequences in the qPCR target regions. A: *A. centrale* (Ac) and *A. marginale* (Am) *groEL* gene sequences obtained in this study. B: *msp1β* gene sequence variants in the qPCR target region (SA1–SA11) obtained in this study. The number in brackets after each sequence name indicates the number of samples from which each sequence was obtained. The primer and probe regions are indicated by arrows. Identical nucleotides are shown by white text on a black background while sequence variations are represented by black text on a white background.

The *A. centrale groEL* sequences were identical to published sequences including those with accession numbers AF414867 (Vaccine strain, South Africa), AF414866 (L strain, South Africa) and ACIS_00394 in the complete genome sequence, CP001759 (Israel strain); while the *A. marginale groEL* sequences were similar to the St. Maries (USA) sequence (AM944 in CP000030). For *msp1β,* 151 different sequences (partial gene sequence; approximately 420 bp in length) were obtained from a total of 183 sequences from 40 samples. Individual samples contained between one and 11 different *msp1β* sequences. Eleven variants (designated as SA1–SA11) were identified in the qPCR target area (**Fig. 2.1B**). Single nucleotide polymorphisms (SNPs) were identified at six positions in the primer and/or probe regions; individual variants contained one to three of these SNPs. Variants SA1, SA2, SA3, SA4, SA5, SA8 and SA9 were identified in multiple samples, while variants SA6, SA7, SA10 and SA11 occurred in only one sample each. The most common variants were SA2 and SA9, identified from 25 samples each. Both of these variants were widespread in South Africa; SA9 occurred in seven provinces, while SA2 was identified in eight provinces. The greatest number of variants (eight) was identified in samples from the Western Cape.

Variants SA1, SA2, SA4, SA5 and SA7 were cloned and their sequences confirmed by Sanger sequencing. Plasmid DNA from clones of these five variants could be detected by the qPCR assay (**Fig. 2.1A**). The qPCR assay efficiency for detection of variant SA1 was evaluated in a previous study (Chaisi et al., 2017). Evaluation of the efficiency of the qPCR assay in detecting the two variants (SA2 and SA4) containing the most differences (3 SNPs) in the primer and/or probe regions indicated that the SNPs did not have any effect on the efficiency of the assay (**Fig. 2.1B**).

2.4.2 Detection of low *A. centrale* loads in duplex qPCR

Serial dilutions of a known amount of *A. centrale* blood vaccine was used in the duplex qPCR to establish our ability to detect low pathogen loads in blood samples (**Fig. 2.2C**). We could detect as few as 10 infected red blood cells (10 iRBCs) per 20 µl reaction. When working directly from genomic DNA extracted from a blood sample, the efficiency of the qPCR becomes 119%. This apparent increase in efficiency compared to the assay applied to plasmids (E = 103%, **Fig. 2.2B**) is likely due to inhibitors co-extracted with the genomic DNA.

2.4.3 Detection of *A. marginale* and *A. centrale* infections in field samples by the duplex qPCR assay

FAM fluorescence (530 nm) was generated in *A. marginale*-positive samples and LC-610 (610 nm) signals were generated in *A. centrale*-positive samples. No amplification was detected from the negative control. The qPCR assay detected *A. marginale* and *A. centrale* in 56.8% and 17.2% of the samples (n = 517), respectively. Eighty-one (15.3%) samples had mixed infections. *Anaplasma marginale*-positive cattle were identified in all provinces except Northern Cape (**Fig. 2.3**). Most of the *A. marginale*-positive samples were identified in KwaZulu-Natal (100%), Western Cape (88.5%) and Mpumalanga (77.4%), while most of the *A. centrale*-positive cattle were from KwaZulu-Natal (76.7%) and Western Cape (69.2%). *Anaplasma centrale* was not identified in samples from the Eastern Cape and Northern Cape.

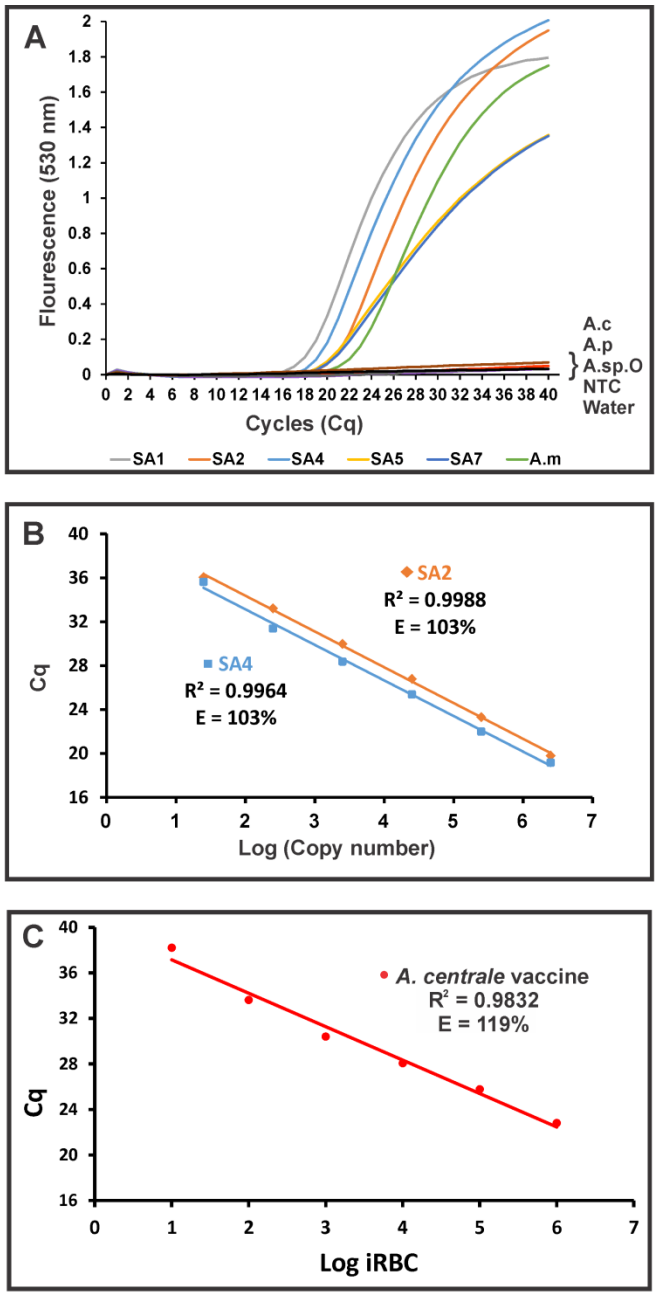

Figure 2.2: qPCR amplification of *A. marginale msp1β* variants. A: qPCR amplification of plasmid DNA ($2.5x10^7$ copies) of *A. marginale msp1β* variants (SA1, SA2, SA4, SA5, SA7). Genomic DNA (gDNA) from sample C14 was used as a positive control for *A. marginale* (A.m) and water as a negative control. gDNA from the *A. centrale* (A.c) vaccine strain, *A. phagocytophilum* (A.p), *Anaplasma* sp. (Omatjenne) (A.spO) and a no temple control (NTC) were included in the analysis. B: Detection of tenfold serial dilutions ($2.5x10^7$–$2.5x10^2$ copies) of plasmid DNA of *A. marginale* variants SA2 and SA4. C: Detection of tenfold serial dilutions of *A. centrale* vaccine strain (10^6–10^1 iRBCs) genomic DNA. Cq, quantification cycle; R^2, regression co-efficient; E, assay efficiency.

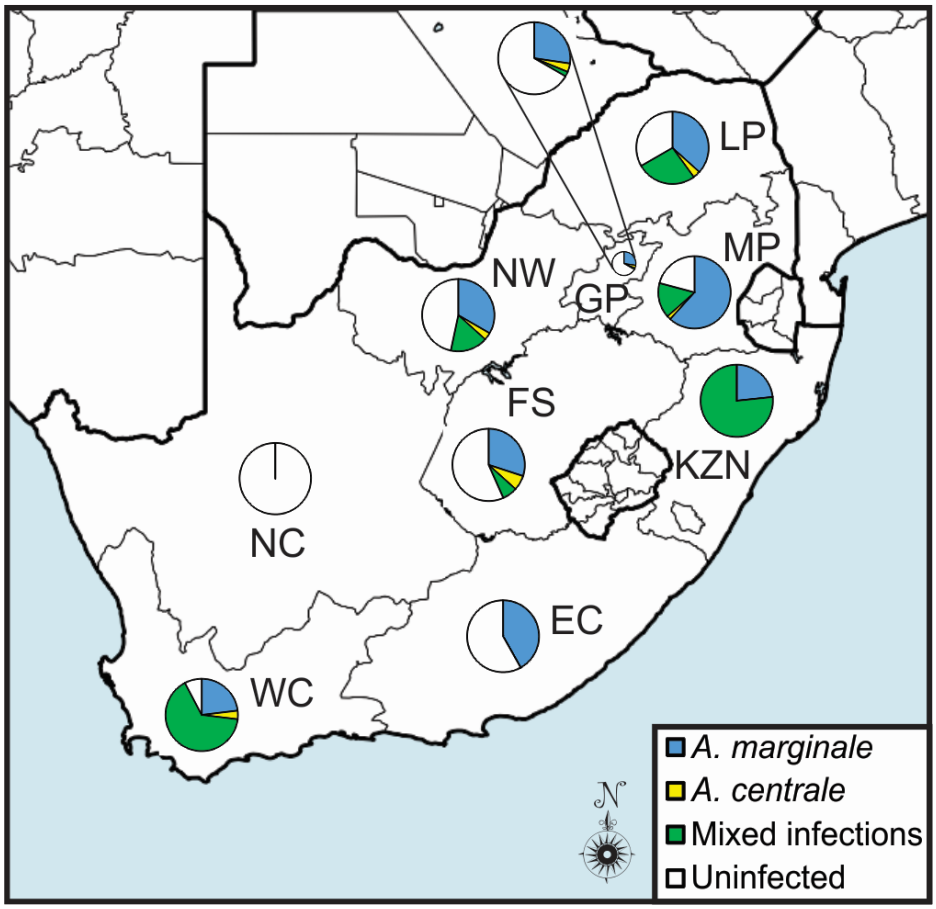

Figure 2.3: Map of South Africa showing the occurrence of *A. marginale* and *A. centrale* in cattle. DNA extracted from blood samples from cattle from all nine provinces of South Africa were tested for *A. marginale* and *A. centrale* using the duplex qPCR assay (Carelli et al., 2007; Decaro et al., 2008). The pie charts indicate the proportion of samples in each province that were positive, negative or which contained mixed infections. GP, Gauteng Province; EC, Eastern Cape; FS, Free State; KZN, KwaZulu-Natal; LP, Limpopo Province; MP, Mpumalanga Province; NC, Northern Cape; NW, North West; WC, Western Cape.

2.4.4 *Msp1α* genotyping and sequence analysis of *A. marginale* Msp1a repeats identified in this study

In order to examine the *A. marginale* strain diversity in the sample set, *msp1α* genotypes were determined in samples that were shown to be *A. marginale*-positive using the duplex qPCR. In our study, a total of 143 genotypes were found from 627 *msp1α* sequences, which were generated from 85 samples from across South Africa. An average of 10.5 samples were analysed per province and an average of 27.8 genotypes were identified per province. Thirty-six Msp1a repeats that have not previously been reported were found, and these were designated UP1–UP36 (**Fig. 2.4**). The novel repeats were 28–29 amino acids in length, with

the exception of UP12 which was found to have an arginine (R) insertion at position 12, making it the longest repeat at 30 amino acids. Alignment of 234 published repeats shows that Serine (S) residues tend to be highly conserved (data not shown). Interestingly, S residues in the repeat region are thought to be O-glycosylated and to facilitate the adhesion function of the Msp1a protein (Garcia-Garcia et al., 2004). The 36 novel repeats (**Fig. 2.4**) all contained variations in the previously reported immunodominant and linear B-cell epitope SSAGGQQQESS (positions 4–14), the neutralisation-sensitive B-cell epitope Q/EASTSS (positions 21–26) and the T-cell epitope VSSQSDQASTSSQLG (positions 15–29) (Allred et al., 1990; Brown et al., 2002; Garcia-Garcia et al., 2004; Cabezas-Cruz et al., 2013). The former B-cell epitope varied at 7 out of 11 positions: 4(S/W), 7 (G/S), 8 (G/N/D/C), 9 (Q/H), 12 (E/G), 13 (S/V) and 14 (S/G/V), while the latter varied at 3 out of 6 positions: 21 (Q/E/G/D/S/P), 22 (A/T) and 23 (S/G). The T-cell epitope had variations at 11 out of 15 positions: 16 (S/L/P), 17 (S/P), 18 (Q/Y), 19 (S/Q/T), 20 (D/G/S), 21 (Q/E/G/D/S/P), 22 (A/T) and 23 (S/G), 27 (Q/K/R/H), 28 (L/F/S), 29 (G/R/E).

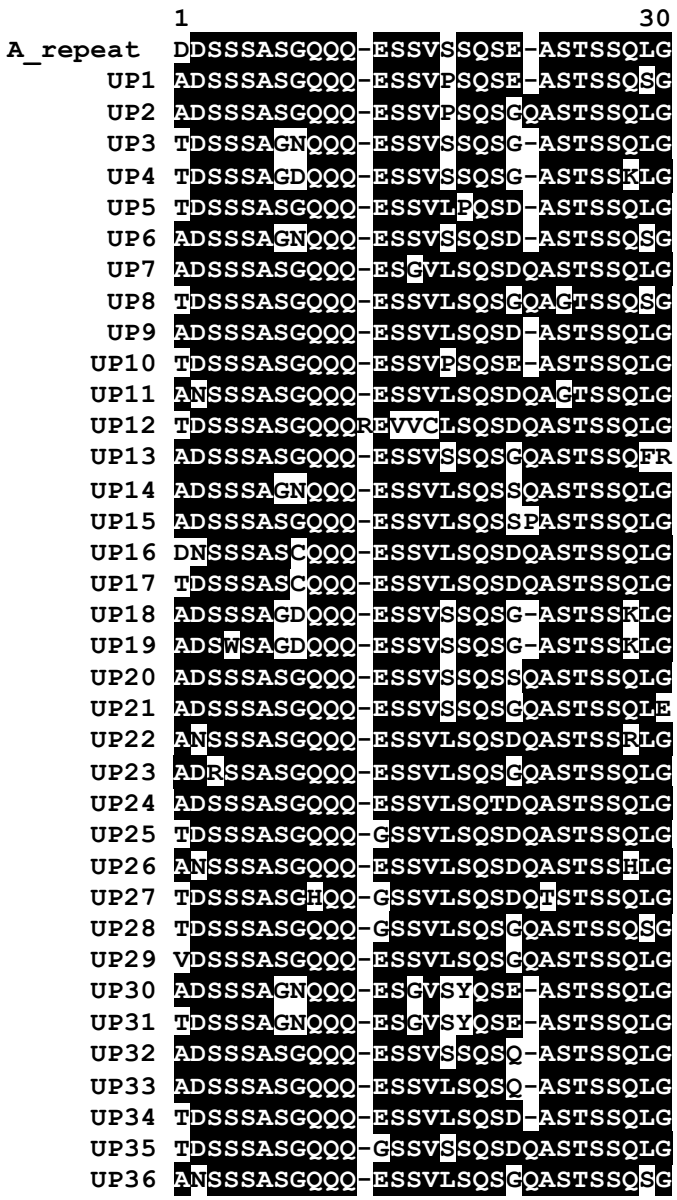

Figure 2.4: Novel Msp1a sequences repeats found in this study. Thirty-six unique repeats were identified in this study (UP1–UP36) and aligned against the A repeat (Allred et al., 1990), using the AlignX module of Vector NTI (Invitrogen). Identical amino acid residues in the alignment are shown by white text on a black background; variable residues are indicated by black text on a white background.

2.4.5 Analysis of Msp1a repeats and *msp1α* genotypes using RepeatAnalyzer

For all South African Msp1a data collected to date, from this and previous reports (Mutshembele et al., 2014; Mtshali et al., 2007), the frequency distribution of Msp1a repeats resembled a power-law distribution (**Fig. 2.5A**). Unique repeats (those observed only once in all *A. marginale* genotypes in South Africa) were observed in 48 instances; examples of such repeats are G, 39, 44, T, UP29, 83, 145, and 154. Six Msp1a repeats, 13, 37, 34, 27, 4 and 3,

were found to be most common in South Africa, occurring between 37 and 78 times. There was a normal distribution of *msp1α* genotype lengths (**Fig. 2.5B**) ($\mu = 4.26$; $\sigma = 1.48$), which ranged from one to nine repeats. *Msp1α* genotypes in South Africa most frequently contained four or five repeats; these occurred 53 (27.9%) and 49 (25.8%) times, respectively (**Fig. 2.5B**). The frequency of genotypes per sample (**Fig. 2.5C**) was found to be positively skewed. A total of 78.8% of the samples contained one (28.2%), two (23.5%) or three (27.1%) genotypes per sample. Four to nine genotypes per sample were also observed, but much less frequently.

To date, a total of 99 Msp1a repeats (**Fig. 2.6A**) have been described in South African *A. marginale* genotypes, 71 (71.7%) of which are unique to the country (**Table 2.2**). These repeats are found in a total of 190 *msp1α* genotypes (**Fig. 2.6B),** the majority of which appear to be unique to South Africa (**Table 2.3**). In general, repeats were fairly evenly distributed around the country (**Fig. 2.6A**). The most abundant strains found in this study are also reported previously (Mtshali et al., 2007; Mutshembele et al., 2014). These include SW112: 42 43 25 31 (occurring 12 times in five provinces, Mpumalanga, Eastern Cape, Limpopo, KwaZulu-Natal and North West), SW32: 34 13 13 37 (occurring 6 times in five provinces, Western Cape, Mpumalanga, Gauteng, Limpopo, KwaZulu-Natal) and NW-C1-160312: 34 13 3 36 38 (occurring 8 times in five provinces, Mpumalanga, Limpopo, KwaZulu-Natal, Free State). Some *msp1α* genotypes were found in more than one province, while low abundance genotypes which appeared only once in one province were also detected (**Fig. 2.6B**).

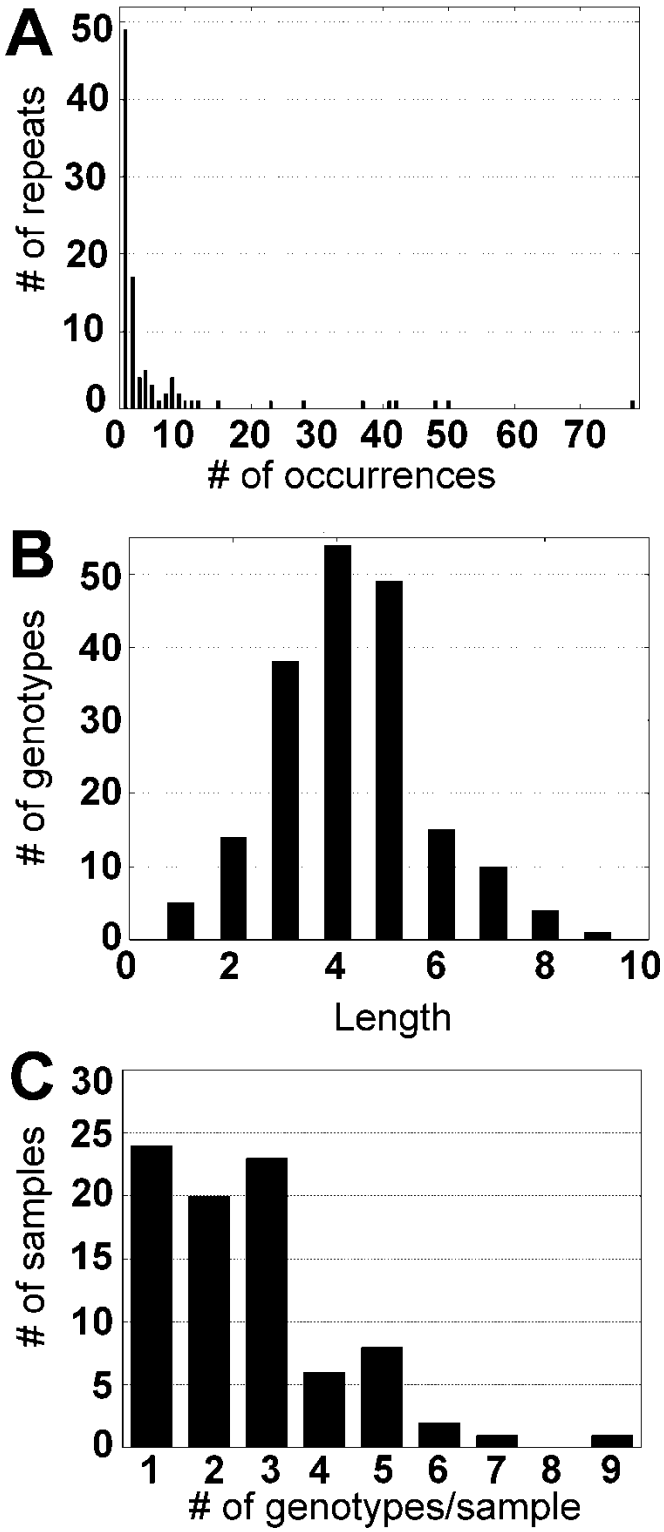

Figure 2.5: Msp1a repeat and *msp1α* genotype metrics. A: Frequency distribution of repeats in Msp1a sequences in South Africa generated by RepeatAnalyzer (Catanese et al., 2016). B: Genotype-Length distribution of Msp1a repeats in South Africa generated by RepeatAnalyzer. C: The frequency of *A. marginale msp1α* genotypes found per animal in this study (n = 85).

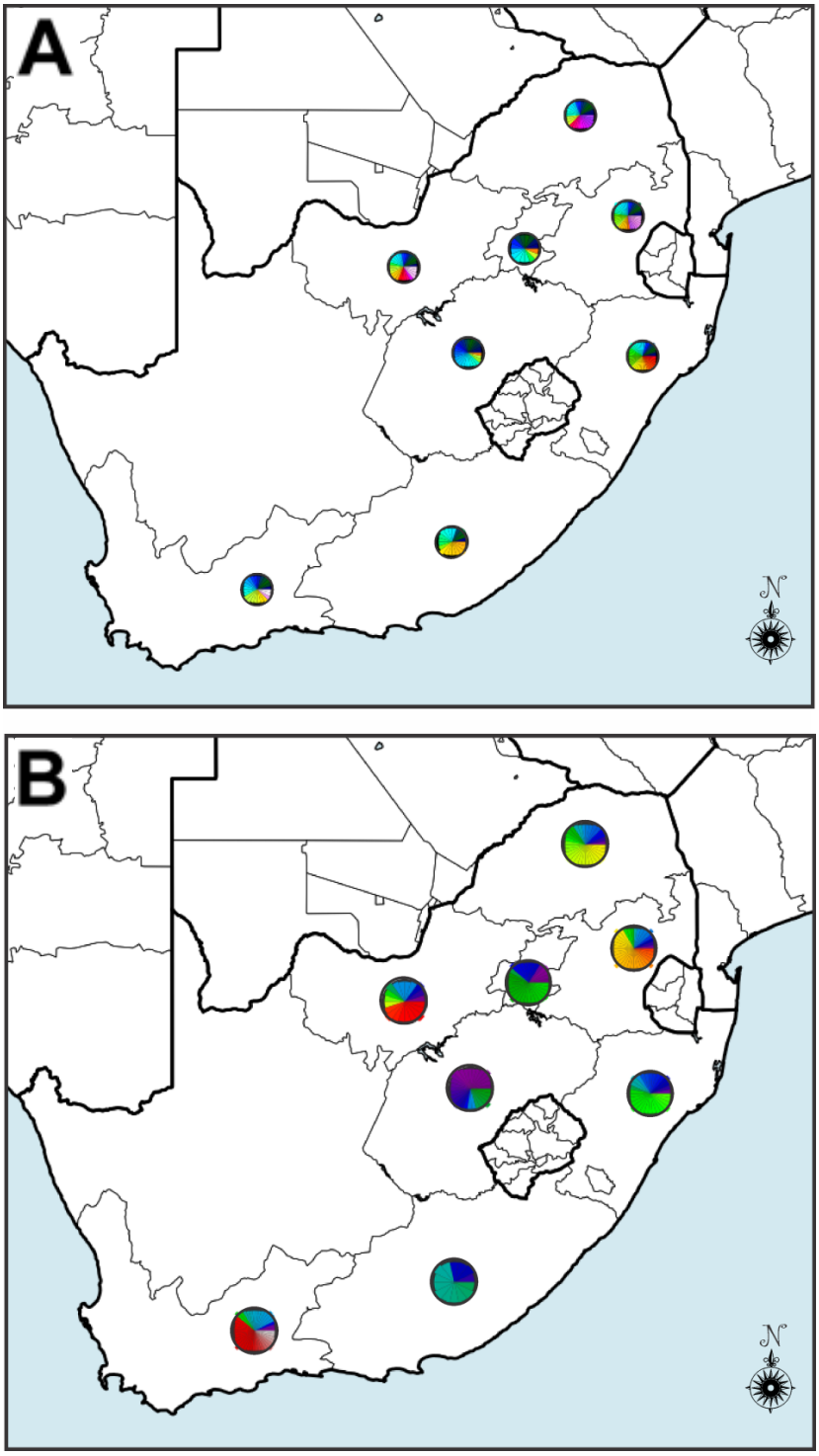

Figure 2.6: Geographical distribution of Msp1a repeats and strains from *A. marginale* in South Africa. A: Distribution of 99 Msp1a repeats from *A. marginale* identified in South Africa in this and previous studies. Different colours in each circle represent different repeats, with more colours indicating a higher repeat diversity in each region. B: Distribution of 190 *A. marginale* strains identified in South Africa in this and previous studies. Different colours in each circle represent different strains, with more colours indicating a higher strain diversity in each region. Results were generated in RepeatAnalyzer (Catanese et al., 2016).

Msp1a repeats and *msp1α* genotypes occurring in five selected countries, Brazil, Argentina, Mexico, South Africa and USA, were compared. The percentage of repeats specific to each country (unique repeats) (**Table 2.2**) was consistently lower than the percentage of unique genotypes (**Table 2.3**). The highest percentage of unique repeats (71.7%) was found in South Africa, while the lowest (18.2%) was in Brazil (**Table 2.2**).

Table 2.2: Msp1a repeat analysis for different geographical locations, using RepeatAnalyzer.

	Location				
	Brazil	**USA**	**Argentina**	**Mexico**	**South Africa**
Number of unique Msp1a repeats	6	10	12	27	71
Total number of Msp1a repeats	33	22	33	64	99
% unique repeats	18.2	45.5	36.4	42.2	71.7
Other locations with repeats in common[a]	Arg, Mex, SA, USA	Arg, Brz, Mex, SA	Brz, Mex, SA, USA	Arg, Brz, SA, USA	Arg, Brz, Mex, USA
Common repeats appearing in four or more countries	F	F	F	F	F
	M	M	M	M	M
	13	−	13	13	13
	15	−	15	15	15
	18	−	18	18	18
	27	−	27	27	27
	B	B	B	B	−
	C	C	C	C	−
	Q	−	Q	Q	Q
	τ	−	τ	τ	τ

[a] Arg – Argentina; Brz – Brazil; Mex – Mexico; SA – South Africa; USA – United States of America

The most common repeats, which appeared in all of the countries examined, were F and M. Eight other common repeats were found to be present in four of the five countries (**Table 2.2**). Although many of the Msp1a repeats identified were found in all five countries examined (an average of 42.8% of Msp1a repeats were unique to each country), very few genotypes were present in more than one country (an average of 91.0% of the *msp1α*

genotypes were unique to each country). The highest proportions of unique genotypes were found in USA (100%) and South Africa (99.0%), with Brazil (78.3%) having the lowest observed value (**Table 2.3**).

Table 2.3: *Msp1α* genotype analysis for different geographical locations, using RepeatAnalyzer.

	Location				
	Brazil	**USA**	**Argentina**	**Mexico**	**South Africa**
Number Of Unique *Msp1α* Genotypes	18	43	15	84	188
Total Number Of *Msp1α* Genotypes	23	43	18	89	190
% Unique Genotypes	78.3	100.0	83.3	94.4	99.0
Other Locations With Genotypes In Common[a]	Mex, Arg, SA	–	Brz, Mex	Brz, Arg	Brz
Genotypes Occurring In More Than One Country	α β β β β γ	–	α β β β β γ	α β β β β γ	–
	–	–	α β β β γ	α β β β γ	–
	α β β γ	–	–	α β β γ	–
	τ 57 13 18	–	–	–	τ 57 13 18
	τ 10 15	–	τ 10 15	τ 10 15	–
	13 27 27[b]	–	–	–	13 27 27[b]
	–	–	–	13 13[b]	–

[a] Arg – Argentina; Brz – Brazil; Mex – Mexico; SA – South Africa; USA – United States of America

[b] also found in the Philippines

More *msp1α* genotypes have been identified in South Africa (190 *msp1α* genotypes) than in any other country, although this likely due to sampling density. Only two genotypes that are previously been identified in other countries were identified in samples from South Africa: (i) τ 57 13 18, found in strain Minas-11 (Minas Gerais, Brazil) (Mtshali et al., 2007; Pohl et al., 2013) was identified in two samples from KwaZulu-Natal and (ii) 13 27 27, found in strain UFMG-2 (Minas Gerais, Brazil) (Mtshali et al., 2007; Pohl et al., 2013) (also found in the Philippines (Ybañez et al., 2014)) was identified in samples from Eastern Cape and

Mpumalanga. The genotypes common between South Africa, Brazil and the Philippines represent only 1% of the total number of genotypes described thus far in South Africa.

2.5 Discussion

We have recently shown that the duplex qPCR assay (Decaro et al., 2008) is a more sensitive method of detecting *A. marginale* and *A. centrale* infections in cattle in South Africa than the RLB (Bekker et al., 2002) or nPCR (Molad et al., 2006) assays. We also detected sequence variation in the *msp1β* gene in the target region of one of the nPCR internal primers in South African *A. marginale* strains (Chaisi et al., 2017). The *msp1β* multigene family encodes the Msp1b protein, which has been shown to exhibit variation between strains of *A. marginale* (Brayton et al., 2009; Ybañez et al., 2014). Variation of 0.9–1.4% between Msp1b peptide sequences has been shown, but Msp1b is stable during the bovine and tick stages of the *A. marginale* lifecycle within a given strain (Bowie et al., 2002). This variation could be detrimental when it is used as a target for detection of the pathogen by diagnostic tests such as the *A. marginale*-specific qPCR (Carelli et al., 2007). Sequence analysis of the *msp1β* gene in the target region of the qPCR assay in the current study indicated that the *msp1β* gene of *A. marginale* from cattle in South Africa was highly variable, many samples had multiple *msp1β* variants (when considering the full-length of the amplicon sequence), and SNPs were present at six nucleotide positions in the primer and probe target areas of the qPCR assay. Eleven *msp1β* variants were identified in the qPCR target area.

It has been demonstrated that mismatches located towards the 3' end of a PCR primer are potentially detrimental to PCR amplification as they can significantly affect annealing of the primer to the template, leading to underestimation of the initial copy number, or even a complete failure of amplification (Stadhouders et al., 2010). However, the SNPs identified in this study did not appear to decrease the efficiency of the qPCR assay. The efficiency of the qPCR assay in detection of variants SA2 and SA4 (with the most SNPs) compared well with that of the qPCR assay in detection of SA1 (Chaisi et al., 2017) in which there is no variation in the qPCR target region. Nevertheless, the sensitivity of the qPCR assay could still be compromised if there is more variation in the field than we have detected in this study. Moreover, *A. marginale* has been identified from wildlife in South Africa (Khumalo et al., 2016) but the sequence variation in the *msp1β* gene of the rickettsia in these hosts is unknown.

It should be noted that there are two full and three partial length copies of the *msp1β* gene in *A. marginale,* that have been described and annotated in each of the Florida and St. Maries strains (Barbet et al., 1987; Barbet & Allred, 1991), and the primers and probe used in the duplex qPCR assay can amplify the target region in both copies. This would explain the large number of samples containing multiple *msp1β* gene variants, since many samples contained multiple *A. marginale* strains (as shown by *msp1α* genotyping), and each strain could contain two different *msp1β* copies. The presence of multiple different copies within a sample could increase the likelihood of detecting *A. marginale* since it increases the chance of a single sample containing a variant that can be detected by the qPCR.

The *groEL* gene of prokaryotes, homologous to the heat-shock protein gene in eukaryotes (Sumner et al., 1997), is highly conserved, but contains variable regions that can be useful in differentiating closely related organisms (Yu et al., 2001; Rymaszewska, 2008). In contrast to the *A. marginale msp1β* gene, the *groEL* genes of *A. centrale* and *A. marginale* were highly conserved in the target region of the qPCR assay, although SNPs in other regions of this gene were identified. Since the sequence differences targeted by the qPCR primers and probes were highly conserved in all *A. centrale* and *A. marginale groEL* sequences examined, the *groEL* gene is therefore a good marker for the detection of *A. centrale* infections in cattle in South Africa. However, in a recent study on the occurrence of tick-borne infections in cattle samples from Uganda (Byaruhanga et al., 2016), the RLB assay detected more *A. centrale* infections than the qPCR assay, indicating the possibility of *groEL* gene variants which cannot be detected by the qPCR assay. This highlights the necessity for testing the assay in each region in which it is to be deployed. Further, the detection limits are shown to be approximately 10 iRBC/reaction; although this is not being used as a quantitative assay, this can be used as a guideline for field sample detection.

