Development of a high-throughput diagnostic screening tool to monitor the status of amitraz resistance and genotype in the cattle tick, *Rhipicephalus microplus*

by

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Submission declaration

I, **Laura Bishop**, declare that the thesis/dissertation, which I hereby submit for the degree **Master of Science (MSc) Genetics** at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signature:

Date: 15/02/2021

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University of Pretoria

Full names of student: Laura Bishop

Student number: 14053790

Topic of work: Development of a high-throughput diagnostic screening tool to monitor the status of amitraz resistance and genotype in the cattle tick, *Rhipicephalus microplus*

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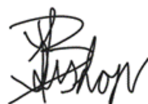
Signature: 

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Summary of project

Acaricide resistance against all major classes of acaricides has been reported in tick populations around the world. In South Africa, amitraz is one of the most commonly used acaricides, with resistance being reported in both of the *Rhipicephalus* cattle tick species, namely *Rhipicephalus microplus* and *R. decoloratus*. Conventional methods to detect acaricide resistance, such as larval packet tests or adult immersion tests, are time-consuming assays that do not provide any information on tick genotype. This is essential for the identification of areas where resistance is emerging to allow for early intervention before homozygous resistant tick populations are established. Due to these limitations, new rapid detection approaches are needed to streamline resistance screening protocols to ensure knowledge-based intervention.

In this study, the current amitraz resistance status of *R. microplus* ticks in the Mnisi communal area was investigated, where it was found that 87% of the population was heterozygous. Results indicated that the heterozygous genotype is being maintained in the population despite the application of a continued selection pressure over time, which suggests that there could be a fitness cost associated with amitraz resistance. In addition, TaqMan SNP genotyping assays were designed and tested as a high-throughput diagnostic screening tool to improve the turnover time of genetic testing, where hundreds of samples can be analysed for an array of resistance associated genetic markers within hours. Two TaqMan SNP genotyping assays were designed to genotype the two SNPs in the octopamine/tyramine receptor gene that have been linked to amitraz resistance. The Oct 2 assay shows potential for the diagnostic screening of *R. microplus* field populations, although further testing is required. An ITS2 TaqMan SNP genotyping assay was also designed with the aim of rapidly differentiating between *R. microplus* and *R. decoloratus* ticks. This assay did not prove to be successful in initial testing but the concept may still hold potential for future studies.

This project will allow us to expand our database on the current status of amitraz resistance in the Mnisi communal area to put forward a strategy for improved acaricide use in collaboration with government and our industry partners. This will benefit the agricultural industry as well as the South African economy by reducing capital losses to farmers as they will be able to purchase the correct acaricides and use them in a more effective way. This will also impact veterinary health disciplines in South Africa by decreasing the tick burden on cattle, which will hinder the spread of resistant ticks and potentially lessen the transmission of tick-borne disease.

List of abbreviations

AMPA	α-amino3-hydroxy-5-methyl-4-isoxazolepropionic acid
αAOR	α-adrenergic-like octopamine receptor
βAOR	B-adrenergic-like octopamine receptor
AIT	Adult immersion test
AS-PCR	Allele-specific PCR
ABC transporters	ATP-binding cassette transporters
bp	Base pairs
CNS	Central nervous system
cAMP	Cyclic adenosine monophosphate
DNA	Deoxyribonucleic acid
°C	Degrees Celsius
ddH20	Double distilled water
EC	Eastern Cape
ELT	Egg laying test
ER	Estimated reproduction of adult females
FAM	Fluorescein amidites
FASTA	Fast all
FMD	Foot-and-Mouth Disease
gDNA	Genomic DNA
Gbp	Giga base pairs
GSTs	Glutathione-S-transferases
GPCR	G-protein coupled receptor
g	grams
HRM analysis	High resolution melt curve analysis
hrs	Hours
ITS2	Internal transcribed spacer 2
Kb	Kilobases
LIT	Larval immersion test
LPT	Larval packet test
LTT	Larval tarsal test
m/v	Mass per volume
Tm	Melting temperature
mRNA	Messenger RNA
µL	Microliters
µM	Micromolar
mg	Milligrams
mL	Millilitres
mM	Millimolar
Mya	Millions of years ago
MAFFT	Multiple sequence alignment fast fourier transform
NMDA	N-methyl-D-aspartate
ng	Nanograms
NCBI	National Centre for Biotechnology Information
NTC	No template control

N. i.	Not indicated
OCT/Tyr receptor	Octopamine/tyramine receptor
pmol	Picomole
PCR	Polymerase chain reaction
PCR-RFLP	Polymerase chain reaction restriction fragment length polymorphism
RET	Reproductive estimate test
RI	Reproductive index
RF	Resistance factor
R. microplus	*Rhipicephalus microplus*
R. decoloratus	*Rhipicephalus decoloratus*
RNAse	Ribonuclease
RNA	Ribonucleic acid
SNP	Single nucleotide polymorphism
Taq	*Thermus aquaticus*
tRNA	Transfer RNA
TAE	Tris-acetate EDTA
TE	Tris-EDTA
Tris	Tris(hydroxymethyl) aminomethane
USD	United States dollars
U	Units
VIC	Victoria
Zim	Zimbabwe

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Chapter 1

Rhipicephalus microplus ticks and amitraz resistance: a review

1. Ixodida ticks: taxonomy and evolution

Ticks are ectoparasites that affect a wide variety of vertebrates including humans, livestock, wildlife, birds, amphibians and reptiles through blood-sucking and the transmission of tick-borne diseases. For the latter, it has been proposed that ticks transmit a greater variety of infectious agents than any other arthropod vector (Wikel 2018; Walker 2003; Jongejan and Uilenberg 1994). Ticks are classified in the phylum Arthropoda, class Arachnida and order Ixodida (Sonenshine 1991). Under the order of Ixodida, there are three families namely Argasidae, Nutalliellidae and Ixodidae which consist of 193, 1 and 702 species respectively (Guglielmone *et al*. 2010; Horak *et al*. 2002). Argasid ticks are also referred to as soft ticks as they lack the hard scutum present in hard ticks (ixodid) (Sonenshine 1991). *Nuttalliella namaqua*, the only species in the Nutalliellidae family, has been described to be the missing link between the hard tick and the soft ticks because it possesses features of both argasid and ixodid ticks (Mans *et al*. 2013). A taxonomic overview of ticks is depicted in Figure 1 (Beati and Klompen 2018). As this study focuses on two *Rhipicephalus* species, all further discussion will be on the Ixodidae and subfamily Rhipicephalinae.

Ixodid ticks have been around for millions of years during which they have continuously evolved and adapted (Mans *et al.* 2011; Mans *et al.* 2012; Brites-Neto *et al.* 2015). A recent study on the phylogeography of ixodid ticks was performed in 2018 by Beati and Klompen to gain insight into the origin of this family. Their findings proposed that ixodid ticks are monophyletic with *Nutalliellidae namaqua* as a sister lineage to all other ticks. The Rhipicephalinae were also monophyletic (Figure 1) (Beati and Klompen 2018). In addition, it was found that the origin of Ixodida was at least 195 million years ago (mya) and that the Ixodidae separated from Argasidae 178 mya (Figure 2) (Beati and Klompen 2018). Rhipicephalinae split between the genus *Dermacentor* and the *Rhipicephalus-Hyalomma* clade approximately 36 mya (Beati and Klompen 2018). These results support other analyses based on 18S rDNA analyses (Black *et al*. 1997; Klompen *et al.* 2007; Klompen *et al.* 1996; Mans *et al.* 2012), but it should be noted that the conclusions that can be drawn from this data are limited as only one gene was examined. Furthermore, the current information on some tick genera is still incomplete with most sequences originating from Australia and South America. The use of fossil data would be most advantageous to evolutionary genetics studies, but due to the difficulties

associated with genomic DNA extraction and valid fossil calibration this data remains unavailable (Beati and Klompen 2018).

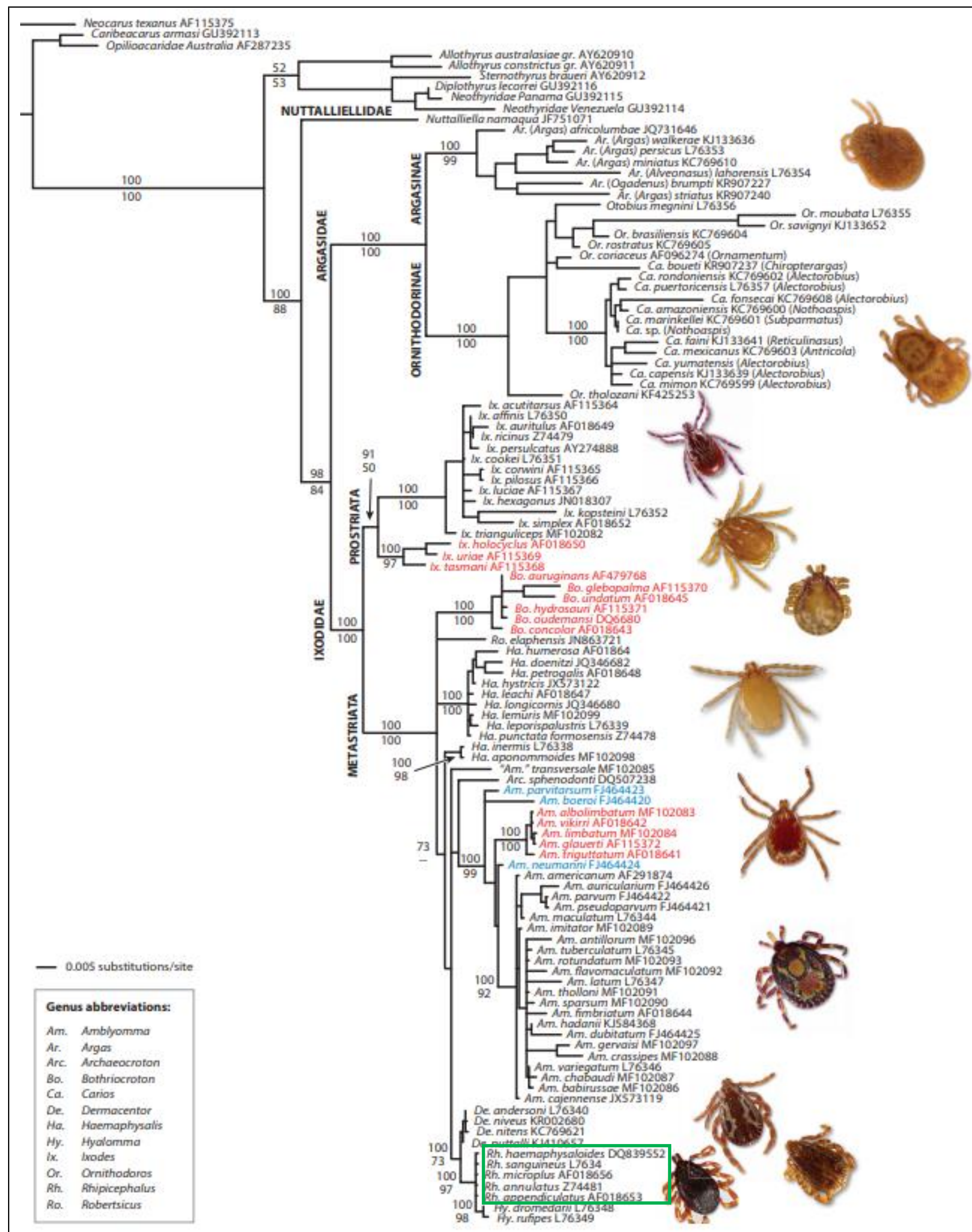


Figure 1: **Bayesian phylogeny of Ixodida based on the analysis of 18S rDNA sequences (taken from Beati and Klompen 2018).** Numbers above the branches indicate posterior probabilities and numbers below the branches indicate maximum parsimony bootstrap values. GenBank accession numbers are shown after the species names. Endemic Australian lineages are in red and South American lineages in blue. *Rhipicephalus* species are indicated in the green box.

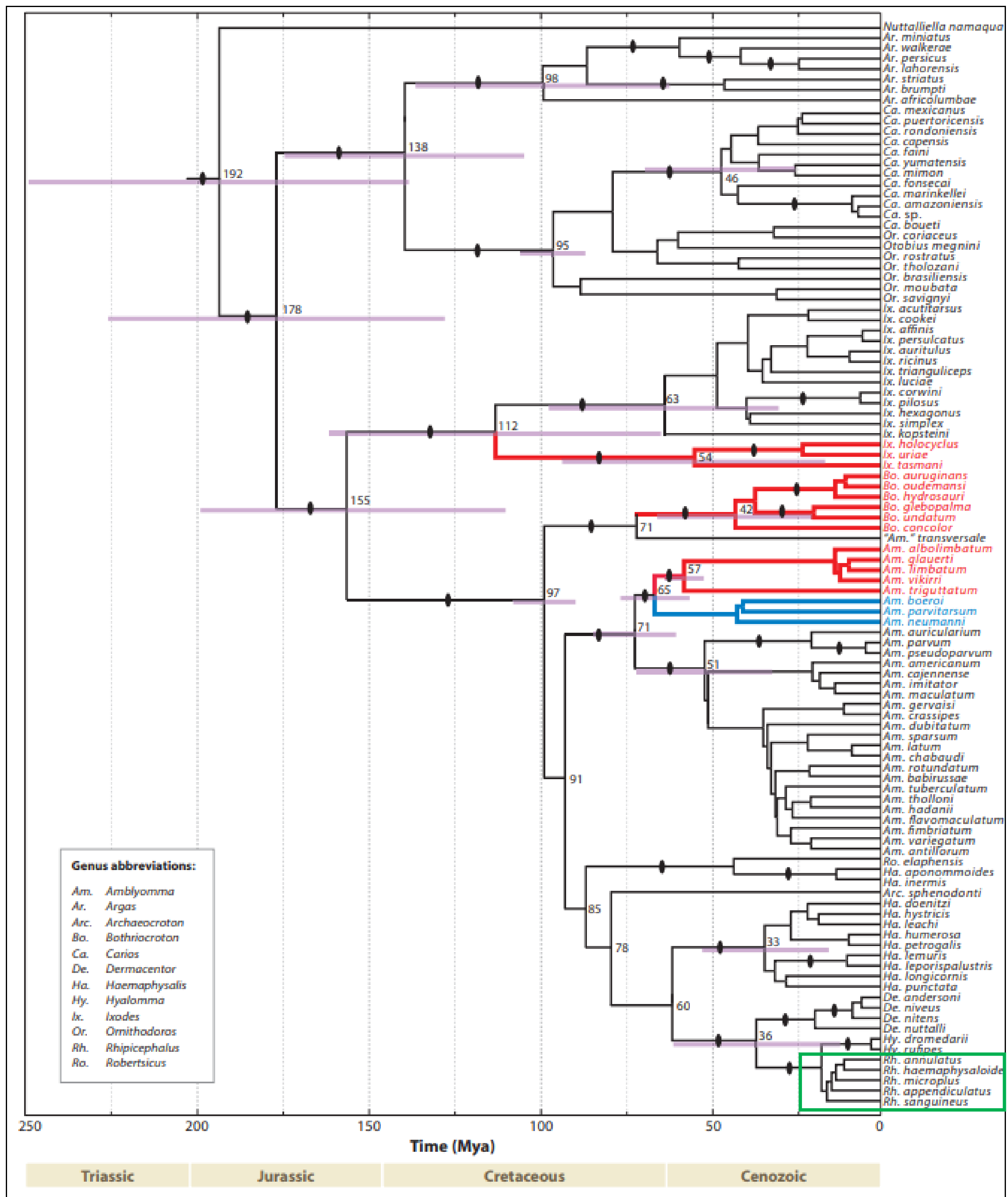


Figure 2: Node dating of Ixodida using BEAST (taken from Beati and Klompen 2018). A relaxed molecular clock was applied. Dates of radiation are indicated near the nodes. Black ovals= nodes supported by >90% posterior probability. Purple bars correspond to confidence intervals. Endemic Australian lineages are in red and South American lineages in blue. *Rhipicephalus* species are indicated in the green box.

2. The cattle tick: *R. microplus*

2.1. Introduction to the species

This focus of this study is the cattle tick *R. microplus* (also known as the southern cattle fever tick). *Rhipicephalus microplus* is classified under the genus *Rhipicephalus* (Figure 1 and 2), which consists of 82 different species (Guglielmone *et al.* 2010; Horak *et al.* 2002). *Rhipicephalus microplus* is a hard tick that appears almost blue in appearance and it is characterized by a dorsal shield (scutum) and a protruding hexagonal mouthpart (capitulum) (Figure 3).

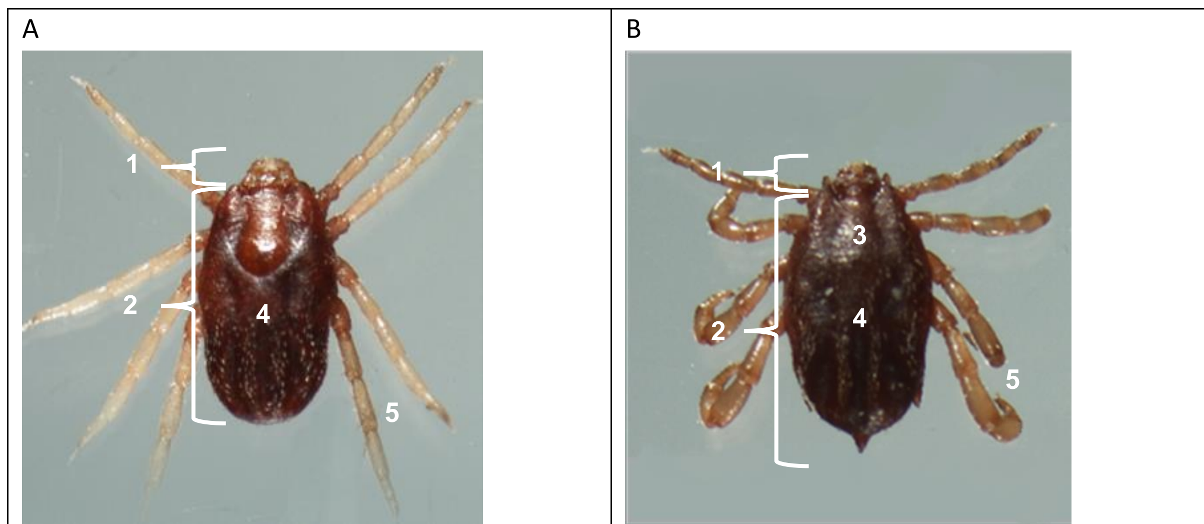


Figure 3: External dorsal view of a freshly moulted (A) female and (B) male *R. microplus* adult tick. Numbers correspond to the (1) capitulum, (2) idiosoma, (3) scutum, (4) alloscutum and (5) terminal segments of legs.

Phylogenetic analyses of *Rhipicephalus* ticks show a species complex of *R. annulatus*, *R. australis* and two clades of *R. microplus* (Figure 4) (Burger *et al*. 2014). The two *R. microplus* clades, also known as the "*R. microplus* complex" contains *R. microplus* species from Southern China and Northern India (clade B), which are more closely related to *R. annulatus* than to *R. microplus* species from Asia, South America and Africa (clade A) (Burger *et al*. 2014). This suggests that the species from Southern China and Northern India may be a cryptic species (Burger *et al*. 2014), but additional studies are needed to confirm these findings as only a very limited number of geographical areas were included.

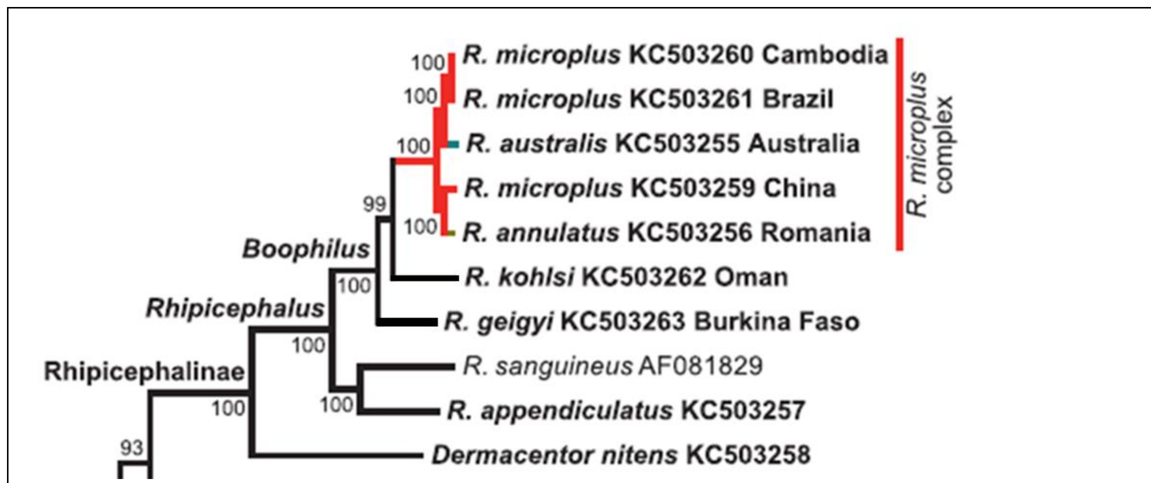


Figure 4: Maximum likelihood tree inferred from mitochondrial genome sequences (adapted from Burger *et al*. 2014). The clade containing the *R. microplus* complex is indicated in red where *R. australis* is indicated in blue and *R. annulatus* is indicated in green.

Rhipicephalus microplus is found in South, East and South-East Asia as well as Australia, countries in East and Southern Africa and South and Central America (Burger *et al*. 2014). In South Africa, *R. microplus* and *R. decoloratus* are present in every province except for the Northern Cape, with acaricide resistant ticks already described in Limpopo, Mpumalanga, Free State and KwaZulu-Natal (RuVASA Report January 2019). *Rhipicephalus microplus* and *R. decoloratus* transmit redwater and anaplasmosis. *Rhipicephalus microplus* transmits both severe/lethal Asiatic babesiosis (also known as redwater) as well as endemic babesiosis (African redwater) which is less severe and not associated with high morbidity and mortality. *R. decoloratus* only transmits African redwater. Asiatic redwater has been detected in every province in South Africa (except for the Northern Cape) (RuVASA Report January 2019). The expansion of *R. microplus* in South Africa is likely due to a number of reasons, including climate change, the uncontrolled movement of cattle and adaptation to new habitats (Nyangiwe *et al*. 2017). In addition, literature reflects that *R. microplus* is increasingly displacing *R. decoloratus* (Horak *et al*. 2009; Nyangiwe *et al*. 2013; Tønnesen *et al*. 2004; Yawa *et al*. 2019), which is likely due to *R. microplus* having a more rapid lifecycle and thus allowing for the outbreeding of *R. decoloratus* (discussed below). This emphasises the economic importance of studying *R. microplus* ticks and highlights the need to develop effective tick control strategies to prevent the spread of acaricide resistant tick populations.

2.2. Economic importance

Rhipicephalus microplus is considered to be the most economically important ectoparasite of livestock in the world (Cunha *et al.* 2012). This can be ascribed to a consequence of tick burden, where both

direct and indirect effects are incurred. Direct effects due to tick feeding include weight loss, leather hide damage, secondary infections at the feeding sites as well as a reduction in meat and milk production (Benitez *et al.* 2012). Indirect effects include the transmission of protozoan, rickettsial and viral diseases as well as the cost of tick control measures (Benitez *et al.* 2012; Madder 2018). In 2016 it was estimated that global losses in the cattle industry caused by *R. microplus* are 22–30 billion US dollars (USD) per annum (Lew-Tabor and Valle 2016). This amount was extrapolated from the 1996 cost estimate per head of cattle for control expenses and damages which was 13.9-18.7 USD (de Castro 1997). Considering an inflation rate of 52.3% from 1996-2015, and the estimated world cattle population in 2016 which was 1.47 billion, the figure of 22-30 billion USD was thus calculated (Lew-Tabor and Valle 2016). It should, however, be noted that this amount may not be entirely accurate as it is an extrapolation from costs estimated in the 1960's which only included direct costs and not indirect costs. A more representative amount remains to be calculated.

2.3. *Rhipicephalus microplus*: a vector of high morbidity and mortality diseases

Babesia species are economically the most significant agricultural pathogen transmitted by arthropods in the world. *R. microplus* is a vector for the transmission of two *Babesia* species which are the causative agents for bovine babesiosis, also commonly referred to as cattle fever or redwater (Ristic 1981; Underwood 2015; Almazan *et al*. 2018). Asiatic babesiosis is caused by the transmission of *Babesia bovis* while African redwater is caused by *B. bigemina*, both being intraerythrocytic protozoan parasites (Underwood 2015; Ristic 1981). The most severe and commonly lethal form of babesiosis (Asiatic redwater) is caused by *B. bovis* (Guerrero *et al* 2014; Ristic 1981), while African babesiosis is considered a mild form of disease and is associated with endemic stability in South Africa (Mtshali and Mtshali 2013). The lifecycle of *Babesia* parasites can be summarised as follows: the parasites are transmitted to the blood of the host when infected ticks acquire their bloodmeal, the parasites subsequently infect and replicate within the erythrocytes of the host, which finally leads to cell lysis when new parasites are released (Chauvin *et al*. 2009). The excessive breakdown of erythrocytes causes bilirubinaemia, haemoglobinuria as well as liver and kidney failure (Underwood 2015). Acute encephalitis may also occur but less commonly, where symptoms include: fever, ataxia, depression, mania, convulsions, coma and death in immunocompromised cattle (Underwood 2015).

Rhipicephalus microplus is also a vector for the transmission of bovine anaplasmosis, also known as gall sickness or yellow fever (Aubrey and Geale 2010). Bovine anaplasmosis is caused by an array of *Anaplasma* species which are rickettsial parasites that also infect red blood cells (Ristic 1977). *Rhipicephalus microplus* is associated with the transmission of *A. marginale* which occurs virtually in

all regions of the world including South Africa, Asia, Australia, Europe, South America and North America (Hove *et al.* 2018). Infected red blood cells are subsequently phagocytosed by the bovine reticuloendothelial system, which results in clinical symptoms such as jaundice and severe anaemia (Ristic 1977; Potgieter and Stoltsz 2004). Other symptoms may include fever, weight loss, abortion, lethargy and death (Kocan *et al.* 2003). Anaplasmosis is typically treated with antibiotics and with the growing number of reports on antibiotic resistance in cattle this is of great concern (Almazan *et al.* 2018). The frequent use of antibiotics has also resulted in reduced acceptance of meat and milk in the South African export market, resulting in further capital losses to farmers (Smit 2008; RuVASA Report March 2017).

In addition, limited studies have proposed that *R. microplus* may be a vector for the transmission of bovine borreliosis (Smith *et al*. 1978; Callow 1967). Bovine borreliosis is caused by the transmission of the spirochete *Borrelia theileri* (Smith *et al*. 1978; Callow 1967), resulting in symptoms such as mild fever and occasional mild depression with anorexia and anaemia (Smith *et al*. 1978; Callow 1967; McCoy *et al*. 2014). However, it should be noted that the transmission of *B. theileri* by *R. microplus* is mainly reported by studies conducted in the 1980's and prior. More recently, only one paper was published by Cordeiro *et al*. in 2018 where a morphological, molecular and phylogenetic characterization of *B. theileri* was obtained from infected *R. microplus* in Brazil. This study reported an infection rate of 2%, but only one animal was investigated and thus these findings cannot be considered as being statistically significant. Ultimately, there is a lack of validated research for the transmission of *B. theileri* by *R. microplus* and it cannot be determined whether this is a rare occurrence or if it has simply been under-reported. The lack of studies could potentially be due to the low pathogenicity of *B. theileri* in cattle, thus leading to it being perceived as less urgent than studying the transmission of the more concerning *Babesia* and *Anaplasma* species.

Ultimately, the combined disease-vector capabilities of *R. microplus* demonstrate the potential impact that infestations can have on livestock health, with emphasis on *B. bovis* infection due to high morbidity associated with this disease. Currently, Asiatic redwater has been reported in every province in South Africa (RuVASA Report January 2019) which is thus of great concern and emphasizes the need for effective tick control strategies to reduce tick load and hinder the spread of disease.

2.4. Lifecyle and mating structure of *R. microplus*: A driver for displacing endemic *R. decoloratus*?

Rhipicephalus ticks, being classified as hard ticks, have three life stages: larvae, nymph and adult (Jongejan and Uilenberg 1994). Both *R. microplus* and its closely related species *R. decoloratus* have one-host lifecycles, which means that all stages of the lifecycle are spent on one animal (Jongejan and Uilenberg 1994). During this lifecycle, the tick takes one blood meal at each life stage where it needs to ingest enough blood to moult to the next life stage (Jongejan and Uilenberg 1994). After the adult ticks obtained their final bloodmeal, the males become sexually mature and mate with females (Sonenshine 1991). After mating, the engorged females drop to lay eggs and the males usually remain on the host to mate with other females (Sonenshine 1991; Jongejan and Uilenberg 1994). The lifecycle of *R. microplus* is rapid, where three weeks of feeding takes place, followed by one month for egg laying and hatching where approximately 3000 eggs are laid at a time (Barker and Walker 2014; Walker 2003).

Evidence for *Rhipicephalus microplus* displacing the native tick species in South Africa, *R. decoloratus* is growing and of great concern due to its ability to transmit *B. bovis* (Horak *et al*. 2009; Nyangiwe *et al.* 2013; Tønnesen *et al.* 2004; Yawa *et al.* 2019). It is hypothesized that this is due to the shorter lifecycle and higher egg production of *R. microplus* that allows for the outbreeding of *R. decoloratus,* which can take up to two months for egg laying (± 2500 eggs) and hatching (Walker 2003). *Rhipicephalus microplus* ticks may also outcompete *R. decoloratus* due to mating factors. For instance, *R. microplus* ticks have a pangamic mating structure which has not been observed in *R. decoloratus* ticks, where one *R. microplus* female can mate with multiple males (Chevillon *et al*. 2007b; Cutullé *et al.* 2010). This pangamic mating structure allows for greater genetic diversity in *R. microplus* populations. In addition, cross mating between *R. microplus* and *R. decoloratus* is also possible (Spickett and Malan 1978; Tønnesen *et al*. 2004; Horak *et al.* 2013), although male ticks preferentially mate with females from their own species (Norval and Sutherst 1986). In most cases *R. microplus* males have been found to mate with female *R. decoloratus* ticks (Tønnesen *et al*. 2004; Horak *et al.* 2013). This is possibly due to *R. microplus* males becoming sexually mature a few days earlier than *R. decoloratus* males (Londt and Arthur 1975), providing them with a competitive mating advantage over *R. decoloratus* males in cases of co-infestation of a host. It was, however, reported that the mating of *R. decoloratus* females with *R. microplus* males results in the production of sterile eggs (Spickett and Malan 1978). This would further allow for the displacement of *R. decoloratus* (Horak *et al.* 2009).

It should, however, be noted that more recent research has proposed that hybridization may occur between *R. microplus* and *R. decoloratus* tick species and that the eggs resulting from cross mating may not always be sterile. For instance, two separate studies conducted in the Eastern Cape in South Africa identified larvae which exhibited characteristics of both species, i.e. *R. microplus x R. decoloratus* hybrids (Nyangiwe *et al.* 2013; Nyangiwe *et al.* 2017). The ability of *R. microplus* to hybridize with other species has also been reported between *R. annulatus* and *R. microplus* where hybrids were reared to adults, but they were found to be infertile (Davey and Hillburn 1991). If hybridization between *R. microplus* and *R. decoloratus* does in fact occur, this may also provide a potential explanation for the adaptation and spread of *R. microplus* in South Africa, as they could potentially assimilate characteristics from *R. decoloratus* populations. However, further research into *R. microplus x R. decoloratus* hybrids is required to validate this theory as no adult hybrids have been collected and no molecular characterization of hybrids has been conducted.

2.5. Characteristics of the genome of *R. microplus*

Although ticks may appear to be simple organisms, their genomes are challenging to sequence, assemble and annotate due to their large size and complexity. Tick genomes typically range from 1.04 to 7.1×10^9 base pairs in size and contain a high number of transposable elements and tandem repeats (Valle and Guerrero 2018). Despite the recent evolution of the *Rhipicephalus* species (Figure 2), the total genome size of *R. microplus* is estimated to be approximately 7.1 Gbp (Barrero *et al*. 2017). The current *R. microplus* genome assembly, however, only comprises of a mere 2.0 Gbp. This contributes to the lack of resolution of the *Rhipicephalus* phylogeny (Figure 4). The current *R. microplus* draft genome assembly contains 51.46% repetitive elements, and it was estimated that the total genome would contain approximately 70% repetitive DNA (Barrero *et al*. 2017). These repetitive elements could potentially be beneficial to *R. microplus* by allowing for mutations, such as in genes encoding for acaricide target molecules (discussed below), without always having the decreased cost benefit that is generally associated with mutation. Furthermore, the *R. microplus* draft genome assembly identified 24,758 protein coding genes. A comparison of shared protein families between 12 different species was performed and identified 1,697 protein families and 10,835 proteins unique to *R. microplus* (Barrero *et al.* 2017). It should also be considered that this number is indicative only of annotatable proteins in the partial genome assembly, and that there is likely an even higher number of proteins unique to *R. microplus*, further demonstrating the complexity and uniqueness of the *R. microplus* genome.

3. Acaricide resistance in ticks

3.1. Background and basic principles

Globally, the most common method of tick control in livestock is the use of chemical acaricides as they are cost-effective and act relatively quickly (Guerrero *et al*. 2012a). Almost all acaricides are neurotoxins (except for growth regulators) that cause paralysis and death. Acaricides act on ectoparasites either systemically through the ingestion of the chemical from host tissues (Rodriguez-Vivas *et al*. 2014a) or through physical exposure to the chemical once it has been applied externally to the host (Taylor 2001). Methods of acaricide application to cattle include: dipping stations, spray guns, injections (macrocyclic lactones only), ingestion through an intraruminal bolus or as an ear tag (George *et al*. 2004).

However, the intensive use of acaricides has resulted in tick populations globally that are resistant to all major classes of chemical acaricides, including organochlorines, synthetic pyrethroids, organophosphates, amidines, phenylpyrazoles and macrocyclic lactones (Rodriguez-Vivas *et al.* 2018). In Figure 5, the global extent of acaricide resistance in *R. microplus* is shown.

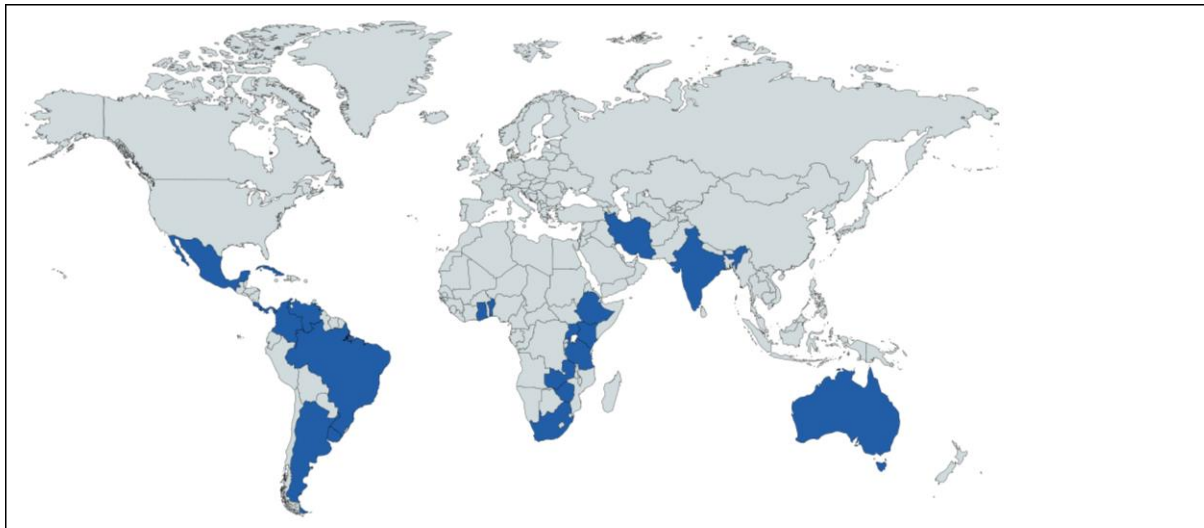


Figure 5: The geographic distribution of acaricide resistant *R. microplus* populations (adapted from Rodriguez-Vivas *et al*. 2018). In blue the countries with reported acaricide resistance are indicated.

In 1992 the World Health Organization defined resistance in arthropods as “an inherited characteristic that imparts an increased tolerance to a pesticide, or group of pesticides, such that the resistant individuals survive a concentration of the compound(s) that would normally be lethal to the species.” It should, however, be noted that there are different types of acaricide resistance. Firstly, a distinction should be made between phenotypic and genotypic resistance. Phenotypic resistance is the level of resistance that a tick displays to the application of an acaricide, and genotypic resistance is the

genotype of the tick which causes the resistant phenotype (Guerrero *et al*. 2014b). It is therefore possible that one resistant phenotype can be caused by multiple different genotypes (Guerrero *et al*. 2014b).

Secondly, there is acquired acaricide resistance, cross-resistance and multiple resistance. Acquired resistance is defined as "resistance that results from heritable decreases in sensitivity to drugs with the passage of time" (Abbas *et al*. 2014). Therefore, acquired resistance is a genetic characteristic of a population that is driven via continued exposure to the same active ingredient in an acaricide (Chapman 1997; Meyer *et al*. 2012). Cross-resistance occurs when the continued exposure of a population to one acaricide results in the selection of mutations that also cause phenotypic resistance to a different acaricide (Rodrigues-Vivas *et al.* 2018; Abbas *et al.* 2014). For example, both carbamates and organophosphates inhibit acetylcholinesterase, and acetylcholinesterase insensitivity results in resistance to both acaricides in multiple strains of *R. microplus* (Li *et al*. 2005; Madder *et al*. 2011; Perez-Gonzalez *et al*. 2014). Multiple resistance, on the other hand, occurs when ticks express multiple resistance mechanisms and are resistant to two or more acaricides, even if they have different targets (Rodriguez-Vivas *et al*. 2018; Abbas *et al*. 2014). For example, strains of *R. microplus* in Mexico have exhibited resistance to chlorinated hydrocarbons, pyrethroids, organophosphates and formamidines (Foil *et al.* 2004). Target site mutations (discussed below) were found in these strains, but it is also possible that the resistance may be due to metabolic detoxification (discussed below) (Sammataro *et al*. 2015; Bielza *et al*. 2007). Further examples of studies where cross-resistance and multiple resistance were found are discussed and summarized in the review by Rodriguez-Vivas *et al.* (2018).

3.2. Detection of acaricide resistant ticks

In vitro bioassays: evaluating dose responses

The most widely used method to detect acaricide resistance is via the use of *in vitro* bioassays (Rodriguez-Vivas *et al*. 2018), which generally involve the evaluation of dose responses to acaricides. Briefly, ticks are exposed to serial dilutions of an acaricide and the lethal concentration (LC) required to kill 50% (LC_{50}), 90% (LC_{90}) and/or 99% (LC_{99}) of the population is determined (Robertson *et al.* 2007). The resistance factor (RF) is then calculated as the LC value of the tested sample divided by the LC value of the reference strain, where the reference strain is a strain of ticks that is susceptible to the acaricide being tested (FAO 1987). For instance, RF_{50} is the RF measured at LC_{50}, RF_{90} is the RF measured at LC_{90} and RF_{99} is the RF measured at LC_{99}.

There are different criteria that have been proposed with regards to RF values when considering a tick population to be resistant or susceptible to a specific acaricide active ingredient. For instance, Beugnet and Chardonnet 1995 consider tick populations to be susceptible when RF50 is <3, tolerant when RF50 is 3-5 and resistant when RF50 is $\geq$5. For ivermectin resistance, Castro-Janer *et al.* 2011 proposed that tick populations are susceptible when RF50 ≤ 1, have low resistance when RF50 > 1 ≤ 2 and resistant when RF50 > 2. Furthermore, considering that synthetic pyrethroid ratios have generally been found to be higher when compared with other acaricides, Rodriguez-Vivas *et al.* 2012 recommended different criteria for synthetic pyrethroids. These criteria are as follows: both the LC50 and LC99 values should be determined, where populations are susceptible when both RF values (RF50 + LC99) are <3, tolerant when both RF values are 3-5 and resistant when both RF values are $\geq$5. Despite the various recommendations, there continues to be large inter-population variation in phenotypic acaricide resistance reported worldwide (Table 1), which highlights inconsistency and inaccuracy in the use of bioassays to detect acaricide resistance.

Table 1: A table summarizing reports of amitraz resistance around the world.