Only two natural isolates of *A. centrale* have been made in South Africa, the original isolate made by Theiler (Theiler, 1911) that is used in the blood vaccine, and a second isolate that was made when unfed adult *Rhipicephalus simus* ticks collected in the Louis Trichardt district of the Northern Transvaal (now Limpopo) were fed on a splenectomized ox and an *A. centrale* infection was transmitted (Potgieter, 1979; Potgieter & van Rensburg, 1987). Very little work has been done on this strain of *A. centrale* although it has been shown to have a close identity to Theiler's *A. centrale* vaccine strain by phylogenetic analysis of the 16S

rRNA and *groEL* genes (Lew et al., 2003). The *groEL* sequence from this strain (accession no. AF414866) (Lew et al., 2003) was included in our analysis, and, as with all the other *A. centrale groEL* sequences analysed, there was no variation in the qPCR target region. It is possible that some of the *A. centrale* infections detected in field samples in this study were due to this strain.

Our results indicated that *A. marginale* is widespread in cattle in eight of the nine provinces of South Africa. As expected, high percentages (>70%) of *A. marginale*-positive samples were identified in KwaZulu-Natal, Western Cape and Mpumalanga, since endemic stability is established in these regions. No *A. marginale* infections were detected in cattle from the Northern Cape; this is consistent with the results from a recent study (Mutshembele et al., 2014) and was expected since the tick vectors do not occur in this province. Interestingly, *A. centrale* was also detected in the cattle, although none of them were known to have been vaccinated, and mixed infections of *A. marginale* and *A. centrale* were common. A high percentage of cattle from KwaZulu-Natal and Western Cape were positive for *A. centrale,* suggesting that this organism is more common in the southern provinces of South Africa. However, it was not detected in cattle samples from the Eastern Cape, but this may have been an artefact of the sampling (43 samples were collected from five farms in two of 39 local municipalities, representing only 3.8% of the area of the Eastern Cape); more samples should therefore be sourced from this province to increase confidence in this result. This is the first comprehensive study on the occurrence of *A. centrale* in cattle in all nine provinces of South Africa using a nucleic acid-based method, although we recently reported on the occurrence of this species in cattle in Bergville, KwaZulu-Natal province, South Africa (Khumalo et al., 2016). Mixed infections of *A. centrale* and *A. marginale* have been reported in cattle and wildlife in South Africa (Khumalo et al., 2016) and in cattle elsewhere (Decaro et al., 2008; Belkahia et al., 2015; Byaruhanga et al., 2016). Although multiplex qPCR assays are recommended for detecting tick-borne pathogens, competitive PCR suppression may occur if infection levels are similar between two or more target species, or are higher in one species/target (Pienaar et al., 2011). This can affect assay sensitivity as has been reported with multiple infections of *T. parva*, *Theileria* sp. (buffalo) and *Theileria* sp. (bougasvlei) in buffalo (Pienaar et al., 2011). Decaro and co-workers (Decaro et al., 2008) partly addressed this problem by increasing the concentration of the *A. centrale* primers in order to increase the chance of detecting this pathogen in mixed infections.

Msp1α genotyping revealed that most qPCR-positive cattle (71.8% of samples) in this study were found to be infected with multiple *A. marginale* strains. This is expected in endemic areas, and has been reported in previous studies in USA and the Philippines (Palmer et al., 2004; Ybañez et al., 2014). Although up to nine *msp1α* genotypes were found per animal, the most abundant genotypes were one to three genotypes per sample. Competition for limited niches or resources in a single host is likely to increase with increasing number of genotypes, and may explain the lower numbers of genotypes per animal. Moreover in South Africa, oxytetracycline and imidocarb are bought over-the-counter by farmers without the need for a veterinary prescription, and these drugs are commonly used to treat babesiosis, heartwater and anaplasmosis, the most common tick-borne diseases in South Africa (de Waal, 2000). Therefore, treatment regimens used by farmers and veterinarians, which have been shown to reduce infection in animals (Potgieter & Stoltsz, 2004; Reinbold et al., 2010), combined with host immunity (Potgieter & Stoltsz, 2004), may play an important role in maintaining lower numbers of genotypes per animal.

Msp1α genotype has been shown to be a surrogate indicator for strain antigenicity, with strains with different *msp1α* genotypes having different *msp2* repertoires (Rodríguez et al., 2005). Futse and co-workers demonstrated that a single unique *msp2* allele was sufficient for a strain to establish superinfection in the face of robust immunity to a primary infecting strain (Futse et al., 2008). Our results may suggest superinfection by genomically distinct *A. marginale* strains, which is thought to be uncommon in the temperate regions of the world but occurs more frequently in the tropics (Esquerra et al., 2014; Ueti et al., 2012; Castañeda-Ortiz et al., 2015). However, superinfection cannot be proven to have occurred in our samples as the infection progress was not monitored in the animals over time, only assessed at one static time point.

Our results demonstrate the importance of cloning all *msp1α* PCR products when genotyping *A. marginale* in order to detect multiple infections per animal. Previous studies have focused on samples with only a single detectable band, and/or have only sequenced one product. To fully explore the diversity of genotypes in a given sample, an investigator must analyse all *msp1α* amplicons obtained. The detection of thirty-six low abundance, previously undescribed *A. marginale* repeats in this study, emphasizes this point. It should be noted however, that, since *msp1α* is a repetitive sequence, errors in PCR are possible if amplification halts and one repeat primes amplification on another, leading to genotypes with

extra repeats. Such a situation may have occurred in up to six samples (7.1%) in this study. Errors may also occur due to *Taq* polymerase slippage early in the PCR, resulting in over- or under-representation of certain repeats. Other error sources may be due to low DNA concentration or poor sample quality, which may arise from improper storage or repeated cycles of freezing and thawing of blood samples (reviewed in Pompanon et al. (2005)).

Worldwide, over 250 highly variable Msp1a repeats have been detected to date (de la Fuente et al., 2007; Cabezas-Cruz et al., 2013; Catanese et al., 2016). The amino acid sequences of the B- and T-cell epitopes that have previously been identified and shown to be necessary to elicit a protective immune response by Msp1a (Allred et al., 1990; Brown et al., 2002; Garcia-Garcia et al., 2004; de la Fuente et al., 2010; Cabezas-Cruz et al., 2013), were found to be variable in the novel Msp1a repeats described in this study, and this variation almost certainly has an effect on the overall epitope structure. Such variations should therefore be considered when testing Msp1a as a protective antigen. Serine residues at positions 4 and 25, however, were found to be highly conserved; these residues are thought to be important for O-glycosylation and the adhesion function of the protein, which is essential for transmission of *A. marginale* (Garcia-Garcia et al., 2004).

We found that 28 out of the 99 (22.3%) Msp1a repeats identified in South Africa are also found in strains in other countries, but this does not translate to many shared genotypes, with only two genotypes out of 190 (approximately 1%) found in common between South Africa and Brazil, and the Philippines. This result is in concordance with a recent study analysing global repeat and strain distribution (Cabezas-Cruz et al., 2013). These data may suggest that new repeats arise independently in different geographical regions, resulting in the emergence of novel genotypes, which arise from new repeat combinations. Interestingly, one of the two genotypes that was found to be common between South Africa and Brazil (τ 57 13 18), had a repeat structure which differed by one repeat from one of the world's most common genotypes, τ 22 13 18, which has been detected seven times in Argentina and Mexico (Cabezas-Cruz et al., 2013) (repeats 57 and 22 differ by eight amino acids). Although the low prevalence of genotypes common between South Africa and the rest of the world may be due to restricted cattle movements, it could also be due to a lack of *A. marginale* genotyping efforts in other parts of Africa and some regions of the world.

We have identified a large number of diverse Msp1a repeats which are fairly evenly dispersed in South Africa. A large proportion of these Msp1a repeats and *msp1α* genotypes are found only in South Africa. High repeat and genotypic diversity, and an even dispersion of repeats is expected in situations where the number of region-specific repeats and genotypes is high (Catanese et al., 2016; Khumalo et al., 2016), which is evident in the South African data. These data may suggest that repeats (and their associated genotypes) are circulating within the country as a whole, a process which may be driven by cattle movement between the high prevalence endemic areas and the presence of tick vectors of *A. marginale* to propagate the pathogen. In fact, more than one genotype was found to be common between three to five provinces, which provides evidence of ongoing movement of cattle between provinces within South Africa. Both artificial and natural selection factors such as the presence and control of competent tick vectors, host immunity and chemotherapy treatment, are strong determinants of *A. marginale* repeat and genotype composition in different areas. This study demonstrates a high genetic variability of the *A. marginale* population in South Africa, which is an important factor to consider in formulating future vaccine design strategies.

2.5 Conclusions

Both *A. marginale* and *A. centrale* are prevalent in South Africa. *Anaplasma centrale* was detected in cattle despite the lack of vaccination with this organism, suggesting that there is a natural transmission cycle of *A. centrale* in South Africa. A total of 190 different *msp1α* genotypes of *A. marginale* have been detected in South Africa, indicating a diversity of genotypes that must be taken into account when developing a vaccine.

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CHAPTER 3

Anaplasma marginale outer membrane protein vaccine candidates are conserved in North American and South African strains

3.1 Abstract

In this study, we assessed the amino acid variation in vaccine candidate outer membrane proteins (OMPs) in South African strains of *Anaplasma marginale*, and also evaluated their immunogenic properties between strains from South Africa and USA. The OMP genes *Am779, Am854, omp7, 8* and *9* were amplified and sequenced from a genetically diverse set of South African samples that were previously *msp1α*-genotyped. Following translation and alignment, amino acid sequences for OMPs Am854 and Am779 were found to be highly conserved, with the former having identical amino acid sequences and the latter changes at only three amino acid positions leading to five variants. Omp7, 8 and 9 were found to possess conserved N- and C- terminals, along with a pronounced, central hypervariable region (HVR). A highly conserved T-cell epitope, FLLVDDAI/V, was found in the conserved N-terminus of these three OMPs. We found South African OMPs to have 79–100% amino acid sequence identity with the corresponding proteins in the USA *A. marginale* St. Maries and Florida strains. For all OMP genes, we selected the two major South African genetic variants and expressed recombinant proteins. Recombinant OMPs were tested by immunoblotting, using bovine test sera derived from cattle inoculated with *A. marginale* cross-linked or non-cross-linked OMPs, *Anaplasma centrale* vaccinates and naturally infected *A. marginale* carrier animals. His-tagged recombinant OMPs Am779, Am854, Omp7, 8 and 9 and their amino acid variants were successfully expressed. All recombinant OMPs were detected by specific bovine test sera from USA and South African vaccinates, for all four test groups; none of the test sera detected related negative control antigens. All 12 post-immune test sera detected the *A. marginale* Proefplaas 1332 crude lysate control and *A. centrale* blood vaccine crude protein lysate control. Furthermore, the immunological cross-reactivity demonstrated between the *A. centrale* and *A. marginale* organisms, indicating significant antigenic and immunological relationships, validates the continued use of the *A. centrale* blood vaccine for immunisation against *A. marginale* infections. Our study provides evidence to suggest that the *A. marginale* OMPs are good vaccine candidates for use in a global vaccine cocktail, although further work on the best formulation and delivery methods is necessary.

3.2 Introduction

Anaplasma marginale is an economically important and globally widespread tick-borne, intra-erythrocytic rickettsial pathogen, which causes bovine anaplasmosis (Kocan et al., 2004; 2010). It is endemic in tropical and subtropical parts of the world and is characterized by fever, weight loss, haemolytic anaemia and even death, which lead to considerable losses in the cattle industry (Kocan et al., 2010). The disease may also be caused by *A. centrale*, a close relative of *A. marginale*, which usually causes mild infections (de Waal, 2000; Potgieter & Stoltsz, 2004), although a few cases of anaplasmosis attributed to *A. centrale* have been reported, most recently in Europe (Carelli et al., 2008).

Various control methods for anaplasmosis exist (Kocan et al., 2003; Merck, 2017), including vaccination with the *A. centrale* live blood vaccine, which is still used in essentially the same form as was conceived by Theiler over 100 years ago (Theiler, 1912; Palmer, 2009). This vaccination strategy takes advantage of the fact that *A. centrale* shares $CD4^+$ T- and B-cell epitopes with *A. marginale* (Brayton et al., 2005; Herndon et al., 2010). This approach is useful for preventing the onset of disease after infection with field strains of *A. marginale*, and is used widely in different parts of the world (Bock & de Vos, 2001; Kocan et al., 2003; 2010). However, due to its inherent drawbacks, ranging from offering only partial protection to challenge by heterologous *A. marginale* strains, to its potential to introduce emerging diseases from endemic to non-endemic areas, it is not used in some parts of the world such as USA. Other vaccination methods such as the use of cultured pathogens and inactivated/ killed pathogens have been developed for bovine anaplasmosis (Pipano, 1995; de la Fuente et al., 2002; Kocan et al., 2003; Hammac et al., 2013). However, besides their having partial efficacy, these vaccines have associated safety issues and are not sufficiently well-developed for reproducible, large-scale production, making them unattractive in their current state (Kocan et al., 2003; 2010; de la Fuente et al., 2017).

An attractive and practical option to the shortcomings of the current blood vaccine may lie in the use of subunit or recombinant vaccines (Palmer et al., 1986; 1988; 1989; 1991; 1999; Tebele et al., 1991; Albarrak et al., 2012; Santos et al., 2013; Ducken et al., 2015). Polypeptides can be produced in a reproducible manner on a large scale using recombinant DNA technology, thus offering a viable means for producing more-or-less uniform vaccine stocks (Kocan et al., 2003; Palmer, 1991). Outer membrane protein (OMP) preparations of *A.*

marginale have been experimentally shown to induce protection against *A. marginale* infection, by limiting the clinical effects of the pathogen in the majority of cattle tested (Palmer et al., 1986; 1988; 1989; 1999; Tebele et al., 1991). It has also been demonstrated that protection against homologous strains of *A. marginale* results in a corresponding increase in IgG2 binding to the pathogen surface (Brown et al., 1998; 2003; Palmer et al., 1999; Han et al., 2010), indicating that an effective vaccine should induce IgG2 that binds OMPs expressed by field strains of *A. marginale*.

Using genomic and proteomic methods, vaccine candidate OMPs that could be effective against *A. marginale* in cattle have been identified. Brayton et al. (2005) predicted 62 OMPs from the *A. marginale* genome sequence data, and some of these were shown to be conserved between *A. centrale* and *A. marginale* (Herndon et al., 2010) using bioinformatics methodologies. Lopez et al. (2005) used 2D electrophoresis, immunoblotting and liquid chromatography tandem mass spectrometry (LC-MS/MS) to show that a subset of 21 of the OMPs identified by Brayton et al. (2005) was immunoreactive with sera that were collected from animals that were protected. The study by Lopez et al. (2005) revealed that IgG from OMP-vaccinated cattle reacted with *A. marginale* OMP preparations. Furthermore, Noh et al. (2008; 2010) showed that immunisation with cross-linked OMP surface complexes induced protection against *A. marginale* challenge that was similar to the protection induced by immunisation with whole OMP preparations. A subset of OMPs was identified in the cross-linked surface complex that could also be found in the *A. marginale* OMP preparations. These included Msp1a, Msp2, Msp3, Msp4, OpAG2, Omp7, Omp8, Omp9, Am779 and Am854. Agnes et al. (2011) also employed a proteomic approach to identify OMP immunogens of *A. marginale* that were recognised by IgG2 raised against the Israel vaccine strain of *A. centrale* in calves; this study revealed, amongst others, five OMPs: Omp7, Omp8, Omp9, Am779, and Am854. These have further been shown to be potential vaccine candidates because of protection they afforded cattle in challenge experiments using the St. Maries strain (Brown et al., 1998; Noh et al., 2008).

The aims of this study were therefore the amplification, sequence analysis and expression of five OMPs (Am779, Am854, Omp7, Omp8, and Omp9) and their major variants from South African strains of *A. marginale* with differing *msp1α* genotypes and to determine, by immunoblotting, whether or not recombinant OMP proteins bind immune sera derived from: 1) cattle immunised with cross-linked and non-cross-linked OMPs derived from USA *A.*

marginale St. Maries strain, 2) cattle immunised with the South African *A. centrale* blood vaccine and 3) cattle naturally exposed to field strains of *A. marginale* from South African.

3.3 Materials and Methods

3.3.1 Amplification and sequencing of OMP genes

Am779, *Am854, omp7, omp8* and *omp9* were amplified from 85 selected *A. marginale*-positive field samples with diverse *msp1α* genotypes [Hove et al. (2018) and Section 2.3.5, Chapter 2]. *omp7, 8* and *9* are encoded as three tandemly arranged genes in an operon in the St. Maries and Florida strains, with 70–75% amino acid sequence identity among the three OMPs (Brayton et al., 2005; Noh et al., 2006). Primer pairs Am779 F and R (*Am779*), Am854 F and R (*Am854*), OMP7 F and R (*omp7*), ALL F and OMP8 R (*omp8*), and ALL F and OMP9 R (*omp9*) were used to specifically amplify the five OMP genes (**Table 3.1**). Optimal annealing temperatures for the PCRs were determined by gradient PCR and amplification was performed in a 25 µl volume consisting of 1X Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific, Waltham, USA), 0.5 µM primers and 2.5 µl of template DNA (approximately 200 ng). Thermal cycling conditions for *Am779* amplification were 98°C for 10 s, 40 cycles of 98°C for 1 s, 69.5°C for 5 s and 72°C for 18 s, and a final extension at 72°C for 60 s. Thermal cycling conditions for *Am854* amplification were similar to those for *Am779*, the only differences being annealing at 71°C for 5 s and extension at 72°C for 15 s. Cycling conditions for *omp7*, *8*, and *9* were similar to those for *Am779* with the exception of the annealing temperature at 68°C for 5 s.

Table 3.1: Primers for PCR amplification of *A. marginale* OMP genes (*Am779*, *Am854, Omp7, Omp8* and *Omp9*), and expected PCR product sizes.

Primer name	Sequence (5′–3′)	Annealing Temperature	Expected amplicon size
Am779 F Am 779 R	ATG GTA CAT AAA GGT TCT CTG GTG GCT C GTT TGT GGC TTT CAC GCT CCT GAG	69.5°C	1 593 bp
Am779 F PE Am779 R PE	ATG AGC TAT GCT TTT GTC ACC GGG CGA GTG C TTA CCT CAG TAC GTG CTC ACC ATC AAA CCC C	64.0°C	1 265 bp
Am854 F Am854 R	GCT GCA TCG TTG GTT AGC TCT GTG GCC GAA GAA TCC TGT GCC ACT TC	71.0°C	711 bp
Am854 F PE Am854 R PE	ATG TCT GCC GTG ACA GGT TGT GGT CTT TTC AGC TTA TCT TCA GGC GCC GCT TCT TCG	64.0°C	586 bp
OMP7 F* OMP7 R	TCT TTT CTG TTG GGT GCG GTT GTA GAC ACG CGG CAC TGC TCT TTA TAC TC	68.0°C	1 100 bp
OMP7 F PE OMP7 R PE	ATG AGC TTT GGT GGT GAC GAT ACC GAC TTA TTG TGG GGA GAG CTC GTA ACT C	64.0°C	935 bp
ALL F OMP8 R	GGT CTT TTC TGT TGA GCG CGG TTG CGC GCG CTC TGA TAT TTT CCC TT	68.0°C	1 133 bp
OMP8 F PE OMP8 R PE	ATG AGC GAC TTT TAC TTA GGA TTT GGG CTT GCC TTA TTC TTC AGG CGC CGC TTC TTC GGA	64.0°C	950 bp
ALL F OMP9 R	GGT CTT TTC TGT TGA GCG CGG TTG GTG CCT TGA CAT CTT CCC TCT CAA C	68.0°C	1 142 bp
OMP9 F PE OMP9 R PE	ATG TCT GCA GGG TTT GGT GGT GAT GAT ACT GAC TTA TCC ATC GAC AAA AAC CCT AGC CCG	64.0°C	980 bp

* Obtained from Junior et al. (2010).
Shaded rows indicate primary PCR primers used for amplification and sequencing of OMP genes.
Unshaded rows indicate secondary (nested) PCR primers used to generate amplicons for cloning into the pET SUMO expression vector.

PCR amplicons were column-purified using a Qiagen PCR product purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and sent for direct sequencing at Inqaba Biotechnologies (Pretoria, South Africa). DNA sequences were translated into amino acid sequences in frame with reference sequences [St. Maries and Florida sequences obtained from the whole genome sequences (Accession numbers: St. Maries, CP000030 and Florida, CP001079) of these two strains] then assembled and aligned using CLC Genomics Workbench 8.0.3 (Qiagen, Hilden, Germany) and the AlignX module

of Vector NTI (Thermo Fisher Scientific, Waltham, USA). Variant groups (**Table 3.2**) were determined by alignment of amino acid sequences and construction of phylogenetic trees.

3.3.2 Cloning of OMP genes into pET SUMO

For construction of OMP-pET SUMO constructs, truncated *Am779*, *Am854*, *omp7*, *omp8*, and *omp9* sequences that excluded the sequence encoding the signal peptide and represented two variant groups per OMP were amplified using the nested PCR primers shown in **Table 3.1** from selected samples containing different *msp1α* genotypes (**Table 3.2**). The secondary PCR was carried out in a total volume of 25 µl containing 1X DreamTaq PCR Master Mix (Thermo Fisher Scientific, Waltham, USA), 0.5 µM forward and reverse primers ("PE primers", **Table 3.1**) and 2.5 µl of template (primary PCR product diluted 1 in 100 in PCR grade water). The PCR thermal cycling conditions were 95°C for 3 min, 40 cycles of 95°C for 30 s, 64°C for 30 s and 72°C for 1 min, and a final extension at 72°C for 10 min. For each sample, the secondary PCR was carried out in triplicate, with replicates pooled in order to minimize the probability of including *Taq* errors occurring early in any one of the reactions in the PCR products to be cloned. Pooled PCR products were column-purified using the CloneJET PCR Purification Kit (Thermo Fisher Scientific, Waltham, USA) following the manufacturer's instructions.

Purified PCR products were ligated into the pET SUMO protein expression vector (Thermo Fisher Scientific, Waltham, USA) using the pET SUMO TA Cloning® kit (Thermo Fisher Scientific, Waltham, USA) and transformed into Invitrogen One Shot® Mach1^{TM}-T1^{R} chemically competent *Escherichia coli* cells (Thermo Fisher Scientific, Waltham, USA) following the manufacturer's protocol. Positive transformants were screened using colony PCR using the appropriate forward PE primer (**Table 3.1**) and the T7 sequencing/vector reverse primer (5′-TAG TTA TTG CTC AGC GGT GG-3′). Positive colonies were picked into 5 ml Luria-Bertani (LB) broth containing 50 µg/ml kanamycin (Thermo Fisher Scientific, Waltham, USA), grown for 12–16 h and 500 µl of culture was stored in an equal volume of sterile 50% glycerol. The rest of the culture was centrifuged at 8 000 rpm for 30 s to pellet the cells. Plasmid DNA was extracted from the cell pellet and eluted in a final volume of 100 µl of elution buffer (10 mM Tris-HCl, pH 8.5) using the High Pure Plasmid Isolation Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

Table 3.2: Samples chosen for amplification and expression of OMP variants.

OMP (Length in aa[a], MW[b] in kDa[c])	MW of recombinant OMP + SUMO protein in kDa [#]	OMP variant	Name of sample	Origin of sample	*Msp1α* genotype (number of *msp1α* genotypes in the sample)	Amino acid sequence identity to StM[d] & FL[e]
Am779 (421 aa, 46.3)*	59.3	1	C14	Mpumalanga	42 43 25 31 13 27 27 **(2)**	StM– 99.9% FL – 99.9%
		4	127	Western Cape	34 13 37 3 4 37 **(2)**	StM – 99.9% FL – 99.9%
Am854 (195 aa, 21.4)*	34.4	–	136	Mpumalanga	42 43 25 31 34 13 13 37 84 UP19 UP19 78 31 31 31 34 13 3 36 38 **(5)**	StM – 100% FL – 100%
Omp7 (311 aa, 34.2)*	47.2	2A	112	Western Cape	154 **(1)**	StM – 85% FL – 93%
		3A	NW C28	North West	34 36 36 38 **(1)**	StM – 79% FL – 86%
Omp8 (316 aa, 34.8)*	47.8	1C	LPC 46	Limpopo	41 4 37 13 155 37 4 38 41 13 4 37 **(3)**	StM – 90% FL – 99%
		2B	84	Mpumalanga	34 3 3 3 36 38 34 13 13 37 34 36 36 3 36 38 3 37 37 34 3 27 3 UP1 34 UP2 36 38 34 3 34 3 38 **(7)**	StM – 98% FL – 90%
Omp9 (326 aa, 35.9)*	48.9	2A	GP-K C6A	Gauteng	34 13 13 13 37 27 13 18 27 13 3 36 38 **(3)**	StM – 91% FL – 91%
		4A	GP-K C12	Gauteng	34 13 4 4 13 4 27 4 13 4 4 4 37 **(2)**	StM – 92% FL – 92%

* Denotes length in amino acids (aa) and size of truncated, recombinant OMP (kDa).

[#] Denotes molecular weight of truncated, recombinant OMP + SUMO protein (13 kDa).

[a]aa = amino acids, [b]MW = Molecular weight, [c]kDa = molecular weight of proteins in kiloDaltons,

[d]StM = St. Maries strain of *A. marginale*, [e]FL = Florida strain of *A. marginale.*

Plasmid DNA preparations were sent to Inqaba Biotechnologies (Pretoria, South Africa) for sequencing with SUMO forward (5′-AGA TTC TTG TAC GAC GGT ATT AG-3′) and T7 reverse primers. Analysis of the sequence using CLC Genomics Workbench 8.0.3 allowed for selection of positive constructs cloned in-frame with no sequence errors.

3.3.3 Expression and purification of recombinant OMPs

Approximately 10 ng of plasmid DNA from each sample was used to transform Invitrogen *E. coli* BL-21(DE3) One Shot® expression cells, using the heat-shock protocol as specified in the Champion pET SUMO manual (Thermo Fisher Scientific, Waltham, USA). Following this, 500 µl of the overnight culture of transformed BL-21(DE3) cells was used to inoculate 10 ml of fresh LB broth containing 50 µg/ml kanamycin, and grown at 37°C with shaking at 240 rpm for 2 h. After induction of the culture by addition of isopropylthio-β-galactoside (IPTG) at a final concentration of 1 mM the culture was grown for the optimal expression time of 4–5 h. Thereafter, crude protein extracts from each culture were separated by polyacrylamide gel electrophoresis (PAGE) on a 12% Criterion TGX (Midi - 12 + 2 lanes, 45 µl) precast denaturing polyacrylamide stacking gel (BioRad, Hercules, USA) in 1X Tris/Glycine SDS electrophoresis buffer, at 100 V for 1 h. The Precision Plus Protein Kaleidoscope pre-stained standard (BioRad, Hercules, USA) was used as a molecular weight marker. Following this, the polyacrylamide gels were stained in order to visualize proteins using the Coomassie Blue SafeStain according to the supplier's instructions (Thermo Fisher Scientific, Waltham, USA).

Expression of His-tagged recombinant OMPs was confirmed using a standard Western blotting protocol. HisDetector Nickel^{2+} Horse Radish Peroxidase (HRP) (KPL Scientific Inc, Gaithersburg, USA) at a final dilution of 1:5,000 was used, with the 1-Step Tetramethylbenzidine (TMB)-Blotting Solution for Western blotting (Thermo Fisher Scientific, Waltham, USA) as substrate. Soluble and insoluble (from inclusion bodies) protein fractions were extracted from the cell pellets using the Novagen BugBuster Protein extraction kit (Merck, Kenilworth, USA), according to the manufacturer's instructions. Crude proteins were purified using Protino Ni-IDA Nickel^{2+} columns (Machery-Nagel, Duren, Germany), in accordance with the supplier-specified methodology. Purified extracted protein was then run on PAGE for each OMP, as previously described. Thereafter, the polyacrylamide gel was stained using the Coomassie Blue SafeStain (Thermo Fisher Scientific, Waltham, USA) as described above. The amount of purified recombinant protein generated for each OMP and control protein samples were spectrophotometrically quantified using the Bicinchoninic Acid (BCA) method following the protocol in the Pierce BCA kit (Thermo Fisher Scientific, Waltham, USA). Recombinant OMPs were derived mostly from

inclusion bodies and were solubilised using urea and refolded by dilution as previously reported (Ducken et al., 2015).

For crude protein lysate controls, a 200 µl volume of each control blood sample [i. *A. marginale*-infected blood: whole bovine EDTA blood sample with 33% parasitized erythrocytes (9678/2) supplied by the Agricultural Research Council-Onderstepoort Veterinary Research Institute (ARC-OVR), South Africa. Sample 9678/2 was infected with blood originating from an animal at the Proefplaas, the UP experimental farm in Pretoria, South Africa and is designated as Proefplaas strain 1332 and ii. *A. centrale*-infected blood: the bovine blood vaccine produced by the Onderstepoort Biological Products (OBP) (Pretoria, South Africa)] was washed at least five times in 1X phosphate buffered saline (PBS) with centrifugation at maximum speed in a benchtop microcentrifuge, prior to BCA quantification. Thereafter, samples were resuspended in 200 µl of 1X PBS. Untransformed *E. coli* BL-21 (DE3) cells were used as a control for contaminating *E. coli* proteins. Other controls used were the truncated recombinant *Ehrlichia ruminantium* predicted membrane protein, Erum1040 (Collins et al., 2005) and the recombinant SUMO protein.

3.3.4 *Anaplasma marginale* and *A. centrale* test serum samples

Anaplasma marginale and *A. centrale* seropositive and seronegative serum samples were used as controls for the immunoblotting experiments. In order to prepare control sera, a total of 17 bovine blood samples were collected from Proefplaas (11 samples), and East Lynne farm in Bergville, KwaZulu-Natal (six samples). Samples were collected in 9 ml Vacuette EDTA and serum tubes.

Samples were collected from animals at Proefplaas which were previously found to be *A. marginale* positive when tested with duplex real-time quantitative PCR (qPCR) (Decaro et al., 2008; Chaisi et al. 2017). The Bergville animals selected for sampling were young calves (six to eight months old) that had not been vaccinated with the *A. centrale* blood vaccine. After an initial collection (pre-immune) of blood and serum samples from these six animals, they were immediately vaccinated with a subcutaneous injection of 5 ml of the *A. centrale* blood vaccine (OBP, Pretoria, South Africa). Thereafter, animals were given a six-week period to seroconvert, after which blood and serum samples were again collected (post-immune).

Genomic DNA was extracted from all ETDA samples using the Qiagen Blood Mini Kit (Qiagen, Hilden, Germany) and tested using the duplex qPCR assay on the Lightcycler (Roche Diagnostics, Mannheim, Germany), which detects *A. marginale* and *A. centrale* (Decaro et al., 2008) as previously described (Chaisi et al. 2017). Serological testing of all serum samples was done at the ARC-OVR using the Msp5 competitive inhibition enzyme-linked immunosorbent assay (cELISA) (Visser et al., 1992), which detects antibodies to Msp5 antigen of *Anaplasma* spp.

Other test sera to be used for immunoblotting were imported from Dr. Susan Noh (United States Department of Agriculture, Pullman, WA, USA) (Noh et al., 2008; Noh et. al., 2013). The sera were as follows:

1a. Sera from cattle immunised with OMPs derived from the St. Maries strain of *A. marginale*: identity numbers 43071, 43092 and 43100: 2.5 ml x 3,

1b. Pre-immune sera from above cattle: 2.5 ml x 3

2a. Sera from cattle immunised with cross-linked OMPs derived from the St. Maries strain of *A. marginale*: identity numbers C1252, 35100 and 35130: 1.5 ml x 3 and,

2b. Pre-immune sera from above cattle: 1.5 ml x 3.

For immunoblots, anti-*E. coli* antibodies were adsorbed from all of the pre- and post-immune sera (primary antibodies) as described by Ducken et al. (2015). Prior to use in immunoblots, pre-immune sera were pooled in equal volumes for each serum and then diluted 1:100 in 1X PBS. Post-immune sera were tested individually, after dilution at 1:100 in 1X PBS.