Organism:	Location:	Level of Resistance:	Test used:	Reference:
R. microplus	Argentina	RF_{50}= 0.7- 32.5 RF_{99}= 0.1- 4.3	LTT	Lovis *et al*. 2013
R. microplus	Argentina	$RF_{50=}$ 57 and 32.5	LTT	Cutullé et al. 2013
R. microplus	Australia	6 out of 58 farms resistant	LPT	Jonsson *et al*. 2000
R. microplus	Benin	RF_{50}= >10 RF_{99}= >10	LPT	Adehan *et al*. 2016
R. microplus	Brazil	RF_{50}= 1.68- 4.58	LPT	Li *et al*. 2004
R. microplus	Brazil	ER= 0-100%	AIT	Andreotti *et al*. 2011
R. microplus	Brazil	RF_{50}= 2.14- 132	LIT	Mendes *et al*. 2013
R. microplus	Brazil	RF_{50}= 11.907	LPT	Reck *et al.* 2014
R. microplus	Brazil	76.92% of samples resistant	LPT	Klafke *et al*. 2016
R. microplus	Colombia	RF_{50}= 69.99 (LPT) RF_{50}= 15.27 (AIT)	LPT & AIT	Benavides 2006
R. microplus	Colombia	14 and 56% efficacies (RI)	AIT	Lopez-Arias *et al*. 2014
R. microplus	Colombia	97% of samples resistant	AIT	Araque *et al*. 2014
R. microplus	Colombia	10-20% efficacy (RI)	AIT	Puerta *et al*. 2015
R. microplus	Colombia	10-50% efficacy (RI)	AIT	Villar *et al*. 2016
R. microplus	Costa Rica	50% of samples resistant	LIT	Alvarez and Hernandez 2010
R. microplus	India	RF_{95}= 24.78	AIT	Singh *et al*. 2015
Rhipicephalus species	Ghana	80.8% mean mortality	LPT	Kaljouw 2009

R. microplus	Mexico	RF_{99}= 41.9	LIT	Soberanes *et al.* 2002
R. microplus	Mexico	19.4% of samples resistant	LIT	Rodriguez-Vivas *et al*. 2006b
R. microplus	Mexico	RF_{50}= 1.0-23.0	LIT	Rosado-Aguilar *et al*. 2008
R. microplus	Mexico	13.4- 82.7% larval mortality	LPT	Olivares-Pérez *et al.* 2011
R. microplus	Mexico	54.7% of samples resistant	LIT	Fernandez-Salas *et al.* 2012
R. microplus	Mexico	RF_{50}= 2.3	LPT	Rodriguez-Vivas *et al*. 2013
R. microplus	New Caledonia	RF_{50}= 0.2- 9.2 RF_{95}= 0.3- 72	LPT	Chevillon *et al.* 2007a
R. microplus	New Caledonia	RF_{50}= 1.18- 8.09 RF_{90}= 2.57- 29.4	LPT	Ducornez *et al*. 2005
R. microplus	South Africa	2/59 dip tanks emerging resistance; 1/59 dip tanks resistant.	LIT	Ntondini *et al*. 2008
R. microplus	South Africa	RF= 10-100	LPT	Baron *et al*. 2015
R. microplus	South Africa	Frequency of heterozygous resistant genotypes= 0.5161; Frequency of homozygous resistant genotypes= 0.0322.	AS-PCR	Robbertse *et al.* 2016
R. decoloratus	South Africa	N.i.	RET & ELT	Mekonnen *et al.* 2003
R. appendiculatus and *R. decoloratus*	Uganda	12.9% of samples resistant	LPT	Vudriko *et al*. 2016
R. microplus	Uruguay	2009 and 2010 populations resistant.	LPT & LIT	Cuore and Solari 2014
R. microplus	USA	RF_{50}= 1.0- 4.5	LPT	Li *et al*. 2004
R. microplus	Venezuela	N.i.	AIT	Coronado 1999
R. microplus	Zambia	The test population had a lower susceptibility (LD50 0.014 %; LD90 0.023 %) than the reference population (LD50 0.013 %; LD90 0.020 %).	LPT	Muyobela *et al.* 2015
R. microplus	Zimbabwe	Frequency of mutant allele= 0.55; Frequency of heterozygous genotypes= 0.785 (288/367); Frequency of homozygous resistant genotypes= 0.378 (39/103);	AS-PCR	Sungirai *et al.* 2018

RF_{50}= resistance factor at 50%; RF_{95}= resistance factor at 95%; RF_{99}= resistance factor at 99%; ER= estimated reproduction of adult females; RI= reproductive index; LPT= larval packet test; LIT= larval immersion test; AIT= adult immersion test; LTT= larval tarsal test; RET= reproductive estimate test; ELT= egg laying test; AS-PCR= allele-specific PCR; N.i.= not indicated.

Types of *in vitro* bioassays and their associated disadvantages

The first bioassay developed was the larval packet test (LPT) in 1962 by Stone and Haydock, where tick larvae are exposed to chemically impregnated filter papers and their subsequent mortality is determined after 24 hours. Secondly, the larval immersion test (LIT) was developed in 1966 by Shaw, which involves immersing tick larvae (approximately seven to ten days old) in different dilutions of an acaricide and their mortality is determined after 24 hours. Thirdly, the adult immersion test (AIT) developed in 1973 by Drummond *et al*. entails the treatment of engorged female ticks with a dilution range of an acaricide which is compared to untreated ticks in order to assess the dose mortality responses of the ticks as well as the effects of the treatment on fecundity and fertility. More recently, the larval tarsal test (LTT) was developed in 2011 by Lovis *et al*. to be more sensitive and time-effective than the LPT. The LTT has comparable resistance ratios with the LPT, but the methodology is more simplified as it does not involve the handling of larvae and multi-well plates, and resistance to multiple acaricides can be determined at a time (Lovis *et al*. 2011).

Although these bioassays are portable and have low costs, they are associated with many disadvantages. For instance, the LPT is labour-intensive and it takes up to 6 weeks to obtain results (Guerrero *et al*. 2014b). Even though the LTT has simplified the testing process for the LPT, initial testing results are still only obtained after approximately three weeks, whereafter tests may still be repeated in triplicate to obtain wider mortality ranges. In addition, the AIT has been shown to be inaccurate due to large differences in LC values with wide confidence intervals (Jonsson *et al*. 2007). In addition, these bioassays only diagnose the resistance phenotype of a tick population and provide no information about the resistance genotype (Rodriguez-Vivas *et al*. 2018). This does not allow for a distinction to be made between homozygous or heterozygous resistant genotypes for acaricide resistance. Consequently, information cannot be provided about whether resistance may be emerging in a tick population as would be evident when heterozygous individuals are present (Abbas *et al*. 2014). This ultimately prevents farmers from making timely and informed choices about acaricide management and/or integrated control strategies to prevent acaricide resistance from occurring.

Synergistic assays: detecting metabolic enzymes in acaricide resistance

Synergistic assays have also been used in conjunction with LPTs to detect the involvement of metabolic enzymes in acaricide resistance. Examples of synergists include: triphenyl phosphate (TPP) which inhibits esterase activity, piperonyl butoxide (PBO) which inhibits cytochrome p450 oxidase and diethylmaleate (DEM) which inhibits glutathione-S-transferases (Li *et al*. 2007; Guerrero *et al*. 2012a).

However, synergistic assays are also associated with disadvantages. For instance, results are also only obtained after 6 weeks. In addition, although synergistic assays have proven successful in identifying the activity of metabolic enzymes, these enzymes are also involved in other cellular mechanisms and expression levels may therefore be elevated in response to other influencing factors, not only acaricide exposure. Consequently, synergistic assays alone cannot be used to detect resistance. However, synergistic assays are still useful as they can be used in conjunction with other resistance detection methods such as bioassays or PCR-based assays (discussed below) to monitor the expression levels of metabolic enzymes and to contribute to our understanding of the mechanisms of acaricide resistance.

PCR-based assays and associated benefits

Due to the disadvantages associated with bioassays, a more rapid and accurate approach is needed for acaricide resistance detection. PCR-based assays have many advantages over *in vitro* bioassays and have proven to be useful molecular tools in detecting the resistance status of ticks. PCR-based assays are being designed to be completed within a day, where the specific resistance mechanism of ticks (discussed below) can be determined and the assay can be performed from a single tick. These assays are generally designed around the detection of validated SNPs (single nucleotide polymorphisms) that are associated with target site resistance (discussed below). Screening for SNPs is a more effective way to rapidly genotype acaricide resistance-associated mutations in tick populations that does not require live ticks (Aguilar *et al*. 2018; Sungirai *et al.* 2018) and ticks can be screened at any life stage which reduces the time and cost associated with completing life cycles in the laboratory (Morgan *et al.* 2009). Examples of PCR-based assays to detect target site resistance have been developed for synthetic pyrethroids in *R. microplus* (Guerrero and Pruett 2003) as well as other species such as *Spodoptera frugiperdan* (fall armyworm) (Carvalho *et al.* 2013) and *Varroa destructor* (parasitic mite of honey bees) (Millán-Leiva *et al*. 2018).

The Ticks and Tick-borne Diseases research group at the University of Pretoria has also made significant progress in the detection of SNPs associated with acaricide resistance and has already reduced diagnostic testing to two days per sample (Baron *et al.* 2015; Baron *et al*. 2018; Robbertse *et al.* 2016; van Wyk *et al*. 2016). For instance, Baron *et al.* 2015 used allele-specific PCR (AS-PCR) as well as a quick and affordable restriction fragment length polymorphism (RFLP) based diagnostic technique to assess amitraz resistance in *R. microplus* field samples in South Africa. In addition, Robbertse *et al*. 2016 determined both amitraz and pyrethroid resistance status in South African *R. microplus* field

strains from 12 dip stations using AS-PCR to detect SNPs. Sungirai *et al.* (2018) also genotyped *R. microplus* populations in Zimbabwe for amitraz, pyrethroid and organophosphate resistance using molecular markers and the same method described by Baron *et al.* (2015) for detecting amitraz resistance.

The main disadvantage of PCR-based assays is that they require more advanced equipment to conduct tests in contrast to bioassays. In addition, PCR-based assays can only detect resistance that is linked to known mutations and no information is provided on metabolic resistance or the resistance phenotype. However, bioassays can be used in conjunction with PCR-based assays to confirm acaricide resistance in tick field populations and synergistic assays can also be implemented to identify metabolic resistance. This will ultimately allow for the genotypic as well as the phenotypic resistance of *R. microplus* ticks to be determined, which will enable the laboratory to advise farmers on the correct acaricides to purchase as well as on effective long-term acaricide management strategies.

Quantitative PCR-based assays

Quantitative PCR-based assays have previously been implemented in diagnostic screening. For instance, Klafke *et al*. (2019) recently designed a novel method for the detection of SNPs in the *para*-sodium channel gene of *R. microplus* that are associated with pyrethroid resistance. This method is based on quantitative PCR high-resolution melt (HRM) analysis. Although this method was successful in rapidly detecting different genotypes, it may not be suitable for high-throughput screening. For instance, the fluorescence difference that was observed between different genotypes (homozygous, heterozygous and wild type) in the melt curves obtained with the HRM assays was in many cases very close, with differences in the curves occurring only between intervals of 2°C. This could potentially allow for large margins of error should this test be upscaled for screening in *R. microplus* outbreak populations.

Another example of qPCR-based assays that have been used for the diagnostic detection of resistance-associated SNPs are TaqMan SNP genotyping assays. For instance, Morgan *et al.* (2009) developed a diagnostic qPCR assay using allele-specific TaqMan minor groove-binding (MGB) probes to detect a SNP in the para-sodium channel gene that is associated with resistance to synthetic pyrethroid acaricides. To date, there is no TaqMan SNP genotyping assays that have been developed for the detection of the two amitraz-resistance associated SNPs in the OCT/Tyr gene.

TaqMan SNP genotyping assays: the future of diagnostic screening?

TaqMan SNP genotyping assays could prove to be a promising way forward for the rapid and high throughput detection of acaricide resistance-associated mutations in *R. microplus* ticks. TaqMan SNP genotyping assays contain two primers for specific amplification of a gene fragment containing the SNP of interest and two TaqMan minor-groove binding (MGB) probes for the detection of the SNP alleles (Morgan et al. 2009; Hartsthorne 2013). Each probe is fluorescently labelled with a reporter dye at the 5' end, either VIC (Victoria) or FAM (Fluorescein amidites), where the two probes will have different labels (Hartsthorne 2013). For instance, the allele 1 probe will be labelled with VIC and the allele 2 probe will be labelled with FAM. At the 3' end each probe will have an MGB as well as a nonfluorescent quencher (Hartsthorne 2013). The MGB binds to the minor groove of the DNA molecule which increases the probe melting temperature (T_m) (Hartsthorne 2013) and enhances the probe hybridization efficiency (de Kok *et al.* 2002; Latif *et al.* 2006) by stabilizing the binding of the probe to the template DNA. This ultimately allows for the design of shorter probes and improved allelic discrimination. The quencher suppresses the reporter dye fluorescence when the probe is intact, primarily by Förster-type energy transfer (ThermoFisher Scientific 2017).

Both probes are then multiplexed in a qPCR reaction (Morgan *et al.* 2009). During polymerization, one of the TaqMan probes will anneal specifically to the complementary target sequence and the reporter dye will be cleaved from the probe by the 5' nuclease activity of the *Taq* polymerase (Hartsthorne 2013; Walker *et al.* 2007; ThermoFisher Scientific 2017). The separation of the reporter dye from the quencher results in the fluorescence of the reporter dye, which will increase as PCR product accumulates in the qPCR reaction (Walker *et al.* 2007; ThermoFisher Scientific 2017). Only probes that hybridize to the target sequence will be cleaved and the probe that is not complementary will remain intact. The amount of fluorescence of one fluorophore relative to the other becomes an X, Y bivariate, of which the values indicate the likelihood of one or both of the genotypes being present in the sample (De la Vega *et al.* 2005; Callegaro *et al.* 2006). Statistical algorithms classify the fluorescence values to the correct allele utilising cluster analysis and maximum likelihood estimation, allowing for the genotype "calling" of the alleles (Callegaro *et al.* 2006). The ultimately allows for allele-specific discrimination of a single base pair (Hawthorne 2013; Callegaro *et al.* 2006). This process is summarized in Figure 6.

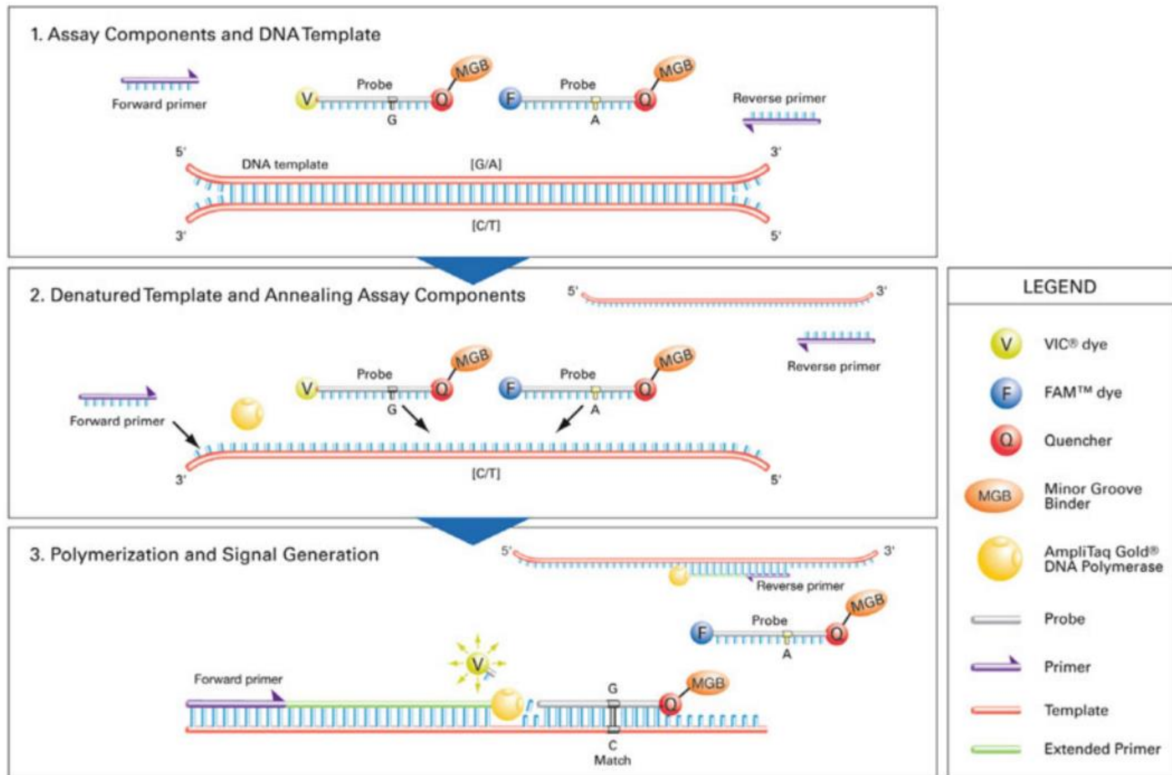


Figure 6: Schematic presentation of the principles underlying a TaqMan SNP genotyping assay (taken from Hartsthorne 2013). (1) The assay components are combined with the target double-stranded DNA template where the SNP of interest is indicated in brackets. (2) During the initial denaturation step, the single-stranded DNA template is accessible for the annealing of the complementary primers and probes. Each probe is fluorescently labelled with a reporter dye at the 5' end and an MGB and a nonfluorescent quencher at the 3' end. (3) PCR-mediated synthesis of the second DNA strand is performed where the *Taq* polymerase amplifies the target gene using the forward primer as a starting point, thus forming the extended primer. One of the probes will hybridize to a complementary sequence between the gene-specific forward and reverse primers. During DNA elongation, the reporter dye will be cleaved from the probe by the *Taq* polymerase resulting in a fluorescent signal that will increase as PCR product accumulates. This will ultimately allow for allele-specific discrimination.

The qPCR reactions containing the TaqMan SNP genotyping assay/s can be conducted on the QuantStudio® 12K Flex Real-Time PCR System (ThermoFisher Scientific, USA), which will allow for up to 384 samples to be genotyped for SNPs simultaneously within hours. This screening platform can also potentially be upscaled to a TaqMan® OpenArray® platform where over 110,000 samples can be genotyped in a day.

The QuantStudio® 12K Flex v1.3 software (ThermoFisher Scientific, USA) records and interprets the fluorescent signals generated by the qPCR reactions. This allows for the analysis of the data in a variety of ways, including but not limited to: the amplification of each PCR product on an amplification plot, the real time fluorescent signal produced by each probe on a multicomponent plot and the normalized fluorescence levels (ΔRn) for each allele in each qPCR reaction. The amplification plots allow for the confirmation of PCR product amplification, the multicomponent plots allow for the manual calling of

SNP genotypes and the ΔRn values can be utilized to determine the optimal input DNA concentration from a dilution series (Brocannello *et al.* 2018).

The data from the QuantStudio® 12K Flex v1.3 software on the TaqMan SNP genotyping assays can then be imported into the TaqMan® Genotyper Software Version 1.5.0 available from Life Technologies (ThermoFisher Scientific, USA) to produce an allelic discrimination plot (also known as a "cluster plot" or "AD plot") as indicated in Figure 7. The allelic discrimination plot will allow for discrimination between genotypes within or between samples by comparing the fluorescent signal obtained using the allele-specific, dye-labelled probes.

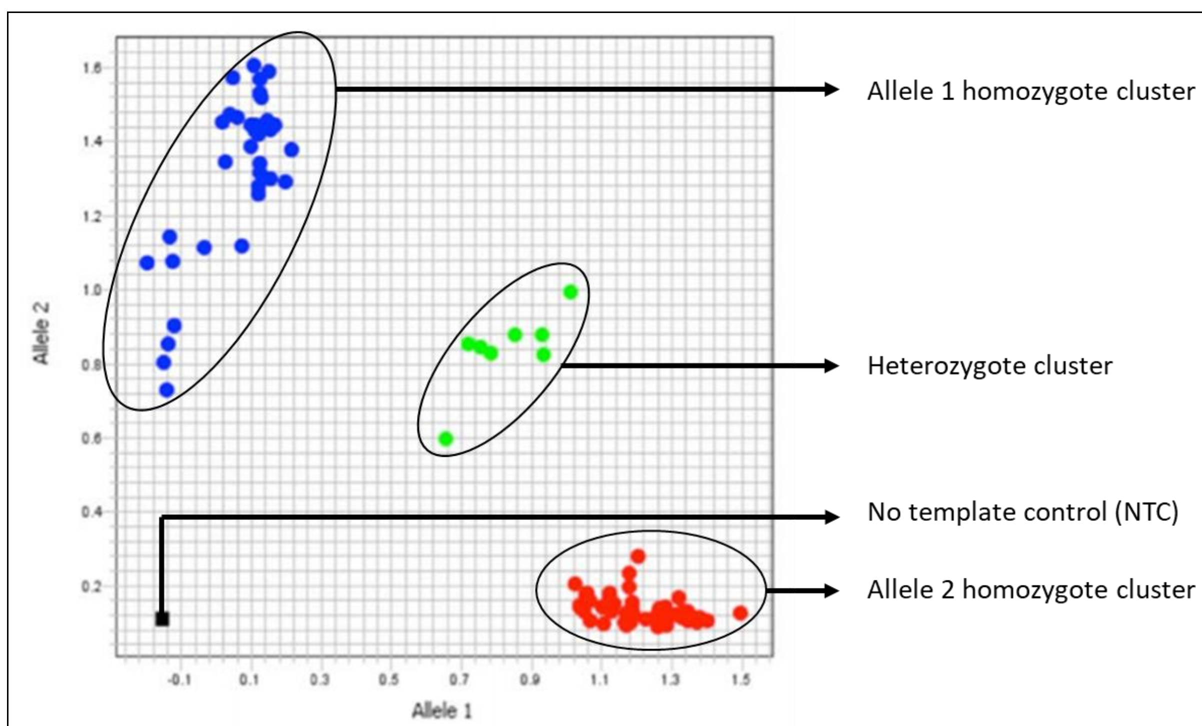


Figure 7: An allelic discrimination plot typically derived from a TaqMan SNP genotyping assay (adapted from Brocannello *et al*. 2018). Each dot corresponds to an individual/sample. The red and blue dot clusters represent the two possible homozygous genotypes, while the green cluster represents the heterozygous genotypes. The black square indicates the results of a no template control (NTC) reaction.