3.3.5 Immunoblotting of recombinant OMPs using anti-*A. centrale* and *A. marginale* bovine sera

Approximately 200 ng of each recombinant OMP and control proteins [crude protein extracts from *E. coli* (BL-21 (DE3)) cells, and recombinant *E. ruminantium* OMP Erum1040 and SUMO proteins] was loaded onto precast Criterion TGX (BioRad, Hercules, USA) denaturing, gradient polyacrylamide gels (4 to 15%) and electrophoresed at 120 V for 50 min. Thereafter, the recombinant OMPs and controls were transferred onto a PVDF membrane using a wet transfer, according to standard methodology. The *A. marginale* (Proefplaas strain 1332 with *msp1α* genotype 3 37) and *A. centrale* blood vaccine (OBP, Pretoria, South Africa) crude lysate controls were loaded onto a single gel and, once

transferred onto PVDF membranes, were cut into strips which were used in immunoblots. PVDF membranes were blocked overnight at 4°C in 20 ml of 5% non-fat skimmed milk (BioRad, Hercules, USA), with gentle shaking. Test sera (primary antibody) diluted 1:100 (and 1:50 for sera from *A. marginale* naturally infected animals) in 1X PBS were incubated for 1 h at room temperature, with the PVDF membranes containing the recombinant OMPs and control proteins, followed by three washes in 1X PBS-Tween20. The membrane was then incubated for 30 min with polyclonal, HRP-conjugated, rabbit anti-bovine (secondary) antibodies (Dako A/S, Glostrup, Denmark) at a final dilution of 1:2,000, in 2.5% non-fat skimmed milk. After three 5 min washes in 1X PBS-Tween20, the PVDF membranes were immersed in TMB substrate, for 5–10 min in order to facilitate signal detection on PVDF membranes. Pictures were taken using the G:Box Chemi-XT4 GENESys (Syngene, India) gel documentation system.

3.4 Results

3.4.1 Amplification and sequencing of OMP genes

Sequences of OMP Am854 exhibited minimal variation in the 85 *A. marginale*-positive field samples examined. Three single nucleotide polymorphism (SNP) mutations detected for Am854 were synonymous and thus did not result in any amino acid changes. Thus, no amino acid variants were detected for Am854 over the 220 amino acid residues analysed, and South African OMP Am854 was found to have 100% identity with OMP Am854 from the St. Maries and Florida strains of *A. marginale* from USA (**Table 3.2**).

Of the 85 samples sequenced for *Am779*, 62 gave sequences which could be analysed. Very few amino acid variations were detected in the Am779 sequences examined; there were changes at only 3 amino acid positions, leading to 5 variants. Variations occurred at 3 positions out of 420 total residues (0.55%), with 4 of 5 variants having a single amino acid change relative to the St. Maries and Florida sequences. Variants 1 (RTA) and 4 (HTA) were the most abundant at 59.7% and 30.6% representation, with the other 3 variants (2, 3 and 5) each showing less than 5% abundance (**Table 3.3**). By virtue of abundance, samples representing major variants 1 and 4 were chosen for expression. South African OMP Am779 variants 1 and 4 were found to have 99.9% amino acid sequence identity with the St. Maries and Florida strains of *A. marginale* from USA (**Table 3.2**).

Out of a total of 85 samples, 38, 58, and 46 DNA sequences were obtained for *omp7, 8* and *9,* respectively. Omp7, 8 and 9 were found to have 86 (of 321; 26.8%), 68 (of 337; 20.2%) and 51 (of 341; 15.0%) positions where amino acids varied, respectively (**Figures 3.1, 3.2 and 3.3**). All three OMPs were found to have conserved N- and C- terminals and a central prominent HVR. The T-cell epitope FLLVDDAI/VV recently described by Deringer et al. (2017) was present in all of the South African Omp7, 8 and 9 sequences.

Table 3.3: Amino acid variation in Am779, in 62 South African field samples.

Locus Variant	Amino acid change at position			# of variants detected	% Representation
	67	113	442		
1	R	T	A	37	59.7%
2	R	T	V	2	3.2%
3	R	A	A	3	4.8%
4	H	T	A	19	30.6%
5	H	T	V	1	1.6%

Omp7 (**Fig. 3.1**) had the least variants, with 14 variants in four major variant groups, even though it had the highest number of amino acid changes relative to its length (26.8%). Groups 1 and 2 of the Omp7 variants were similar to St. Maries and Florida sequences, respectively. South African Omp7 variant 2A was found to have 85% and 93% amino acid sequence identity with USA strains St. Maries and Florida, whereas South African variant 3A had 79% and 86% identity, respectively (**Table 3.2**).

On the other hand, Omp8 (**Fig. 3.2**) was found to have the highest number of variants, with 29 variants in two major variant groups. This OMP had amino acid changes at 68 positions of the 337 residues analysed (20.2%). For Omp8, the Florida sequence was found to be similar to group 1 and St. Maries to group 2. South African Omp8 variant 1C was found to have 90% and 99% amino acid sequence identity with USA strains St. Maries and Florida, while South African variant 2B had 98% and 90% identity, respectively (**Table 3.2**).

Omp9 was found to have the second highest number of variants, with 20 variants in four major groups. Interestingly for Omp9 (**Fig. 3.3**), Florida and St. Maries sequences were both similar to group 1. This OMP had the lowest percentage of amino acid changes at 51 out of

341 positions (15.0%). South African Omp9 variant 2A was found to have 91% amino acid sequence identity with USA strains St. Maries and Florida, whereas South African variant 4A was shown to have 92% identity with the two USA strains (**Table 3.2**).

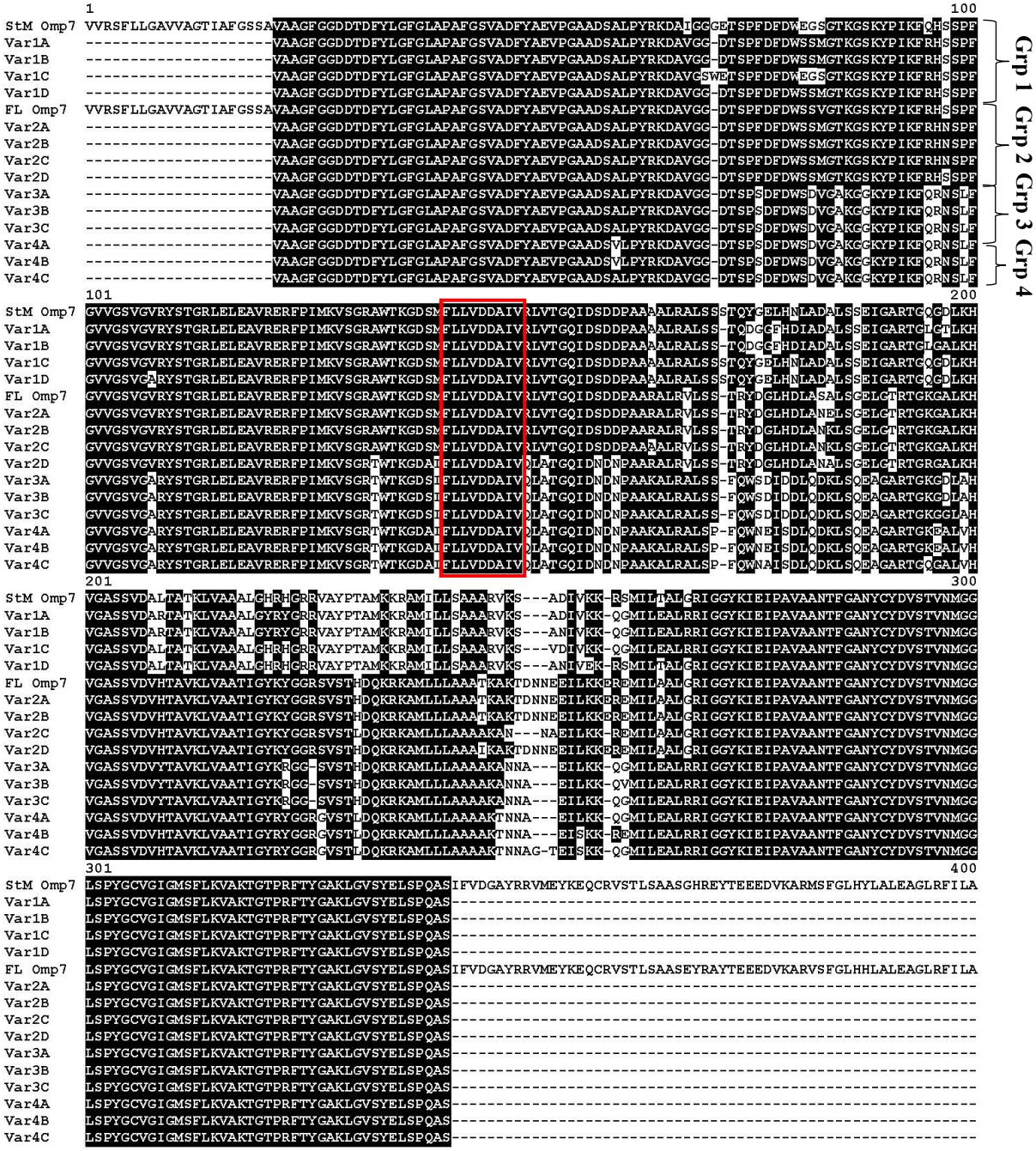
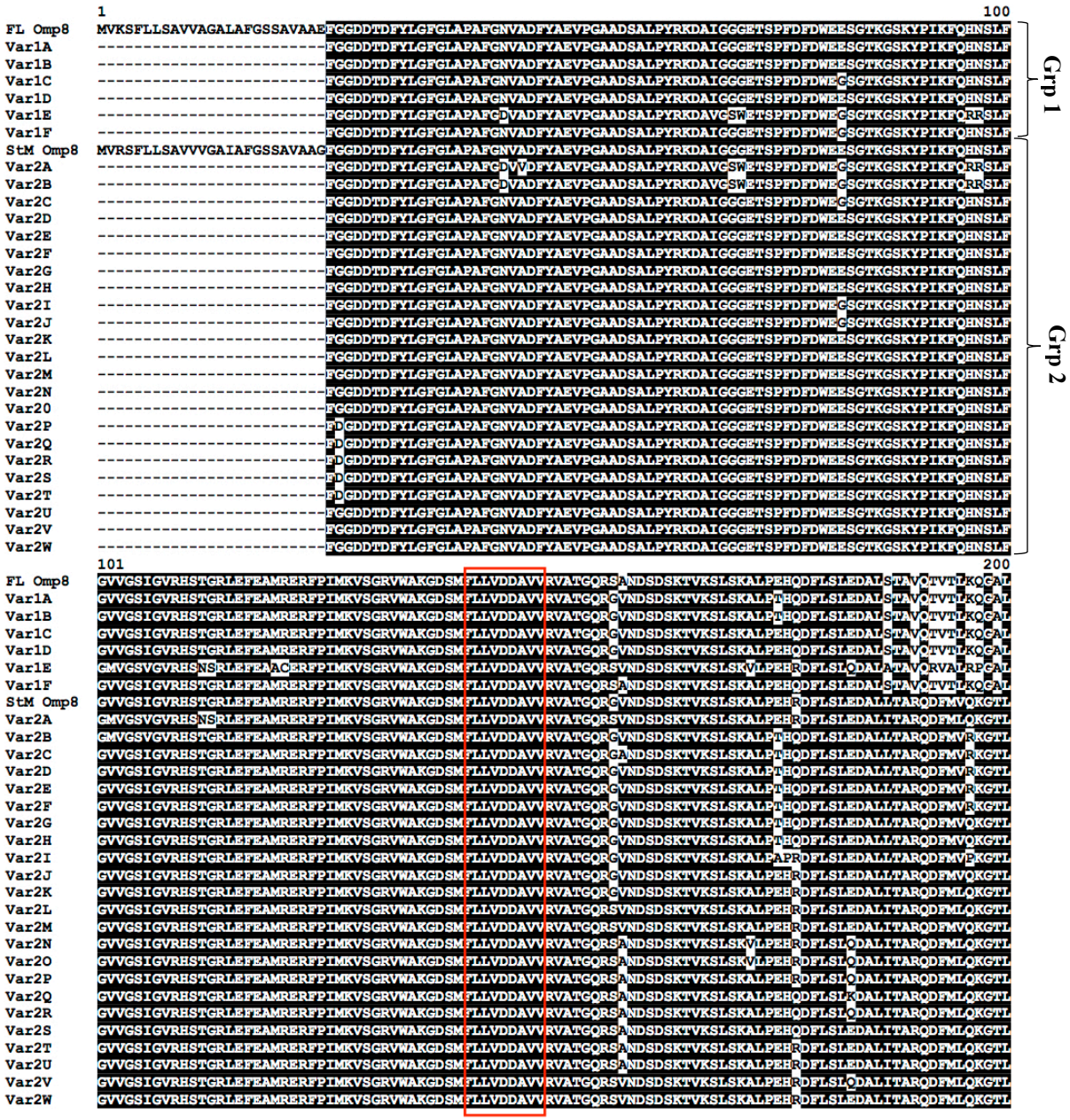


Figure 3.1: Amino acid sequence variation in Omp7, shown by alignment using the AlignX module of Vector NTI (Thermo Fisher Scientific, Waltham, USA). Identical amino acid residues in the alignment are shown by white text on a black background; variable residues are indicated by black text on a white background. The T-cell epitope FLLVDDAIV is highlighted by a red rectangle. Grp = Variant group.



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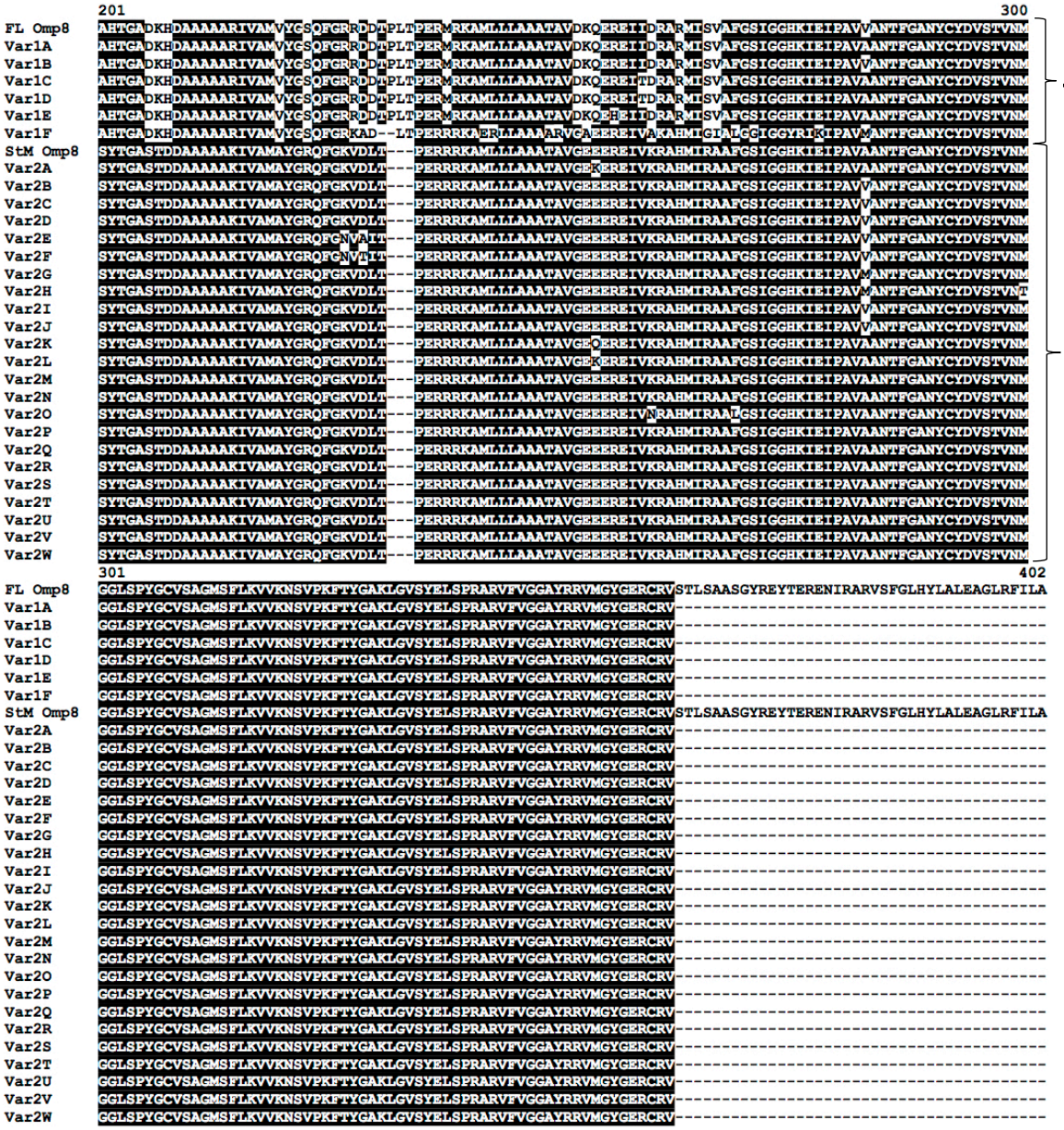
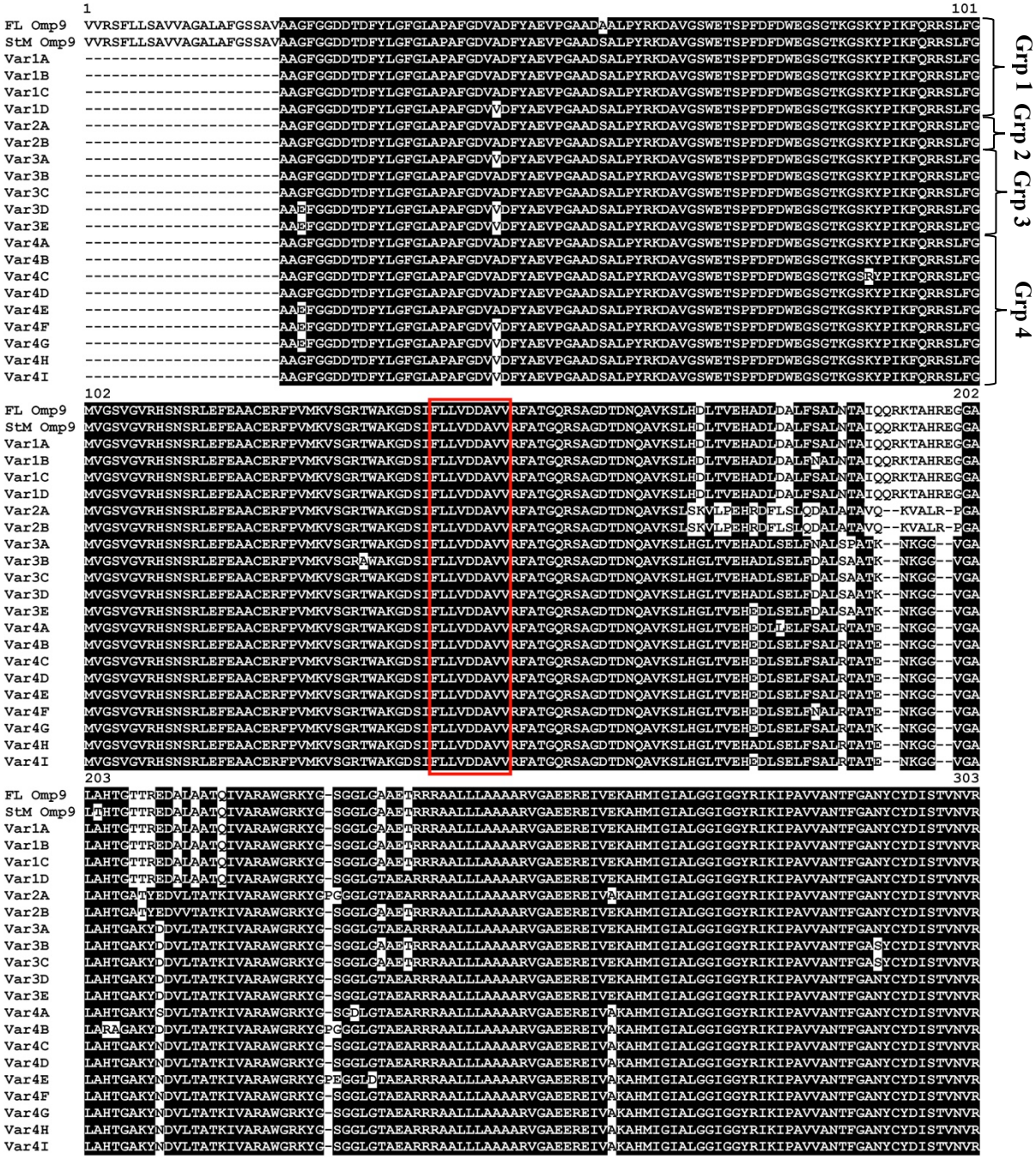


Figure 3.2: Amino acid sequence variation of Omp8, shown by alignment using the AlignX module of Vector NTI (Thermo Fisher Scientific, Waltham, USA). Identical amino acid residues in the alignment are shown by white text on a black background; variable residues are indicated by black text on a white background. The T-cell epitope FLLVDDAVV is highlighted by a red rectangle. Grp = Variant group.



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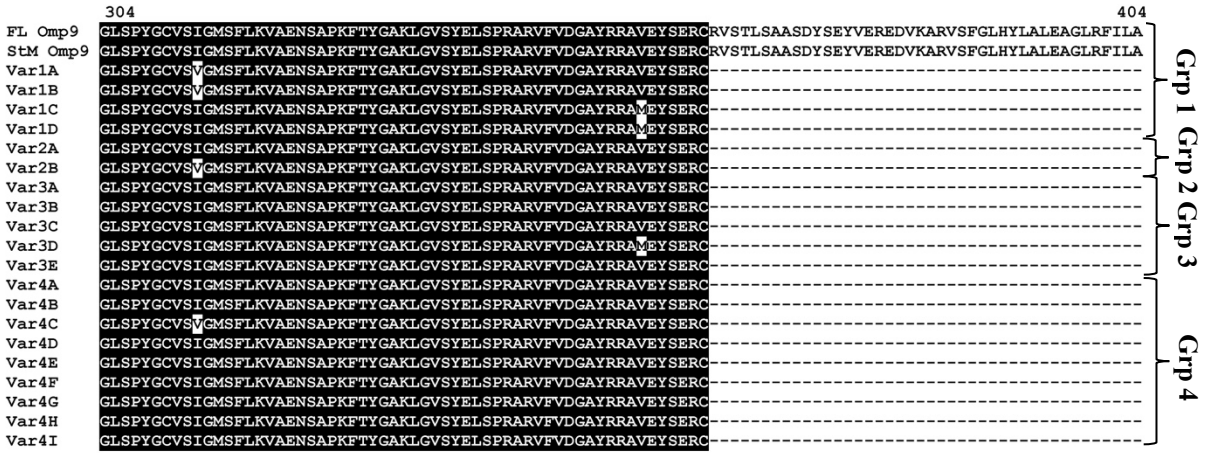

Figure 3.3: Amino acid sequence variation of Omp9, shown by alignment using the AlignX module of Vector NTI (Thermo Fisher Scientific, Waltham, USA). Identical amino acid residues in the alignment are shown by white text on a black background; variable residues are indicated by black text on a white background. The T-cell epitope FLLVDDAVV is highlighted by a red rectangle. Grp = Variant group.

3.4.2 Immunoblotting of recombinant OMPs with anti-*A. centrale* and *A. marginale* bovine sera

The five OMPs and their variants were successfully expressed with sizes 37 to 60 kiloDaltons (kDa) (**Table 3.2**), as confirmed by detection of the His-tags on the N-terminus of the OMPs using HRP-labelled antibodies (**Fig. 3.4**). However, molecular weights observed for recombinant OMPs were higher than the theoretical values. The recombinant OMPs were then used for immunoblots as described in section 3.3.4, the results of which are shown in **Figures 3.5–3.8**.

Generally, strong signals corresponding to the expected molecular weight of the recombinant proteins generated were detected for all sera tested. Our results (**Figures 3.5–3.8**) show that all five recombinant OMPs and their variants were recognised by anti-*A. marginale* and *A. centrale* bovine (post-immune) sera from both USA and South Africa. Sera derived from cattle immunized with cross-linked or non-cross linked OMPs mostly resulted in multiple bands in *A. marginale* or *A. centrale* crude lysate lanes, whereas sera derived from *A. marginale* or *A. centrale*-infected cattle gave multiple bands with smearing in these lanes in the immunoblots.

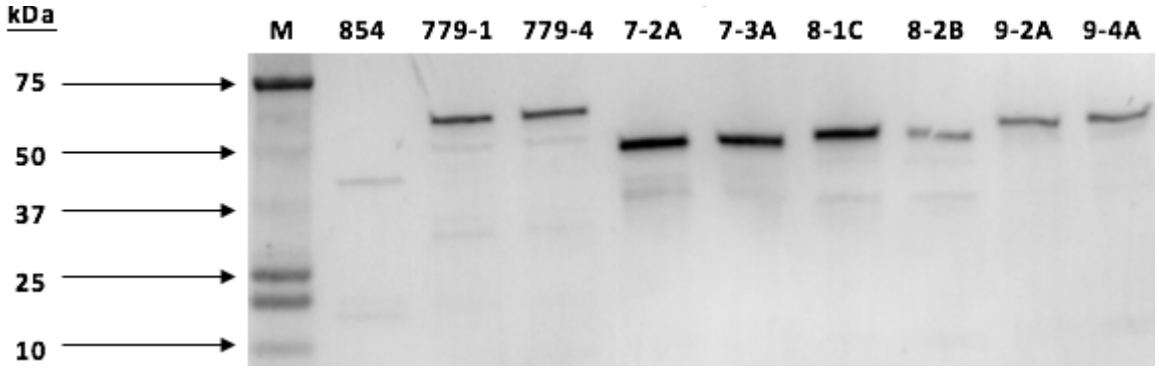

Figure 3.4: His-tagged colorimetric detection of nine recombinant OMPs using HRP-labelled His-detector Nickel^{2+} conjugate (KPL Scientific Inc, Gaithersburg, USA). M = Precision Plus Protein Kaleidoscope pre-stained size standard with size indicated in kiloDaltons (kDa) (BioRad, Hercules, USA), 854 = Am854, 779-1 = Am779 variant 1, 779-4 = Am779 variant 4, 7-2A = Omp7 variant 2A, 7-3A = Omp7 variant 3A, 8-1C = Omp8 variant 1C, 8-2B = Omp8 variant 2B, 9-2A = Omp9 variant 2A, 9-4A = Omp9 variant 4A.

Recognition of the *A. marginale* Proefplaas 1332 strain (AmS) infected blood crude lysate control by the test sera was high, as all of the anti-*A. marginale* and anti-*A. centrale* sera, from both South Africa and USA, detected multiple bands appearing as distinct bands and smears (**Figures 3.5** to **3.8**) from approximately 20 kDa to >75 kDa. These bands likely indicate that these sera contained antibodies directed against the immunodominant immunogens Msp2 and Msp3 (approximately 36 kDa and 74 kDa respectively). The *A. centrale* blood vaccine crude lysate control (AcV) was also recognised by all of the sera with strong multiple bands, most prominent at approximately 37 to >75 kDa, were observed in the AcV lane for all serum samples. No signal was detected for the *E. ruminantium* and SUMO recombinant proteins. All pooled pre-immune sera groups (**Figures 3.5D, 3.6D and 3.8D**) gave no signal for both the recombinant OMPs and the controls.

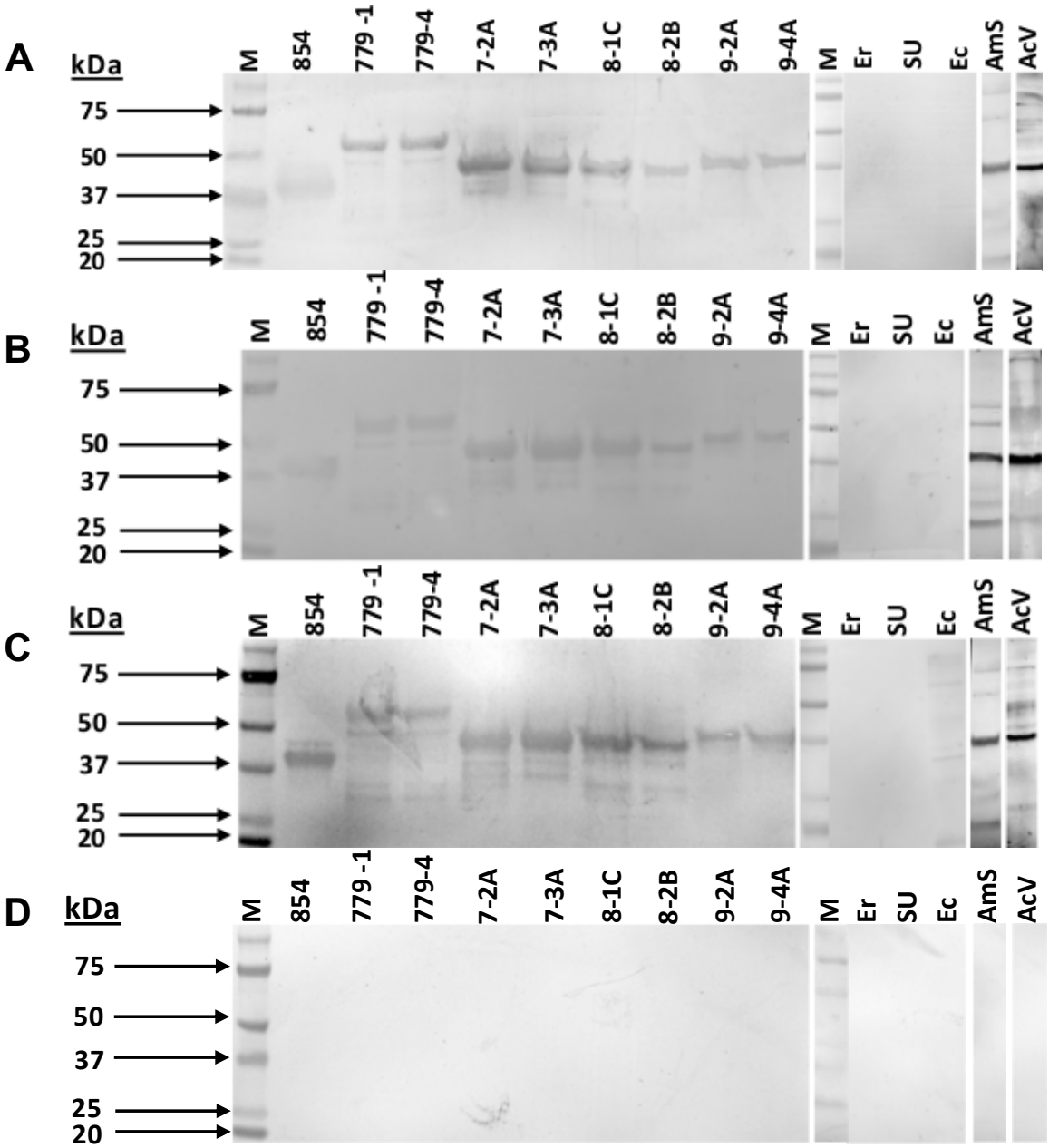

Figure 3.5: Immunoblots done using sera derived from *A. marginale* (St. Maries strain) non-cross-linked OMP bovine vaccinates 43071 (A), 43092 (B) and 43100 (C) from USA, as well as pooled pre-immune sera from the same three animals (D). M = Precision Plus Protein Kaleidoscope pre-stained size standard with size indicated in kiloDaltons (kDa) (BioRad, Hercules, USA), 854 = Am854, 779-1 = Am779 variant 1, 779-4 = Am779 variant 4, 7-2A = Omp7 variant 2A, 7-3A = Omp7 variant 3A, 8-1C = Omp8 variant 1C, 8-2B = Omp8 variant 2B, 9-2A = Omp9 variant 2A, 9-4A = Omp9 variant 4A. AmS = *A. marginale* Proefplaas 1332 crude protein lysate, AcV = *A. centrale* blood vaccine (OBP, Pretoria, South Africa) crude protein lysate, Er = *E. ruminantium* recombinant outer membrane protein Erum1040, SU = recombinant SUMO protein, Ec = *E. coli* BL-21(DE3) expression strain crude protein lysate.