Ideally, these plots show three distinct clusters for the three different genotypes as well as a no template control (NTC). Samples that are homozygous for allele 1 labelled with VIC dye will cluster in the lower right corner, samples that are homozygous for allele 2 labelled with the FAM dye will cluster near the upper left corner and samples that contain both allele 1 and 2, i.e., heterozygous samples, will cluster in the top right corner approximately midway between the allele 1 and 2 clusters (ThermoFisher Scientific 2020). The NTC control should be indicated in the bottom left corner of the

plot and samples that are undetermined (i.e., a genotype cannot be determined) may cluster anywhere on the plot (ThermoFisher Scientific 2020).

TaqMan SNP genotyping assays have the potential to largely improve the turnover time of genetic testing. Hundreds of samples could be screened for all genetic markers within a few hours where screening can be conducted for individual samples on a 384-well qPCR plate. If successful, this screening platform could also potentially be upscaled for commercial use in the future, where TaqMan® OpenArray® Genotyping Plates enable the highest sample throughput for mid-density genotyping at over 110,000 genotypes in a single day with the QuantStudio™ 12K Flex Real-Time PCR System (ThermoFisher Scientific 2017). Furthermore, sequencing and sequence analysis is not required as testing can be conducted directly from genomic DNA that will allow for rapid resistance genotyping of samples simultaneously due to the allele-specific discrimination abilities of the TaqMan SNP genotyping assays.

3.3. The molecular basis of acaricide resistance

Molecular mechanisms employed by ticks

Ticks have developed different mechanisms to enable them to become resistant to the toxic effects of acaricides, including penetration resistance, target site resistance and metabolic resistance. An overview of these defence mechanisms is depicted in Figure 8.

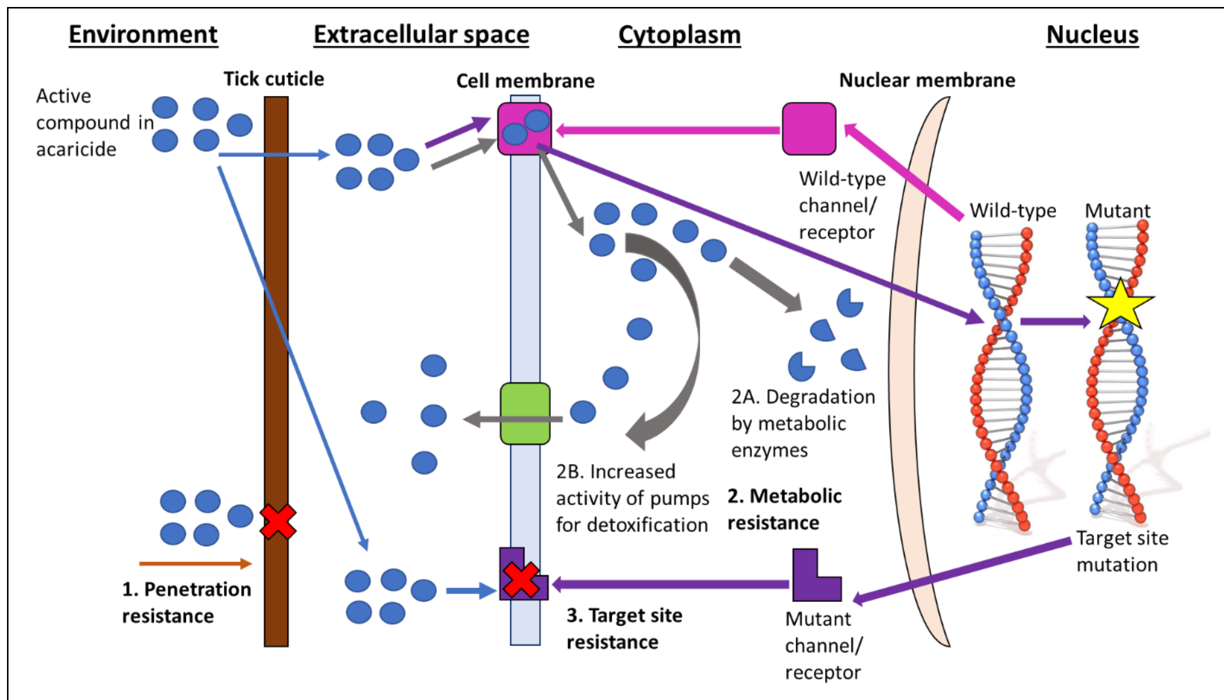


Figure 8: An overview of the resistance mechanisms employed by ticks against chemical acaricides (adapted from Reinecke 2015). The blue circles indicate the active compound in the acaricide and the blue arrows indicate the normal entry/penetration of the acaricide into the tick. (1) Penetration resistance (indicated by the orange arrow) can be caused by a thicker cuticle around the tick's body, reducing/preventing the penetration/entering of acaricides. After penetration/entry occurs the acaricide can enter the cytoplasm of the cell via a wild-type protein channel/receptor on the cell membrane which is yielded by the process of transcription and translation from a wild-type gene (indicated in pink). (2) Metabolic resistance (indicated by the grey arrows) is caused by enhanced metabolic detoxification which caused by degradation of the acaricide by metabolic enzymes in the cytoplasm (2A) or upregulation of pumps (indicated by a green rectangle) on the cell membrane whereby the acaricide is exported from the cell (2B). (3) Target site resistance (indicated by the purple arrows) is caused when a mutation occurs in the gene encoding the protein target channel/receptor of the acaricide (indicated by the yellow star), which will yield a mutated protein that will prevent the uptake of the acaricide into the cytoplasm of the cell (indicated by the purple shape).

Penetration resistance (Figure 8 number 1, indicated in orange) is the tick's first line of defence, where there are alterations in the ability of an acaricide to penetrate or otherwise enter an individual that has been treated with the acaricide. Penetration resistance was mostly reported to be observed in the 1980's in arthropods such as *Plutella xylostella* (diamondback moth) (Noppun *et al*. 1989) as well as in *R. microplus* (Schnitzerling *et al.* 1983). In *R. microplus*, the cuticle around the tick's body was found to become thicker after prolonged acaricide exposure, and as such it was hypothesized to reduce the absorption of acaricide(s). There have, however, not been any recent or more validating investigations into this resistance mechanism in *R. microplus*, and as such the specific details of this mechanism and its specificity towards a specific class of acaricide(s) requires further validation.

Metabolic resistance (Figure 8, number 2, indicated in grey) can occur through the detoxification of acaricide active compounds by metabolic enzymes (Figure 8, 2A). There are three major enzyme families, including: esterases (Jamroz *et al*. 2000; Baffi *et al*. 2005; Gupta *et al*. 2016; Yessinoua *et al*. 2018), glutathione-S-transferases (GSTs) (Yessinoua *et al*. 2018; Chigure *et al.* 2018) and cytochrome P450 monooxygenases (Chevillon *et al*. 2007a; Cossio-Bayuger *et al*. 2008; Yessinoua *et al*. 2018).

These enzymes induce/enable acaricide resistance/detoxification both through the increased levels of their expression and/or potentially through mutations. In *R. microplus*, both mutations and increased levels of expression of monoamine oxidases have been detected and as such it has been proposed to be a mechanism of resistance, although there is little supporting evidence available to date (Jonsson *et al.* 2018). Studies in other species, such as mosquitoes (Hemingway and Karunaratne 1998; Hemingway *et al*. 2004), moths and flies (Young *et al*. 2006) have suggested that mutations may alter a metabolic enzyme in a way that increases the rate at which it metabolizes acaricides, or the gene encoding for the metabolic enzyme may undergo enhanced expression. For example, in *Aedes aegypti* it was found that a mutation in a *trans*-acting repressor element underlies the enhanced expression of δ-class GSTs (Grant and Hammock 1992). In addition, it was found that in insecticide-resistant *Drosophila* strains, a mutation in a *trans*-acting repressor causes over-expression of Cyp6a8 (Maitra *et al.* 1996, 2000 and 2002). This has, however, not been investigated in ticks.

Metabolic resistance can also occur through the increased activity of pumps to transport xenobiotic compounds (acaricides) out of the cytoplasm and into the extracellular space (Figure 8, 2B) (Jonsson *et al.* 2018). In *R. microplus*, a study found that expression of ATP-binding cassette (ABC) transporters is increased in amitraz resistant ticks in comparison to amitraz susceptible ticks (Lara *et al*. 2015). It was also determined that the ABCB10 glycoprotein is responsible for the transport of amitraz into the hemosome (Lara *et al*. 2015). This supports the findings from an earlier study which was conducted on *R. microplus* cell cultures (Koh-Tan *et al*. 2016) that demonstrated a higher ABCB10 expression level in an amitraz-resistant cell line known as BME/CTVM6. Similar findings have been published in other species as well, for example ABC transporters have been associated with anthelmintic resistance in the parasitic nematode, *Cooperia oncophora* (De Graef *et al.* 2013).

Another mechanism of resistance that is common in *R. microplus* is target site resistance (Figure 8, number 3, indicated in purple). Target site resistance occurs due to point mutations and frameshift mutations in genes that code for the protein targets of the respective acaricide active compound. These protein changes ultimately render the acaricide less effective or in some cases completely ineffective (Baffi *et al*. 2007; Guerrero *et al*. 2012a; Hernandez *et al*. 2002). These mutations often occur as a result of replication slippage and recombination, which drives genetic diversity (Guerrero *et al*. 2012a). This diversity is then furthered by random mating without selection (Chevillon *et al*. 2007b; Budeli *et al*. 2009). Since *Rhipicephalus microplus* ticks are known to have a high degree of genetic diversity due to their large genome size (as described previously), their pangamic mating structure and their rapid lifecycle (as described previously), these mechanisms can occur alone or in combination with each other to confer resistance (Hemingway *et al.* 2004). Combined this

demonstrates the well-adapted resistance mechanisms employed by ticks and the various targets for the development of a comprehensive panel of resistance diagnostic markers.

The common drivers of population genetics: impact on acaricide resistance

In 2018 Rodriguez-Vivas *et al*. referred to the emergence of acaricide resistance as an evolutionary process, where it is subject to mutation, migration, selection and drift, i.e. the common drivers of population genetics. Typically, an acaricide applied at a lethal concentration will kill most ticks in a susceptible population. However, it is possible that resistance alleles (alleles with mutations that are associated with resistance) are already present in a susceptible population prior to acaricide exposure, most likely in heterozygote individuals (Nolan 1987; Mitchell 1996; Chapman 1997). These resistance alleles will then be selected for and will increase in frequency within the population after continued exposure to the same active drug ingredient, i.e. acquired resistance (Nolan 1987; Alonso-Diaz *et al*. 2013; Aguilar-Tipacamu *et al*. 2011; Rodriquez-Vivas *et al*. 2011). Once the number of heterozygotes in a population passes a threshold, fully resistant (homozygous) ticks will begin to emerge (Mitchell 1996). After prolonged exposure to the same acaricide, eventually all susceptible individuals will be removed from the population and only the resistant individuals will remain (Lin *et al*. 2009; Feng *et al*. 2011), i.e. a process of selection for resistance occurs (Abbas *et al.* 2014). Such adaptations are expected to occur through directional selection (Kaplan 1989), where if a mutant allele increases the fitness of some individuals in the population, natural selection will act to increase the frequency of the mutant allele (Fisher 1930; Williams 1966).

However, it is possible that directional selection may not always be applicable to *R. microplus* field populations and that in some cases positive balancing selection occur, where the mutations in the acaricide target gene are maintained in the population (Nordburg and Innan 2002). For instance, a study conducted by Robbertse *et al.* (2016) on *R. microplus* populations in the Mnisi communal area in South Africa revealed that despite a continued weekly amitraz dipping regime, 52% of the population was heterozygous for amitraz resistance. It was thus hypothesized that the weekly dipping regime at the Mnisi communal area dip stations serves as a selection pressure to maintain the amitraz resistance-associated alleles (discussed below) in a heterozygous state, although more data is required to confirm this.

According to Orr (2009) the change in allele frequency due to natural selection depends only on the difference in relative fitness between two alleles. The abundance of heterozygosity observed for the amitraz resistance-associated alleles in *R. microplus* populations could thus potentially be due to the homozygous resistant genotype posing a fitness cost to *R. microplus* tick. The homozygous genotype

may be associated with a significant disadvantage in comparison to heterozygous or homozygous susceptible individuals in a population, as has been reported for insects (Kliot and Ghanim 2012). A study in 2006 was conducted on organophosphates and formamidines to evaluate this hypothesis, but the magnitude of statistical differences in reproductive parameters between resistant and susceptible ticks was not enough to conclude that the resistant strains were biologically less fit than the susceptible strains and the results were thus inconclusive (Davey *et al*. 2006). More research is therefore still required to validate this hypothesis.

Natural selection is also influenced by the dominance of resistance alleles, their mode of inheritance and the number of genes involved (Georghiou and Taylor 1977; Mulchandani *et al*. 1998). A resistance phenotype in *R. microplus* can be inherited as a dominant, partially dominant or recessive trait (ffrench-Constant and Roush 1990; Roush and McKenzie 1987). For example, a study showed through reciprocal crosses of a susceptible and a resistant *R. microplus* strain that resistance to synthetic pyrethroids is inherited as a partially dominant trait for cypermethrin when the female tick is resistant but when the male tick is resistant for flumethrin and deltamethrin, resistance is inherited as a complete recessive trait (Aguilar-Tipacamu *et al*. 2008). The results of studies investigating the mode of inheritance for amitraz resistance have shown to be variable. Earlier studies by Li *et al.* (2004) and Li *et al*. (2005) reported that amitraz resistance was likely inherited as an incomplete recessive trait, whilst later studies by Fragoso-Sanchez *et al.* (2011) and Corley *et al.* (2013) found that amitraz resistance has a recessive mode of inheritance. As such this indicates that the observed resistance phenotype of ticks may also vary depending on the mode of inheritance of a particular form of resistance (George *et al.* 2004). It should also be considered that the mode of inheritance that is determined in a laboratory may not always correspond with the mode of inheritance that is observed in the field (ffrench-Constant and Roush 1990; Roush and McKenzie 1987).

Drift can also potentially increase the frequency of a mutation, as it allows mutant allele frequencies to change randomly and independently of selection, eventually allowing for mutations to either become fixed or eradicated from a population (Fisher 1930; Williams 1966). The probability of fixation taking place is typically dependant on the initial frequency of the mutant allele in a population (Fisher 1930; Williams 1966). Drift has, however, not been extensively investigated in tick populations. According to Rodriguez-Vivas *et al*. (2018), drift is likely to be relevant in tick strains maintained in culture and it may play a role in outbreak populations in areas that were not previously infested with ticks

Lastly, migration can impact resistance through the spread of resistant tick populations in previously uninfested areas. For example, shared local *R. microplus* infestations on cattle and white-tailed deer

in southern Texas has resulted from the frequent short-distance migration of *R. microplus* ticks from the tick eradication quarantine area between Mexico and the United states as well as from long-distance human-mediated migrations from populations outside of this area (likely from Mexico) (Busch *et al.* 2014).

3.4. The influence of environmental factors on acaricide resistance

Operational factors and human error

Resistance can be influenced by errors in human-controlled operations of cattle hosts in the environment. For instance, the movement of cattle without prior dipping and testing for acaricide resistance may lead to the spread of resistant tick populations. This is common in rural areas of developing countries where the movement of cattle is not strictly regulated. For example, during the war in Zimbabwe in the 1970's there was a lack of cattle dipping, a lack of testing for acaricide resistance and the movement of cattle was not properly regulated. This allowed for the spread of resistant tick populations and tick-borne disease consequently became widespread, which resulted in the death of approximately one million cattle (Norval 1979).

Another contributing factor to the development of resistance is acaricide application. It has been proposed that ticks are likely to become resistant if they are exposed to (a) low/insufficient concentrations of an acaricide over a prolonged time (Lin *et al*. 2009) and (b) the frequency of applications (Kunz and Kemp 1994). To date, multiple studies have found that ticks are more likely to become resistant if the same acaricide is applied five or more times in a year. For example, a study on *R. microplus* in Australia showed that more than five acaricide treatments per season is a positive risk factor for resistance (Jonsson *et al.* 2000). In Mexico, a study on *R. microplus* found that there is a high probability of resistance developing on farms where acaricides were applied six or more times in a year (Rodriguez-Vivas *et al*. 2006b). This is consistent with an earlier study from 1979 that found that there is a higher probability of resistance developing when acaricides were applied six times a year as opposed to four or five times a year (Sutherst 1979).

However, the scientific evidence provided by studies on dipping frequency is limited and is based purely on phenotypic observations and as such this data alone is not sufficient to advise on appropriate acaricide dipping regimes. The availability of genotypic data would be invaluable to better understand the development of resistance mechanisms employed by ticks (discussed previously), taking into account the resistance genotype (e.g. sequence of resistance associated SNPs) at the onset of the study and the influence of the selection pressure that is applied by the relevant acaricide dipping

regime. This highlights the need for DNA sequencing data to identify and track acaricide resistance status in the field so that more accurate recommendations can be made on the frequency and/or timing of acaricide applications whilst taking into account the selection pressure that will be imposed on tick populations. This will enable farmers to implement more appropriate control strategies that are informed by both phenotypic and genotypic data.

Improved management of acaricide use to reduce resistance

There are several control strategies that can be implemented after diagnostic testing has been conducted to identify and/or confirm acaricide resistance status in the field. Firstly, the selection and use of an appropriate acaricide(s) to which the tick population is susceptible. Thereafter, to maintain a level of susceptibility, rotation of acaricides can take place. By alternating two or more acaricides that contain different active components and as such affect different target sites within the tick, the selection pressure to any specific compound will be reduced (Abbas *et al*. 2014; Rodriguez-Vivas *et al*. 2014b). For example, a study on amitraz resistant *R. microplus* ticks in Australia showed that Spinosad (a natural substance made by a soil bacterium that is toxic to insects) used in rotation with amitraz every 2 months over 4 years resulted in the loss of amitraz resistance and ticks returned to a more susceptible phenotype (Jonsson *et al*. 2010).

Another strategy to prevent resistance from emerging is to reduce the frequency of acaricide application. Application of acaricides every three weeks during the tick season is common, but frequent application poses a risk factor for the emergence of resistance (Sugimoto and Osakebe 2013; Jonsson *et al.* 2000; Kunz and Kemp 1994). From available studies on treatment frequency (as discussed above) it can be suggested that acaricide treatments should not exceed more than five per season (Rodriguez-Vivas *et al*. 2006b; Jonsson *et al.* 2000; Sutherst 1979). However, to more accurately track whether resistance is emerging in a specific tick population, regular monitoring should be performed in conjunction with a laboratory (Sun *et al.* 2011). Another way to reduce frequency of applications is to combine the use of acaricides with alternative control agents (discussed below) as part of an integrated control strategy.

The use of synergists has also been used in tick control to potentiate acaricidal effects. Synergists that have commonly been used to potentiate the effects of acaricides are piperonyl butoxide (PBO), triphenyl phosphate (TPP) diethyl maleate (EM) and verbutin (Li *et al.* 2007). These synergists are inhibitors of metabolic enzymes that serve to decrease the metabolic detoxification of acaricides by ticks (as described below) thus reducing tick metabolic resistance. For example, Li *et al.* (2010)

demonstrated that PBO and verbutin potentiate the effects of permethrin, coumaphos and amitraz. In 2013, Rodriguez-Vivas *et al.* demonstrated that a mixture of cypermethrin, amitraz and PBO was effective for killing resistant *R. microplus* ticks both *in vitro* and *in vivo*. Although these studies show promising results, the use of synergists in developing African countries may not prove viable due to increased costs. Li *et al.* (2007) also demonstrated that pyrethroids used in combination with amitraz have a synergistic effect against resistant *R. microplus* ticks. This same effect has been observed in other insects as well, including *Helicoverpa zea, Spodoptera frugiperda* and *Agrotis ipsilon* (Usmani and Knowles 2001). Although the use of acaricide mixtures is effective in the short-term, it can disadvantage tick control in the long-term by increasing the chance of developing cross resistance or multiple resistance in a tick population. Thus, a more effective approach would be the rotation of acaricides with different modes of action rather than mixing them together, with regular monitoring from a diagnostics laboratory.

4. Amitraz resistance

This study will be focusing on amitraz, as it is one of the acaricides that is most commonly used by both small-scale and commercial farmers in South Africa for the control of *R. microplus* ticks (Jonsson *et al*. 2018). This is most likely due to its low cost and availability alongside cost-effective pyrethroids. For instance, TAKTIC® cattle spray (amitraz 12.5 % m/v) is available from multiple online suppliers and costs around ZAR 255 (including VAT) per 1 L which can be diluted into 500 L for dipping of numerous cattle. This is much more affordable in comparison to other acaricides such as macrocyclic lactones, for example IVOTAN® (Ivermectin 1% m/v) which costs ZAR 425 (including VAT) per 500 mL.

Amitraz was first introduced in Australia in the 1970's (Nolan 1981). It can be used in full body dipping of cattle, a spray or pour-on formulation for the control of mites, lice, fleas and various tick species (Taylor 2001). The use of amitraz to control ticks on cattle has a rapid short-lasting effect where detachment from the host occurs within one hour (mostly cleared within 7 hours) and is followed by tick mortality or reduced egg production from treated females (Davey *et al.* 1984; Haigh *et al*. 1980). An advantage associated with amitraz is its low toxicity levels for bees, spiders, birds, fish and mammals when it is used at the correct concentrations (Chang and Knowles 1977; Schuntner and Thompson 1978). Amitraz poisoning has occurred under non-conventional conditions in dogs and cats through the accidental ingestion of collars and in humans, although far fewer than in animals, of which most intoxications were in children and a limited number in adults (Avsarogullari *et al.* 2006; Yilmaz and Yildizdas 2003).

Excessive use of amitraz in tick control has, however, resulted in many reports of resistant *R. microplus* populations around the world, as summarized in Table 1. The levels of amitraz resistance in South American countries such as Brazil and Mexico are notably high, indicated by high resistance factors in multiple studies. For example, a study in Brazil showed that some *R. microplus* tick populations were up to 100% resistant to amitraz (Andreotti *et al*. 2011). The levels of resistance in South Africa are lower, where a study conducted in 2016 by Robbertse *et al.* reported that the frequency of heterozygous resistant genotypes was 0.52 and the frequency of homozygous resistant genotypes was 0.03. However, this study was conducted on ticks collected in 2012/2013 and as mentioned above, resistant *R. microplus* ticks have been detected in multiple provinces in South Africa and amitraz resistance is continuously emerging (RuVASA 2019) and thus needs to undergo regular monitoring.

4.1. The molecular targets of amitraz in arthropods

Amitraz, *N*'-(2,4-dimethylphenyl)-*N*-[(2,4-dimethylphenyl) iminomethyl]-*N*-methylmethanimidamide, is a triazapentadine compound that is a member of the formamidine class of pesticides (Figure 9). Two targets of amitraz have been proposed, namely the octopamine receptor and the monoamine oxidase enzyme. Studies from the 1970's hypothesized that amitraz targets the monoamine oxidase enzyme (Aziz and Knowles 1973; Knowles and Roulston 1973; Atkinson et al. 1974), but more recent literature shows that the toxicity of amitraz is not due to the inhibition of this enzyme and that amitraz is most likely an agonist of the octopamine receptor (Baxter and Barker 1999; Li *et al*. 2005; Chen *et al*. 2007; Jonsson and Hope 2007). Recent studies also show that amitraz and its metabolites, namely N^2-(2,4-dimethylphenyl)-N1-methyformamidine (DPMF), may differentially activate α- and β-adrenergic-like octopamine receptors (Kita *et al*. 2016).

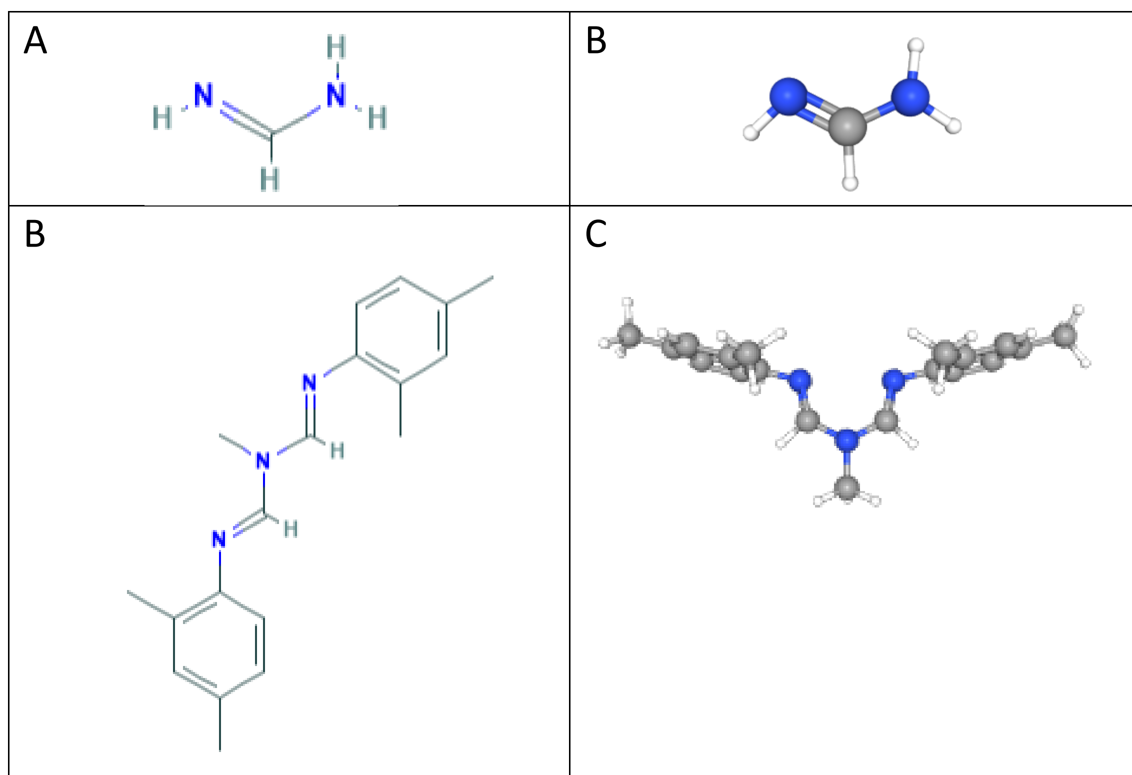


Figure 9: The basic chemical structure of a formamidine (CH_4N_2) (Figure A and B) and of Amitraz ($C_{19}H_{23}N_3$) (Figure C and D) (taken from PubChem Open Chemistry Database, https://pubchem.ncbi.nlm.nih.gov/compound/68047 and https://pubchem.ncbi.nlm.nih.gov/compound/36324 respectively). Figure A and C show the two-dimensional chemical structure. Figure B and D show the ball and stick interactive chemical structure models.

Amitraz thus competes with octopamine (a neurotransmitter) for binding of the octopamine receptor. The octopamine receptor is a G-protein coupled receptor (GPCR) (Baxter and Barker 1999) that plays a role in cyclic adenosine monophosphate (cAMP) second messenger pathways, and calcium signalling pathways (Gilman 1987; Lees and Bowman 2007). There are three different classes of octopamine receptors that have been identified in arthropods. Firstly, the α-adrenergic-like octopamine receptor (αAOR) which elevates intracellular calcium concentrations (Han *et al*. 1998). Secondly, the octopamine/tyramine (OCT/Tyr) receptor which in most cases is preferentially activated by tyramine over octopamine (Nagaya *et al*. 2002; Roeder *et al*. 2002). Lastly, the β-adrenergic like octopamine receptor (βAOR) which is preferentially activated by octopamine rather than tyramine, which causes an increase in intracellular cAMP concentration rather than calcium (Evans and Maqueira 2005). In ticks, the binding of octopamine or tyramine to the receptors excites neurons in the abdominal ganglion, which is related to an increase in motor activity (Evans and Gee 1980). On the other hand, when the octopamine receptor is bound by amitraz it causes ticks to experience uncoordinated motor activity, which prevents them from attaching to their hosts, and ultimately results in paralysis and death (Evans and Gee 1980).

4.2. Mutations in the molecular targets of amitraz associated with resistance in *R. microplus*

Resistance-linked SNPs in the amitraz target genes are a promising prospect for the molecular diagnosis of amitraz resistance through distinct molecular markers (Kumar 2019). According to literature, target site insensitivity can occur in the OCT/Tyr receptor and βAOR genes of *R. microplus* ticks as potential mechanisms of amitraz resistance (Jonsson *et al*. 2018). The OCT/Tyr receptor was first sequenced in 1999 for both amitraz resistant and susceptible *R. microplus* ticks (Baxter and Barker 1999), where no differences were found in the sequences. Eight years later, Chen *et al.* (2007) sequenced the same putative sequence for the OCT/Tyr receptor gene examined by Baxter and Barker in 1999, from a *R. microplus* ticks from America, Brazil and Mexico. The American sequences were found to be almost identical to the Australian sequences, but two nucleotide substitutions were identified in the Brazilian and Mexican sequences that result in amino acid substitutions (Chen *et al.* 2007).

These mutations occur at nucleotide position 157 where an adenine is substituted by a cytosine and at nucleotide position 200 where thymine is substituted by cytosine (Chen *et al.* 2007). This resulted in two amino acid substitutions, T8P and L22S respectively, both occurring in loops between transmembrane domains (Chen *et al*. 2007). In 2015, these same two SNPs (T8P and L22S) were confirmed to be associated with amitraz resistance in *R. microplus* strains in South Africa where susceptible genotypes were classified as AA/TT, heterozygous as AC/TC and resistant as CC/CC (Baron *et al*. 2015). Additional studies were also conducted in the Mnisi communal area in South Africa (Robbertse *et al.* 2016) and Zimbabwe (Sungirai *et al*. 2018) to genotype *R. microplus* field populations on the basis of the presence of these two SNPs as summarized in Table 1. The two amitraz resistance-linked SNPs in the OCT/Tyr receptor gene are summarized in Table 2.

Table 2: The two validated resistance-linked SNPs in the OCT/Tyr receptor in amitraz resistant *R. microplus* tick strains.

SNP position:	Nucleotide substitution:	Amino acid change:	Protein domain:	Susceptible genotype:	Heterozygous genotype:	Resistant genotype:
157	A→C	T8P	Loops between transmembrane domains	AA	AC	CC
200	T→C	L22S		TT	TC	CC

In addition, the βAOR gene of amitraz resistant *R. microplus* was also sequenced and a mutation was discovered at nucleotide position 181 where adenine is substituted by thymine, which resulted in an I61F substitution in the first transmembrane domain of the receptor, which is a highly conserved

region (Corley *et al*. 2013). This mutation has only been linked to resistance in the amitraz resistant Ultimo strain of *R. microplus* from central Queensland, Australia, and was not found in North and Southeast regions of Australia (Corley *et al*. 2013). Furthermore, this I61F mutation was found to be selected for by amitraz treatment over seven generations of *R. microplus* in the field and that there was a positive correlation between amitraz resistance and the frequency of the I61F homozygous resistant genotype (r=0.90) (Corley *et al*. 2013). Jonsson *et al*. (2018) also confirmed the presence of the I61F mutation in amitraz-resistant South American isolates of *R. microplus*. The resistance-linked SNP in the βAOR is summarized in Table 3.

Table 3: The resistance-linked SNP in the βAOR in amitraz resistant *R. microplus* tick strains.

SNP position:	Nucleotide substitution:	Amino acid change:	Protein domain:	Susceptible genotype:	Heterozygous genotype:	Resistant genotype:
181	A→T	I61F	First transmembrane domain	AA	AT	TT

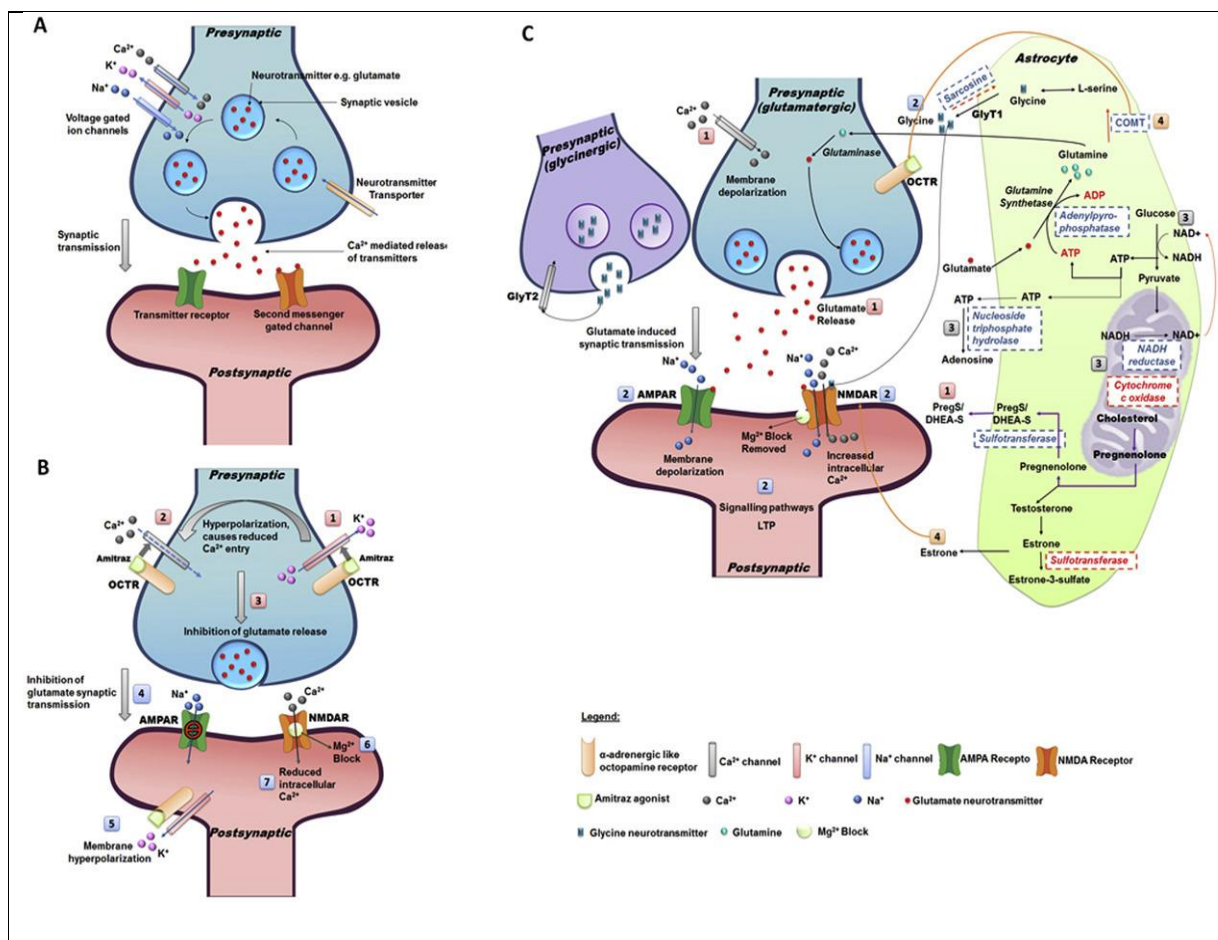
Seven other SNPs in the βAOR gene have been identified in *R. microplus* ticks from multiple countries, including Brazil, Mexico, Australia, Thailand and South Africa, which are summarized in Table 4 (Jonsson *et al*. 2018). However, the results from this study have only been briefly discussed in a review by Jonsson *et al.* (2018) and the primary findings have not yet been published. As such the association of these SNPs with amitraz resistance cannot be verified. Once association with resistance has been validated, these SNPs could also potentially be used as additional molecular markers in the future molecular diagnosis of amitraz resistance in *R. microplus* populations.

Table 4: A table showing several SNPs remaining to be validated that were identified in the βAOR gene of amitraz resistant *R. microplus* ticks from diverse locations around the world (Jonsson *et al*. 2018).

Position	SNP	Amino Acid
123	T→C	Synonymous
126	C→T	Synonymous
181	A→T	I→F
185	T→C	I→T
225	A→G	Synonymous
263	A→C	Y→S
264	C→A	Y→S

4.3. Mechanisms of amitraz resistance in *R. decoloratus*

In addition to *R. microplus*, amitraz is also commonly used for the control of *R. decoloratus* ticks. A recent study by Baron *et al.* (2018) proposed a model of amitraz resistance in *R. decoloratus* (Figure 10) where in susceptible ticks, the presence of amitraz inhibits the entry of calcium into cells and subsequently inhibits membrane hyperpolarization, which in turn prevents the release of neurotransmitters. In resistant ticks it was hypothesized that this mechanism is overcome by ionotropic glutamate receptors, the N-methyl-D-aspartate (NMDA) and α-amino3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, to enhance synaptic transmission and plasticity in the presence of amitraz (Baron *et al.* 2018). This model, however, remains to be validated and has not yet been investigated in *R. microplus* ticks. Upon validation in *R. microplus*, this model can serve as a template for the identifying of novel therapeutics.



<u>Figure 10:</u> Proposed model for amitraz resistance in *R. decoloratus* ticks (taken from Baron *et al.* 2018). (A) indicates susceptible ticks with no amitraz exposure, (B) indicates susceptible ticks exposed to amitraz and (C) indicates resistant ticks in the presence of amitraz. Enzymes that were upregulated in amitraz resistant ticks are indicated in blue and those that were downregulated are indicated in red. In figures (B) and (C), the AMPA receptors are shown in green and the NMDA receptors in orange.

5. Alternative strategies for tick control

5.1. Genetic breeding, plant extracts and biological control

There are many alternative control measures that have been investigated to date, such as genetic breeding of tick resistant bovines (Biegelmeyer *et al*. 2015; Shyma *et al*. 2015), the use of plant extracts or biological control using nematodes or fungi (Kiss *et al*. 2012). Breeding of resistant bovines focuses on the breeding of naturally resistant hosts. For instance, *Bos indicus* cattle are more resistant to infestation by *R. microplus* than *Bos taurus* cattle (O'Kelly and Spiers 1976). Crossbreeding of *B. taurus* and *B. indicus* was also attempted to improve cattle resistance to *R. microplus* infestation, where the interbred cattle were found to exhibit an intermediate level of resistance (Brossard 1998). Some plant extracts have exhibited acaricidal properties, such as *Melia azedarach* (Borges *et al.* 1994 and 2003; De Sousa *et al.* 2008) and *Azadirachta indica* (neem) (Williams 1993; Broglio-Micheletti *et al*. 2009 and 2010), but there are still many limitations around their implementation. For instance, the complex formulation preparation process, the variation in chemical compositions among individual plants of the same species and the lack of information regarding active compounds with acaricidal effects (Borges *et al*. 2011). Biological pesticides, on the other hand, have mostly been shown to be impractical with minimal benefits at a high cost (Kiss *et al.* 2012). Biological pesticides can also potentially harm non-target invertebrates (Domingos *et al*. 2013).