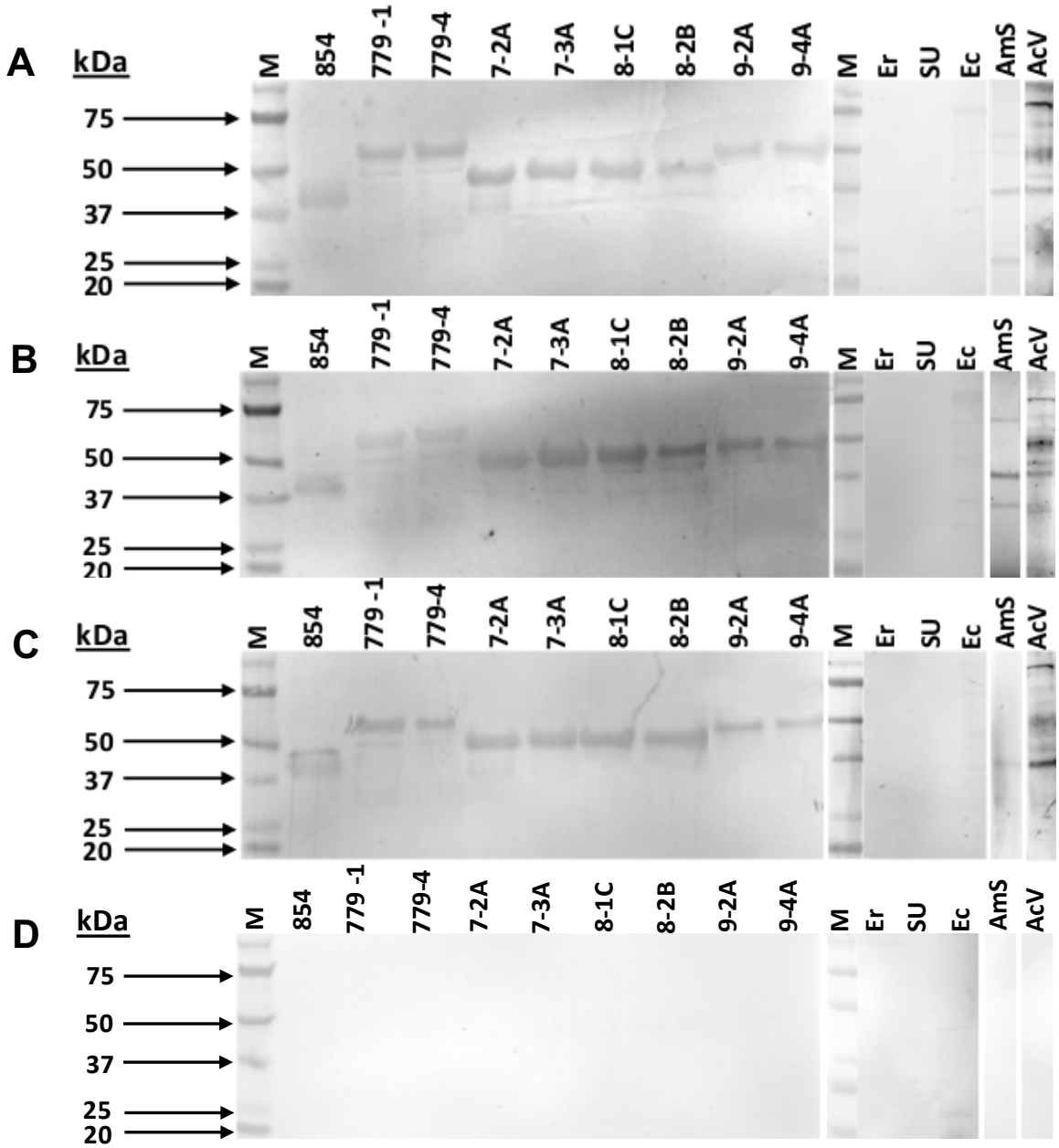

Figure 3.6: Immunoblots done using sera derived from *A. marginale* (St. Maries strain) cross-linked OMP bovine vaccinates C1252 (A), 35100 (B) and 35130 (C) from USA, as well as pre-immune sera pooled for the same three animals (D). M = Precision Plus Protein Kaleidoscope pre-stained size standard with size indicated in kiloDaltons (kDa) (BioRad, Hercules, USA), 854 = Am854, 779-1 = Am779 variant 1, 779-4 = Am779 variant 4, 7-2A = Omp7 variant 2A, 7-3A = Omp7 variant 3A, 8-1C = Omp8 variant 1C, 8-2B = Omp8 variant 2B, 9-2A = Omp9 variant 2A, 9-4A = Omp9 variant 4A. AmS = *A. marginale* Proefplaas 1332 crude protein lysate, AcV = *A. centrale* blood vaccine (OBP, Pretoria, South Africa) crude protein lysate, Er = *E. ruminantium* recombinant outer membrane protein Erum1040, SU = recombinant SUMO protein, Ec = *E. coli* BL-21(DE3) expression strain crude protein lysate.

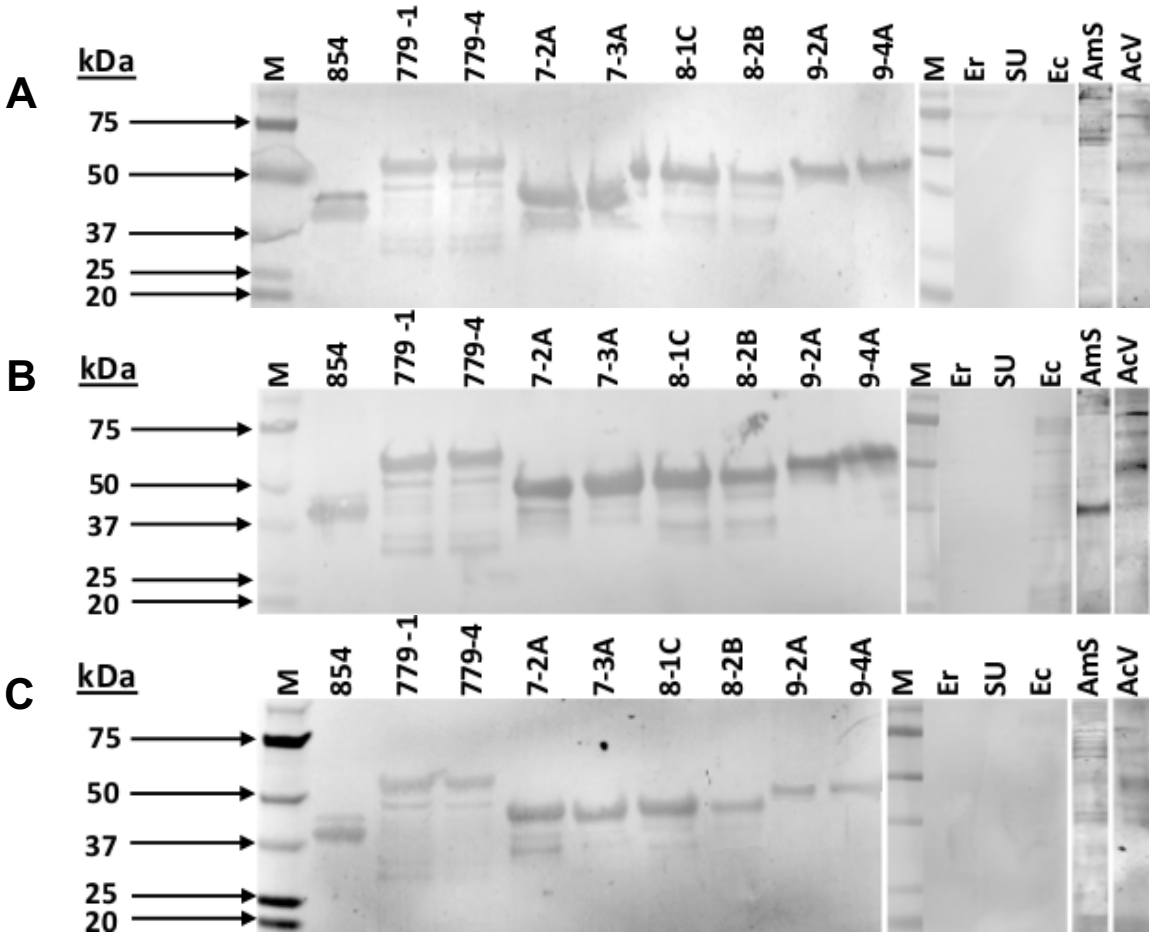

Figure 3.7: Immunoblots done using sera derived from three South African bovines naturally infected with *A. marginale*, 1303 (A), 1313 (B) and 1315 (C). Since these were field samples, no pre-immune sera were available for these three animals. M = Precision Plus Protein Kaleidoscope pre-stained size standard with size indicated in kiloDaltons (kDa) (BioRad, Hercules, USA), 854 = Am854, 779-1 = Am779 variant 1, 779-4 = Am779 variant 4, 7-2A = Omp7 variant 2A, 7-3A = Omp7 variant 3A, 8-1C = Omp8 variant 1C, 8-2B = Omp8 variant 2B, 9-2A = Omp9 variant 2A, 9-4A = Omp9 variant 4A. AmS = *A. marginale* Proefplaas 1332 crude protein lysate, AcV = *A. centrale* blood vaccine (OBP, Pretoria, South Africa) crude protein lysate, Er = *E. ruminantium* recombinant outer membrane protein Erum1040, SU = recombinant SUMO protein, Ec = *E. coli* BL-21(DE3) expression strain crude protein lysate.

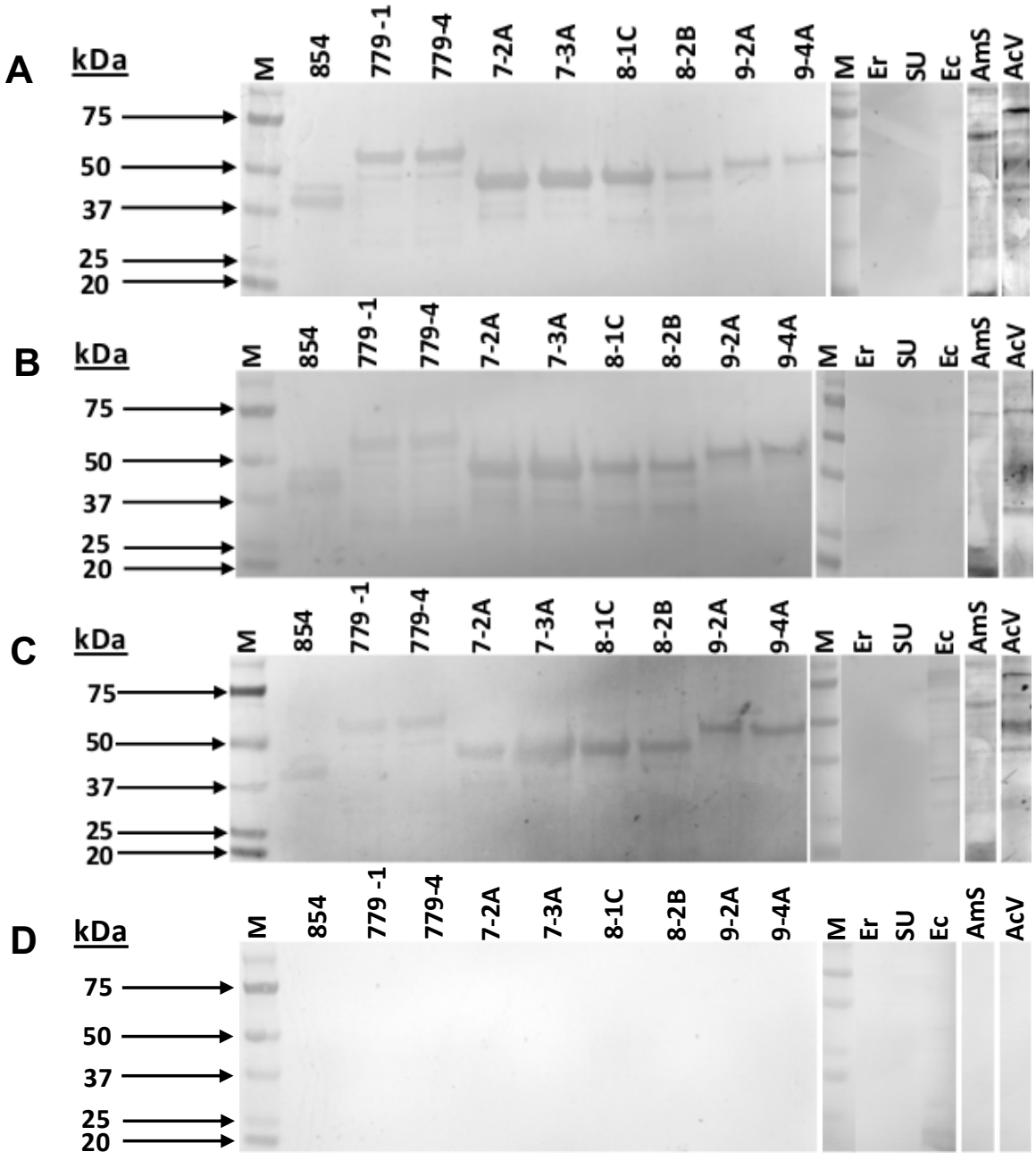

Figure 3.8: Immunoblots done using sera derived from three *A. centrale* (OBP, Pretoria, South Africa, *Anaplasma centrale* vaccine) vaccinates from South African bovines 2503 (A), 2505 (B), 2523 (C), as well as pre-immune sera pooled for the same three animals (D). M = Precision Plus Protein Kaleidoscope pre-stained size standard with size indicated in kiloDaltons (kDa) (BioRad, Hercules, USA), 854 = Am854, 779-1 = Am779 variant 1, 779-4 = Am779 variant 4, 7-2A = Omp7 variant 2A, 7-3A = Omp7 variant 3A, 8-1C = Omp8 variant 1C, 8-2B = Omp8 variant 2B, 9-2A = Omp9 variant 2A, 9-4A = Omp9 variant 4A. AmS = *A. marginale* Proefplaas 1332 crude protein lysate, AcV = *A. centrale* blood vaccine (OBP, Pretoria, South Africa) crude protein lysate, Er = *E. ruminantium* recombinant outer membrane protein Erum1040, SU = recombinant SUMO protein, Ec = *E. coli* BL-21(DE3) expression strain crude protein lysate.

3.5 Discussion

Determining the correct immunogens for inclusion in a recombinant vaccine cocktail that offers lasting and broad spectrum immune protection from infections by the highly genetically diverse *A. marginale* is challenging. In South Africa, high genetic diversity of *A. marginale* has been demonstrated for field strains (Mtshali et al., 2007; Mutshembele et al., 2014; Hove et al., 2018) and by extrapolation, high antigenic variation of immunogenically significant genes might be expected. Three of the five OMPs in this study revealed high numbers of genetic variants as expected from the high diversity in *msp1α* genotypes in the samples used to generate them.

The anomalies in molecular weights of recombinant OMPs observed in this study, which were higher than their theoretical molecular weights, may be due to a process known as 'gel shifting', attributable to several factors associated with SDS-PAGE of membrane proteins. These include post-translational modification of membrane proteins such as phosphorylation and glycosylation, binding of SDS molecules to the protein and partial unfolding of the protein after denaturing (Rath et al., 2009; Guan et al., 2015).

In this study, like that of Ducken et al. (2015), we show that the Am854 antigen is identical in sequence, and is recognised by sera from bovines immunised with both cross-linked and non-cross-linked *A. marginale* OMPs, and also by sera from *A. centrale* vaccinates, making it a promising vaccine candidate. However, work by Ducken et al. (2015) revealed that it will be necessary to overcome the obstacle of production of low levels of antibodies after vaccination with individual recombinant proteins, compared with much higher titres in OMP vaccinates, before this immunogen can be used in a vaccine cocktail. Antibody titres may be influenced by many complex factors such as choice of adjuvant and folding of antigens during recombinant protein vaccine formulation (Ducken et al. 2015).

Although we have shown that the subdominant surface antigen Am779 varies at three amino acid residues, it can still be regarded as highly conserved (Albarrak et al., 2012). Residue 67 for variants 1 to 3 of Am779, arginine (R), is a positively charged polar amino acid, which is substituted with histidine (H), a positive, polar amino acid, in variants 4 and 5 (**Table 3.3**). It is known that R substitutes well with other positively charged amino acids. However, H is a polar amino acid with unique chemical properties and does not substitute well with other

amino acids (Betts & Russell, 2007). This is a potential source of change in the overall three-dimensional (3D) structure of the protein, which in-turn may affect the structure and function of B- or T- cell epitopes that are essential in eliciting a strong immune response. For position 113, variants 1, 2, 4 and 5 possess a threonine (T) residue, which is substituted by an alanine (A) in variant 3. Alanine, a non-polar residue with low hydrophobicity and T, a polar amino acid, are relatively unreactive, small amino acids that tolerate change with other amino acids (Betts & Russell, 2007). Thus, it is expected that this substitution will not affect the overall 3D structure of the OMP. At position 442, variants 1, 3 and 4 have an A residue, whereas 2 and 5 have valine (V), which is a small and hydrophobic residue that prefers to be buried inside hydrophobic protein cores (Betts & Russell, 2007). As V and A are both small and hydrophobic, they substitute well with one another, with no expected changes in 3D structure. Overall, the 3 amino acid substitutions appeared to have minimal effects on the antigenicity of the OMP, since we were able to show that both major variants of Am779 were recognised by *A. marginale* and *A. centrale* antisera. This therefore supports the selection of Am779 as an immunogen for inclusion in a recombinant vaccine cocktail. This view is supported by Albarrak and co-workers, who showed that the Am779 antigen possesses, through targeted immunisation, the ability to prime the immune system; a trait which is highly desirable for a good vaccine candidate (Albarrak et al., 2012).

Despite the high levels of amino acid variation we found for the closely related antigens, Omp7, 8, and 9 (i.e. up to 29 genetic variants for the *omp8* gene), the two major variants we selected and expressed for each OMP were detected by all post-immune sera, suggesting that the variations we observed in these three OMPs may not be significant enough to alter the immunologically important regions in these antigens. High levels of conservation have previously been reported, in both *A. marginale* and *A. centrale,* of the T-cell epitope FLLVDDAI/VV for Omp7, 8 and 9, recently described by Deringer et al. (2017). In the present study, we also found this T-cell epitope to be highly conserved in all our sequences; it was found to be FLLVDDAIV for Omp7 and FLLVDDAVV for Omp8 and 9 (100% identity with St. Maries and Florida strains). These T-cell epitopes were located in the conserved regions, generally upstream of the hypervariable region (HVR) for *A. marginale* in Omp7, 8 and 9 in all of the amino acid sequences we generated. This is in contrast with the data reported by Abbott et al. (2004), who looked at another surface protein Msp2, and showed that T-cell epitopes are uniformly distributed between conserved regions and HVRs of the Msp2 antigen in *A. marginale*. Furthermore, we show high levels of conservation by the

amino acid sequence identity of between 79-100%, for these five OMPs in *A. marginale* strains from South Africa and the St. Maries and Florida strains from USA. The data we report in this study may suggest that these three OMPs are good vaccine candidates as they remain highly conserved in terms of T-cell epitopes and react with sera from geographically distant regions of the world, for both *A. marginale* and *A. centrale*.

Noh et al. (2008; 2013) previously showed that both non-cross-linked and cross-linked immunogens resulted in generation of high antibody titres. Thus, the strong binding of sera from *A. marginale* cross-linked and non-cross-linked OMP vaccinates to both *A. marginale* crude lysate and recombinant proteins was expected [3/3 (100%) of the sera from cross-linked OMP vaccinates and 3/3 (100%) of the sera from non-cross-linked OMP vaccinates gave strong signals]. Noh et al. (2008; 2013) found that cross-linking OMPs enhanced immunisation by inducing higher levels of immunoglobulins in bovines, compared to non-cross-linked antigens. This difference was, however, not apparent in our data. This may be attributable to methodological differences in performing the immunoblots between our work and that of Noh et al. (2008; 2013), including but not limited to, choice of immunoblotting membrane (PVDF vs Nitrocellulose) and signal detection (colometric - TMB vs chemiluminescence - ECL).

Bovine serum samples obtained from *A. marginale* carrier cattle from Proefplaas, South Africa, were expected to have low antibody titres, due to a significant drop in antibody titres during persistent infection (Palmer et al., 1999). Nonetheless, these sera bound strongly to proteins in both *A. marginale* crude lysate and the recombinant OMPs (both of South African origin), indicating that low antibody titres in these sera did not adversely affect the performance of the Western blot.

Sera from *A. centrale* vaccinates were shown to strongly bind all recombinant OMPs and like all the sera from the *A. marginale* vaccinates (South Africa and USA), also gave signal for *A. centrale* crude lysate. These data could suggest that the OMPs may be important in the protective immune response, given that antigens shared between the two organisms result in the cross-protection afforded by the *A. centrale* vaccine against infections by field strains of *A. marginale*. Interestingly, *A. marginale omp7, 8* and *9* have close relatives in *A. centrale*, although these are collapsed into one coding DNA sequence in *A. centrale* (Herndon et al., 2010). *A. marginale* Am854 and Am779 have sequence identity of between 81 and 84% with

A. centrale sequences (Herndon et al. 2010). These data emphasize the strong antigenic relationship between *A. marginale* and *A. centrale*, a finding that was exploited by Theiler to develop the *A. centrale* live vaccine still used with little modification to this day (Theiler, 1912; Potgieter, 1979; Palmer, 2009). However, the potential risks associated with vaccination using the *A. centrale* live blood vaccine have led to some countries not using the vaccine. Our results provide evidence to support the continued use of this vaccine in countries that allow its distribution.

The detection of *A. marginale* recombinant immunogens by both anti-*A. centrale* and anti-*A. marginale* sera from USA and South Africa, point to significant and complex immunological and antigenic relationships between these two pathogens and may therefore be preliminary evidence in support of the inclusion of the recombinant OMPs Am779, Am854, Omp7, Omp8 and Omp9, in a global rather than region-specific recombinant vaccine against anaplasmosis.

3.6 Conclusions

The data generated in this study, supported by previous studies, reveal that the *A. marginale* OMPs Am779, Am854, Omp7, Omp8 and Omp9 are good vaccine candidates for inclusion in vaccines against the rickettsia, *A. marginale*. Additionally, data presented in this study reveal sequence conservation and antigenic similarities between South African and USA strains of *A. marginale,* making a case for a global recombinant vaccine against bovine anaplasmosis. However, further work into vaccine composition and efficient delivery mechanisms still needs to be explored before recombinant proteins become viable as working vaccines.

3.7 References

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CHAPTER 4

Tick cell culture and attempts to initiate *Anaplasma marginale in vitro* cultures from bovine blood samples

4.1 Abstract

Tick cell cultures are an essential tool for the study of tick-borne pathogens, due to the ability of tick cell lines to harbor and propagate these obligate intracellular pathogens. In this study, we report the culture of ISE6 and IDE8 tick cell lines, to generate stocks for infection with field samples of *Anaplasma marginale*. Infection of the ISE6 and IDE8 tick cell lines with *A. marginale* positive blood inocula prepared from 17 bovine EDTA blood samples (from persistently infected cattle as well as from clinical cases), was attempted over 160 days, with infection progress monitored by duplex quantitative real-time PCR (qPCR) starting from 60 days post inoculation. Blood samples used to infect tick cell lines were screened for *A. marginale* infection using light microscopic examination of Giemsa-stained thin blood smears and duplex qPCR. All blood samples were found to be qPCR positive for *A. marginale* and negative for *Anaplasma centrale*. These blood samples were shown to have a 1 to 2% *A. marginale* rickettsaemia by examination of Giemsa-stained thin blood smears. However, the ISE6 and IDE8 cell lines were found to be qPCR negative for *A. marginale* infection 150 days post-infection and did not exhibit any signs of infection. This was probably attributable to the low infection rate of the blood samples and the resultant lowered number of viable rickettsiae in the inocula used in the infection trials. In order to increase the probability of success, *A. marginale* cultures have been initiated in ISE6 and IDE8 tick cells using inocula with high rickettsaemia (25 and 33%), derived from two splenectomised bovine calves which were artificially infected with *A. marginale* positive bovine blood. Preliminary results, after 60 days of culture suggest that the cultures are positive for *A. marginale*. Cultures were qPCR positive, with Ct values of approximately 23, with a few, small colonies observed. The progress of infection in these cultures will continue to be monitored as these results are still inconclusive.

4.2 Introduction

In vitro cultures of cells originating from tick tissues have been available for many years now (Munderloh et al., 1994) and have been invaluable in the study of many pathogens of global economic importance (Bell-Sakyi et al., 2007). They have been used for various purposes and in numerous studies, including but not limited to: viral and bacterial tick-borne pathogen isolation and characterization (Munderloh et al., 1996; Bell-Sakyi, 2004; 2012; 2015; Passos, 2012; Baêta et al., 2015), acaricide resistance studies (Cossio-Bayugar et al., 2002), and genomic and proteomic studies of ticks (Barbet et al., 1999; Bell-Sakyi et al., 2007). They have also helped to elucidate complex host-vector-pathogen relationships, pathogen biology and have given insights into disease and pathogen management, which is particularly true in the case of tick-borne diseases (Bell-Sakyi et al., 2007; Bastos et al., 2009; Esteves et al., 2009; Passos, 2012).

Most cell lines of tick origin are derived from embryonic cells and their use offers several advantages (Bell-Sakyi et al., 2007; Passos, 2012). Firstly, the use of tick cell culture systems reduces or even eliminates the need for live animals for vaccine or immunogen generation (Kocan et al., 2003; Marcelino et al., 2012), and makes these processes considerably cheaper compared to using live animals (Blouin et al., 2000). Additionally, tick cell lines are favoured for their potential to generate biobanks of pathogen field strains and their antigens for immunological studies, due to their relatively rapid growth in conditions that are easily reproducible using standardized media and conditions (Munderloh & Kurtti, 1989; Munderloh et al., 1994; Blouin et al., 2000; Bell-Sakyi et al., 2007). Furthermore, because of the growth rates of tick cells in culture, they require less regular subculture and attention (Blouin et al., 2000; Bell-Sakyi et al., 2007; Passos, 2012). Some tick cell lines have also been reported to be viable after 12 years of liquid nitrogen cryopreservation (Munderloh et al., 1994). However, short term storage in refrigeration between 4 and 12°C is preferred, as tick cells can be stored viably for up to one month and are easy to resuscitate under these conditions (Bell-Sakyi et al., 2007; Lallinger et al., 2010).

To date, a total of 56 tick cell lines have been developed from 16 tick species (14 ixodid and 2 argasid) for culture of tick-borne pathogens (Bell-Sakyi et al., 2007; Passos, 2012). Cell lines have been demonstrated to be more easily generated from some tick genera, compared to others. *Rhipicephalus* and *Ixodes* have yielded the most cell lines to date with more than 30

reported (more than 50% of all cell lines), compared to other genera such as *Dermacentor*, which has the least of any that have been established in culture with six described cell lines (11% of all cell lines) (Munderloh et al., 1994; Bell-Sakyi et al., 2007; Passos, 2012). An important advancement in tick cell culture has been the formulation of standardized growth supplements, which has facilitated the production of more consistent batches of media (Munderloh & Kurtti, 1989; Munderloh et al., 1994).

There are several reports which document the successful isolation and propagation of *A. marginale* strains and other *Anaplasma* spp. in tick cell lines (Munderloh et al., 1996; Blouin et al., 2000; Zweygarth et al., 2006; Bastos et al., 2009; Esteves et al., 2009; Silaghi et al., 2011; Passos, 2012; Baêta et al., 2015). This therefore formed the basis of this study, where the main objective was to isolate and maintain *in vitro*, field strains of *A. marginale* in ISE6 or IDE8 tick cell lines.

4.3 Materials and methods

4.3.1 Tick cell lines and culture media

Cell lines from *Ixodes scapularis* (southern) embryos isolated on day 6 after onset of oviposition (ISE6) and *I. scapularis* (deer-collected) embryos isolated on day 8 after onset of oviposition (IDE8), developed by Munderloh et al. (1994), were supplied by Dr. Lesley Bell-Sakyi from the Tick Cell Biobank at the Pirbright Institute, United Kingdom. Uninfected IDE8 and ISE6 cell lines were propagated in 5 ml of Leibovitz's L-15B and L-15B 300 liquid medium, respectively [supplemented with 5% heat-inactivated foetal bovine serum (FBS), 10% tryptose phosphate broth (TPB) and 0.1% bovine lipoprotein concentrate, with pH adjusted to 7.15–7.20 using NaOH], (Munderloh & Kurtti, 1989; Munderloh et al., 1994), using TPP® 25 cm^2 culture flasks (Sigma-Aldrich, Buchs, Switzerland), and incubated at 32°C. Medium was changed at bi-weekly intervals by removing 3 ml of spent medium and replenishing it with 3 ml of fresh medium. The cells were checked using a light microscope and once confluent (after 8–12 weeks), they were passaged and split at a 1:5–1:10 ratio in new cell culture flasks. Once cells were infected, they were grown in L15B (IDE8 cell lines) or L15B 300 (ISE6 cell lines) medium supplemented with $NaHCO_3$ (0.1%) and HEPES (10 mM), with pH adjusted to 7.4–7.6 using NaOH (Munderloh et al., 1996).

4.3.2 Blood samples

Blood samples were collected in Vacuette EDTA tubes (Greiner Bio-One, Kremsmünster, Austria), from the coccygeal vein of cattle that were at least one-year old. This was done in accordance with the University of Pretoria's animal ethics code.

4.3.2.1 Collection of blood samples from carrier cattle and clinical cases

Blood samples were collected from animals that were:

i. carrier state Fresian cattle at the University of Pretoria's Experimental farm (Proefplaas), which were previously tested for the presence of *A. marginale* and *A. centrale* using the duplex qPCR assay (**Table 4.1**) or,

ii. exhibiting fever [a general prophylactic treatment with intramuscular injections of Oxytetracycline (20 mg/kg body mass) and Diminazine (3.5 mg/kg body mass) against major endemic tick-borne diseases (anaplasmosis and babesiosis, respectively) had been given to the cattle initially but the animals subsequently developed fever and anaemia], which were supplied by Dr. Jannie Crafford (Department of Veterinary Tropical Diseases, University of Pretoria) (**Table 4.1**). The blood samples from these animals were also tested for *A. marginale* infection using the duplex qPCR.

4.3.2.2 Infection of two splenectomised calves with *A. marginale*-positive blood

Two splenectomised Hereford bovine calves, determined to be negative for *A. marginale* and *A. centrale* by qPCR, with identity numbers 9672/5 and 9678/2 were infected intravenously via the jugular vein, with approximately 200 ml of *A. marginale* positive blood containing 20% (v/v) acid citrate dextrose (ACD) anticoagulant, derived from the two Proefplaas animals, 1313 and 1332, respectively. Any signs of anaphylactic shock in the calves were treated by injection of 2 ml adrenalin. Samples 1313 and 1332 were determined to be *A. marginale* positive using qPCR and also screened for other pathogens by reverse line blot hybridisation (RLB). *Msp1α* genotyping was done as described previously (**section 2.3.5, Chapter 2**) to establish the number of *A. marginale* strains infecting each Proefplaas bovine. The infection of the splenectomised calves was carried out by a qualified animal health technician and veterinarian at the Agricultural Research Council – Onderstepoort Veterinary Research Institute (ARC-OVR). The calves were housed in insect-free facilities at the ARC-OVR where their clinical parameters such as packed cell volume (PCV), percentage

parasitized erythrocytes (PPE) and body temperature were monitored and recorded daily. The prepatent period for *A. marginale* infection in the calves was determined.

4.3.2.3 Screening of blood samples for haemoparasite infections

All blood samples were screened for *A. marginale* infection by light microscopy examination of Giemsa-stained, thin blood smears. Thin blood smears were fixed in methanol and subsequently Giemsa-stained according to standard protocols (Donovan-Myhand et al., 1984). Microscope slides were examined and analysed using an Olympus BX50 light microscope, and the analySIS software version 3.2 (Olympus Corporation, Tokyo, Japan).

Using methodology described previously in chapter 2, the blood samples were tested using a duplex qPCR assay (Carelli et al., 2007; Decaro et al., 2008) (targeting the *msp1β* gene of *A. marginale* and the *groEL* gene of *A. centrale)*, with minor modifications for the LightCycler real-time machine (Roche Diagnostics, Mannheim, Germany) as described by Chaisi et al. (2017). Genomic DNA extracted from field samples C83 and 9410 were used as *A. centrale* positive controls [*A. centrale* infection status confirmed by amplification and sequence analysis of *groEL, msp2* and 16S rRNA genes (Chaisi et al., 2017)]. Field samples C14, C83 or 208291 (obtained from cattle in the Mnisi Community area, Mpumalanga province and Dr. Dirk Verwoerd, Karan Beef) were used as positive controls for *A. marginale* (*A. marginale* infection status confirmed by amplification and sequence analysis of the *msp1β* gene), and molecular grade water as a negative control. The data were analysed using LightCycler software version 4.0 (Roche Diagnostics, Mannheim, Germany). For detection of *A. marginale* and *A. centrale* in the test samples, the LightCycler instrument was used to measure the fluorescence emission signal from probe hydrolysis; the *A. marginale* probe at 530 nm (FAM dye) and the *A. centrale* probe at 610 nm (LC-610 dye) wavelengths.

Genomic DNA extracted from the Proefplaas blood samples 1230, 1305, 1313, 1332 and 13121 was also tested for other haemoparasites using the RLB assay, which is based on simultaneous PCR amplification of the 18S or 16S rRNA gene of related species, followed by hybridization to species-specific probes (Gubbels et al., 1999; Bekker et al., 2002). In short, *Theileria* and *Babesia* species-specific primers, RLB-F2 and R2 (Nijhof et al., 2003) along with *Ehrlichia* and *Anaplasma* species-specific primers, E HR-F and R (Bekker et al., 2002) were used to PCR amplify, respectively, the V4 or V1 hypervariable regions of the small subunit rRNA genes of these pathogens. Resulting PCR products were analysed by RLB

hybridisation, using genus and species-specific probes covalently attached to a biodyne C membrane, thus detecting the above-mentioned pathogens to genus and/or species level (Gubbels et al., 1999). This was done to rule out the possibility of using inocula that were heavily infected with other haemoparasites such as *Theileria* spp. and *Babesia* spp. Where possible, samples that were positive for *A. marginale* only were used for *A. marginale* culture initiation.

Table 4.1: Origin of blood samples tested for infection trials.

Origin	Sample identity	Number of samples	Type of infection
University of Pretoria (UP), Experimental farm (Proefplaas), Hillcrest	1230, 1305, 1313, 1332, 13121	5	Carrier
University of Pretoria (UP), Department of Veterinary Tropical Diseases, Onderstepoort	97, 123, 124, 128, 134, 138, 139, 145, 148, 150, 153, 154, 165	13	Clinical case
Agricultural Research Council-Onderstepoort Veterinary Research Institute (ARC-OVR)	9672/5, infected with 200 ml blood from 1313 9678/2, infected with 200 ml blood from 1332	2	Experimental infection
Total		**20**	

4.3.2.4 Preparation of blood samples for infection of tick cells

For storage of *A. marginale*-infected blood samples prior to infection of tick cell lines, EDTA blood samples were centrifuged at 700 x g for 10 min and washed three times in 1X phosphate buffered saline (PBS) without calcium and magnesium (Sigma-Aldrich, Buchs, Switzerland), to remove the white blood cells. Washed blood samples were aliquoted into 1.5 ml volumes, with DMSO (Sigma-Aldrich, Buchs, Switzerland) added to a final concentration of 10%, then frozen at -80°C for 48 hours.

4.3.3 Infection of tick cell monolayers

Washed *A. marginale*-infected blood samples were thawed in a 37°C water bath and centrifuged at 9 000 x g for 10 min to generate a pellet, which was used as inoculum for infection of ISE6 and IDE8 tick cell lines in TPP® 25 cm^2 culture flasks (Sigma-Aldrich,

Buchs, Switzerland). This was carried out using a protocol adapted from Baêta et al. (2015). Cultures were grown at 32°C in medium for infected cultures (**section 4.3.1**), with an initial change of medium 24 hours after initiation of infection, followed by removal and replacement twice a week of 3 ml of medium as described for the uninfected cells (**section 4.3.1**). The latter was carried out in order to remove carry over DMSO. The cultures were checked for infection by qPCR and light microscopy using the FLoid Cell Imaging Station (Life Technologies, California, USA), 60 days post-infection and weekly to fortnightly thereafter, up to 150 days post-infection. Infected cell cultures were split as was described for uninfected cells in **section 4.3.1** when a few of the cells were observed to be detaching and floating or at about 8–12 weeks.

4.3.4 Testing of infected cultures using the duplex quantitative real-time PCR (qPCR)

To check the infection status of the tick cells using qPCR, they were suspended in the 5 ml of growth media by mechanically agitating the cell culture flasks. A 0.5 ml volume of cell suspension was aliquoted and centrifuged at 300 x g for 10 min, to remove the medium. Genomic DNA was then extracted from the cell pellet using the QIAamp DNA Mini Kit (cultured cells protocol) (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Genomic DNA was eluted in 100 µl elution buffer and stored at -20°C. Genomic DNA from the tick cell cultures was tested using the previously described duplex qPCR assay.

4.4 Results

Blood from carrier cattle was used in an initial attempt to initiate *A. marginale* cultures. Subsequently, blood samples from cattle showing clinical symptoms of bovine anaplasmosis were used. Finally, splenectomised calves were infected with blood from *A. marginale* positive carrier cattle and blood was taken at peak rickettsaemia for initiation of cultures. A summary of all the attempts to initiate *A. marginale* cell cultures during this study is shown in the flow diagram in **Fig. 4.1**.

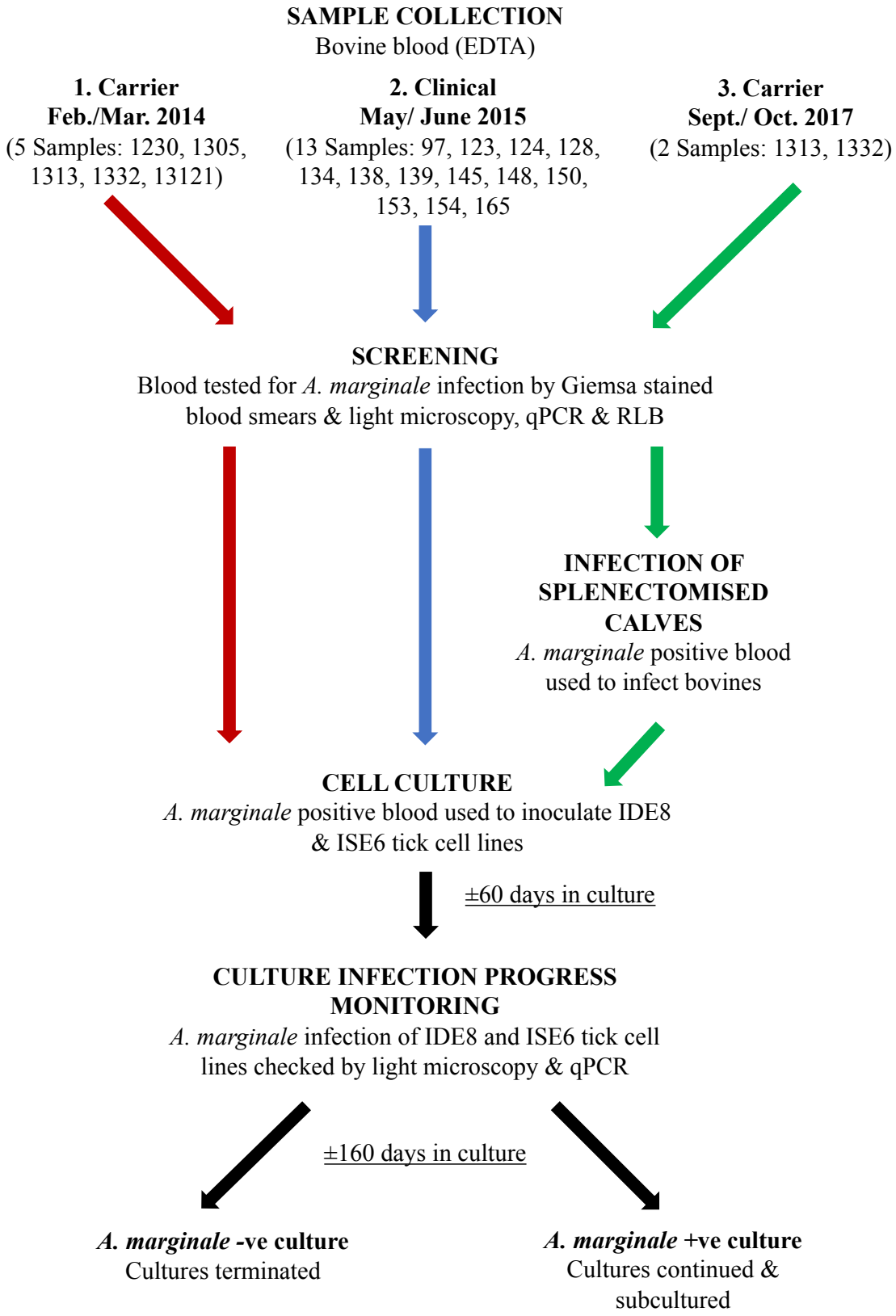

Figure 4.1: Schematic diagram of methodology used for initiation of *A. marginale* cultures in ISE6 and IDE8 tick cell lines from bovine blood samples. +ve = positive, -ve = negative, ± = approximately.

4.4.1 Screening of blood samples from carrier cattle and clinical cases

In all samples, *A. marginale* organisms were observed microscopically except in sample 1230. Sample 145 had the highest density of *A. marginale* inclusion bodies observed by light microscopy. **Figure 4.2** shows Giemsa-stained blood smear samples 97, 145, 148 and 154 used to attempt initiation of *A. marginale* cell cultures. Deep purple stained 'marginal points' (Theiler, 1910) or *A. marginale* colonies were located mostly on the margins of red blood cells, and are characteristic of *A. marginale*-infected bovine erythrocytes. By counting the number of infected erythrocytes occurring in 50 microscopic fields, at 1 000 x magnification, most samples were determined to be at a rickettsaemia of 1–2%.

Results from the qPCR analysis of the samples used as culture inoculum or to infect splenectomised calves are shown in **Table 4.2** and **Fig. 4.3**. All qPCR controls performed as expected. Crossing point values for the genomic DNA extracts from the blood samples that were to be used for initiation of *A. marginale* cell cultures ranged from 20.36 to 26.70. Samples 145 and 1230 were found to have the lowest and highest Cp values (20.36 and 26.25) respectively, and this was in correlation with the light microscopy results.

All the carrier animals from Proefplaas as well as the clinical cases from DVTD were found to be *A. marginale* positive by qPCR. The Proefplaas samples were also tested by RLB, and gave a positive signal for the *A. marginale*-specific probe and *Ehrlichia*/*Anaplasma* genus-specific probes (**Table 4.2**). Though genomic DNA from samples 1305 and 13121 was shown be *A. marginale*-positive by qPCR, these samples were not selected for cell culture because the RLB assay showed that they were also infected with *Babesia bigemina*. Samples 1230, 1313 and 1332 were used in attempts to initiate *A. marginale* cell cultures from carrier animals, and samples 1313 and 1332 were chosen to infect splenectomised calves. RLB showed that samples 1230 and 1313 contained *A. marginale* only. Even though *T. buffeli* was detected in sample 1332, it is unlikely to grow in ISE6 and IDE8 tick cells using culture conditions for *A. marginale.*

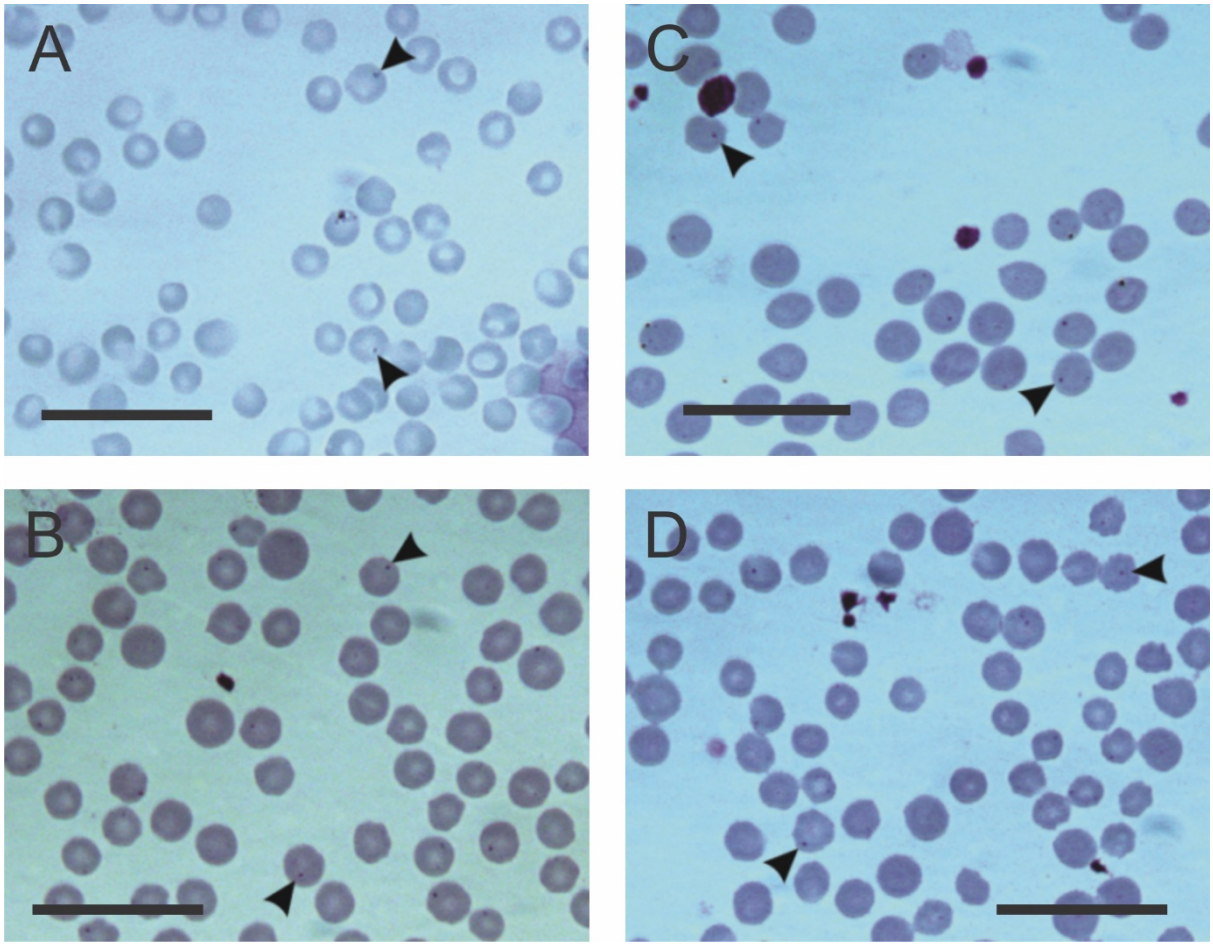

Figure 4.2: **Giemsa-stained thin blood smears of bovine blood samples** 97 (A), 145 (B), 148 (C) and 154 (D) showing *A. marginale* infected erythrocytes at 1 000 x magnification. The horizontal bar in the bottom corner represents 20 µm. Arrow-heads indicate colonies of *A. marginale*, which are located on the periphery of erythrocytes.

Table 4.2: Duplex qPCR and RLB results for bovine blood samples from carrier cattle and clinical cases tested for *A. marginale* and *A. centrale* infection.

Location and Sample Number	qPCR result		RLB result
	A. marginale **Cp value[#]**	***A. centrale*** **Cp value[#]**	
UP, Proefplaas, Hillcrest			
1230	+(26.25)	–	*Ehrlichia*/*Anaplasma* catch-all, *A. marginale*
1305	+(21.88)	–	*Ehrlichia*/*Anaplasma* catch-all, *Theileria*/ *Babesia* catch-all, *A. marginale, Babesia bigemina*
1313	+(25.47)	–	*Ehrlichia*/*Anaplasma* catch-all, *A. marginale*
1332	+(24.69)	–	*Ehrlichia*/*Anaplasma* catch-all, *Theileria*/ *Babesia* catch-all, *A. marginale, Theileria buffeli*
13121	+(18.61)	–	*Ehrlichia*/*Anaplasma* catch-all, *Theileria*/ *Babesia* catch-all, *A. marginale, Babesia bigemina*
UP, DVTD[a], Onderstepoort			
97	+(24.80)	–	NT[b]
123	+(23.42)	–	NT
124	+(23.34)	–	NT
128	+(25.58)	–	NT
134	+(23.57)	–	NT
138	+(25.25)	–	NT
139	+(23.79)	–	NT
145	+(20.36)	–	NT
148	+(26.59)	–	NT
150	+(26.70)	–	NT
153	+(25.56)	–	NT
154	+(22.71)	–	NT
165	+(22.59)	–	NT

+ denotes a positive sample; – denotes a negative sample.

[#] The Cp value given in the bracket (also known as the Cq value, *i.e.* quantification cycle) is the crossing point value given by the Lightcycler instrument, and this is the cycle at which fluorescence from amplification exceeds the background fluorescence.)

[a]UP, DVTD = University of Pretoria, Department of Veterinary Tropical Diseases; [b]NT = Not tested.

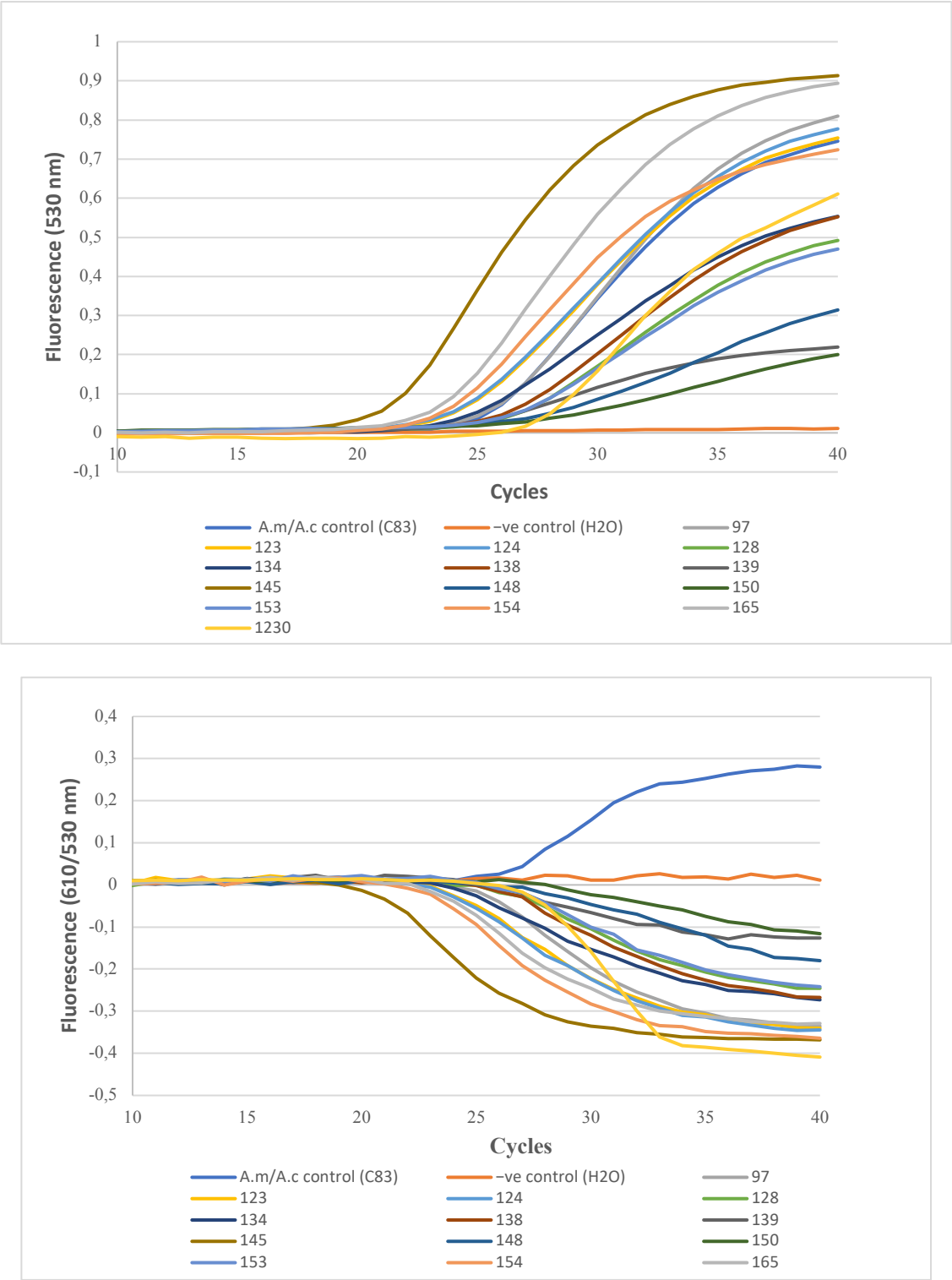

Figure 4.3: Duplex qPCR assay results for genomic DNA extracts from blood samples used to initiate *in vitro* cultures in ISE6 and IDE8 cells. All samples were positive for *A. marginale* (**A**), and negative for *A. centrale* (**B**). The fluorescence signal measured at 530 nm corresponded to the FAM dye (*A. marginale*), and at 610 nm to the LC-610 dye (*A. centrale*). A.m = *A. marginale*, A.c = *A. centrale.*

4.4.2 Infection of splenectomised calves

Using a qPCR standard curve (data not shown), sample 1313 (infected with *A. marginale* only) that was used to infect splenectomised calf 9672/5, was estimated to have a rickettsaemia of approximately 3 x 10^6 infected erythrocytes per ml of blood. Sample 1332 (infected with *A. marginale* and *T. buffeli*) used to infect splenectomised calf 9678/2 was estimated to have a rickettsaemia of approximately 6 x 10^6 infected erythrocytes per ml. The prepatent period post-infection with *A. marginale* positive blood, was nine days for both animals. The PPE reached 25% for 9672/5 and 36% for 9678/2, 17 days post-infection and blood stabilates and cell culture inocula were prepared. A summary of the post-infection clinical parameters of animals 9672/5 and 9678/2 (PPE, PCV and body temperature) for selected days, is given in **Table 4.3**.

Table 4.3: Summary of clinical parameters from days 9 and 12–17 for two splenectomised animals, 9672/5 and 9678/2 experimentally infected with *A. marginale* positive blood from Proefplaas animals 1313 and 1332, respectively.

Animal number	Clinical parameters	Days post-infection						
		9	**12**	**13**	**14**	**15**	**16**	**17**
9672/5	T (°C)	38.8	38.5	39.0	38.9	38.9	39.1	38.9
	PCV	30	30	29	29	28	28	27
	PPE	± 1/1 000	± 2/500	± 2/500	± 2%	8%	15%	25%
9678/2	T (°C)	38.8	39.1	39.1	39.1	39.4	40.6	39.4
	PCV	30	28	28	29	27	26	25
	PPE	± 1/500	± 1%	± 2%	± 9%	18%	33%	± 36%

T (°C) = body temperature measured in degrees Celsius; PCV = packed cell volume; PPE = percentage parasitised erythrocytes

The carrier cattle and inocula prepared from infected splenectomised calves to be used for culture initiation were found to be singly infected with *A. marginale* genotypes; 1313 and 9672/5 by a strain with *msp1α* genotype 27 4 4 37, and 1332 and 9678/2 by a strain with *msp1α* genotype 3 37. These genotypes will be compared with the *A. marginale* genotypes that will be established in culture.

4.4.3 Uninfected ISE6 and IDE8 tick cell culture

Uninfected ISE6 and IDE8 tick cell lines were successfully propagated *in vitro* using standard culture conditions (**Fig. 4.4**).

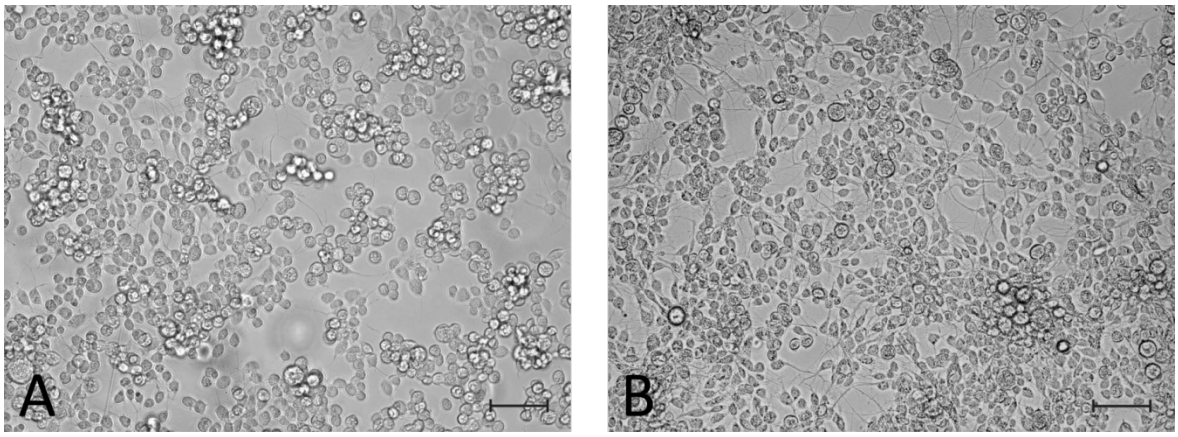

Figure 4.4: **Uninfected IDE8 (A) and ISE6 (B) tick cell lines** as observed by light microscopy. The horizontal bar represents 100 μm.

The IDE8 cells exhibited morphology that deviated noticeably from the expected **(Fig. 4.4A)**, as they showed slightly spherical cell shapes (which may be indicative of slightly unhealthy tick cells); although some had tapered ends, with appendage-like extensions on the terminal ends of the cells, which is observed in healthy cells. On the other hand, ISE6 cells were characterized by cell morphology observed in literature for healthy cells, that is, they were tapered at both ends with many appendage-like structures extending outwards from their terminal ends **(Fig. 4.4B).** The ISE6 cell line was also observed to have a higher growth rate, and consequently slightly higher cell densities, than the IDE8 cell line. In general, high growth densities and slow growth rates over time were observed for both cell lines.

4.4.4 Infection of ISE6 and IDE8 tick cell cultures with *A. marginale*-positive inocula

For tick cell lines infected with inocula prepared from carrier cattle and clinical cases, there was no evidence to indicate that the cells were infected with *A. marginale* (such as the formation of inclusion bodies indicating infection) over an 8–12-week growth period in culture, although there was a notable increase in density of both tick cell lines (**Fig. 4.6**).

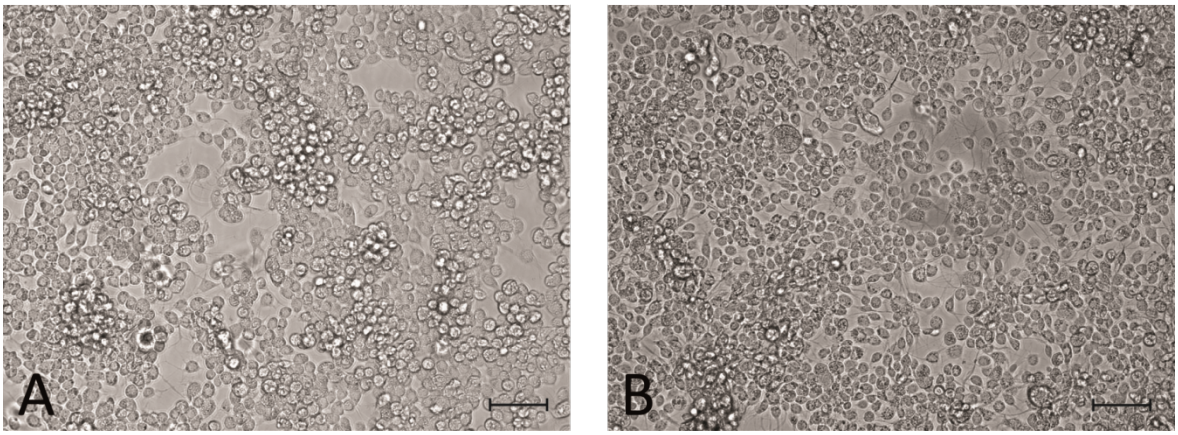

Figure 4.5: IDE8 (A) and ISE6 (B) tick cell lines in which infections were attempted, after more than 150 days post-infection as observed by light microscopy. The horizontal bar at bottom right corner represents 100 μm.

Genomic DNA extracts from the ISE6 and IDE8 cells were negative by qPCR for both *A. marginale* and *A. centrale* (**Fig. 4.6**). Only the two positive controls showed amplification. An amplification curve above the minimum threshold (background signal) was not generated from any of the cultures, *i.e.* none of the cultures were found to be positive.

Due to time constraints, it is unlikely that the final results of the attempted initiation of *A. marginale* cultures in tick cells using inocula from splenectomised cattle will be ready for reporting in this thesis although preliminary results look promising. A few small colonies have been observed at 60 days in culture and qPCR results suggest that the cultures are positive for *A. marginale* (see **Table 4.4**). We will continue to monitor these cultures and the results will be reported separately at a later date.

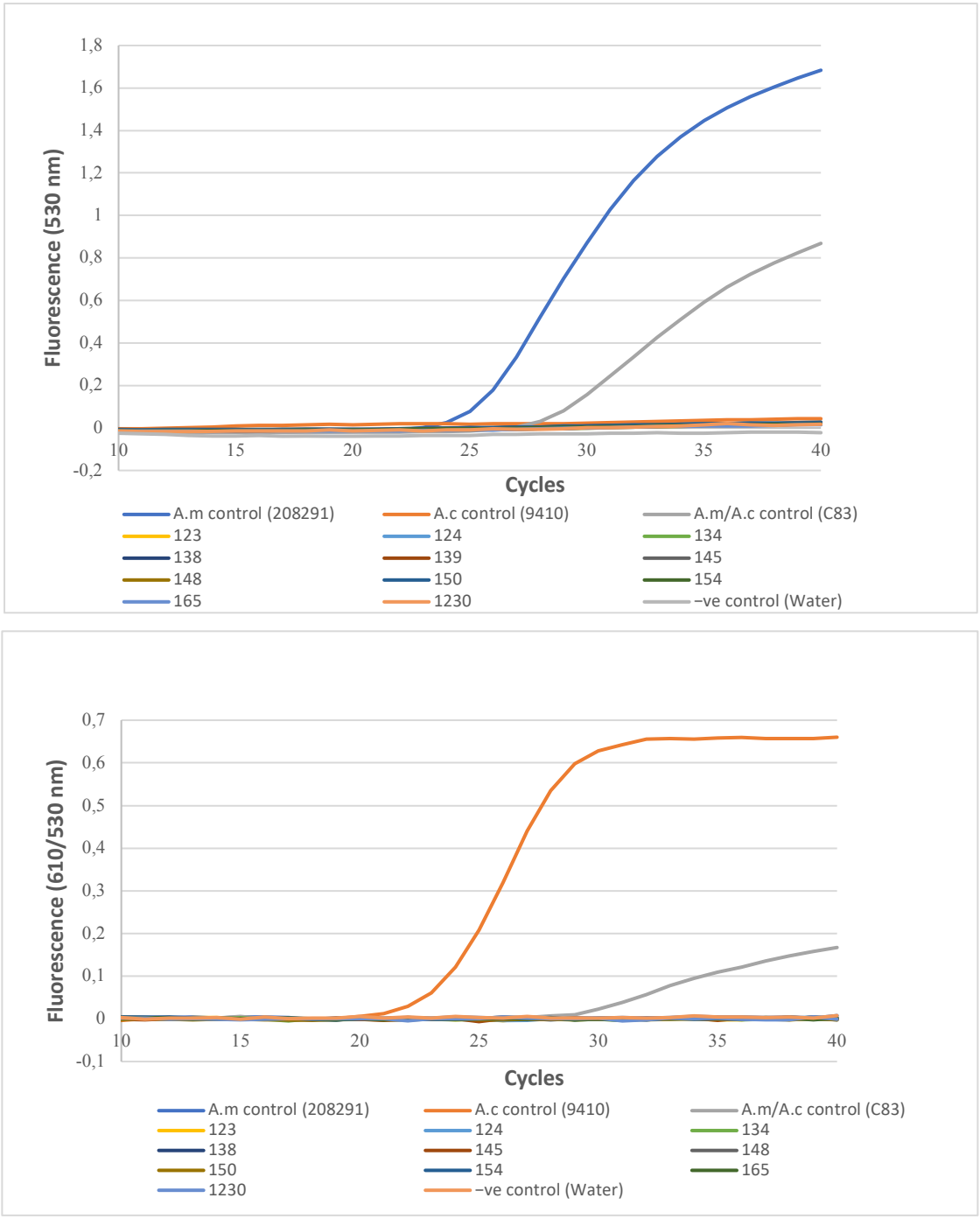

Figure 4.6: Duplex qPCR assay results for genomic DNA extracts from tick cell cultures infected with *A. marginale*-positive blood from carrier animals and clinical cases, after more than 150 to160 days post-infection. All samples were negative for both *A. marginale* (**A**), and *A. centrale* (**B**). The fluorescence signal measured at 530 nm corresponded to the FAM dye (*A. marginale*), and at 610 nm to the LC-610 dye (*A. centrale*). A.m = *A. marginale*, A.c = *A. centrale.*

Table 4.4: Summary of the outcome of attempted initiation of cell culture of *A. marginale,* in ISE6 and IDE8 tick cell lines, using 17 inocula, and their current status after 150–160 days in culture.

Location & Sample number	Number of infected iRBCs[c] per ml of blood estimated by qPCR/ PPE[#]	Number of days in culture	qPCR result (Cp value)	Culture status
UP, Proefplaas, Hillcrest				
1230	5.13 x 10^6	150	Negative	Discontinued
1313	9.45 x 10^6	150	Negative	Discontinued
1332	1.93 x 10^5	150	Negative	Discontinued
UP, DVTD[a], Onderstepoort				
97	1.74 x 10^5	160	Negative	Discontinued
123	6.11 x 10^5	160	Negative	Discontinued
124	6.57 x 10^5	160	Negative	Discontinued
128	8.61 x 10^4	160	Negative	Discontinued
134	5.33 x 10^5	160	Negative	Discontinued
138	1.16 x 10^5	160	Negative	Discontinued
139	4.36 x 10^5	160	Negative	Discontinued
145	9.81 x 10^6	160	Negative	Discontinued
148	3.44 x 10^4	160	Negative	Discontinued
150	3.12 x 10^4	160	Negative	Discontinued
153	8.77 x 10^4	160	Negative	Discontinued
154	1.16 x 10^6	160	Negative	Discontinued
ARC-OVR[b], Onderstepoort				
9672/5	25%[#]	60	Positive (23.91)	Ongoing
9678/2	33%[#]	60	Positive (23.54)	Ongoing

[a]UP, DVTD = University of Pretoria, Department of Veterinary Tropical Diseases,
[b]ARC-OVR = Agricultural Research Council – Onderstepoort Veterinary Research Institute,
[c]iRBCs = infected red blood cells,[#]PPE = percentage parasitised erythrocytes. PPE is determined by microscopic examination of Giemsa stained thin blood smears.