5.2. Vaccines

The preferred method of future tick control, and per association the transmission of tick-borne diseases, would be via vaccination (Valle and Guerrero 2018). A large number of antigens have been tested for the development of a vaccine against *R. microplus*, where to date the most protective antigen that has been identified is Bm86 (Valle and Guerrero 2018). Bm86 is a GPI-linked membrane protein that is expressed on the surface of tick gut cells which is recognised by antibodies in vaccinated hosts which taken up into the tick gut with a bloodmeal (Willadsen *et al.* 1989; Willadsen and Jongejan 1999). These host antibodies then bind to Bm86 and cause lysis of the tick gut cells, disrupting the lining of the gut and causing leakage of host blood into the tick's body cavity, which ultimately prevents digestion and nutrient uptake needed for ovipositioning (Willadsen *et al*. 1989). The Bm86 antigen was used in the first generation of commercial tick vaccines developed in the 1990's namely TickGARD® and TickGARD PLUS® from Australia. Both of these were discontinued. In Latin America Bm86 was used for the vaccines GAVAC® and GAVAC Plus® which are still available in some South American countries (Liao *et al*. 2007; Stutzer *et al.* 2018). More recently, Bm86 was used for the development of the Bovimune Ixovac vaccine in Mexico (Blecha *et al.* 2018) and is still available for purchase from

a company called Lapisa (Bovine Ixovac, 2018). However, Bm86-based vaccines exhibit different levels of efficacy against *R. microplus* in different geographical areas and as such its acceptance as a vaccine remains limited (de la Fuente and Kocan 2003; Cunha *et al.* 2012; Tellam *et al*. 1992; Canales *et al*. 1997).

There is only one other commercially produced tick vaccine against *R. microplus* called Go-Tick® or Tick-Vac® that is available in Latin America (Stutzer *et al.* 2018). The manufacturer claims that this vaccine has an efficacy of approximately 80%, but no research was published in support of these claims. Furthermore, no major antigens are stated by the manufacturer and currently it is understood that this vaccine is derived from fractionated larval extracts (Stutzer *et al.* 2018). Ultimately, more research is still required on the development of a novel, effective vaccine for the control of *R. microplus* tick infestations that can be used as part of an integrated tick control strategy.

6. Conclusion

The increasing levels and spread of acaricide resistance in *R. microplus* populations in South Africa and in other countries around the world is of great concern, especially when taking into consideration the transmission and spread of high morbidity and mortality diseases such as Asiatic redwater. This study therefore aims to determine the current amitraz resistance status of *R. microplus* ticks in the Mnisi communal area in South Africa using conventional PCR and sequencing to genotype the validated SNPs in the OCT/Tyr gene of *R. microplus* ticks linked to amitraz resistance. A previous study was conducted by our research group in the same area (Robbertse *et al*. 2016), which provides a unique opportunity for the investigation of the change in resistance genotype frequencies over time. In addition, to improve the turnover time of our genetic testing and to overcome the disadvantages associated with current resistance detection methods, this project aims to design and test a rapid high-throughput screening tool for the detection of amitraz resistance-associated SNPs in the OCT/Tyr gene of *R. microplus* ticks utilizing TaqMan® SNP genotyping assays. This will allow for 384 individual ticks to be screened simultaneously for amitraz resistance markers within hours using the QuantStudio® 12K Flex Real-Time PCR System (ThermoFisher Scientific, USA). This will ultimately help small-scale and commercial farmers in South Africa to save money and to reduce the emergence and spread of resistant *R. microplus* ticks through purchasing the correct acaricides and implementing of timely, knowledge-based acaricide management strategies.

7. Project aim and objectives

7.1. Aim

1. To determine the genotype frequencies of the amitraz resistance-associated SNPs in the OCT/Tyr gene of *R. microplus* ticks in the Mnisi communal area, South Africa using PCR and DNA sequencing.
2. To design and test TaqMan® SNP genotyping assays for the detection of SNPs associated with amitraz resistance in the OCT/Tyr gene of *R. microplus* ticks.

7.2. Objectives

Part 1: To determine the current amitraz resistance status of *R. microplus* ticks in the Mnisi communal area, South Africa as well as the change in resistance over time.

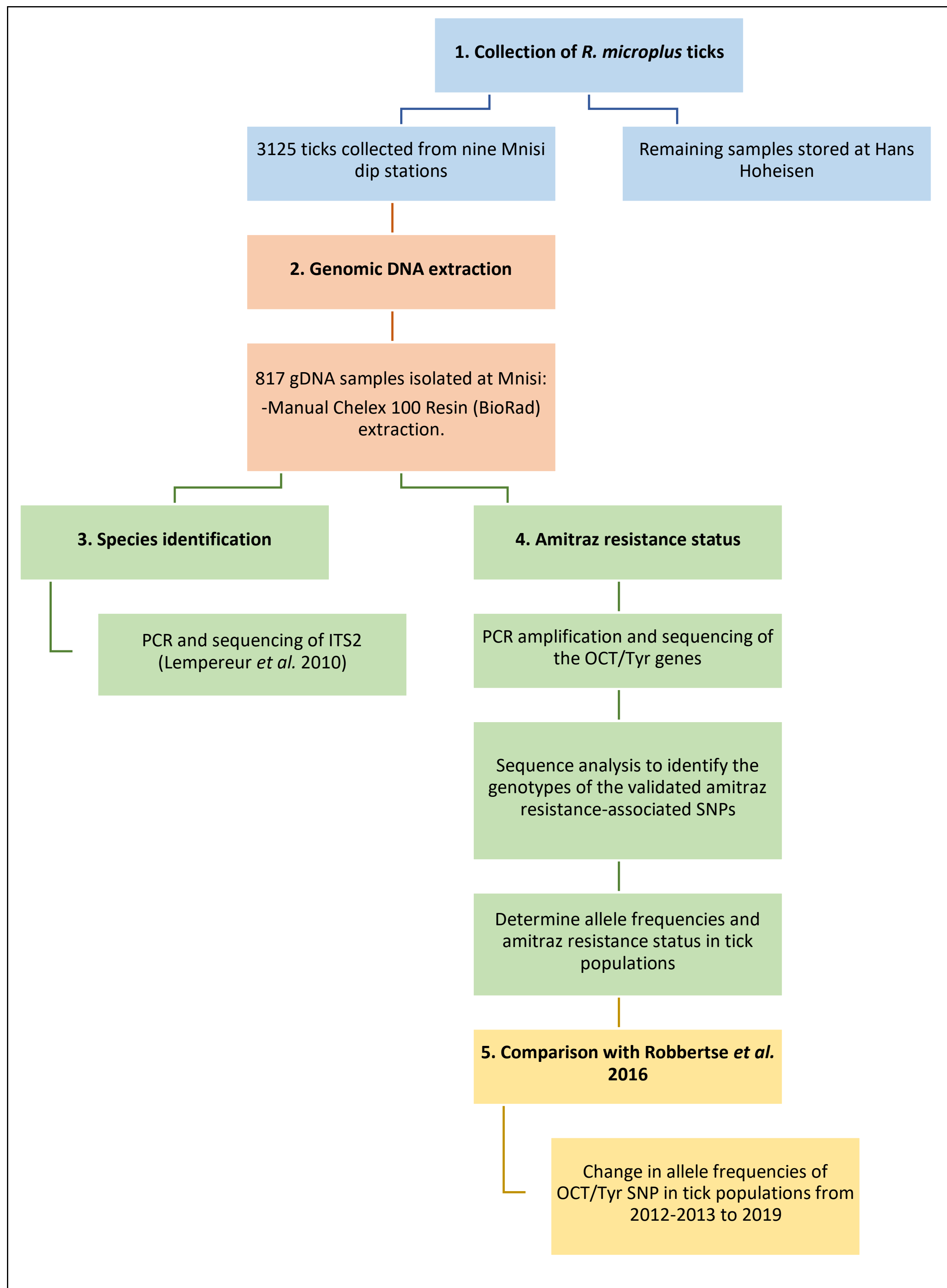
1. To collect *R. microplus* tick samples from cattle dip stations in the Mnisi area, South Africa.
2. To extract genomic DNA from *R. microplus* field samples.
3. To perform species identification via PCR amplification and sequencing of ITS2.
4. To determine the genotypes of the amitraz resistance-associated SNPs in tick populations via PCR amplification and sequencing of the OCT/Tyr gene.
5. To determine the amitraz resistance status in tick populations by determining the allele frequencies of the amitraz resistance-associated SNPs.
6. To determine changes in the genotype frequencies of the amitraz resistance-associated SNPs in the Mnisi communal area to a previous study by Robbertse *et al.* 2016.

Part 2: To establish a rapid, high-throughput diagnostic screening test for the detection of amitraz resistance in *R. microplus* ticks.

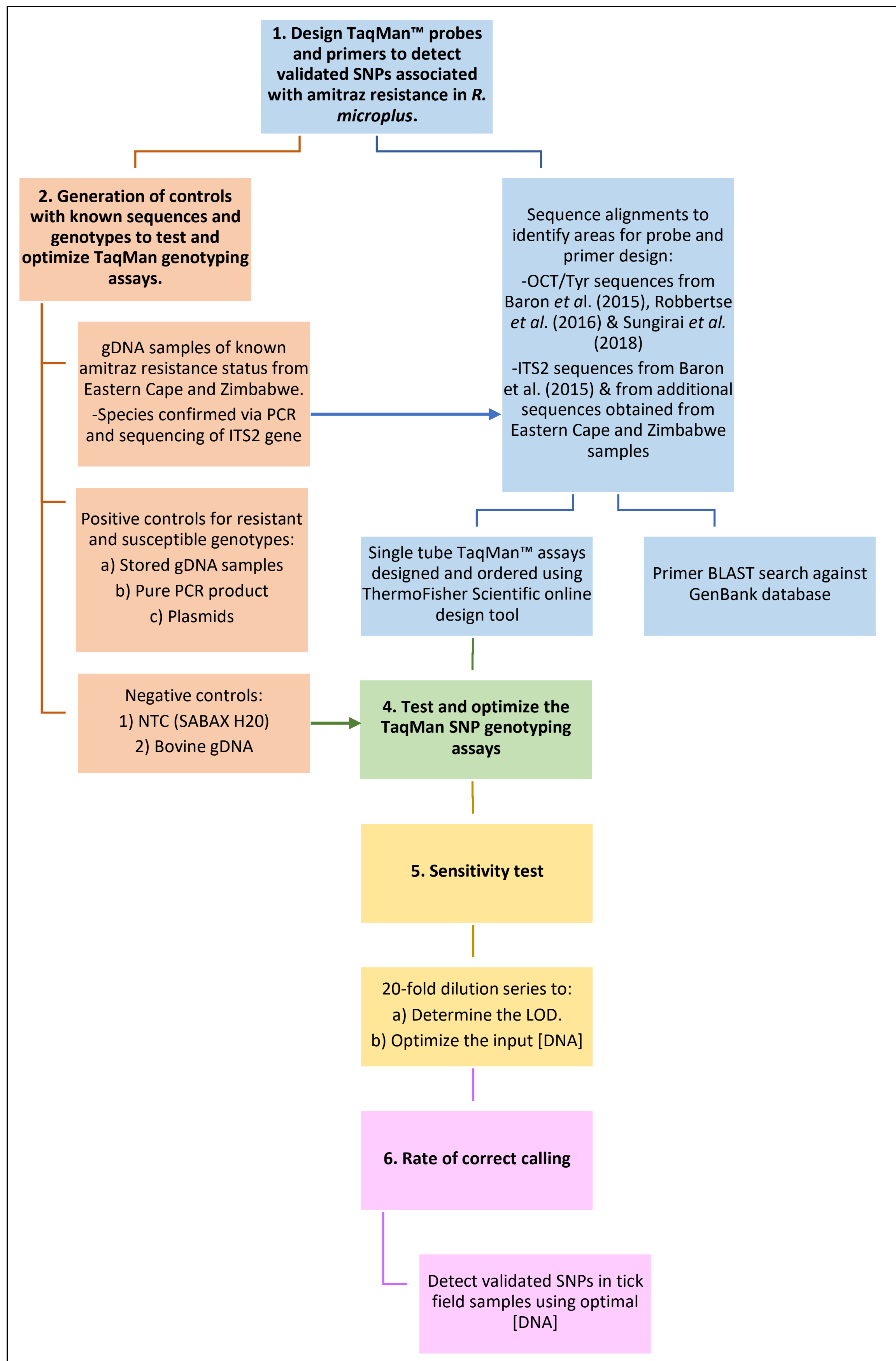
1. To collect *R. microplus* tick samples from cattle farms across South Africa.
2. To extract genomic DNA from these ticks to submit for analysis using TaqMan™ SNP genotyping assays designed by Life Technologies™.
3. To design TaqMan™ probes and primers for the TaqMan™ SNP genotyping assays to detect validated SNPs in the OCT/Tyr receptor that are associated with amitraz resistance in *R. microplus*.
4. To establish positive controls (plasmids containing the region of interest) representing homozygous resistant, homozygous susceptible and heterozygous genotypes.
5. To optimise the TaqMan® genotyping assays using the positive controls.

6. To test the TaqMan™ assays on field samples of known genotype and determine the rate of correct genotype calling.

8. Method overview: Part 1



Part 2



9. Intellectual property

All intellectual property used in and/or produced by this study will adhere to the University of Pretoria Intellectual Property Policy (see Section 21(1) (d) of the Copyright Act, Act No. 98 of 1978, as amended). A patent is held on the novel SNPs and associated RFLP test for rapid identification of amitraz resistance by the University of Pretoria. The diagnostic screening test discussed in this report is protected by intellectual property rights of the University of Pretoria and is not to be disclosed. A patent regarding this research may later be filed.

Intellectual property is defined as "registrable and non-registrable inventions and intellectual property creation, expertise, trademarks, trade secrets, copyrights, designs and plant breeders' rights which have come about through the mental efforts, insight, imagination, knowledge and creativity of humans" (Intellectual Property, 2018). In accordance with the Intellectual Property Rights from the Publicly Financed Research and Development Act 51, 2008, the University as a recipient of public funds for research has both the authority and the responsibility to protect and own the intellectual property that is generated/produced by research at the University of Pretoria (Intellectual Property, 2018). The University of Pretoria Technology Transfer Office (TTO) is the responsible agency at the University of Pretoria that will give effect to these objectives and protect the rightful interests of the University and its staff, students and partners in respect of intellectual property (Intellectual Property, 2018).

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Chapter 2

Comparison of the amitraz resistant SNP allele frequencies in *Rhipicephalus microplus* ticks in the Mnisi communal area over a 6-year period

1. Abstract

Rhipicephalus microplus is a hematophagous ectoparasite that is of great economic importance due to its negative impact on the cattle industry worldwide. The major method of tick control is the use of chemical acaricides, where amitraz is one of the most commonly used acaricides in South Africa. However, amitraz resistance in *R. microplus* has been reported in numerous studies globally. The Mnisi communal area offers a unique opportunity to investigate the amitraz resistance status of *R. microplus* ticks in South Africa as it is a controlled Foot-and-Mouth Disease (FMD) area and as such the movement of cattle is controlled. A previous study by our research group on the acaricide resistance status of *R. microplus* ticks in the Mnisi communal area in 2012/2013 (Robbertse *et al.* 2016) was conducted using PCR and DNA sequencing of two single nucleotide polymorphisms (SNPs) in the octopamine/tyramine receptor (OCT/Tyr) gene that have previously been linked to amitraz resistance (Baron *et al*. 2015). The aims of this study were to (a) sample additional dipping stations and (b) to evaluate change(s) in the allele frequencies of the SNPs between 2012/2013 and 2019. The results show that the frequencies of susceptible (AA/TT), heterozygous (AC/TC) and resistant (CC/CC) genotypes were 0.07, 0.87 and 0.06, respectively. The frequency of the homozygous susceptible genotype has largely decreased over the past 6 years (from 0.45 to 0.09) whilst the heterozygous genotype has largely increased (from 0.52 to 0.85). Only a slight increase was observed for the homozygous resistant genotype (from 0.03 to 0.06), although this is double the frequency that was observed previously. The observed shift to a heterozygous population over the past years is of concern and as such monitoring of these amitraz resistance-associated SNPs are essential as long as amitraz is being used at the dip stations.

2. Introduction

Rhipicephalus microplus, also known as the southern cattle fever tick, is considered to be the most economically important ectoparasite of livestock in the world (Cunha *et al.* 2012). *Rhipicephalus microplus* has mainly been shown to infest cattle, where it has recently been estimated that global losses in the cattle industry are 22–30 billion US Dollars per annum (Lew-Tabor and Valle 2016) as a consequence of tick burden. This is due to direct effects such as weight loss, leather hide damage, secondary infections as well as a reduction in meat and milk production (Benitez *et al.* 2012) and indirect effects such as the transmission of pathogenic species, including bovine babesiosis (Ristic 1981; Underwood 2015; Almazan *et al*. 2018), anaplasmosis (Aubrey and Geale 2010) and bovine borreliosis (Smith *et al.* 1978; Callow 1967). In South Africa, cattle represent a major source of nutrition as well as economic income to both small-scale and commercial farmers. However, the productivity and sustainability of the cattle industry is largely hindered by ticks and tick-borne diseases, where the most severe form of bovine babesiosis, Asiatic redwater, has been detected in every province in South Africa except for the Northern Cape (RuVASA Report January 2019).

The most common method of tick control is the use of chemical acaricides (Guerrero *et al*. 2012a). In South Africa, amitraz is one of the most commonly used acaricides by both small-scale and commercial farmers due to its relatively low cost and availability (Jonsson *et al*. 2018). Amitraz competes with octopamine (a neurotransmitter) for binding of the octopamine receptor, which is a G-protein coupled receptor (GPCR) (Baxter and Barker 1999) that plays a role in cyclic adenosine monophosphate (cAMP) second messenger pathways, and calcium signalling pathways (Gilman 1987; Lees and Bowman 2007). In ticks, the binding of octopamine or tyramine to the receptors excites neurons in the abdominal ganglion, which is related to an increase in motor activity (Evans and Gee 1980). On the other hand, when the octopamine receptor is bound by amitraz it causes ticks to experience uncoordinated motor activity, which prevents them from attaching to their hosts, and ultimately results in paralysis and death (Evans and Gee 1980).

However, the intensive use acaricides has resulted in tick populations that are resistant to all major classes of chemical acaricides worldwide (Rodriguez-Vivas *et al.* 2018). Although the reported levels of amitraz resistance are lower in South Africa than other countries, there is still the threat that it can further emerge. For instance, Robbertse *et al*. (2016) reported that 3% of a South African *R. microplus* population was genotypically resistant to amitraz. In comparison, the levels of amitraz resistance in South American countries is notably high, where a study in Brazil showed that some *R. microplus* tick populations were up to 100% resistant to amitraz (Andreotti *et al.* 2011). This therefore illustrates the

importance of regular monitoring of amitraz resistance to allow for the implementation of informed tick control strategies to prevent an increase in resistant *R. microplus* populations.

Amitraz resistance has previously been linked to two validated SNPs in the OCT/Tyr gene (Baron *et al.* 2015), where nucleotide substitutions occur at positions 22 (A > C) and 65 (T > C) resulting in amino acid changes of threonine to proline and leucine to serine, respectively (Chen *et al.* 2007). These substitutions have been correlated to amitraz resistance in *R. microplus* using larval packet tests (LPTs) but the functional implications of these substitutions remain to be elucidated (Baron *et al.* 2015). In 2016, Robbertse *et al*. reported the frequency of acaricide resistance-associated genotypes in *R. microplus* ticks in the Mnisi communal area in South Africa, where it was found that the genotype frequencies of the tick population were 45% susceptible (AA/TT), 52% heterozygous (AC/TC) and 3% resistant (CC/CC) for amitraz resistance. Due to the abundance of heterozygosity observed in the population, it was suggested that amitraz resistance was on the rise, as the *R. microplus* population that was investigated seemed to be acquiring resistance alleles.

The Mnisi communal area offers a unique and ideal opportunity to investigate amitraz resistance in *R. microplus* ticks. The Mnisi communal area is located in Bushbuckridge at a wildlife-livestock interface in a sub-tropical climate where there is a high cattle host density, which provides optimal breeding conditions for various tick species, including *R. microplus*. The movement of cattle in the Mnisi communal area is controlled due to it being classified as a FMD area. All of the cattle farmers in this area make use of communal dip tanks, where weekly dipping applications of cattle takes place, as they are required by regulation to bring their cattle to be dipped and inspected for FMD on a weekly basis. The communal dip tanks are subsidised by the government and the acaricides are selected by the state veterinarians. Currently, Delete® ALL (Amitraz 2,0 % m/v, Deltamethrin 0,50 % m/v, and Piperonyl Butoxide 2,0 % m/v) is used which is a mixture of amitraz and synthetic pyrethroids as active ingredients.

In this study, PCR and sequencing of the OCT/Tyr gene was conducted to investigate the frequencies of the amitraz resistance-linked alleles and to consequently determine the current amitraz resistance status of *R. microplus* ticks in the Mnisi communal area. The genotype frequencies that were obtained were subsequently used to investigate the change in amitraz resistance over the past 6 years with reference to the data published by Robbertse *et al.* (2016). This study provides novel insights on the current amitraz resistance status in the Mnisi communal area as well as the development of amitraz resistance in a controlled setting where a continued selection pressure is applied. The results of this study can be utilized to advise on future use of amitraz in the Mnisi communal area and to formulate improved tick control strategies.