4.5 Discussion

The uninfected IDE8 tick cells did not exhibit typical growth shapes and morphology, unlike the ISE6 cell lines which showed similar morphology to that reported by Munderloh et al. (1994). The IDE8 cells seemed less healthy than the ISE6 cells and did not appear to adjust well to the growth conditions in culture in our laboratory. This difference was despite the preparation of growth medium for both cell lines from the same L15 stock medium and reagents. Despite the IDE8 cell line's unexpected growth morphology, the cells still retained their ability to proliferate and grow to high densities when grown in L15B media and passaged, and this was comparable to that of the healthier-looking ISE6 cells. The unusual morphology and the ability of the IDE8 cell lines to retain their ability to proliferate may be explained by the fact that tick cell lines are composed of mixed tick tissues, which make them highly robust (Bell-Sakyi et al., 2007). These and other traits make them highly desirable and effective in tick-borne disease research and adaptable to growing a variety of tick-borne pathogens (Passos, 2012).

Giemsa staining of thin blood smears combined with light microscopic examination has a detection limit of 0,03% or 10^6 erythrocytes infected with *A. marginale* per millilitre of blood (Gale et al., 1996). This is not a desirable method for testing carrier state animals for *A. marginale* infection because of its low sensitivity and specificity, and even the most experienced practitioners may give inaccurate results (Salih et al., 2015). However, we still utilised this method to detect the infected red blood cells in our cell culture inocula, as it is a rapid and low cost initial screening method that directly detects the pathogen (Donovan-Myhand et al., 1984). Because most molecular and serological methods used to date are a means of indirect detection of the rickettsiae, direct observation by light microscopy of the pathogen still retains its utility in detection of *Anaplasma* spp., as it identifies the actual bacterial colonies in red blood cells (Gale et al., 1996; Salih et al., 2015; Battilani et al., 2017).

The shortfalls of Giemsa staining and light microscopy were mitigated by also employing duplex qPCR to confirm detection of *A. marginale* prior to commencing cell culture. The qPCR assay results corresponded well with the light microscopy results, with samples that showed the highest rickettsaemias yielding the lowest Cp values. As shown by sample 1230, which was qPCR positive, but did not show any *A. marginale* bacteria in the examination of

the Giemsa-stained thin blood smear, caution should be taken by employing another, more sensitive method in addition to microscopy to confirm the infection status of blood samples. It should be noted, however, that the qPCR indirectly detects nucleic acid from the pathogen, and not intact, whole rickettsiae in the host blood. The combination of techniques can thus be recommended as an effective means for screening *A. marginale* blood samples for cell culture, as has been shown in previous studies (Blouin et al., 2000; Passos, 2012; Baêta et al., 2015). However, it is important to note that qPCR and direct microscopic detection of the *A. marginale* rickettsiae, does not necessarily translate to viable *A. marginale* cells that can be grown in culture, as was proven by our initial *A. marginale* cell culture attempts.

The reported *Anaplasma* spp. culture success rate is as low as 5%. Successful cell culture has previously been carried out from blood samples collected from two naturally infected bovines with a rickettsaemia of 10% (Baêta et al., 2015), and these animals exhibited clinical signs of *A. marginale* infection. Other studies used blood samples with rickettsaemias as high as 30 to 45% to achieve persistent infection of tick cell lines with *A. marginale* (Blouin et al., 2000; Felsheim et al., 2010). However, this level of rickettsaemia was achieved by splenectomising calves and artificially infecting them with *A. marginale*. It is thus advisable to use blood with high rickettsaemia to enable successful establishment and propagation of the bacteria in tick cells (Bell-Sakyi et al., 2007; Silaghi et al., 2011). This therefore, points to the difficulty of achieving positive culture of *A. marginale* field samples, which is aggravated by attempting culture from animals with low rickettsaemia.

It was eventually concluded that our initial attempts to infect the tick cells with *A. marginale* from carrier cattle and clinical cases were not successful, as the tick cells did not show the development of any inclusions (Munderloh et al., 1996), nor did the qPCR give any positive results. The two methods most drawn upon to establish successful *A. marginale* cultures are either acquiring inocula (infected blood) from clinically sick animals with high rickettsaemia or artificially infecting splenectomised animals with *A. marginale* (Blouin et al., 2000; Bell-Sakyi et al., 2007). This may likely explain the failure of our initial attempts to establish successful infection of *A. marginale* in ISE6 and IDE8 cell cultures after as many as 160 days post-infection.

Besides the two above-mentioned methods for increasing the probability of initiating *A. marginale* in tick cell culture, another alternative methodology may be explored. As a

potential solution to overcoming the effects of low rickettsaemia in the initiation of culture *A. marginale*, Prof. U. Munderloh (personal communication) suggested preparation of inoculum with a higher concentration of *A. marginale* rickettsiae, through lysis and collection of rickettsiae from larger volumes of infected blood. A slight adjustment to the protocol used in this study, which follows the suggestion by Munderloh et al. (1996) may also be considered. They suggested attempting to initiate culture from washed and frozen blood stabilate, but without initially using antibiotics in the primary culture attempt, since the antibiotics may hinder the initial invasion of tick cells and progressive cell to cell invasion. Using this line of reasoning, it is also plausible that the infections failed to establish in culture because of carry-over of antimicrobial chemotherapeutics in many of the blood samples from the animals used in this study. In fact, the animals sampled from the Department of Veterinary Tropical Diseases were subjected to prophylactic treatments against tick-borne diseases and only when they relapsed and showed clinical symptoms after treatment, did we collect blood samples from them. Therefore, this may have compounded the negative effect of low rickettsaemia, and worked against the colonisation and establishment of *A. marginale* in the tick cells. This is a significant factor to consider in future when collecting *A. marginale*-positive blood samples for cell culture.

4.6 Conclusions

Giemsa staining of thin blood smears, combined with duplex qPCR, is an effective methodology for detecting *A. marginale* infected blood samples for use in initiating cell cultures. However, samples to be used for establishment and propagation of *A. marginale* in tick cell culture are best drawn from animals with a high rickettsaemia, which may be acquired from non-treated, clinically sick animals or experimentally infected animals which have been splenectomised. We have chosen the latter methodology, since initial attempts to initiate *A. marginale* cultures using blood from carrier cattle and clinical cases failed. Experimental infection of splenectomised calves allowed for the production of high rickettsaemias in inocula for initiation of *A. marginale* in cell culture. Preliminary results look promising: a few small colonies have been observed and positive qPCR results suggest that the cultures are positive for *A. marginale*.

4.7 References

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CHAPTER 5

General Discussion, Conclusions and Recommendations

5.1 Molecular detection and genetic diversity of *A. marginale* in South Africa

Bovine anaplasmosis is an economically important tick-borne disease worldwide, although its direct economic impact in South Africa has not been assessed to date (de Waal, 2000). Of the total cattle deaths in South Africa, 18% are due to the tick-borne diseases babesiosis, heartwater and anaplasmosis, with 3% attributed to the latter (de Waal, 2000). Thus, based on the 2015 figure of 13.7 million cattle in South Africa, (Directorate: Statistics and Economic Analysis, 2016), and a yearly average mortality rate of 3% of cattle (Scholtz & Bester, 2010), we estimate the cost due to mortalities from anaplasmosis to be approximately R115 million ($US9.6 million) per year. This study highlights the continued utility of molecular detection and genotyping assays, along with DNA sequencing methods for the elucidation of *Anaplasma marginale* strain variation and genetic variation in immunologically significant genes.

We have shown that a duplex quantitative real-time PCR (qPCR) (Carelli et al., 2007; Decaro et al., 2008) is an effective screening tool for the detection of *A. marginale* and *Anaplasma centrale* in field samples (Hove et al., 2018; Khumalo et al., 2016). Furthermore, the duplex qPCR proved to be a more sensitive assay than nested PCR (Molad et al., 2006; Decaro et al., 2008) and reverse line blot (RLB) hybridisation (Gubbels et al., 1999; Bekker et al., 2002), making it the detection tool of choice (Chaisi et al., 2017). The qPCR assay was able to assay all of the variants detected in our study when we employed a deep sequencing approach of PCR amplicons (Hove et al., 2018). The endemic status of anaplasmosis in South Africa (de Waal, 2000; Mtshali et al., 2007; Mutshembele et al., 2014) was confirmed by qPCR in this study.

Approximately 57% and 17% of cattle samples examined in this study were found to be infected with *A. marginale* and *A. centrale,* respectively, with 15% found to be co-infected with both *A. marginale* and *A. centrale*. Both organisms were present in cattle from all provinces of South Africa except the Northern Cape where tick vector is absent. These data corroborate reports of these two being among the most important tick-borne pathogens in

South Africa (Potgieter, 1981; Khumalo et al., 2016; Hove et al., 2018). The absence of the tick vector in the Northern Cape of South Africa may change as the important tick vector *R. microplus* is undergoing range expansion (Nyangiwe et al., 2013; 2017), which has direct implications for the spread of anaplasmosis to new areas in South Africa. These findings also support previous reports that have highlighted the presence of anaplasmosis in the country and its importance among the major tick-borne diseases (including babesiosis and heartwater), since it acts in synergy with them to cause mortality and morbidity in cattle (de Waal, 2000). Therefore, treatment and control measures for anaplasmosis together with other tick-borne diseases should be conducted by implementation of a holistic management approach, which controls tick infestations by acaricide use and chemotherapy treatment of the rickettsial pathogen. Furthermore, since endemic stability of anaplasmosis is probably important in preventing outbreaks of the disease in South Africa, complete eradication is not advised (Potgieter & Stoltsz, 2004).

We also demonstrated the usefulness of *msp1α* genotyping (Lew et al., 2002) for characterising *A. marginale* field strains. The method should include cloning of PCR products and sequencing of at least ten clones to assess *msp1α* diversity (Hove et al., 2018), as this maximises the chances of detecting low abundance genotypes in field strains. Our research was assisted by the use of the newly developed software, RepeatAnalyzer (Catanese 2016), which we used for identification, mapping, analysis and curation of sequences. However, there is still an urgent need for a centralised repository of Msp1a sequences of *A. marginale*. The high genetic diversity of *A. marginale* in South Africa, revealed by the highest number of *msp1α* genotypes (190) described in any country to date and 36 novel repeats (Hove et al., 2018), points to a high rate of evolution in this rickettsial pathogen. Nearly 72% of cattle samples examined in this study were infected with multiple strains (2 to 9 strains per animal) of *A. marginale* and this may point to superinfection with *A. marginale.* Furthermore, our study revealed that there were only two genotypes shared between South Africa and the rest of the world. This may be due to strict controls that hinder free animal movement and spread and sharing of strains from other regions of the world. This high *msp1α* diversity should also be considered in recombinant OMP vaccine development since it is a surrogate indicator of antigenic diversity, as strains with different *msp1α* genes have different *msp2* repertoires, and immune evasion through Msp2 variation is one of the most important factors for this pathogens antigenic diversity (Rodríguez et al., 2005).

5.2 Diversity of *Anaplasma marginale* outer membrane proteins in South Africa compared to two USA strains and evaluation of their immunological relationships

Outer membrane proteins (OMPs) of *A. marginale* have been shown to be possible vaccine candidates (Lopez et al., 2005; Noh et al., 2008; 2013). OMPs have been shown to induce a level of protection in field challenges with *A. marginale* mainly in studies in USA (Brown et al., 1998; Noh et al., 2008; Tebele, et al., 1991). Sequence conservation in antigenically significant genes has been assessed in strains of *A. marginale*, also in USA (Dark et al., 2011; Deringer et al., 2017; Ducken et al., 2015). However, a study evaluating the genetic variability and immunogenicity of *A. marginale* OMPs was lacking in South Africa.

This section of the study therefore endeavoured to assess the genetic variation in five OMPs that are known to be part of the protective OMP immunogen: Am779, Am854, Omps 7, 8 and 9, in South African strains of *A. marginale* and compared them with sequences from two USA strains (St. Maries and Florida); further emphasising the utility of molecular methods in conducting such studies. We showed that the five OMPs in South African strains had amino acid sequence identities of between 79 and 100% to the USA strains. We also found the highly conserved T-cell epitope FLLVDDAI/V which has been described previously (Deringer et al., 2017). Although our study revealed variation in these OMPs, all the recombinant OMPs we expressed were recognised by antibodies from immunised animals from USA and South Africa. This provides the first line of evidence for a close immunogenic relationship between *A. marginale* strains from USA and South Africa, suggesting the possible utility of the OMPs in a global rather than regional recombinant OMP vaccine. However, such a conclusion should be bolstered by further data from similar studies conducted in other regions of the world, and much work will have to be done to assess various vaccine formulations and delivery methods.

5.3 Attempted culture of South African *A. marginale* field strains

The *in vitro* culture of tick-borne pathogens such as *A. marginale* in tick-derived cell lines has many uses in modern research, which range from bio-banking of field strains of the pathogen to generation of sample material for genomic and proteomic analyses and preparation of challenge material for vaccine trials (Bell-Sakyi et al., 2007). This study intended to culture *A. marginale* from field samples for the primary purpose of isolating and

bio-banking of field strains. This would then enable future studies including next generation sequencing of *A. marginale*, therefore facilitating comparative genomic analyses of South African and US strains.

As mentioned previously, we showed that qPCR is useful for detection of infection in field samples and can be used effectively as a screening method for *A. marginale*. Giemsa staining and light microscopy, along with RLB can also be used to detect other pathogens in field samples when used in conjunction qPCR; as was the case for cell culture samples. It also proved to be a rapid method for monitoring of infection status of the cultures we attempted. Our study reveals that cell culture of *A. marginale* in tick cell lines is a process requiring a lot of time and patience. It is best carried out with samples that have a high parasitaemia as evidenced by the low success rate in this study, which has been reported previously at 5% (Bell-Sakyi et al., 2007; Silaghi, et al., 2011). This point is further strengthened by the fact that we have two promising cultures from the samples derived from splenectomised animals with 25% and 33% PPE. These were qPCR positive (with Ct values of approximately 23) and we have observed what we suspect to be small *A. marginale* colonies, after 60 days of culture. Culture of these two samples will be continued and progress monitored by qPCR and microscopic analysis, with the hope of observing more distinctive *A. marginale* colonies.

5.4 Conclusions

In conclusion, this study highlights the importance of the combined use of molecular and other conventional tools for the detection and characterisation of *A. marginale* and other pathogens, from field samples in cattle samples. In addition to this, molecular methods can also be used to effectively assess genetic variation in *A. marginale* strains and also to assess immunogenic relationships between geographically distant strains. Though genotyping was based on a single gene locus, *msp1α,* we found *A. marginale* to be highly genetically diverse, though retaining a high level of amino acid conservation in the OMPs Am854 and Am779, as well as the T-cell epitope, FLLVDDAI/V, of OMPs 7, 8 and 9. It will be crucial to consider genetic variation and immunological relationships between strains in the development of a recombinant OMP vaccine. We therefore conclude that the five vaccine candidates Am779, Am854, Omp7, Omp7 and Omp9 are promising vaccine candidates and warrant further study for consideration in the context of a global recombinant vaccine to mitigate the effects *A. marginale* infections of cattle.

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APPENDIX 1

Scientific Publications

The following scientific publications were generated during the course of this research study and either emanated directly from this study, or from the research outputs of colleagues in which this study had a direct or indirect input:

Hove, P., Khumalo, Z. T. H., Chaisi, M. E., Oosthuizen, M. C., Brayton, K. A., Collins N. E. (2018). Detection and characterisation of *Anaplasma marginale* and *A. centrale* in South Africa. MDPI *Veterinary Sciences* Special Issue: "One Health—9th Tick and Tick-borne Pathogen Conference and 1st Asia Pacific Rickettsia Conference". 5(*1*), 26. https://doi.org/10.3390/VETSCI5010026.

Hove, P., Chaisi, M. E., Brayton, K. A., Ganesan, H., Catanese, H. N., Mtshali, M. S., Mutshembele, A. M., Oosthuizen, M. C., & Collins, N. E. (2018). Co-infections with multiple genotypes of *Anaplasma marginale* in cattle indicate pathogen diversity. *Parasites & Vectors*, *11*(5), 1–13. https://doi.org/10.1186/s13071-017-2595-5

Chaisi, M. E., Baxter, J. R., Hove, P., Choopa, C. N., Oosthuizen, M. C., Brayton, K. A., Khumalo, Z. T.H., Mutshembele, A. M., Mtshali, M. S., & Collins, N. E. (2017). Comparison of three nucleic acid-based tests for detecting *Anaplasma marginale* and *Anaplasma centrale* in cattle. *Onderstepoort Journal of Veterinary Research*, *84*(1), 1–9. https://doi.org/10.4102/ojvr.v84i1.1262

Khumalo, Z. T. H., Catanese, H. N., Liesching, N., Hove, P., Collins, N. E., Chaisi, M. E., Gebremedhin, A. H., Oosthuizen, M. C., & Brayton, K. A. (2016). Characterization of *Anaplasma marginale* subsp. *centrale* strains by use of *msp1aS* genotyping reveals a wildlife reservoir. *Journal of Clinical Microbiology*, *54*(10), 2503–12. https://doi.org/10.1128/JCM.01029-16

APPENDIX 2

Animal Ethics Approval

Letter of approval for the PhD study 'Molecular characterisation of potential vaccine candidates from *Anaplasma marginale* strains in South Africa' issued by the Animal Ethics Committee, University of Pretoria.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Molecular characterization of vaccine candidates from *Anaplasma marginale* strains in South Africa**
PROJECT NUMBER	**V032-13**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr. N Collins**

STUDENT NUMBER (where applicable)	**----------**
DISSERTATION/THESIS SUBMITTED FOR	**Academic**

ANIMAL SPECIES	**Cattle (Phase 1)**	
NUMBER OF ANIMALS	**300**	
Approval period to use animals for research/testing purposes		**June 2013–December 2016**
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 **27 May 2013**
CHAIRMAN: UP Animal Ethics Committee	Signature

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

Extension No. 1

PROJECT TITLE	**Molecular characterization of vaccine candidates of *Anaplasma marginale* strains in South Africa**
PROJECT NUMBER	**V067-13**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Mr. P Hove**

STUDENT NUMBER (where applicable)	**UP 134 157 36**
DISSERTATION/THESIS SUBMITTED FOR	**PhD**

ANIMAL SPECIES	**Cattle**	**Tick cell lines**
NUMBER OF ANIMALS	**600**	**IDE8 and ISE6**
Approval period to use animals for research/testing purposes		**January 2017- January 2018**
SUPERVISOR	**Prof. M Oosthuizen**	

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 2 February 2017
CHAIRMAN: UP Animal Ethics Committee	Signature

S4285-15

APPENDIX 3

DAFF Section 20 Permit

Permission to do research in terms of Section 20 of the Animal Diseases Act, 1984 (Act number 35 of 1984) for the research project 'Molecular characterisation of potential vaccine candidates from *Anaplasma marginale* strains in South Africa' issued by the Department of Agriculture, Forestry and Fisheries (DAFF), Pretoria, South Africa.

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Dr Mpho Maja • Tel: +27 12 319 7456 • Fax: +27 12 329 7218 • E-mail: Mpho.Maja@daff.gov.za
Reference: 12/11/1/1/6

Mr Paidashe Hove and Dr Mamohale Chaisi
Department of Veterinary Tropical Diseases
Faculty of Veterinary Science
University of Pretoria
Private Bag X04, Onderstepoort

Dear Mr Hove and Dr.Chaisi,

RE: Permission to do research in terms of Section 20 of the ANIMAL DISEASES ACT, 1984 (ACT NO. 35 of 1984)

Your fax / memo / letter/ Email dated 30 September 2013, requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers.

I am pleased to inform you that permission is hereby granted to perform the following research/study, with the following conditions :

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. As samples will be collected from disease controlled areas, ALL field samples collected for this study must be transported under Red Cross Permits issued by the responsible State Veterinarians directly to TADP for deactivation of FMD virus prior to being released to the University of Pretoria
3. Importation of sera and *Anaplasma marginale* in vitro culture grown in tick cells from the USA is subject to obtaining a Veterinary Import Permit(s) prior to importation
4. Permission in terms of the Genetically Modified Organisms Act, 1997 (Act No. 15 of 1997) may be needed
5. Study may not commence prior to having obtained Animal Ethics approval. Copy of approval to be supplied to DAFF.

Title of research/study: Molecular characterization of vaccine candidates from *Anaplasma marginale* strains in South Africa.
Researcher (s): Mr Paidashe Hove and Dr Mamohale Chaisi
Institution: Faculty of Veterinary Science
Your Ref./ Project Number:
Our ref Number: 12/11/1/1/6

Kind regards,

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date: 2013 -10- 11

APPENDIX 4

DAFF Serum Import Permit

Veterinary Import Permit for the transportation of 37 ml of cattle-derived serum samples from the United States of America to South Africa, for the research project 'Molecular characterisation of potential vaccine candidates from *Anaplasma marginale* strains in South Africa' issued by the Department of Agriculture, Forestry and Fisheries (DAFF), Pretoria, South Africa.

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

DEPARTMENT OF AGRICULTURE
DIRECTORATE: ANIMAL HEALTH
PRIVATE BAG X 138
2016 -09- 12
PRETORIA 0001
FORESTRY & FISHERIES

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: 13/1/1/30/2/0-201609001695
Valid from: 2016-09-12
Expiry date: 2017-12-12

IMPORTER:
MR PAIDASHE HOVE / DR NICOLA COLLINS
DEPARTMENT OF VETERINARY TROPICAL DISEASES.FACULTY OF VETERINARY SCIENCE
UNIVERSITY OF PRETORIA
PRIVATE BAG X04
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 37 ML OF CATTLE DERIVED SERA (IN 19 CONTAINERS) into Republic of South Africa:

From: THE UNITED STATES OF AMERICA
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 THE UNITED STATES OF AMERICA.
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 MOLECULAR BIOLOGY LABORATORY, DEPARTMENT OF VETERINARY DISEASES, FACULTY OF VETERINARY SCIENCE, UNIVERSITY OF PRETORIA under the personal supervision of PAIDASHE HOVE;
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 OR 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:

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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]

(IMP.PATH.CE.10/2013)

Hove *et al. Parasites & Vectors* (2018) 11:5
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Parasites & Vectors

RESEARCH Open Access

Co-infections with multiple genotypes of *Anaplasma marginale* in cattle indicate pathogen diversity

Paidashe Hove[1,2], Mamohale E. Chaisi[1,3], Kelly A. Brayton[1,4], Hamilton Ganesan[5], Helen N. Catanese[6], Moses S. Mtshali[3,7], Awelani M. Mutshembele[3,8], Marinda C. Oosthuizen[1] and Nicola E. Collins[1*]

Abstract

Background: Only a few studies have examined the presence of *Anaplasma marginale* and *Anaplasma centrale* in South Africa, and no studies have comprehensively examined these species across the whole country. To undertake this country-wide study we adapted a duplex quantitative real-time PCR (qPCR) assay for use in South Africa but found that one of the genes on which the assay was based was variable. Therefore, we sequenced a variety of field samples and tested the assay on the variants detected. We used the assay to screen 517 cattle samples sourced from all nine provinces of South Africa, and subsequently examined *A. marginale* positive samples for *msp1a* genotype to gauge strain diversity.

Results: Although the *A. marginale msp1β* gene is variable, the qPCR functions at an acceptable efficiency. The *A. centrale groEL* gene was not variable within the qPCR assay region. Of the cattle samples screened using the assay, 57% and 17% were found to be positive for *A. marginale* and *A. centrale*, respectively. Approximately 15% of the cattle were co-infected. *Msp1a* genotyping revealed 36 novel repeat sequences. Together with data from previous studies, we analysed the Msp1a repeats from South Africa where a total of 99 repeats have been described that can be attributed to 190 *msp1a* genotypes. While 22% of these repeats are also found in other countries, only two South African genotypes are also found in other countries; otherwise, the genotypes are unique to South Africa.

Conclusions: *Anaplasma marginale* was prevalent in the Western Cape, KwaZulu-Natal and Mpumalanga and absent in the Northern Cape. *Anaplasma centrale* was prevalent in the Western Cape and KwaZulu-Natal and absent in the Northern Cape and Eastern Cape. None of the cattle in the study were known to be vaccinated with *A. centrale*, so finding positive cattle indicates that this organism appears to be naturally circulating in cattle. A diverse population of *A. marginale* strains are found in South Africa, with some *msp1a* genotypes widely distributed across the country, and others appearing only once in one province. This diversity should be taken into account in future vaccine development studies.

Keywords: *msp1a*, *msp1β*, *groEL*, qPCR, Next-generation amplicon sequencing

Background

Bovine anaplasmosis is one of the most economically important tick-borne diseases of ruminants the world over [1–3]. The causative agent of the disease is the rickettsia *Anaplasma marginale*, a gram-negative, obligate intra-erythrocytic pathogen of the order Rickettsiales and family *Anaplasmataceae* [2, 4–6]. *Anaplasma marginale* is the most prevalent vector-borne pathogen and is found on all six inhabited continents [5, 7–9]. Approximately 20 tick species worldwide have been implicated as biological vectors of the pathogen, although mechanical and transplacental transmission has also been reported [2, 3, 10–15]. *Anaplasma centrale*, considered by some authors to be a subspecies of *A. marginale*, generally causes a milder, less virulent form of the disease, with occasional clinical cases [16]. Infection with

* Correspondence: nicola.collins@up.ac.za
[1]Vectors and Vector-borne Diseases Research Programme, Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Pretoria, South Africa
Full list of author information is available at the end of the article

A. centrale confers immunity to *A. marginale*. *Anaplasma centrale* has therefore been employed as a live vaccine [2, 17]. In South Africa, bovine anaplasmosis is found in most of the cattle farming regions and is an economically important tick-borne disease [2, 3, 17]. It is endemic in eight of the nine provinces of the country [3], except the Northern Cape where the tick vectors are absent. Five tick species, namely *Rhipicephalus decoloratus*, *R. microplus*, *R. evertsi evertsi*, *R. simus* and *Hyalomma marginatum rufipes*, have been shown experimentally to be capable of transmitting *A. marginale* in South Africa [12].

Recently we compared three nucleic acid-based tests for detecting *A. marginale* and *A. centrale* [18]. The nested polymerase chain reaction (nPCR) assay (which targets the *msp1β* gene of *A. marginale* and *msp2* of *A. centrale* [19, 20]) detected fewer *A. marginale* positive samples than the duplex quantitative real-time PCR (qPCR) (which detects *msp1β* of *A. marginale* and *groEL* of *A. centrale* [20, 21]). This discrepancy was found to be due to sequence variation in the *msp1β* gene in the target region of one of the internal PCR primers. The reverse line blot (RLB) hybridization assay [22], in which species-specific sequences in the 16S rRNA gene of *Anaplasma* and *Ehrlichia* species are detected, was found to be less sensitive than the qPCR and nPCR assays. The qPCR assay was thus shown to be the most appropriate assay for detection of *A. marginale* in blood samples from cattle [18]. However, the identification of *msp1β* gene sequence variants indicates the need to assess sequence variation in the target regions of the qPCR assays, to ensure that all *A. marginale* and *A. centrale* genetic variants are detected.

A genotyping method based on the *msp1α* gene [23–26], which encodes major surface protein 1a (Msp1a) [27, 28], has been developed for characterizing *A. marginale* strains in positive samples and has been applied throughout the world. *Anaplasma marginale msp1α* genotyping is not only useful for understanding the genetic diversity of the pathogen but has also been used to elucidate host-pathogen interactions and co-evolution [8, 25, 29–32]. *Msp1α* genotyping relies on variation in tandem repeats at the 5′ end of the gene that varies both in number and sequence. Msp1a repeats are identified in the deduced amino acid sequence and are given alphanumeric names to distinguish between sequence variants; the Msp1a repeat structure determines the *msp1α* genotype of a strain. Over 250 Msp1a repeats have been described, making it a useful marker for discriminating *A. marginale* strains [24–26, 28, 31, 33, 34]. In the South African context, *msp1α*-based genotyping has revealed diversity in *A. marginale* strains across the country, and novel repeats have been identified, although other repeats are identical to those detected in Europe and the USA [24, 25]. Although infection exclusion was thought to result in only one *A. marginale* genotype in individual cattle and ticks [35], more recently, infections with multiple distinct *msp1α* and *msp2* genotypes have been identified in herds in endemic areas with high infection rates [36–40].

In this study, we used next-generation amplicon sequencing to assess the level of variation in the qPCR target regions of the *msp1β* (*A. marginale*) and *groEL* (*A. centrale*) genes from field samples in order to ensure that the duplex qPCR assay [20, 21] was able to detect all *A. centrale* and *A. marginale* genetic variants in South Africa. The assay was then used to screen cattle samples from all nine provinces of the country for the presence of these organisms, followed by *msp1α* genotyping from selected positive samples. We cloned *msp1α* PCR amplicons and sequenced multiple clones to maximize the diversity of *A. marginale* genotypes detected from individual animals.

Methods

Blood sample collection and genomic DNA extraction

A total of 517 EDTA blood samples were obtained from mixed breeds of cattle from all nine provinces of South Africa (Table 1). These consisted of fresh blood samples collected from cattle in the Mnisi communal area (79) and a private farm near Lydenburg (17), Mpumalanga Province, and 148 samples collected from cattle at the University of Pretoria Experimental Farm (Proefplaas, Gauteng Province), as well as 284 frozen cattle blood samples, collected from different parts of South Africa, obtained from the National Zoological Gardens (NZG), Pretoria, South Africa. Blood samples from cattle were collected according to the animal ethics code of the University of Pretoria in 9 ml Vacuette® EDTA tubes (Greiner Bio-One, Kremsmünster, Austria), from the coccygeal vein of cattle that were at least 1 year old. *Anaplasma centrale* blood vaccine was obtained from Onderstepoort Biological Products (Pretoria, South Africa). Genomic DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and DNA was eluted in 100 μl elution buffer and stored at -20 °C.

Table 1 Number and origin of cattle field samples used in the study

Province	No. of samples
Limpopo	30
Mpumalanga	115
Gauteng	183
North West	30
Free State	30
KwaZulu-Natal	30
Northern Cape	30
Eastern Cape	43
Western Cape	26
Total	517

Next-generation amplicon sequencing of *msp1β* and *groEL genes*

Next-generation sequencing (NGS) was used to determine the extent of variation in amplicons of a part of the *msp1β* and *groEL* genes of *A. marginale* and *A. centrale* in, respectively, 40 and 25 known positive field samples from across South Africa. Twenty *A. marginale msp1β* gene sequences from GenBank (accession numbers: M59845, AF110808–AF110810, AF112479, AF112480, AF111195, AF111197, AF221692, AF348137, AF348138, AY841153, KU647713–KU647720) were aligned using CLC Genomics Workbench 7.5.1 (https://www.qiagenbioinformatics.com) and used to design primers Msp1β_F (5′-GAT GAA GCA CCT GAC ACT GGT GAG-3′) and Msp1β_R (5′-CGC GTC GAT TGC TGT GC-3′) in areas conserved in all of these sequences. The primers amplify a 419 bp fragment of the *msp1β* gene spanning the qPCR primer and probe area. The primer pair groEL-ACF and groEL-ACR [20] was used to amplify a 522 bp fragment of the *groEL* gene from both *A. marginale* and *A. centrale*. The primers were modified by adding Illumina-specific adaptor sequences to allow for barcoding of each amplicon and were synthesized at Inqaba Biotechnical Industries (Pretoria, South Africa). The PCRs were performed in a total volume of 25 μl containing 1× Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific, Waltham, USA), 1.5 μM of each primer and 2.5 μl genomic DNA (approximately 200 ng). For amplification of the *msp1β* amplicon, the PCR thermal cycling conditions were 98 °C for 10 s, 40 cycles of 98 °C for 5 s, 67 °C for 15 s, 72 °C for 15 s, and a final extension at 72 °C for 1 min. The same cycling conditions were used for amplification of the *groEL* amplicon, except that the annealing temperature was 66 °C. The amplicons were purified using the QIAquick gel extraction kit (Qiagen) according to the manufacturer's instructions.

Plasmid controls were included in determining the *Taq* and sequencing error rate, to distinguish sequence artefacts from real sequence variants [38]. Multiple strains of *A. marginale* are known to be present in South African samples [24, 25], but the relative incidence of different strains in each sample is unknown, and some strains may be present at very low levels. Amplicons were therefore generated from plasmid controls F48a (*A. marginale msp1β*), 9410c (*A. centrale groEL*) and C14c (*A. marginale groEL*) to determine the frequency of sequence artefacts (including *Taq* or sequencing errors) expected in the field samples. The positive control plasmids were generated previously from field samples that were positive for *A. marginale* (F48 and C14) and *A. centrale* (9410) [18].

Resulting amplicons were gel purified, end repaired and Illumina-specific adapter sequences were ligated to each amplicon. Following quantification, the samples were individually indexed, and another purification step was performed. Indexed, adapter-ligated amplicons were then sequenced on Illumina's MiSeq platform, using a MiSeq v3 (600 cycles) kit (San Diego, California, USA). About 20 MB of data (2 × 300 bp long paired-end reads) were produced for each sample.

Quality filtering was performed on the MiSeq platform, using standard procedures. Only reads that mapped to *A. centrale groEL* 9410c, *A. marginale groEL* C14c and *A. marginale msp1β* F48a reference sequences [18] were incorporated into the subsequent analysis. The sequences were analysed by first merging corresponding Illumina R1 and R2 reads, and only merged sequences were analysed further. Again, the *groEL* and *msp1β* amplicon sequences were mapped to their respective *A. marginale* or *A. centrale* reference sequences. For each set of merged reads, a clustering based on sequence identity was performed. For the *groEL* control plasmid clone 9410c included to determine the frequency of artefacts, the highest proportion of sequences (47.6%) was identical to the 9410c reference sequence. All other sequences (artefacts) were present at an abundance of less than 1.5% each, but collectively made up 52.4% of the sequences. For the *msp1β* plasmid clone F48c, 63.8% of the sequences were identical to the F48c reference sequence, and all other sequences were present at an abundance of less than 1.4%, collectively making up 36.2% of the sequences. Therefore, for the field samples, sequences present at less than 1.5% of the total after cluster analysis were disregarded as *Taq* or sequencing errors. In each cluster, sequences that were present at ≥ 1.5% of the total number of sequences were therefore considered to be true variants and were aligned with published sequences using CLC Genomics Workbench 7.5.1.

Confirmation of *msp1β* variants by Sanger sequencing

The *msp1β* variants identified by NGS were confirmed by Sanger sequencing in eleven samples. Primers AM456 and AM1164 [19] were used to amplify a 750 bp region of the *msp1β* gene flanking the qPCR target area. The reaction mixture contained 1× Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific), 0.5 μM of each primer, 2.5 μl of template DNA (approximately 200 ng) and molecular grade water to a final volume of 25 μl. The PCR thermal cycling conditions were 95 °C for 3 min, 40 cycles of 94 °C for 10 s, 60 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 7 min. The PCR products were purified, quantified and cloned using the CloneJET PCR Cloning Kit (Thermo Fisher Scientific). Recombinant plasmids were screened by colony PCR using vector-specific primers pJET1.2F and pJET1.2R. Plasmid DNA was extracted from recombinants using the High Pure Plasmid Isolation kit (Roche Diagnostics, Basel, Switzerland). Plasmids containing the correct insert were sequenced bidirectionally on an ABI Prism 3100 Genetic Analyzer (Applied

Biosystems, Foster City, California, USA) at Inqaba Biotechnical Industries. Sequences were assembled and aligned using CLC Genomics Workbench 7.5.1.

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

A duplex qPCR assay with minor modifications for the LightCycler real-time machine (Roche Diagnostics) targeting the *msp1β* gene of *A. marginale* and the *groEL* gene of *A. centrale*, was used to detect *Anaplasma* spp. in genomic DNA samples as previously described [18]. DNA extracted from the *A. centrale* vaccine strain (Onderstepoort Biological Products, Pretoria, South Africa) or field sample 9410 (confirmed to be infected with *A. centrale* by amplification and sequence analysis of the *groEL, msp2* and 16S rRNA genes [18]) were used as positive controls. Field samples C14 or C57 (obtained from cattle in the Mnisi Community area) were used as positive controls for *A. marginale*, and molecular grade water as a negative control. To determine *A. centrale* loads, DNA was extracted from 10-fold serial dilutions of vaccine prepared in uninfected bovine blood. The data were analysed using LightCycler Software version 4.0. (Roche Diagnostics). The linear range of detection and assay efficiency of selected variants were determined as previously described [18].

Amplification, cloning and sequencing of the *msp1α* gene

The repeat-containing region of the *msp1α* gene 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′) [41]. Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific) reactions were set up as for *msp1β*. Cycling conditions were 98 °C for 10 s, 30 cycles of 98 °C for 1 s, 69.1 °C for 5 s and 72 °C for 18 s, and a final extension at 72 °C for 1 min. If these PCR conditions failed to generate an amplicon for a sample, the PCR was repeated using the Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific), and the cycling conditions reported by [41] except that a pre-PCR denaturation at 94 °C for 3 min and *Taq* activation at 98 °C for 10 s were included. Samples were analysed on a 1.5% agarose gel and those displaying a single, strong band were purified using the Qiagen PCR product purification kit (Qiagen) according to the manufacturers' instructions. Samples containing multiple PCR products and PCR products that produced mixed sequences were cloned into pJET 1.2 (Thermo Fisher Scientific). Recombinant clones and amplicons were sequenced at Inqaba Biotechnical Industries as described above.

Analysis of Msp1a repeats to determine strain type

Sequences were assembled and aligned using CLC Genomics Workbench 7.5.1. RepeatAnalyzer [42] was used to identify, curate, map and analyse Msp1a repeats and *A. marginale* strains. New names (UP1 to UP36) were given to novel repeats that were not recognized by RepeatAnalyzer. All South African Msp1a repeats and *msp1α* genotypes ([24, 25] and this study) were pooled and analysed using RepeatAnalyzer, generating diversity metric scores [42]. For comparison, similar analyses on previously published data from Argentina, Brazil, Mexico, the Philippines and USA, were also carried out.

Results

Next-generation sequencing of the *groEL* and *msp1β* genes

A total of 39 *A. centrale* and 40 *A. marginale* partial *groEL* sequences (approximately 520 bp in length) were obtained from 25 bovine samples. The *A. centrale groEL* sequences were conserved within the qPCR target region. The *A. marginale groEL* sequences were also conserved and differed from the *A. centrale groEL* sequences at six nucleotide positions in the probe area and three nucleotide positions in the reverse primer region (Fig. 1a). The *A. centrale groEL* sequences were identical to published sequences including those with accession numbers AF414867 (Vaccine strain, South Africa), AF414866 (L strain, South Africa) and ACIS_00394 in the complete genome sequence, CP001759 (Israel strain); while the *A. marginale groEL* sequences were similar to the St. Maries (USA) sequence (AM944 in CP000030). For *msp1β*, 151 different sequences (partial gene sequence; approximately 420 bp in length) were obtained from a total of 183 sequences from 40 samples. Individual samples contained between one and 11 different *msp1β* sequences. Eleven variants (designated as SA1-SA11) were identified in the qPCR target area (Fig. 1b). Single nucleotide polymorphisms (SNPs) were identified at six positions in the primer and probe regions; individual variants contained one to three of these SNPs. Variants SA1, SA2, SA3, SA4, SA5, SA8 and SA9, were identified in multiple samples, while variants SA6, SA7, SA10 and SA11 occurred in only one sample each. The most common variants were SA2 and SA9, identified from 25 samples each. Both of these variants were widespread in South Africa; SA9 occurred in seven provinces, while SA2 was identified in eight provinces. The greatest number of variants (eight) was identified in samples from the Western Cape.

Variants SA1, SA2, SA4, SA5 and SA7 were cloned and their sequences confirmed by Sanger sequencing. Plasmid DNA from clones of these five variants could be detected by the qPCR assay (Fig. 2a). qPCR assay efficiency for detection of variant SA1 was evaluated in a previous study [18]. Evaluation of the efficiency of the qPCR assay in detecting the two variants (SA2 and SA4)

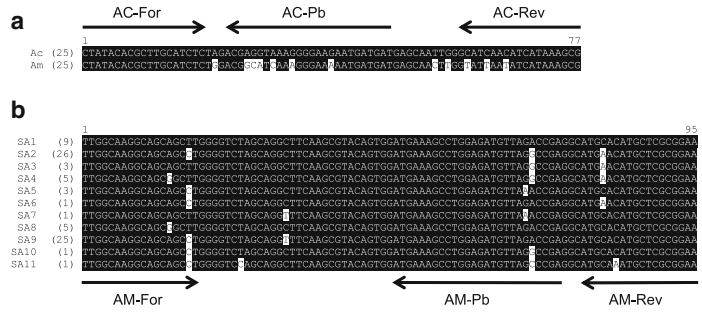

Fig. 1 Sequence alignment of groEL and *msp1β* sequences in the qPCR target regions. **a** *A. centrale* (Ac) and *A. marginale* (Am) *groEL* gene sequences obtained in this study. **b** *msp1β* gene sequence variants in the qPCR target region (SA1–SA11) obtained in this study. The number in parentheses after each sequence name indicates the number of samples from which each sequence was obtained. The primer and probe regions are indicated by arrows. Identical nucleotides are shown by white text on a black background while sequence variations are represented by black text on a white background

containing the most differences (3 SNPs) in the primer and probe regions indicated that the SNPs did not have any effect on the efficiency of the assay (Fig. 2b).

Detection of low *A. centrale* loads in duplex qPCR

Serial dilutions of a known amount of *A. centrale* blood vaccine was used in the duplex qPCR to establish our ability to detect low parasite loads in blood samples (Fig. 2c). We could detect as few as ten infected red blood cells (10 iRBCs) per 20 μl reaction. When working directly from genomic DNA extracted from a blood sample, the efficiency of the qPCR becomes 119%. This apparent increase in efficiency compared to the assay applied to plasmids (E = 103%, Fig. 2b) is likely due to inhibitors co-extracted with the genomic DNA.

Detection of *A. marginale* and *A. centrale* infections in field samples by the duplex qPCR assay

FAM fluorescence (530 nm) was generated in *A. marginale*-positive samples and LC-610 (610 nm) signals were generated in *A. centrale*-positive samples. No amplification was detected from the negative control. The qPCR assay detected *A. marginale* and *A. centrale* in 56.8% and 17.2% of the samples ($n = 517$), respectively. Eighty-one (15.3%) samples had mixed infections. *Anaplasma marginale*-positive cattle were identified in all provinces except Northern Cape (Fig. 3). Most of the *A. marginale*-positive samples were identified in KwaZulu-Natal (100%), Western Cape (88.5%) and Mpumalanga (77.4%), while most of the *A. centrale*-positive cattle were from KwaZulu-Natal (76.7%) and Western Cape (69.2%). *Anaplasma centrale* was not identified in samples from the Eastern Cape and Northern Cape.

Msp1a genotyping and sequence analysis of *A. marginale*

Msp1a repeats identified in this study

To examine the *A. marginale* strain diversity in the sample set, *msp1α* genotypes were determined in samples that were shown to be *A. marginale*-positive using the duplex qPCR. In our study, a total of 143 genotypes were found from 627 *msp1α* sequences, which were generated from 85 samples from across South Africa. An average of 10.5 samples was analysed per province, and an average of 27.8 genotypes was identified per province. Thirty-six Msp1a repeats that have not previously been reported were found, and these were designated UP1-UP36 (Fig. 4). The novel repeats were 28–29 amino acids in length, except UP12 which was found to have an arginine (R) insertion at position 12, making it the longest repeat at 30 amino acids. Alignment of 234 published repeats shows that Serine (S) residues tend to be highly conserved (data not shown). Interestingly, S residues in the repeat region are thought to be O-glycosylated and to facilitate the adhesion function of the Msp1a protein [43]. The 36 novel repeats (Fig. 4) all contained variations in the previously reported immunodominant and linear B-cell epitope SSAGGQQQESS (positions 4–14), the neutralisation-sensitive B-cell epitope Q/EASTSS (positions 21–26) and the T-cell epitope VSSQSDQASTSSQLG (positions 15–29) [28, 31, 43, 44]. The former B-cell epitope varied at 7 out of 11 positions: 4(S/W), 7 (G/S), 8 (G/N/D/C), 9 (Q/H), 12 (E/G), 13 (S/V) and 14 (S/G/V), while the latter varied at 3 out of 6 positions: 21 (Q/E/G/D/S/P), 22 (A/T) and 23 (S/G). The T-cell epitope had variations at 11 out of 15 positions: 16 (S/L/P), 17 (S/P), 18 (Q/Y), 19 (S/Q/T), 20 (D/G/S), 21 (Q/E/G/D/S/P), 22 (A/T) and 23 (S/G), 27 (Q/K/R/H), 28 (L/F/S), 29 (G/R/E).

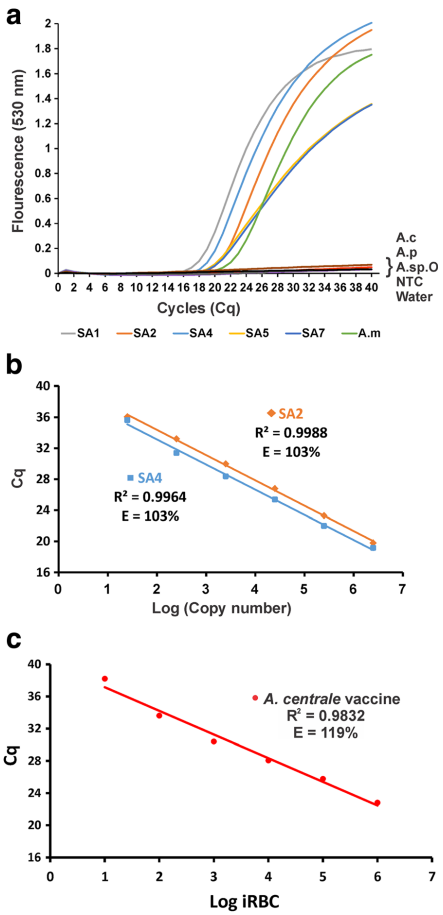

Fig. 2 qPCR amplification of *A. marginale msp1β* variants. **a** qPCR amplification of plasmid DNA (2.5×10^7 copies) of *A. marginale msp1β* variants (SA1, SA2, SA4, SA5, SA7). Genomic DNA (gDNA) from sample C14 was used as a positive control for *A. marginale* (A.m) and water as a negative control. gDNA from the *A. centrale* (A.c) vaccine strain, *A. phagocytophilum* (A.p), *Anaplasma* sp. (Omatjenne) (A.spO) and a no temple control (NTC) were included in the analysis. **b** Detection of tenfold serial dilutions (2.5×10^7 – 2.5×10^2 copies) of plasmid DNA of *A. marginale* variants SA2 and SA4. **c** Detection of tenfold serial dilutions of *A. centrale* vaccine strain (10^6–10^1 iRBCs) genomic DNA. *Abbreviations*: C_q, quantification cycle; R^2, regression coefficient; E, assay efficiency

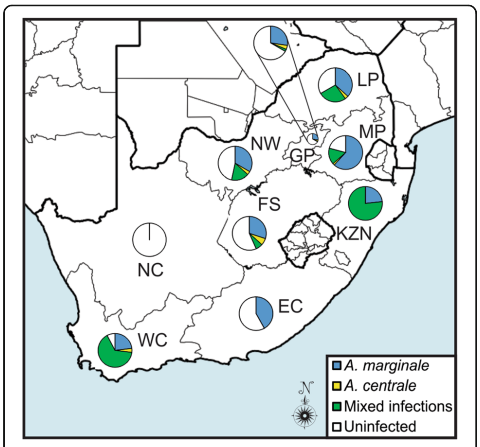

Fig. 3 Map of South Africa showing the occurrence of *A. marginale* and *A. centrale* in cattle. DNA extracted from blood samples from cattle from all nine provinces of South Africa were tested for *A. marginale* and *A. centrale* using the duplex qPCR assay [20, 21]. The pie charts indicate the proportion of samples in each province that were positive, negative or which contained mixed infections. *Abbreviations*: GP, Gauteng; EC, Eastern Cape; FS, Free State; KZN, KwaZulu-Natal; LP, Limpopo; MP, Mpumalanga; NC, Northern Cape; NW, North West; WC, Western Cape

Analysis of Msp1a repeats and *msp1a* genotypes using RepeatAnalyzer

For all South African Msp1a data collected to date, from this and previous reports [23, 24], the frequency distribution of Msp1a repeats resembled a power-law distribution (Fig. 5a). Unique repeats (those observed only once in all *A. marginale* genotypes in South Africa) were observed in 48 instances; examples of such repeats are G, 39, 44, T, UP29, 83, 145, and 154. Six Msp1a repeats, 13, 37, 34, 27, 4 and 3, were found to be most common in South Africa, occurring between 37 and 78 times. There was a normal distribution of *msp1α* genotype lengths (Fig. 5b) ($\mu = 4.26$; $\sigma = 1.48$), which ranged from one to nine repeats. *Msp1α* genotypes in South Africa most frequently contained four or five repeats; these occurred 53 (27.9%) and 49 (25.8%) times, respectively (Fig. 5b). The frequency of genotypes per sample (Fig. 5c) was found to be positively skewed. A total of 78.8% of the samples contained one (28.2%), two (23.5%) or three (27.1%) genotypes per sample. Four to nine genotypes per sample were also observed, but much less frequently.

To date, a total of 99 Msp1a repeats (Fig. 6a) have been described in South African *A. marginale* genotypes, 71 (71.7%) of which are unique to the country (Table 2). These repeats are found in a total of 190 *msp1α* genotypes (Fig. 6b), the majority of which appear to be unique to South Africa (Table 3). In general, repeats were fairly evenly distributed around the country (Fig. 6a). The most abundant strains found in this study have been reported previously [24, 25]. These were SW112. 42 43 25 31

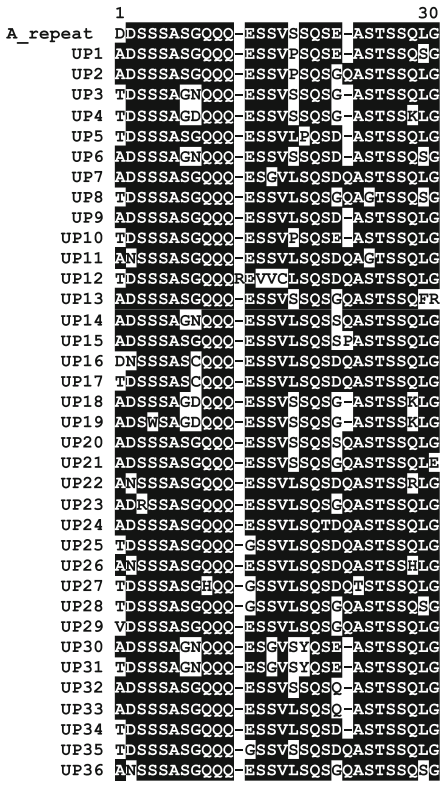

Fig. 4 Novel Msp1a sequences repeats found in this study. Thirty-six unique repeats were identified in this study (UP1-UP36) and aligned against the A repeat [28], using the AlignX module of Vector NTI. Identical amino acid residues in the alignment are shown by white text on a black background; variable residues are indicated by black text on a white background

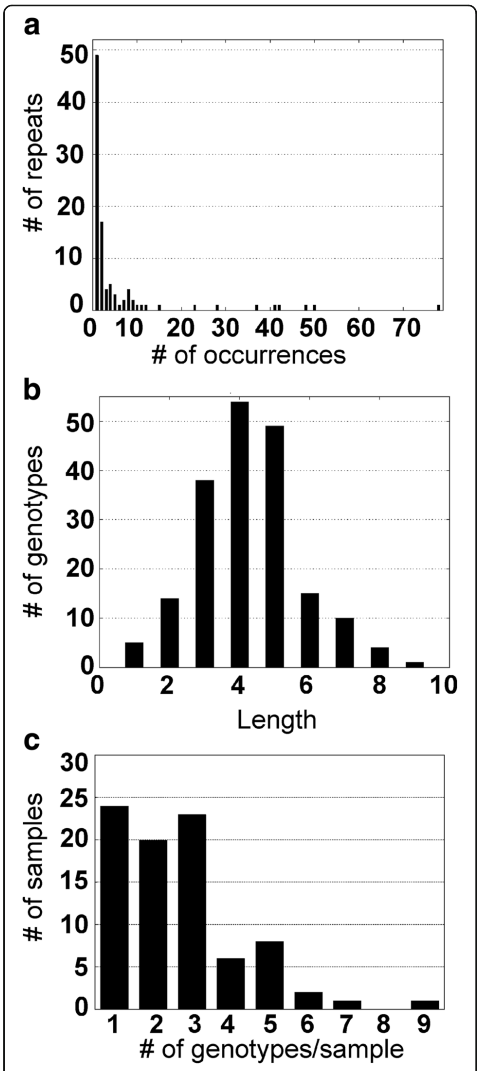

Fig. 5 Msp1a repeat and *msp1a* genotype metrics. **a** Frequency distribution of repeats in Msp1a sequences in South Africa generated by RepeatAnalyzer [42]. **b** Genotype-length distribution of Msp1a repeats in South Africa generated by RepeatAnalyzer. **c** The frequency of *A. marginale msp1a* genotypes found per animal in this study ($n = 85$)

(occurring 12 times in five provinces, Mpumalanga, Eastern Cape, Limpopo, KwaZulu-Natal and North West), SW32. 34 13 13 37 (occurring 6 times in five provinces, Western Cape, Mpumalanga, Gauteng, Limpopo, KwaZulu-Natal) and NW-C1-160312. 34 13 3 36 38 (occurring 8 times in five provinces, Mpumalanga, Limpopo, KwaZulu-Natal, Free State). Some *msp1α* genotypes were found in more than one province, while low abundance genotypes which appeared only once in one province were also detected (Fig. 6b).

Msp1a repeats and *msp1α* genotypes occurring in five selected countries, Brazil, Argentina, Mexico, South Africa and USA, were compared. The percentage of repeats specific to each country (unique repeats) (Table 2) was consistently lower than the percentage of unique genotypes (Table 3). The highest percentage of unique repeats (71.7%) was found in South Africa, while the

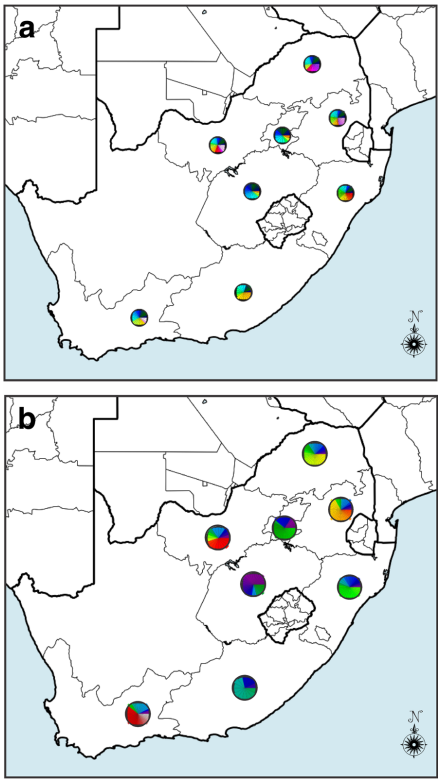

Fig. 6 Geographical distribution of Msp1a repeats and strains from *A. marginale* in South Africa. **a** Distribution of 99 Msp1a repeats from *A. marginale* identified in South Africa in this and previous studies. Different colours in each circle represent different repeats, with more colours indicating a higher repeat diversity in each region. **b** Distribution of 190 *A. marginale* strains identified in South Africa in this and previous studies. Different colours in each circle represent different strains, with more colours indicating a higher strain diversity in each region. Results were generated in RepeatAnalyzer [42]

lowest (18.2%) was in Brazil (Table 2). The most common repeats, which appeared in all of the countries examined, were F and M. Eight other common repeats were found to be present in four of the five countries (Table 2). Although many of the Msp1a repeats identified were found in all five countries examined (an average of 42.8% of Msp1a repeats were unique to each country), very few genotypes were present in more than one country (an average of 91.0% of the *msp1α* genotypes were unique to each country). The highest proportions of unique genotypes were found in USA (100%) and South Africa (99.0%), with Brazil (78.3%) having the lowest observed value (Table 3). More *msp1α* genotypes have been identified in South Africa (190 *msp1α* genotypes) than in any other country, although this likely due to sampling density. Only two genotypes that have previously been identified in other countries were identified in samples from South Africa: (i) τ 57 13 18, found in strain Minas-11 (Minas Gerais, Brazil) [24, 45] was identified in two samples from KwaZulu-Natal; and (ii) 13 27 27, found in strain UFMG-2 (Minas Gerais, Brazil) [24, 45] (also found in the Philippines [39]) was identified in samples from Eastern Cape and Mpumalanga. The genotypes common between South Africa, Brazil and the Philippines represent only 1% of the total number of genotypes described thus far in South Africa.

Discussion

We have recently shown [18] that the duplex qPCR assay [20] is a more sensitive method of detecting *A. marginale* and *A. centrale* infections in cattle in South Africa than RLB [22] or nPCR [19] assays. We also detected sequence variation in the *msp1β* gene in the target region of one of the nPCR internal primers in South African *A. marginale* strains [18]. The *msp1β* multigene family encodes the Msp1b protein, which has been shown to vary between strains of *A. marginale* [7, 39]. Variation of 0.9–1.4% between Msp1b peptide sequences has been shown, but Msp1b is stable during the bovine and tick stages of the *A. marginale* life-cycle within a given strain [34]. This variation could be detrimental when it is used as a target for detection of the parasite by diagnostic tests such as the *A. marginale*-specific qPCR [21]. Sequence analysis of the *msp1β* gene in the target region of the qPCR assay in the current study indicated that the *msp1β* gene of *A. marginale* from cattle in South Africa was highly variable, many samples had multiple *msp1β* variants (when considering the full-length of the amplicon sequence), and SNPs were present at six nucleotide positions in the primer- and probe-target areas of the qPCR assay. Eleven *msp1β* variants were identified in the qPCR target area.

It has been demonstrated that mismatches located towards the 3′ end of a PCR primer are potentially detrimental to PCR amplification as they can significantly affect annealing of the primer to the template, leading to underestimation of the initial copy number, or even a complete failure of amplification [46]. However, the SNPs identified in this study did not appear to decrease the efficiency of the qPCR assay. The efficiency of the qPCR assay in detection of variants SA2 and SA4 (with the most SNPs) compared well with that of the qPCR assay in detection of SA1 [18] in which there is no variation in the qPCR target region. Nevertheless, the sensitivity of the qPCR assay could still be compromised if there is more variation in the field than we have

Table 2 Msp1a repeat analysis for different geographical locations, using RepeatAnalyzer

	Location				
	Brazil	USA	Argentina	Mexico	South Africa
Number of unique Msp1a repeats	6	10	12	27	71
Total number of Msp1a repeats	33	22	33	64	99
% unique repeats	18.2	45.5	36.4	42.2	71.7
Other locations with repeats in common	Arg, Mex, SA, USA	Arg, Brz, Mex, SA	Brz, Mex, SA, USA	Arg, Brz, SA, USA	Arg, Brz, Mex, USA
Common repeats appearing in four or more countries	F	F	F	F	F
	M	M	M	M	M
	13	–	13	13	13
	15	–	15	15	15
	18	–	18	18	18
	27	–	27	27	27
	B	B	B	B	–
	C	C	C	C	–
	Q	–	Q	Q	Q
	τ	–	τ	τ	τ

Abbreviations: *Arg* Argentina, *Brz* Brazil, *Mex* Mexico, *SA* South Africa, *USA* United States of America

detected in this study. Moreover, *A. marginale* has been identified from wildlife in South Africa [47], but the sequence variation in the *msp1β* gene in the parasite in these hosts is unknown.

It should be noted that there are two copies of the *msp1β* gene in *A. marginale* [48, 49], and the primers and probe used in the duplex qPCR assay can amplify the target region in both copies. This would explain a large number of samples containing multiple *msp1β* gene variants since many samples contained multiple *A. marginale* strains (as shown by *msp1α* genotyping), and each strain could contain two different *msp1β* copies. The presence of multiple different copies within a sample could increase the likelihood of detecting *A. marginale* since it increases the chance of a single sample containing a variant that can be detected by the qPCR.

The *groEL* gene of prokaryotes, homologous to the heat-shock protein gene in eukaryotes [50], is highly conserved but contains variable regions that can be useful in differentiating closely related organisms [51, 52]. In contrast to the *A. marginale msp1β* gene, the *groEL* genes of *A. centrale* and *A. marginale* were highly conserved in the target region of the qPCR assay, although SNPs in other regions of this gene were identified. Since the sequence differences targeted by the qPCR primers and probes were highly conserved in all *A. centrale* and

Table 3 *Msp1a* genotype analysis for different geographical locations, using RepeatAnalyzer

	Location				
	Brazil	USA	Argentina	Mexico	South Africa
Number of unique *msp1a* genotypes	18	43	15	84	188
Total number of *msp1a* genotypes	23	43	18	89	190
% unique genotypes	78.3	100.0	83.3	94.4	99.0
Other locations with genotypes in common	Mex, Arg, SA	–	Brz, Mex	Brz, Arg	Brz
Genotypes occurring in more than one country	α β β β β γ	–	α β β β β γ	α β β β β γ	–
	–	–	α β β β γ	α β β β γ	–
	α β β γ	–	–	α β β γ	–
	τ 57 13 18	–	–	–	τ 57 13 18
	τ 10 15	–	τ 10 15	τ 10 15	–
	13 27 27[a]	–	–	–	13 27 27[a]
	–	–	–	13 13[a]	–

Abbreviations: *Arg* Argentina, *Brz* Brazil, *Mex* Mexico, *SA* South Africa, *USA* United States of America
[a]Also found in the Philippines

A. marginale groEL sequences examined, the *groEL* gene is, therefore, a good marker for the detection of *A. centrale* infections in cattle in South Africa. However, in a recent study on the occurrence of tick-borne infections in cattle samples from Uganda [53], RLB assay detected more *A. centrale* infections than the qPCR assay, indicating the possibility of *groEL* gene variants which cannot be detected by the qPCR assay. This highlights the necessity for testing the assay in each region in which it is to be deployed. Further, the detection limits are shown to be approximately ten iRBC/reaction; although this is not being used as a quantitative assay, this can be used as a guideline for field sample detection.

Only two natural isolates of *A. centrale* have been made in South Africa, the original isolate made by Theiler [54] that is used in the blood vaccine, and a second isolate that was made when unfed adult *Rhipicephalus simus* ticks collected in the Louis Trichardt district of the Northern Transvaal (now Limpopo) were fed on a splenectomized ox and an *A. centrale* infection was transmitted [17, 55]. Very little work has been done on this strain of *A. centrale* although it has been shown to have a close identity to Theiler's *A. centrale* vaccine strain by phylogenetic analysis of the 16S rRNA and *groEL* genes [56]. The *groEL* sequence from this strain (accession no. AF414866) [56] was included in our analysis, and, as with all the other *A. centrale groEL* sequences analysed, there was no variation in the qPCR target region. It is possible that some of the *A. centrale* infections detected in field samples in this study were due to this strain.

Our results indicated that *A. marginale* is widespread in cattle in eight of the nine provinces of South Africa. As expected, high percentages (> 70%) of *A. marginale*-positive samples were identified in KwaZulu-Natal, Western Cape and Mpumalanga, since endemic stability is established in these regions. No *A. marginale* infections were detected in cattle from the Northern Cape; this is consistent with the results from a recent study [25] and was expected since the tick vectors do not occur in this province. Interestingly, *A. centrale* was also detected in the cattle, although none of them was known to have been vaccinated, and mixed infections of *A. marginale* and *A. centrale* were common. A high percentage of cattle from KwaZulu-Natal and Western Cape were positive for *A. centrale,* suggesting that this organism is more common in the southern provinces of South Africa. However, it was not detected in cattle samples from the Eastern Cape, but this may have been an artefact of the sampling (43 samples were collected from five farms in two of 39 local municipalities, representing only 3.8% of the area of the Eastern Cape); more samples should, therefore, be sourced from this province to increase confidence in this result. This is the first comprehensive study on the occurrence of *A. centrale* in cattle in all nine provinces of South Africa using a nucleic acid-based method, although we recently reported on the occurrence of this species in cattle in Bergville, KwaZulu-Natal province, South Africa [47]. Mixed infections of *A. centrale* and *A. marginale* have been reported in cattle and wildlife in South Africa [47] and in cattle elsewhere [20, 53, 57]. Although multiplex qPCR assays are recommended for detecting tick-borne pathogens, competitive PCR suppression may occur if infection levels are similar between two or more target species, or are higher in one species/target [58]. This can affect assay sensitivity as has been reported with multiple infections of *T. parva*, *Theileria* sp. (buffalo) and *Theileria* sp. (bougasvlei) in buffalo [58]. Decaro et al. [20] partly addressed this problem by increasing the concentration of the *A. centrale* primers to increase the chance of detecting this pathogen in mixed infections.

Msp1α genotyping revealed that most qPCR-positive cattle (71.8% of samples) in this study were found to be infected with multiple *A. marginale* strains. This is expected in endemic areas and has been reported in previous studies in the USA and the Philippines [36, 39]. Although up to nine *msp1α* genotypes were found per animal, the most abundant genotypes were one to three genotypes per sample. Competition for limited niches or resources in a single host is likely to increase with increasing number of genotypes and may explain the lower numbers of genotypes per animal. Moreover, in South Africa, oxytetracycline and imidocarb are bought over-the-counter by farmers without the need for a veterinary prescription, and these drugs are commonly used to treat babesiosis, heartwater and anaplasmosis, the most common tick-borne diseases in South Africa [3]. Therefore, treatment regimens used by farmers and veterinarians, which have been shown to reduce infection in animals [2, 59], combined with host immunity [2], may play an important role in maintaining lower numbers of genotypes per animal.