3. Methodology

3.1. Collection of *R. microplus* ticks

A permit under Section 20 of the Animal Disease Act 1984 (Act No. 35 of 1984) to perform a research project or study was approved by the Department of Agriculture, Forestry and Fisheries (DAFF) on 2019-09-19 titled "Molecular diagnostic screening of acaricide resistance genes in *Rhipicephalus* tick species from South Africa and Zimbabwe". Ethical clearance from the University of Pretoria Ethics Committees (reference number NAS186/2019) for tick collection was obtained.

Rhipicephalus microplus engorged adult female ticks were collected from nine communal cattle dip stations in the Mnisi communal area. Tick collection took place in November 2019 via random sampling, where a minimum of ten *Rhipicephalus* ticks were collected per bovine. If there were less than 10 ticks per animal the collected ticks were pooled to generate a single sample for the dip station. Upon collection, ticks were placed in 70% ethanol and were stored at the biobank repository at the University of Pretoria Hans Hoheisen Wildlife Research Station (HHWRS).

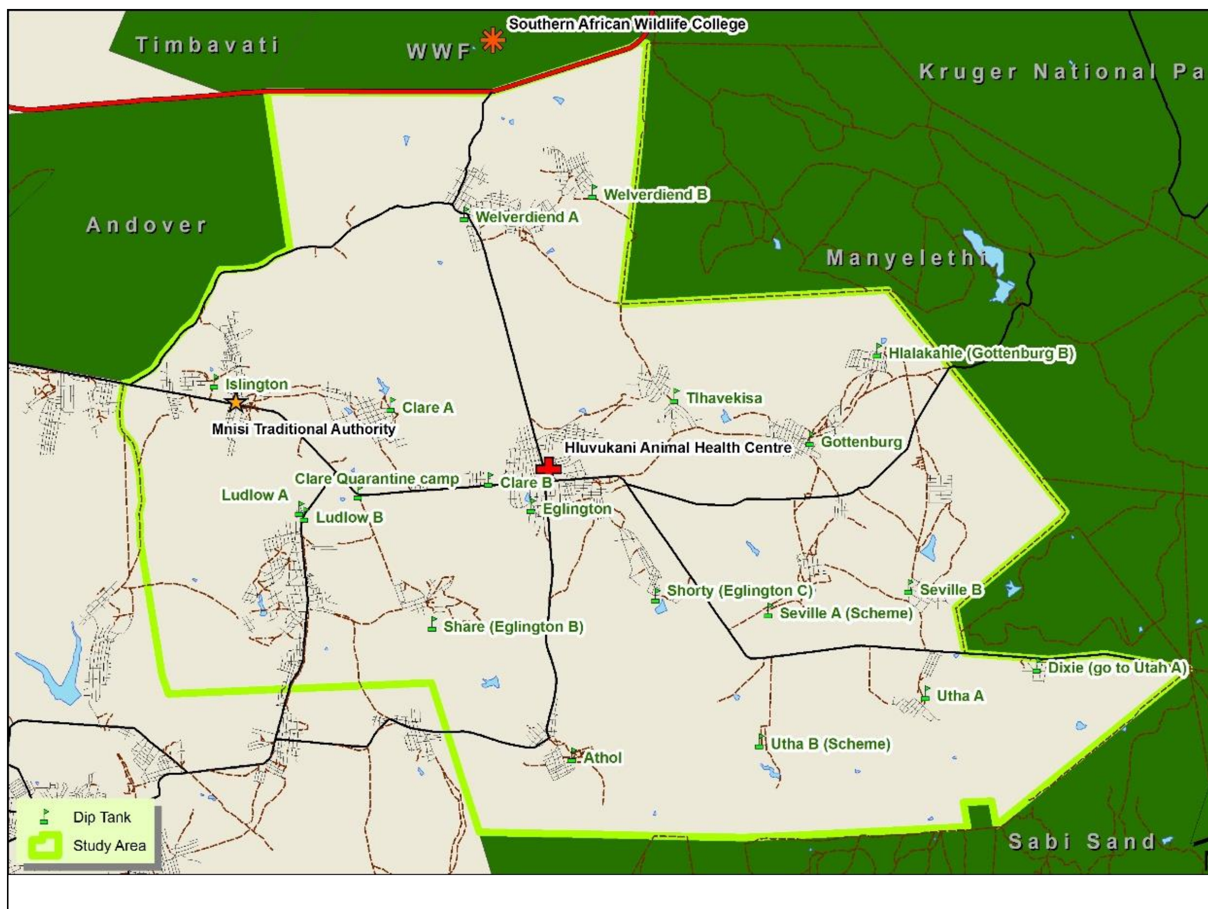


Figure 1: A map of the Mnisi communal area. The green border indicates the study area, and the green flags indicate cattle dip tanks.

3.2. Cataloguing of samples and genomic DNA isolation

For the isolation of genomic DNA (gDNA), a manual Chelex 100 Resin (BioRad) DNA isolation protocol was adapted from Lienhard and Schäffer (2019). Adult ticks were opened with a scalpel and all tissue removed, leaving behind the exoskeleton. The tissue was placed in a 2 ml Eppendorf tube containing 300 µl of 5% Chelex solution in triple distilled water and approximately 20 small glass beads with a diameter of 1.0 mm (Sigma Aldrich 2250473). Thereafter, 2 µl of Proteinase K (50 µg/mL) (Promega) was added and the sample incubated for 30 minutes at 56°C at 2000 rpm in an Eppendorf thermomixer. The sample was then incubated for an additional 30 minutes at 56°C with no shaking, centrifuged for 30 seconds at 15,600 *xg* to pellet the debris and Chelex beads. The supernatant was transferred to a clean 2 ml Eppendorf tube and used for downstream analyses. For every 20 gDNA isolation reactions performed, two samples were randomly selected for quality assessment, where 5 µl sample was loaded onto a 1% agarose gel stained with ethidium bromide and electrophoresed at 100 V for 20 minutes. High molecular weight gDNA was visualized using a UV light. gDNA concentrations were determined using a Nanodrop spectrophotometer (Thermo Scientific, USA).

A code was developed for the labelling and cataloguing of the gDNA samples, where an example of one of the sample names is "SAMAT 10-1R", which means "South Africa Mnisi Athol Cow 10 Tick 1 *Rhipicephalus*". A key for the code that was developed is indicated in Table 1.

Table 1: A table listing the dip stations from the Mnisi communal area where tick samples were collected in November 2019 as well as the key for the code that was developed for the labelling and cataloguing of the samples.

Code:	Key:
SA	South Africa
UTA	Utha A
ALA	Allandale A
IS	Islington
SH	Shorty
LUB	Ludlow B
AT	Athol
DU-C	Dumfries C
First number	Cow number (where P=pooled cows)
Second number	Tick number
R	*Rhipicephalus*

3.3. PCR amplification of ITS2 and amitraz resistance-associated genes

ITS2

Published primers were used to amplify the internal transcribed spacer 2 (ITS2) gene from gDNA samples for species identification: forward primer 5' GCC-GTC-GAC-TCG-TTT-TGA 3' and reverse primer 5' TCC-GAA-CAG-TTG-CGT-GAT-AAA 3' (Lempereur *et al*. 2010). PCR amplification was performed in 25 µl reactions containing 10 pmol of each primer, 200 ng gDNA and 12.5 µl One*Taq* Quick-Load 2x Master Mix with Standard Buffer (New England Biolabs, undiluted mastermix contains 20 mM Tris-HCl; 22 mM KCl; 22 mM NH_4Cl; 1.8 mM $MgCl_2$; 5% Glycerol; 0.05% Tween® 20; 0.06% IGEPAL® CA-630; 0.2 mM dNTPs; 25 units/ml One*Taq*® DNA Polymerase; pH 8.9 at 25°C). The PCR cycling parameters were as follows: 94°C for 30 seconds, 30 cycles of 94°C for 30 seconds, 58°C for 30 seconds and 68°C for 1 minute with a final extension at 68°C for 5 minutes. All PCR reactions were performed in a GeneAmp 9700 thermocycler (PE Applied Biosystems, USA).

Oct/Tyr receptor

Published primers were used for PCR amplification of a 417 bp fragment of the OCT/Tyr gene: forward primer 5' GGT TCA CCC AAC CTC ATC TCT GAA 3' and reverse primer 5' GCA GAT GAC CAG CAC GTT ACC G 3' (Chen *et al*. 2007). The PCR amplification of the OCT/Tyr receptor was performed as described by Baron *et al*. (2015). PCR amplification was performed in 25 µl reactions containing 10 pmol of each primer, 200 ng gDNA and 12.5 µl One*Taq* Quick-Load 2x Master Mix with Standard Buffer (New England Biolabs). The PCR cycling parameters were as follows: 94°C for 30 seconds, 40 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 68°C for 1 minute with a final extension at 68°C for 5 minutes.

Electrophoresis and visualization of PCR products

The presence of amplified DNA fragments was confirmed by agarose gel electrophoresis in a Mini ReadySub-Cell™ GT Cell (Bio-Rad) electrophoresis tank utilising a 2% agarose gel prepared in TAE (40 mM Tris acetate, 1 mM EDTA, pH 8.0) containing ethidium bromide (1 µg/ml). DNA was visualized using the Gel Doc™ XR+ System (BioRad, USA) and the relative sizes of the PCR products calculated from the Rf-values of the 1 Kb molecular marker (New England Biolabs).

3.4. Purification of PCR products

PCR products were purified using the Promega SV Wizard Gel and PCR Clean-up Kit according to the manufacturer's protocol. To each PCR sample, an equal volume of Membrane Binding Solution was added to the PCR reaction. The provided mini column was placed in a collection tube and the diluted PCR product transferred to the column, incubated for one minute at room temperature, centrifuged at 15,600 *xg* for one minute and the flowthrough discarded. Membrane Wash Solution (700 µl) was added to the column, centrifuged at 15,600 *xg* for one minute and the flow-through discarded. The wash step was repeated by using 500 µl of Membrane Wash Solution before a final centrifugation step of one minute was performed to remove residual ethanol. The minicolumn was placed in a clean 2 ml collection tube and 30 µl ddH20 (preheated to 37°C) was added and incubated at room temperature for one minute. The DNA was eluted by centrifugation at 15,600 *xg* for one minute. DNA concentrations were determined using a Nanodrop Spectrophotometer (ThermoFisher Scientific).

3.5. Sequencing and sequence analysis

Standard DNA sequencing was performed at Macrogen Europe B. V. (Amsterdam, Netherlands) on an Applied Biosystems (ABI) 3730XL DNA Analyzer. DNA samples (10 µl) was provided to Macrogen with a minimum concentration of 50 ng/µl along with 10 µM of the OCT/Tyr forward primer and the ITS2 reverse primer. The DNA sequences were analysed using the QIAGEN CLC Main Workbench version 20.02.2. Manual curation of the data was conducted to confirm base calling and to remove areas of low confidence. The ITS2 GenBank sequence entries for *R. microplus* (GenBank Accession: U97715.1) and *R. decoloratus* (GenBank Accession: U97716.1) were used as reference sequences. For the OCT/Tyr sequences, sequence alignments were constructed using the GenBank sequence entries for the resistant Santa Luiza *R. microplus* strain (GenBank Accession EF490688) and the Gonzalez susceptible *R. microplus* strain (GenBank Accession: EF490687.1). The amitraz resistance genotype frequencies were calculated as the number of OCT/Tyr sequences with each genotype divided by the total number of sequences (including all genotypes), where AA/TT is homozygous susceptible, AC/TC is heterozygous and CC/CC is homozygous resistant.

4. Results

4.1. Tick collection

A total of 3125 *Rhipicephalus* ticks were collected from nine communal dip stations in the Mnisi communal area (Table 3). Samples were selected for gDNA isolation from different dip stations where samples of ten ticks or more per cow were collected. Pooled samples of individual ticks from different cows were also selected for gDNA isolation where the number of ticks per cow was limited. A total of 810 individual gDNA isolations were performed (Table 2).

Table 2: Summary of *R. microplus* ticks collected from dip stations in the Mnisi communal area in November 2019.

Name of dip station:	**Number of individual cows sampled with 10 ≤ ticks + ticks pooled from multiple cows (<10 ticks per cow):**	**Number of ticks collected (average weight= 0.12 g):**	**Number of samples selected for gDNA isolation (ticks from individual cows + ticks pooled):**	**Number of gDNA samples selected for PCR and sequencing:**
Eglington	24 + ticks pooled	186	110	14
Clare B	Ticks pooled only	16	16	4
Utha A	Ticks pooled only	22	22	4
Allandale A	10 + ticks pooled	298	140	14
Islington	Ticks pooled only	53	20	4
Shorty	25 + ticks pooled	565	170	21
Ludlow B	23 + ticks pooled	263	107	-
Athol	25 + ticks pooled	980	125	21
Dumfries C	24 + ticks pooled	561	100	14
TOTAL	**131 cows (>10 ticks per cow) + ticks pooled (<10 ticks per cow) per dip station**	**2944**	**810**	**96**

*Ticks were pooled from more than one bovine if there were less than 10 ticks present on a single cow/bull. The pooled samples thus represent a sample that contains multiple ticks from an unspecified amount of cattle.

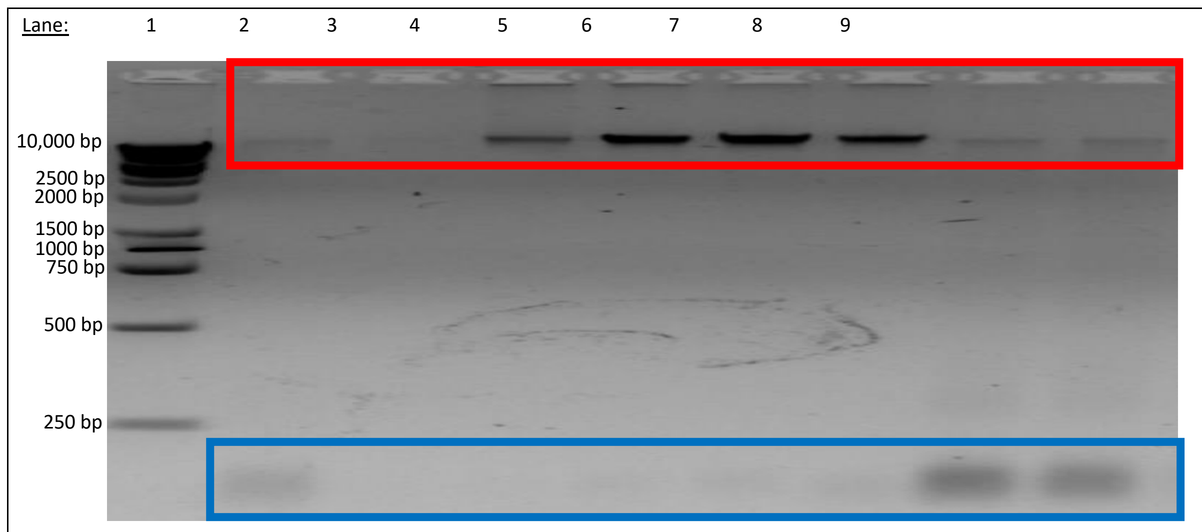


Figure 2: A 1% agarose showing the analyses of crude DNA isolated using Chelex. The red box indicates high molecular weight gDNA and the blue box indicates RNA and/or degraded DNA. Lanes correspond to (1) 1 Kb molecular marker and (2-8) gDNA isolated from a single tick.

4.2. Genomic DNA isolation

High molecular weight gDNA was obtained (Figure 2) with a concentration ranging from 567.3 - 3566.9 ng/ µl per sample. Figure 2 is a representation of a few of the samples that were analysed on a gel, where it is evident that some samples had up to three bands present in a lane (lanes 4-7).

Since no RNase treatment was conducted, the bands observed at the bottom of the gel are likely to be RNA. Alternatively, these bands could also be indicative of degraded gDNA, as it has previously been found by our research group (Reinecke 2015) that including RNase treatment in the DNA isolation protocol did not improve the resolution or integrity of the extracted DNA and the same observed bands remained at the bottom of the gel.

4.3. PCR amplification of ITS2 and the amitraz-resistance associated region of the Oct/Tyr gene segment.

A total of 96 samples were randomly selected for PCR and sequencing (Table 2) to gain insight into the amitraz resistance status in the Mnisi area.

ITS2

The initial ITS2 PCR amplifications performed according to Reinecke (2015) resulted in smears and multiple bands, which was indicative of non-specific amplification (Figure 3, lane 7). The protocol therefore had to be adapted to increase the stringency of the PCRs. Firstly, one PCR was performed with the same conditions as described by Reinecke (2015) but the annealing time was decreased to 20 seconds (Figure 3, lane 2), but non-specific amplification was still observed. Secondly, the number

of cycles were adjusted, where PCRs were performed at 30, 32, 34 and 36 cycles with 30 seconds annealing time (Figure 3, lanes 3-6 respectively). The most stringent amplification was observed at 30 cycles, but there was still a faint smear going downwards from the band indicating non-specific amplification and primer dimers were also observed at the bottom of the gel (Figure 3, lane 3). This indicated that the primer to template ratio was not optimal.

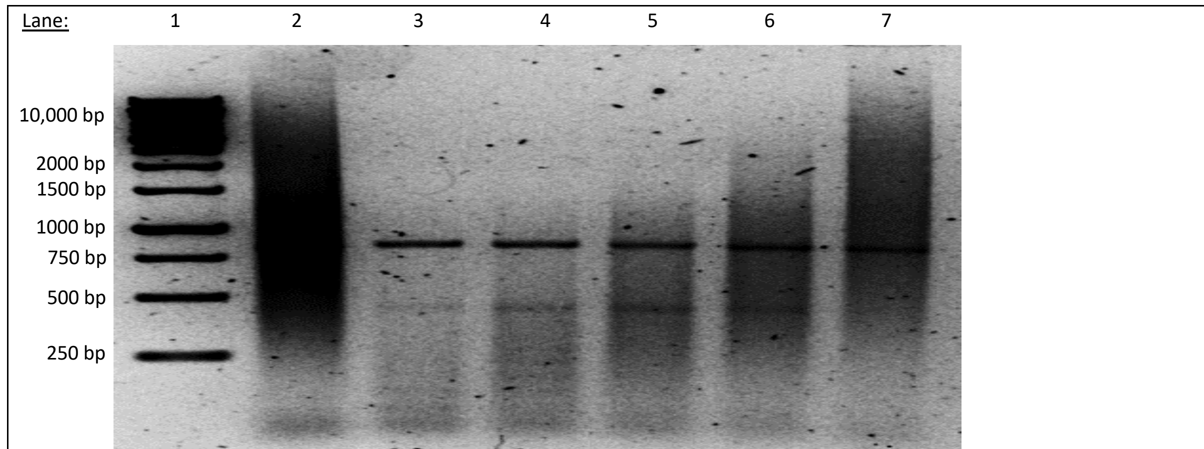


Figure 3: A 2% agarose gel showing the PCR amplification of ITS2 at an expected size of 750-850 bp. Lanes correspond to (1)1 Kb molecular marker, (2) 40 cycles, 20 seconds annealing time, (3) 30 cycles, 30 seconds annealing time, (4) 32 cycles, 30 seconds annealing time, (5) 4 cycles, 30 seconds annealing time, (6) 36 cycles, 30 seconds annealing time and (7) 40 cycles, 30 seconds annealing time.

Thirdly, additional PCRs were performed to optimize the primer to template ratio where concentrations of 200 ng, 150 ng, and 100 ng of template were each tested in combination with a concentration of 10 pmol, 7.5 pmol and 5 pmol of primers (Figure 4). These reactions were performed with 30 seconds annealing time for 30 cycles.