Msp1α genotype has been shown to be a surrogate indicator for strain antigenicity, with strains with different *msp1α* genotypes having different *msp2* repertoires [23]. Futse et al. [60] demonstrated that a single unique *msp2* allele was sufficient for a strain to establish superinfection in the face of robust immunity to a primary infecting strain. Our results may suggest superinfection by genomically distinct *A. marginale* strains, which is thought to be uncommon in the temperate regions of the world but occurs more frequently in the tropics [37, 38, 40]. However, superinfection cannot be proven to have occurred in our samples as the infection progress was not monitored in the animals over time, only assessed at one static time point.

Our results demonstrate the importance of cloning all *msp1α* PCR products when genotyping *A. marginale* to

detect multiple infections per animal. Previous studies have focused on samples with only a single detectable band, and have only sequenced one product. To fully explore the diversity of genotypes in a given sample, an investigator must analyse all *msp1α* amplicons obtained. The detection of 36 low abundance, previously undescribed *A. marginale* repeats in this study, emphasizes this point. It should be noted, however, that since *msp1α* is a repetitive sequence, errors in PCR are possible if amplification halts and one repeat primes amplification on another, leading to genotypes with extra repeats. Such a situation may have occurred in up to six samples (7.1%) in this study. Errors may also occur due to *Taq* polymerase slippage early in the PCR, resulting in over- or under-representation of certain repeats. Other error sources may be due to low DNA concentration or poor sample quality, which may arise from improper storage or repeated cycles of freezing and thawing of blood samples (reviewed in [61]).

Worldwide, over 250 highly variable Msp1a repeats have been detected to date [8, 31, 42]. The amino acid sequences of the B- and T-cell epitopes that have previously been identified and shown to be necessary to elicit a protective immune response by Msp1a [28, 31, 32, 43, 44], were found to be variable in the novel Msp1a repeats described in this study, and this variation almost certainly has an effect on the overall epitope structure. Such variations should, therefore, be considered when testing Msp1a as a protective antigen. Serine residues at positions 4 and 25, however, were found to be highly conserved; these residues are thought to be important for O-glycosylation and the adhesion function of the protein, which is essential for transmission of *A. marginale* [43].

We found that 28 out of the 99 (22.3%) Msp1a repeats identified in South Africa are also found in strains in other countries, but this does not translate to many shared genotypes, with only two genotypes out of 190 (approximately 1%) found in common between South Africa and Brazil, and the Philippines. This result is in concordance with a recent study analysing global repeat and strain distribution [31]. These data may suggest that new repeats arise independently in different geographical regions, resulting in the emergence of novel genotypes, which arise from new repeat combinations. Interestingly, one of the two genotypes that was found to be common between South Africa and Brazil (τ 57 13 18), had a repeat structure which differed by one repeat from one of the world's most common genotypes, τ 22 13 18, which has been detected seven times in Argentina and Mexico [31] (repeats 57 and 22 differ by eight amino acids). Although the low prevalence of genotypes common between South Africa and the rest of the world may be due to restricted cattle movements, it could also be due to a lack of *A. marginale* genotyping efforts in other parts of Africa and some regions of the world.

We have identified a large number of diverse Msp1a repeats which are fairly evenly dispersed in South Africa. A large proportion of these Msp1a repeats and *msp1α* genotypes are found only in South Africa. High repeat and genotypic diversity, and an even dispersion of repeats are expected in situations where the number of region-specific repeats and genotypes is high [42, 47], which is evident in the South African data. These data may suggest that repeats (and their associated genotypes) are circulating within the country as a whole, a process which may be driven by cattle movement between the high prevalence endemic areas and the presence of tick vectors of *A. marginale* to propagate the pathogen. In fact, more than one genotype was found to be common between three to five provinces, which provides evidence of ongoing movement of cattle between provinces within South Africa. Both artificial and natural selection factors such as the presence and control of competent tick vectors, host immunity and chemotherapy treatment, are strong determinants of *A. marginale* repeat and genotype composition in different areas. This study demonstrates a high genetic variability of the *A. marginale* population in South Africa, which is an important factor to consider in formulating future vaccine design strategies.

Conclusions

Both *A. marginale* and *A. centrale* are prevalent in South Africa. *Anaplasma centrale* was detected in cattle despite the lack of vaccination with this organism, suggesting that there is a natural transmission cycle of *A. centrale* in South Africa. A total of 190 different *msp1α* genotypes of *A. marginale* have been detected in South Africa, indicating a diversity of genotypes that must be taken into account when developing a vaccine.

Abbreviations

C_q: Quantification cycle; DNA: Deoxyribonucleic acid; E: Assay efficiency; EDTA: Ethylenediaminetetraacetic acid; gDNA: Genomic DNA; iRBCs: Infected red blood cells; Msp1a: Major surface protein 1a; *msp1α*: Gene encoding major surface protein 1a; *msp1β*: Gene encoding major surface protein 1b; NGS: Next-generation sequencing; nPCR: Nested polymerase chain reaction; PCR: Polymerase chain reaction; qPCR: Quantitative real-time polymerase chain reaction; R^2: Regression coefficient; RLB: Reverse line blot; rRNA: Ribosomal ribonucleic acid; SNP: Single nucleotide polymorphism

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Availability of data and materials
The datasets used and analysed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions
PH and MEC played equal roles in carrying out all of the experimental work, data analysis and manuscript preparation. HG was involved in the generation and analysis of the amplicon sequencing data. HNC developed RepeatAnalyzer and assisted with Msp1a data analysis. MSM and AM collected and supplied most of the samples used in this study. MCO, KAB and NEC were the study leaders. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Permission to perform the research was granted regarding Section 20 Animal Diseases Act, 1984 (Act number 34 of 1984), by the Department of Agriculture Fisheries and Forestry, Reference number 12/11/1/1/6. Ethics approval was also granted by the University of Pretoria's Animal Ethics Committee, project number V067/13.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Publisher's Note

Author details
[1]Vectors and Vector-borne Diseases Research Programme, Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Pretoria, South Africa. [2]Biotechnology Platform, Agricultural Research Council, Onderstepoort, Pretoria, South Africa. [3]Research and Scientific Services Department, National Zoological Gardens of South Africa, Pretoria, South Africa. [4]Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, WA, USA. [5]Inqaba Biotechnical Industries, Hatfield, Pretoria, South Africa. [6]School of Electrical Engineering and Computer Science, Washington State University, Pullman, WA, USA. [7]Present Address: National Research Foundation, Brummeria, Pretoria, South Africa. [8]Present Address: Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria, South Africa.

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Review

Detection and Characterisation of *Anaplasma marginale* and *A. centrale* in South Africa

Paidashe Hove [1,2,*], Zamantungwa T. H. Khumalo [1,2], Mamohale E. Chaisi [3], Marinda C. Oosthuizen [1], Kelly A. Brayton [1,4] and Nicola E. Collins [1]

1 Vectors and Vector-borne Diseases Research Programme, Department of Veterinary Tropical Diseases, University of Pretoria, Pretoria 0110, South Africa; mantungwa98@gmail.com (Z.T.H.K.); marinda.oosthuizen@up.ac.za (M.C.O.); kbrayton@vetmed.wsu.edu (K.A.B.); nicola.collins@up.ac.za (N.E.C.)

2 Biotechnology Platform, Agricultural Research Council, Onderstepoort, Pretoria 0110, South Africa

3 Research and Scientific Services Department, National Zoological Gardens of South Africa; Pretoria 0001, South Africa; mechaisi@yahoo.co.uk

4 Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, WA 99164, USA

* Correspondence: paidah2000@gmail.com; Tel.: +27-(0)12-529 8382

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Abstract: Bovine anaplasmosis is endemic in South Africa and it has a negative economic impact on cattle farming. An improved understanding of *Anaplasma marginale* and *Anaplasma marginale* variety *centrale (A. centrale)* transmission, together with improved tools for pathogen detection and characterisation, are required to inform best management practices. Direct detection methods currently in use for *A. marginale* and *A. centrale* in South Africa are light microscopic examination of tissue and organ smears, conventional, nested, and quantitative real-time polymerase chain reaction (qPCR) assays, and a reverse line blot hybridisation assay. Of these, qPCR is the most sensitive for detection of *A. marginale* and *A. centrale* in South Africa. Serological assays also feature in routine diagnostics, but cross-reactions prevent accurate species identification. Recently, genetic characterisation has confirmed that *A. marginale* and *A. centrale* are separate species. Diversity studies targeting Msp1a repeats for *A. marginale* and Msp1aS repeats for *A. centrale* have revealed high genetic variation and point to correspondingly high levels of variation in *A. marginale* outer membrane proteins (OMPs), which have been shown to be potential vaccine candidates in North American studies. Information on these OMPs is lacking for South African *A. marginale* strains and should be considered in future recombinant vaccine development studies, ultimately informing the development of regional or global vaccines.

Keywords: bovine anaplasmosis; qPCR; *msp1α* genotyping; Msp1a; Msp1aS

1. Introduction

A large number of cattle mortalities in South Africa are due to tick-borne diseases, the most important of which are anaplasmosis, babesiosis, and heartwater [1]. Bovine anaplasmosis (or Gall-sickness, as it was formerly known) is a tick-borne disease of ruminants that is caused by microbial pathogens of the genus *Anaplasma* which are obligate, intra-erythrocytic bacteria of the order Rickettsiales and family *Anaplasmataceae* [2–6]. In South Africa, bovine anaplasmosis is endemic in most of the cattle-farming areas [5,7]. In fact, *Anaplasma marginale* is the most prevalent tick-borne pathogen on a global scale, as it is found on all six inhabited continents [6].

Bovine anaplasmosis was first characterised by Sir Arnold Theiler between 1907 and 1910 in South Africa. He observed, in cattle imported from England and infested with ticks in South Africa,

the intra-erythrocytic, membrane-bound coccus-like bodies of *A. marginale*, which he described as 'marginal points' [2,3,8]. Theiler, through a combination of experimental and epidemiological observations, identified *A. marginale* as the causative agent of bovine anaplasmosis, which had been earlier mistaken as a lifecycle stage of the causative agent of redwater (*Babesia bigemina*). Theiler also identified an organism, which he called *Anaplasma marginale* variety *centrale* (referred to as *A. centrale* hereafter) that generally causes a milder, less virulent form of the disease [5,8]. Infection with *A. centrale* confers some cross-protection against *A. marginale*, and it has therefore been employed as a live vaccine from the time it was first developed as such by Theiler in 1910 [5,8].

Anaplasmosis is one of the most economically important diseases of cattle in South Africa [1,5], with symptoms ranging from fever, icterus, inappetence, weight loss, abortion in pregnant cows, and lowered milk production [5,6,9]. It results in significant productivity losses, and, in some cases, mortality [1,9]. Chemical control and treatment measures in South Africa largely involve the use of acaricides to control tick vectors, and long-acting, rickettsicidal tectracyclines, such as the most commonly used oxytetracycline. In South Africa, as in the world over, the effects of tick-borne diseases on animals are often synergistic, where animals are infected with more than one pathogen at a time [1]. Therefore, studies to quantify the losses that are specifically attributable to bovine anaplasmosis are yet to be carried out in South Africa [1,5], and consequently, studies addressing anaplasmosis have been few and far between. In other parts of the world, costs arising from bovine anaplasmosis have been estimated from $US 300–800 million [10]. Furthermore, economic costs attributable to disease burden and control for babesiosis and anaplasmosis together have been approximated at $US 875 million in South America [11] and $US 30.5 million in Australia [12]. Due to the high economic impact, vaccination with *A. centrale* has been deemed to be cost effective for many countries, despite the risk of transmitting emerging pathogens along with the blood-borne vaccine [5].

2. Classification of *Anaplasma* Species

Historically, *Anaplasma* spp. have been incorrectly classified as anything from viruses to protozoa [13]. A taxonomic reclassification and reorganization of the genus using genetic analyses [4] provided an invaluable contribution to the systematics of the *Anaplasma* spp. *Anaplasma marginale* is currently regarded as the type species for the genus *Anaplasma* [4], which was expanded to accommodate three species that are reclassified from the genus *Ehrlichia* that invade cells of haematopoietic origin (neutrophils and erythrocytes) in their vertebrate host species. These are *A. phagocytophilum* (formerly known as *Ehrlichia phagocytophila*, *E. equi*, and the agent of human granulocytic ehrlichiosis), *A. bovis* (formerly *E. bovis*) and *A. platys* (formerly *E. platys*). Also included in the genus *Anaplasma* is another species, *A. ovis*, that causes mild to severe disease in sheep, deer and goats.

Additional species have been reported that are not formally described, including *Anaplasma* sp. (Omatjenne) [formerly *Ehrlichia* sp. (Omatjenne)] [14] and *A. capra* [15]. The name *A. caudatum* was given to an *A. marginale* strain with appendages that also causes bovine anaplasmosis [5]. While this is formally recognised as a separate species, it is thought to be simply a "tailed" strain of *A. marginale*, but has not been studied in great detail [6].

A. centrale was erroneously classified as a separate species, an error that is attributable Ristic in 1968 [16] who incorrectly stated: "In 1911, Theiler, who first described *A. centrale*, indicated that it was a separate species and thus distinct from *A. marginale*". While some authors recognised this error and continued to refer to *A. centrale* as a variety or subspecies of *A. marginale*, the organism was listed as a separate species in List No. 15 of new names and new combinations previously effectively published outside the International Journal of Systematic Bacteriology [17] and subsequently in Bergey's Manual of Systematics of Bacteriology [18]. It is thus referred to as a separate species in many publications. We have recently shown, through sequence analyses of the 16S rRNA gene, *groEL* and *msp4* from several isolates of *A. marginale* and *A. centrale* from around South Africa, that *A. centrale* consistently forms a separate clade from *A. marginale* [19]. These results, when combined with morphological differences,

and the differences in Msp1a/Msp1aS gene structure [20], as well as genome architecture [21,22], provide evidence to suggest that *A. centrale* is, in fact, a separate species.

Thus, the current classification of *Anaplasma* species can be considered, as shown below (adapted from [13]), with seven formally recognised species and two others that have not yet been formally described.

Superkingdom	Bacteria
Phylum	Proteobacteria
Class	Alpha-proteobacteria
Order	Rickettsiales
Family	*Anaplasmataceae*
Genus	*Anaplasma*
Species	*A. marginale* (type species)
	A. bovis
	A. caudatum
	A. centrale
	A. ovis
	A. phagocytophilum
	A. platys
	Not formally described:
	A. capra
	Anaplasma sp. (Omatjenne)

3. Epidemiology

Bovine anaplasmosis is endemic in South Africa [1,7,23], although the Northern Cape is considered to be free of the disease [1]. In South Africa, the role played by tick species in anaplasmosis transmission has been poorly studied, and it has long been assumed that the one-host tick, *Rhipicephalus decoloratus* is the main disease vector. This is due to the co-occurrence of this tick and the disease in endemic areas of the country [5] (Figure 1). *Rhipicephalus microplus* is spreading in South Africa and is therefore probably increasing in importance as a vector [24]. Experimental transmission studies have demonstrated transstadial transmission of *A. marginale* by *R. decoloratus*, *R. microplus* and *Rhipicephalus simus*, and experimental intrastadial transmission has been demonstrated for these three tick species, as well as *Rhipicephalus evertsi evertsi* and *Hyalomma marginatum rufipes* [1,5]. *R. simus* has also been shown to transmit *A. centrale* transstadially [25]. More recent data from a study conducted between 2014 and 2017, in which ticks were collected and analysed for *A. centrale* infection, suggests that *A. centrale* is also transmitted by the tick vector, *Rhipicephalus appendiculatus* [26]. However, this is yet to be confirmed by performing transmission studies.

Many antelope and other game species are abundant both in game reserves and farming areas in South Africa, and they are likely to play a role in the epidemiology of anaplasmosis. However, the role of wildlife as reservoir hosts of *Anaplasma* spp. has not been extensively studied. Blesbok (*Damaliscus pygargus phillipsi*), common duiker (*Sylvicapra grimmia*), and black wildebeest (*Connochaetes gnou*) have been experimentally infected with *A. marginale* and *A. centrale*, although the infections were subclinical [5,27]. It has also been shown that blesbok are susceptible to *A. centrale* infection [5]. *Anaplasma* spp. have also been recorded in giraffe (*Giraffa camelopardalis*), sable antelope (*Hippotragus niger*), buffalo (*Syncerus caffer*), and black wildebeest (*Connochaetes gnou*) [5,28]. A more complete understanding of the epidemiology of anaplasmosis is important for both domestic and wild animal health.

The recommended approach to the control of tick-borne diseases in South Africa is the integrated strategic use of acaricides and application of vaccines [1]. Acaricides are expensive, they pose an environmental hazard, and acaricide resistance is rapidly developing among tick populations worldwide [29]. Vaccines available to prevent bovine anaplasmosis, which is caused by *A. marginale*,

are currently limited. Infection with *A. centrale* confers cross-protection to *A. marginale*, and *A. centrale* is used in a live blood vaccine in many countries, including South Africa [30]. This vaccine is expensive to produce as live cattle are required, it requires careful maintenance of a cold chain, and carries the risk of unintended introduction of other blood-borne pathogens. The vaccine also does not protect against all field strains of *A. marginale* and can cause severe clinical reactions following vaccination [31].

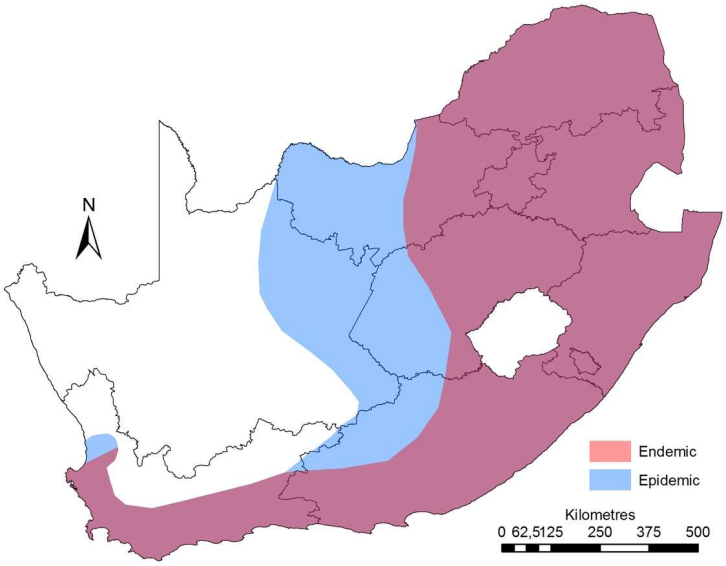

Figure 1. Endemic (red) and epidemic (blue) areas of bovine anaplasmosis disease coverage in South Africa based on historical distribution of vector ticks and areas where the disease has been reported.

A recombinant vaccine would circumvent many of the problems that are associated with live blood vaccines. An effective vaccine needs to induce both high IgG2 titres and possess both $CD4^+$ T- and B-cell epitopes, which produce robust B- and T-cell memory responses during subsequent *A. marginale* infections [32,33]. Highly promising outer membrane protein (OMP) vaccine candidates have recently been identified primarily from North American strains of *A. marginale* [34–38], but it is not known if these candidates are sufficiently conserved to be broadly useful or if vaccine development based on regional pathogen strains is necessary. The OMPs Am202, Am368, Am854, Am936, Am1041, and Am1096, which have been shown to have between 97 and 100% amino acid identity in strains and isolates from different geographical locations, have recently been assessed as vaccine candidates [39]. This study revealed that, although the four most conserved of these OMPs were consistently recognised by sera from animals vaccinated with outer membrane complexes, OMPs Am854 and Am936 were recognised most consistently. Variation in these OMPs has not yet been examined in South Africa.

The antibody-sensitive neutralization epitope, Q(E)ASTSS, as first described by Allred et al. [40], and both T-cell (VSSQSDQASTSSQLG) [41] and B-cell (SSAGGQQQESS) [42] epitopes have been described in the N-terminal repeat region of the Msp1a protein. More recently, Omp7–9 have been reported to possess a T-cell epitope (FLLVDDAI/VV) which is conserved between the three A. *marginale* OMPs across strains from America, Australia and Ghana, as well as A. *centrale* [43]. These epitopes have not been examined in South African strains. Therefore, the detection of different *A. marginale* strains in South Africa is necessary in order to assess the variation in vaccine candidate OMPs and to determine if previously identified epitopes are present in South African strains.

4. Detection of *A. marginale* and *A. centrale* in South Africa

A comparison of routinely utilised detection strategies for *A. centrale* and *A. marginale* in South Africa is shown in Table 1. The oldest method is direct detection by light microscopic observation of the parasite in tissue or organ smears after staining with Giemsa and other Romanowksy stains. Giemsa staining of thin blood smears combined with light microscopic examination are routinely used in the detection of *A. marginale* and *A. centrale* in clinical and field samples in South Africa. An earlier, less advanced form of this methodology was employed by Sir Arnold Theiler in the discovery of *A. marginale* and *A. centrale* [2,3,8]. The method is not very sensitive, and is therefore used in conjunction with other assays to confirm infection. In Giemsa-stained thin film blood smears, *A. marginale*, *A. caudatum* and *A. centrale*, which all infect cattle, appear as dense, deep purple, vacuole-bound, near-circular inclusion bodies, with a diameter ranging from 0.3 to 1 µm. The inclusion bodies are located on the margins of the erythrocytes, except for *A. centrale*, which, as the name implies, has inclusion bodies located centrally [5,6]. Necroscopy accompanied by microscopic examination is also utilized to detect *Anaplasma* in thin films of internal organs such as liver and spleen, along with peripheral blood; smears are stained with dyes, such as toluidine blue, new methylene blue, and acridine orange.

Table 1. Comparison of diagnostic assays currently in use in South Africa for detection of *A. marginale* and *A. centrale.*

Assay	Cost per sample (South African Rand - R)	Average throughput time	Comments on assay sensitivity	Technical skills & expensive equipment needed?
Light microscopic examination of Giemsa-stained smears [5,44]	R113	3 days	Low (10^6 *A. marginale*-infected erythrocytes per ml of blood) Best used during acute phase of infection	Low to Medium No
Msp5 competitive ELISA (cELISA) [5,45]	R140	4 days	Low to Medium Results in false negatives Detects *Anaplasma* to genus level only	Medium to High Yes
Reverse line blot (RLB) hybridisation [46,47]	R445	3 days	Medium to high Similar to PCR & higher than nPCR, but lower than qPCR	Medium to High Yes
Conventional PCR [48,49]	R250	2 days	Medium Similar to RLB	Medium to High Yes
Nested PCR [47,50]	R350	3 days	Medium Fails to detect genetic variant sequences leading to false negatives Less sensitive than RLB & qPCR	Medium to High Yes
Duplex quantitative real-time PCR (qPCR) [47,51,52]	R430	2 days	High (30 *Anaplasma*-infected erythrocytes per ml of blood) Detects parasites at very low levels Most sensitive test available in South Africa	Medium to High Yes

Indirect genus-specific detection of *Anaplasma* species in infected animals is carried out using the following serological tests: major surface protein 5 (Msp5) enzyme-linked immunosorbent assay (ELISA), complement fixation and the card agglutination test [1,5,6,45]. However, the Msp5 ELISA is not able to distinguish between *Anaplasma* spp. Numerous nucleic acid-based assays for the detection of the parasite have been developed and include: conventional polymerase chain reaction

(PCR) [48,49], nested PCR (nPCR) [50,53], quantitative real-time PCR (qPCR) [51,52], and a reverse line blot hybridization (RLB) assay [46]. We recently demonstrated the utility of next-generation PCR amplicon sequencing as a tool for detection and analysis of genetic variation in *A. marginale* and *A. centrale* [54]. These tests have been demonstrated to be effective for inter- and intra-species differentiation and for the detection of low levels of rickettsaemia, which cannot be detected in thin blood smears.

The RLB hybridization assay has been used extensively for the routine screening of cattle and wildlife samples in South Africa and has the ability to detect up to 32 pathogens in one reaction. This technique has been used in the discovery of novel pathogens or genetic variants of known pathogens [55,56]. Its utility lies in its ability to detect *Anaplasma*, *Ehrlichia*, *Babesia* and *Theileria* parasites in a single reaction [46,57], and it is therefore a good screening tool to establish what pathogens might be in a sample. The duplex qPCR test for detection of *A. marginale* and *A. centrale* is a more rapid test than the RLB assay and can be used to confirm the RLB results and for quantification of the infection. We evaluated the performance of three of the nucleic acid-based methods, RLB hybridization, nPCR, and duplex qPCR in the detection of *A. centrale* and *A. marginale* in South African samples [47]. The nPCR assay was shown to give false negative results, due to sequence differences in the internal forward priming region in South African *A. marginale* strains. It was concluded that duplex qPCR is the most sensitive of these three methods, as it detected more *A. marginale* and *A. centrale* positive samples. The duplex qPCR assay has been used in our laboratory for detection and quantification of *A. marginale* and *A. centrale* infections in cattle and wildlife [20,54,55]. Using the qPCR assay, we determined the prevalence of 57% and 17%, respectively, for *A. marginale* and *A. centrale* infections in South African cattle, as well as a co-infection rate of 15%. These studies [20,54] suggest that *A. centrale* is circulating naturally in South African cattle, as it was found in non-vaccinated cattle and wild animals.

5. Genetic Diversity of *A. marginale* and *A. centrale* in South Africa

5.1. msp1α Genotyping of A. marginale

Genotyping efforts using the *msp1α* gene are well advanced in DNA-based strain differentiation of *A. marginale* strains [7,58,59]. *msp1α* is a single copy gene encoding major surface protein 1a (Msp1a). The gene can be used to characterise strain differences due to variations in the number and sequence of tandem repeats at the 5′ end of the gene [40,60] (Figure 2). A complex system has been developed in which the Msp1a repeats are named alphanumerically, in order to distinguish sequence variants, leading to *msp1α* genotypes being described as, for example, J/B/B (the St. Maries strain) or A/B/B/B/B/B/B (the Florida strain) [58]. The current, most widely used PCR-based *msp1α* genotyping protocol is based on the PCR methodology, as described by Lew et al. [53] and de la Fuente et al. [61]. *msp1α* genotyping has elucidated the genotypic variation found in *A. marginale* strains in virtually all the regions of the world that are plagued by anaplasmosis, including South Africa [7,23,54], Asia [15,62], Australia [53], Europe [59,63], South America [64,65] and North America [66,67]. A tool was recently developed to provide analytics for Msp1a repeats which also provides databasing capabilities [68].

A. marginale strains present in different herds show variation in Msp1a repeat structure and it is thought that this can be indicative of sequence variation in other antigenically significant proteins [58,69]. Msp1a has also been shown to contain B-cell and neutralization sensitive epitopes, and, in the repeats, amino acid 20 is thought to be important for binding to tick cells [40,42] (Figure 2).

A parallel genotyping system, based on applying a formula to the number of the microsatellite repeats found between the Shine-Dalgarno sequence (GTAGG) and the initiation codon (ATG) sequence upstream of the *msp1α* coding sequence has been described [63]. However, this genotyping scheme is used much less frequently, and the significance of the genotypes remains unclear.

The first study to examine Msp1a in the South African context demonstrated *msp1α*-based genetic diversity in *A. marginale* strains from the Free State province, and identified Msp1a repeats that are

similar to repeats identified in strains from the United States strains, as well as repeats unique to South Africa [7]. Furthermore, 42% of the Msp1a repeats were shared between South African strains and those from South America, North America, and Europe.

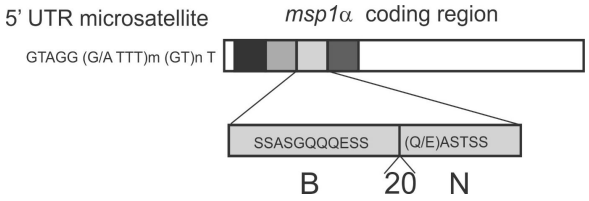

Figure 2. A schematic diagram of the *msp1α* gene. The tandem repeats are shown as grey boxes, with each shade representing a different repeat. The microsatellites in the 5' untranslated region (UTR) used in a second genotyping system are shown. The B-cell (B) and neutralization sensitive (N) epitopes, and amino acid 20, found to be important for binding to tick cell extracts, are shown on an enlarged protein repeat.

Another study used *msp1α* sequence data to examine the epidemiology and genetic diversity of *A. marginale* strains in South Africa and suggested mechanisms for the evolution of *A. marginale* [23]. This study found a 65–100% prevalence of *A. marginale* in different provinces, along with the associated Msp1a genetic diversity in each province. This diversity was highlighted by the 23 novel Msp1a tandem repeats found in South African *A. marginale* strains, which are likely to have evolved from tandem repeat 4. Interestingly, it was also shown that genetic diversity in the highly variable Msp1a was evolving under both positive and negative selection pressure in the South African *A. marginale* population. Using a bioinformatics approach, the authors also showed that Msp1a contains B- and T-cell epitopes, with serine residues that are highly conserved in the repeat region and are thought to be important for the adhesion function of the Msp1a protein. This suggests that Msp1a is a possible vaccine candidate, despite its highly variable amino acid residues. The same B- and T-cell epitopes were also identified in a more recent South African study [54].

We recently assessed South African Msp1a genetic diversity and found 36 novel Msp1a repeats that were contributing to a total of 99 described in the country to date [54]. These 99 repeats are configured to make up 190 genotypes, suggesting that strain variation across South Africa is prevalent. However, caution needs to be taken in interpreting this genetic variation as assessment of genetic diversity using *msp1α* genotypes is based on a single genetic locus, and the inference that this locus is a surrogate reporter for more widespread genomic variation is based on a single study [58].

5.2. msp1aS Genotyping of A. centrale

We developed a novel genotyping system for *A. centrale* based on the Msp1aS protein, a homolog of *A. marginale* Msp1a [20]. The genotyping methodology is similar to *msp1α* genotyping in *A. marginale*, the only difference being that the repeats in Msp1aS are larger than the repeats in Msp1a. A total of 47 Msp1aS repeats were identified in South African cattle, wildebeest, and buffalo, representing 32 *A. centrale* genotypes, which were described for the first time and are distinguishable from the vaccine strain. The study revealed genetic diversity of *A. centrale* strains in cattle and wildlife, and suggested that wildlife could be reservoirs of *A. centrale* infection [20]. The study also showed that Msp1aS could be utilised as a genetic marker for diversity analysis in *A. centrale*.

Both of our recent studies examining *A. marginale* and *A. centrale* in South Africa [20,54] have used the program RepeatAnalyzer [68] to identify, curate, map, and analyse Msp1aS (*A. centrale*) and Msp1a repeats (*A. marginale*). These studies reveal the urgent need for a centralized online repeat genotype/strain repository along with the development of a unified nomenclature for *A. marginale* and *A. centrale*.

6. Conclusions

The South African studies that are outlined in this mini-review, along with other studies elsewhere in the world, highlight the variety of assays employed in detection and evaluation of genetic diversity in *A. marginale* and *A. centrale*. While nucleic acid based assays have been widely used in South Africa, these have to be used judiciously and in conjunction with direct methodologies, such as tissue and organ staining, combined with light microscopy. The *A. marginale msp1α* genotyping studies carried out in South Africa confirm that *A. marginale* is endemic in the country and is a genetically diverse organism that is continuously evolving. Genetic diversity of *A. marginale* and the corresponding variation in OMP genes of immunogenic importance, need to be considered when developing a recombinant vaccine, which is likely to be the future of *A. marginale* control.

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1. Hove P., Brayton K.A., Liebenberg J., Pretorius A., Oosthiuzen M.C., Mtshali M.S., Mutshembele A., Noh S.M., Collins N.E. 2017. Molecular characterization of vaccine candidates from Anaplasma marginale strains in South Africa. 9th Tick and Tick-borne Pathogen Conference & 1st Asia Pacific Rickettsia Conference, Cairns, Australia, 27 August–1 September 2017.
2. Chaisi M.E., Hove P., Oosthuizen M.C., Brayton K.A., Collins N.E. 2017. Anaplasma marginale and A. centrale are widespread in cattle in South Africa. 9th Tick and Tick-borne Pathogen Conference & 1st Asia Pacific Rickettsia Conference, Cairns, Australia, 27 August–1 September 2017.
3. Khumalo, Z.T.H., Collins, N.E., Chaisi, M.E., Brayton, K.A., Quan, M., Chaisi, M.E., Oosthuizen, M.C. 2017. Confirmation of Anaplasma marginale variety centrale (Theiler 1911) as a separate species, Anaplasma centrale (non Theiler 1911) sp. nov., comb. nov (Ristic & Kreier 1984). 9th Tick and Tick-borne Pathogen Conference & 1st Asia Pacific Rickettsia Conference, Cairns, Australia, 27 August–1 September 2017.

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