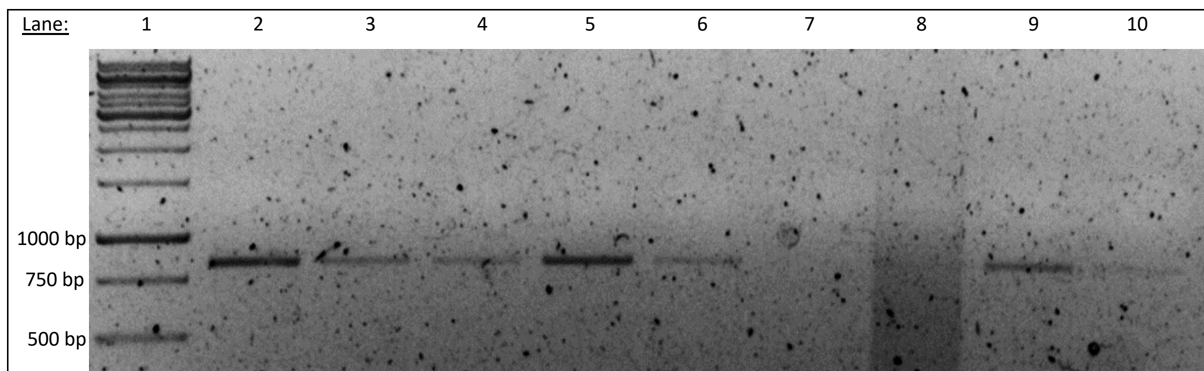


Figure 4: A 2% agarose gel showing the PCR amplification of ITS2 at an expected size of 750-850 bp. Lanes correspond to (1) 1 Kb molecular marker, (2) 200 ng DNA, 10 pmol primers, (3) 200 ng DNA, 7.5 pmol primers, (4) 200 ng DNA, 5 pmol primers, (5) 150 ng DNA, 10 pmol primers, (6) 150 ng DNA, 7.5 pmol primers, (7) 150 ng DNA, 5 pmol primers, (8) 100 ng DNA, 10 pmol primers, (9) 100 ng DNA, 7.5 pmol primers and (10) 100 ng DNA, 5 pmol primers.

It was found that the optimal template and primer combination was 200 ng template with 10 pmol primers and a single band of expected size (750-800 bp) as reported by Lempereur *et al.* (2010) was observed with no smears or primer dimers (lane 2 of Figure 4). This reaction was therefore selected as the optimal PCR amplification of ITS2 and utilized for all downstream experiments (200 ng template, 10 pmol primers, 30 seconds annealing time and 30 cycles).

Two different *Taq* polymerases were also tested to improve the PCR amplification of ITS2 (Figure 5), including AmpliTaq Gold Fast PCR Mastermix (ThermoFisher Scientific) and OneTaq 2x Mastermix (New England Biolabs). Results indicate that both the One*Taq* Quick-Load 2x Master Mix (New England Biolabs) as well as the AmpliTaq Gold (ThermoFisher Scientific) are equally robust for the amplification of the ITS2 gene from gDNA (Figure 5, lanes 5 and 11). A positive plasmid control (as described in Chapter 3, Table 3) (Figure 5, lanes 6 and 12) and a no template control (Figure 5, lanes 7 and 13) was included for each PCR reaction. The One*Taq* Quick-Load 2x Master Mix (New England Biolabs) was chosen for all downstream analyses as it is more cost efficient.

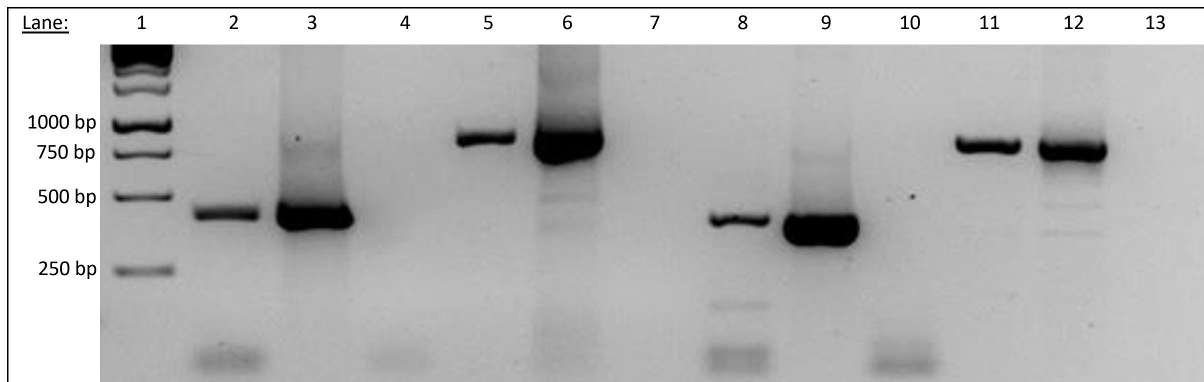
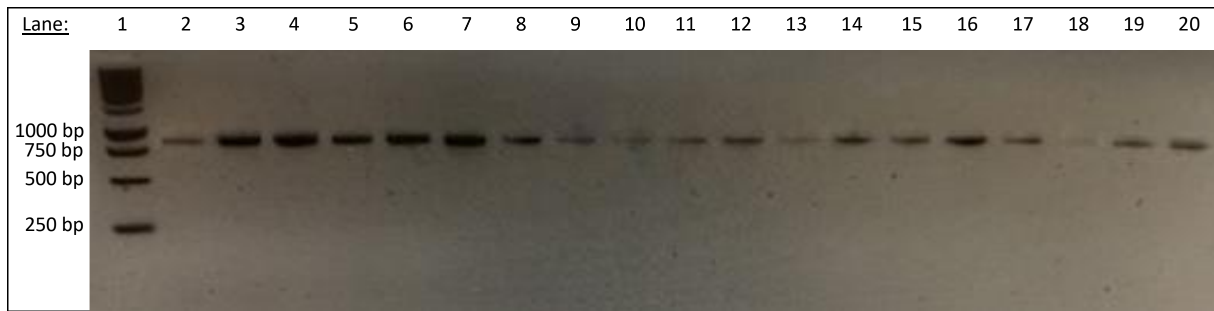


Figure 5: A 2% agarose gel showing the PCR amplification of ITS2 at an expected size of 750-850 bp and the OCT/Tyr gene at an expected size of 417 bp. Each gene was amplified with AmpliTaq Gold Fast PCR Mastermix (ThermoFisher Scientific) (lanes 2-7) as well as OneTaq 2x Mastermix (New England Biolabs) (lanes 8-9). Lanes correspond to (1) 1 Kb molecular marker, (2) OCT/Tyr PCR from gDNA, (3) OCT/Tyr PCR from plasmid, (4) OCT/Tyr PCR negative control, (5) ITS2 PCR from gDNA, (6) ITS2 PCR from plasmid, (7) ITS2 PCR negative control, (8) OCT/Tyr PCR from gDNA, (9) OCT/Tyr PCR from plasmid, (10) OCT/Tyr PCR negative, (11) ITS2 PCR from gDNA, (12) ITS2 PCR from plasmid and (13) ITS2 PCR negative control.

Following optimization, ITS2 was successfully PCR amplified with an expected size of 750-800 bp from 92 of the selected 96 gDNA samples (Figure 6, a selection of products shown). PCR products of the correct size were subsequently purified and submitted for Sanger sequencing.



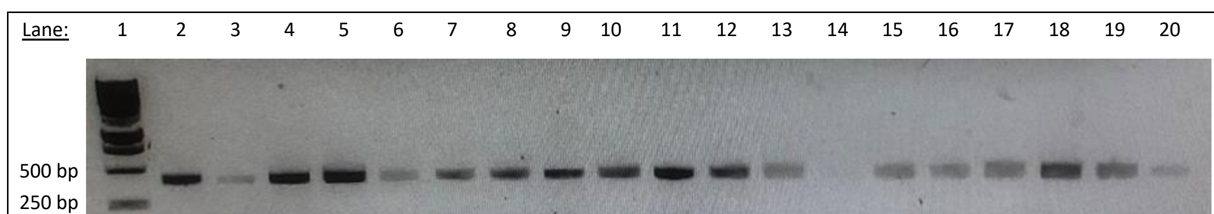
<u>Figure 6:</u> A 2% agarose showing electrophoresis of ITS2 PCR products amplified from a selection of samples. Lanes correspond to (1) 1 Kb molecular marker, (2-20) ITS2 PCR product amplified from a single tick.

However, it should be noted that the amplification of ITS2 still faces some challenges. As can be seen in Figure 3, it is clear that the PCR amplification is largely template dependant, where the same PCR conditions did not work as efficiently for all samples being tested. As a result, a large range of PCR product concentrations was observed (Figure 3). In addition, ITS2 could not be amplified from four of the selected samples despite repeated attempts and optimization. This is likely due to degradation of the gDNA.

OCT/Tyr

Two different *taq* polymerases were tested to determine which would be the most suitable for the PCR amplification of the OCT/Tyr gene. Both the One*Taq* Quick-Load 2x Master Mix (New England Biolabs) as well as the AmpliTaq Gold (ThermoFisher Scientific) were found as equally robust for PCR from genomic DNA (Figure 5, lanes 2 and 8). A positive plasmid control (Figure 5, lanes 3 and 9) and a no template control (Figure 5, lanes 4 and 10) was included for each PCR reaction. The One*Taq* Quick-Load 2x Master Mix (New England Biolabs) was chosen for all downstream analyses as it is more cost efficient.

The OCT/Tyr gene fragment was successfully PCR amplified with an expected size of 417 bp from 91 of the selected 96 gDNA samples (Figure 7, selection of samples shown). PCR products were subsequently purified and submitted for Sanger sequencing.



<u>Figure 7:</u> A 2% agarose gel showing electrophoresis of the OCT/Tyr receptor gene fragment PCR products from a selection of samples. Lanes correspond to (1) 1 Kb molecular marker and (2-20) ITS2 PCR product amplified from a single tick.

As with ITS2, it is also evident that the amplification of the OCT/Tyr receptor gene fragment is template dependent, where there was a range of PCR product concentrations that was observed as well as five samples that would not amplify. This could also be due to degraded gDNA. However, it is also possible that this could be due to a point mutation/s in the binding region of the forward primer on the OCT/Tyr receptor gene fragment, which has been reported previously for a few *R. microplus* samples by Reinecke (2015). These could be evaluated in future studies.

4.4. DNA Sequencing and sequence analysis

ITS2

Sequence data for the ITS2 amplicons were curated and trimmed to a 650 bp region after areas of low confidence were removed. A sequence alignment was constructed with available ITS2 sequences from GenBank for *R. microplus* and *R. decoloratus* to identify the tick species of each individual sample (Figure 8).

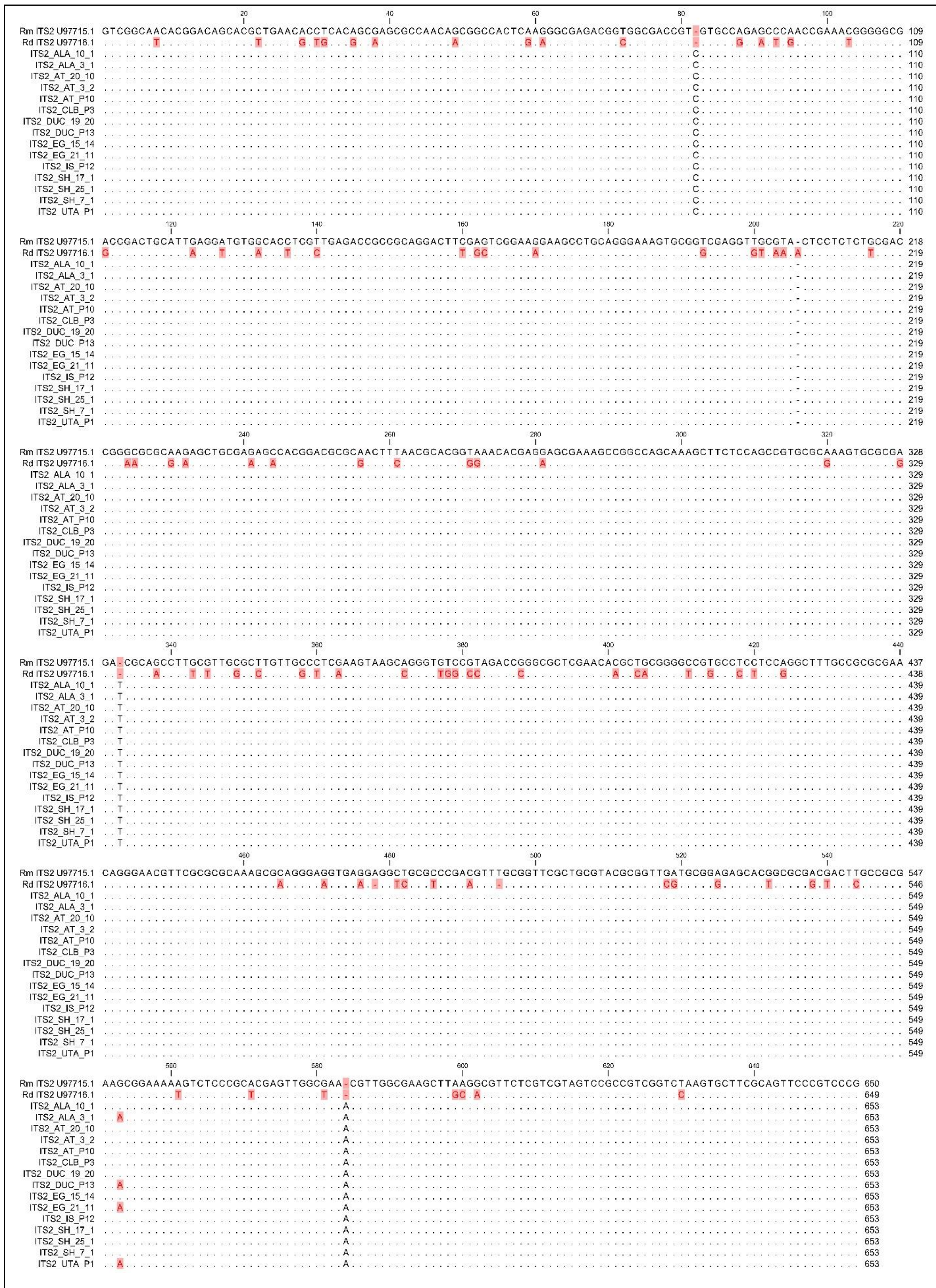


Figure 8: A 650 bp region of the ITS2 sequence alignment of selected sequences. One sequence is included from each sample from each dip station. RmITS2 U97715.1 and RmITS2 U97716.1 were the reference sequences used for *R. microplus* and *R. decoloratus* respectively. The letters indicate the dip station, the first number indicates the cow and the second number indicates the tick. Different residues are indicated in red.

Sequence analysis confirmed 81 samples as *R. microplus*. The remaining 11 sequences were of poor quality and were discarded. These need to be repeated in future studies. Within the 650 bp alignment, three nucleotide insertions can be seen at nucleotide positions 82, 333 and 584 (Figure 8). These are consistent across all of the sequences that were not common with either of the reference sequences. One point mutation that occurs in 12 of the *R. microplus* sequences is also evident at nucleotide position 553. This observed sequence variation is likely due to the geographical variation between different strains of *R. microplus* ticks as the reference sequences used are from Australia.

OCT/Tyr

Sequence data for the OCT/Tyr gene fragments were curated and trimmed to a 230 bp region that contains both of the amitraz-resistance associated SNPs of interest. The sequence alignment confirmed the amplification of the OCT/Tyr receptor gene fragment from 82 *R. microplus* ticks and 9 sequences were of poor quality and were discarded. At the nucleotide positions of the two amitraz-resistance associated SNPs (indicated by red arrows in Figure 9 and 10), a single peak was genotyped as homozygous and a double peak was genotyped as heterozygous from the sequence chromatograms (Figure 10). There were also several additional SNPs that were observed (indicated by blue arrows in Figure 9).

The two amitraz-resistance associated SNPs were evaluated and the frequencies of susceptible (AA/TT), heterozygous (AC/TC) and resistant (CC/CC) genotypes were calculated as 0.09, 0.85 and 0.06, respectively. This correspond to 9% of the investigated population being susceptible to amitraz, 85% heterozygous and 6% as homozygous resistant.

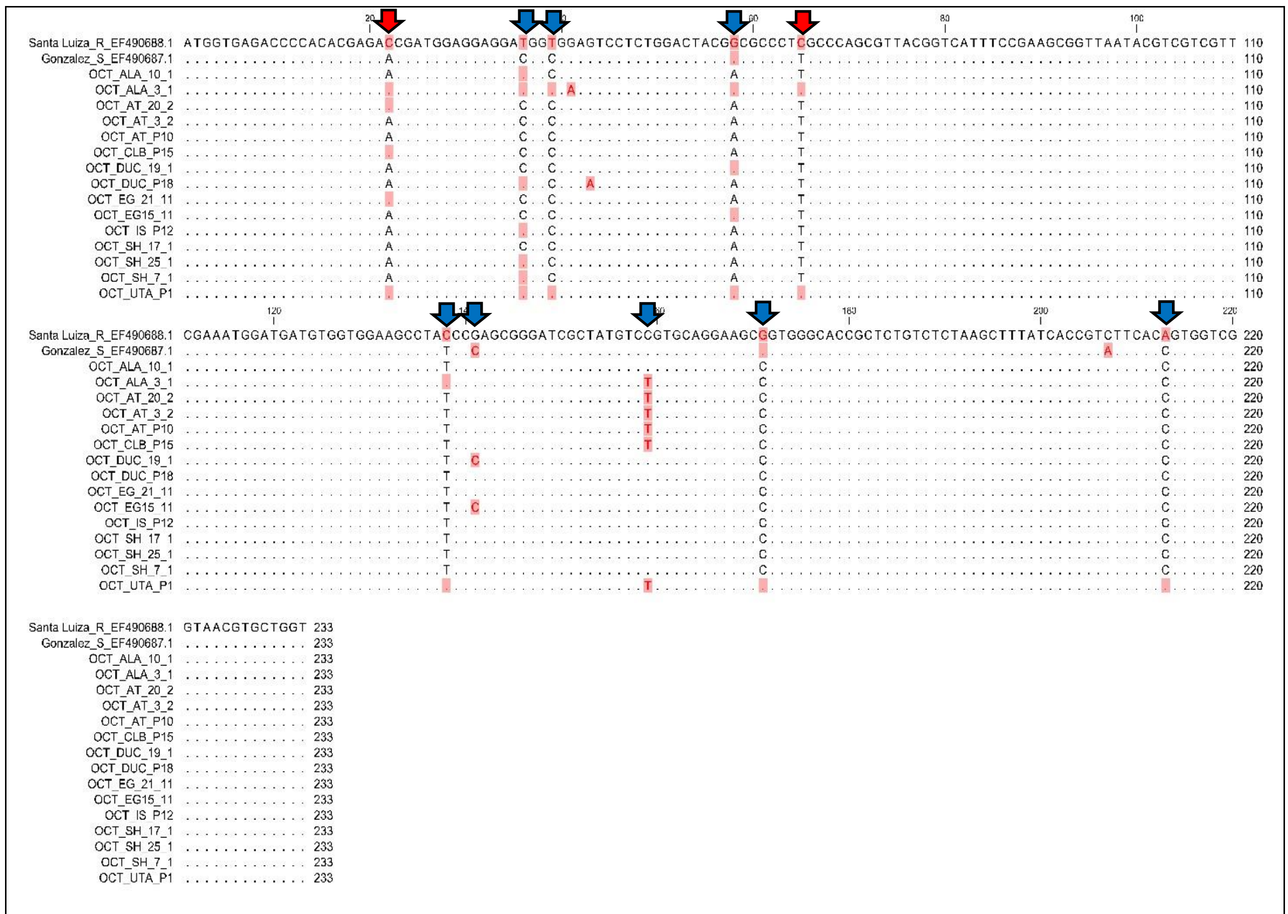


Figure 9: A 230 bp region of the OCT/Tyr gene fragment sequence alignment. The red arrows indicate the positions of the two amitraz-resistance associated SNPs. The blue arrows indicate additional SNPs/point mutations. Santa Luiza_R_EF490688.1 and Gonzalez_S_EF490687.1 are the reference sequences from the resistant Santa Luiza *R. microplus* strain and the Gonzalez susceptible *R. microplus* strain respectively. The letters indicate the dip station, the first number indicates the cow and the second number indicates the tick. Different residues are indicated in red.

Figure 10: An 80 bp region of the sequence chromatograms of the OCT/Tyr gene fragment. The red arrows indicate the positions of the two amitraz-resistance associated SNPs. A single peak indicates a homozygous genotype and a double peak indicates a heterozygous genotype. Santa Luiza_R_EF490688.1 and Gonzalez_S_EF490687.1 are the reference sequences from the resistant Santa Luiza *R. microplus* strain and the Gonzalez susceptible *R. microplus* strain respectively. The letters indicate the dip station, the first number indicates the cow and the second number indicates the tick.

4.5. The change in amitraz resistance over time

The genotype frequencies of the two amitraz-resistance associated SNPs in the OCT/Tyr gene fragment from this study were compared to the frequencies reported by Robbertse *et al.* (2016) (Figure 11) for *R. microplus* ticks collected in 2012/2013 in the Mnisi communal area. Robbertse *et al*. (2016) reported the frequencies of susceptible (AA/TT), heterozygous (AC/TC) and resistant (CC/CC) genotypes as 0.45, 0.52 and 0.03, respectively (Figure 11B).

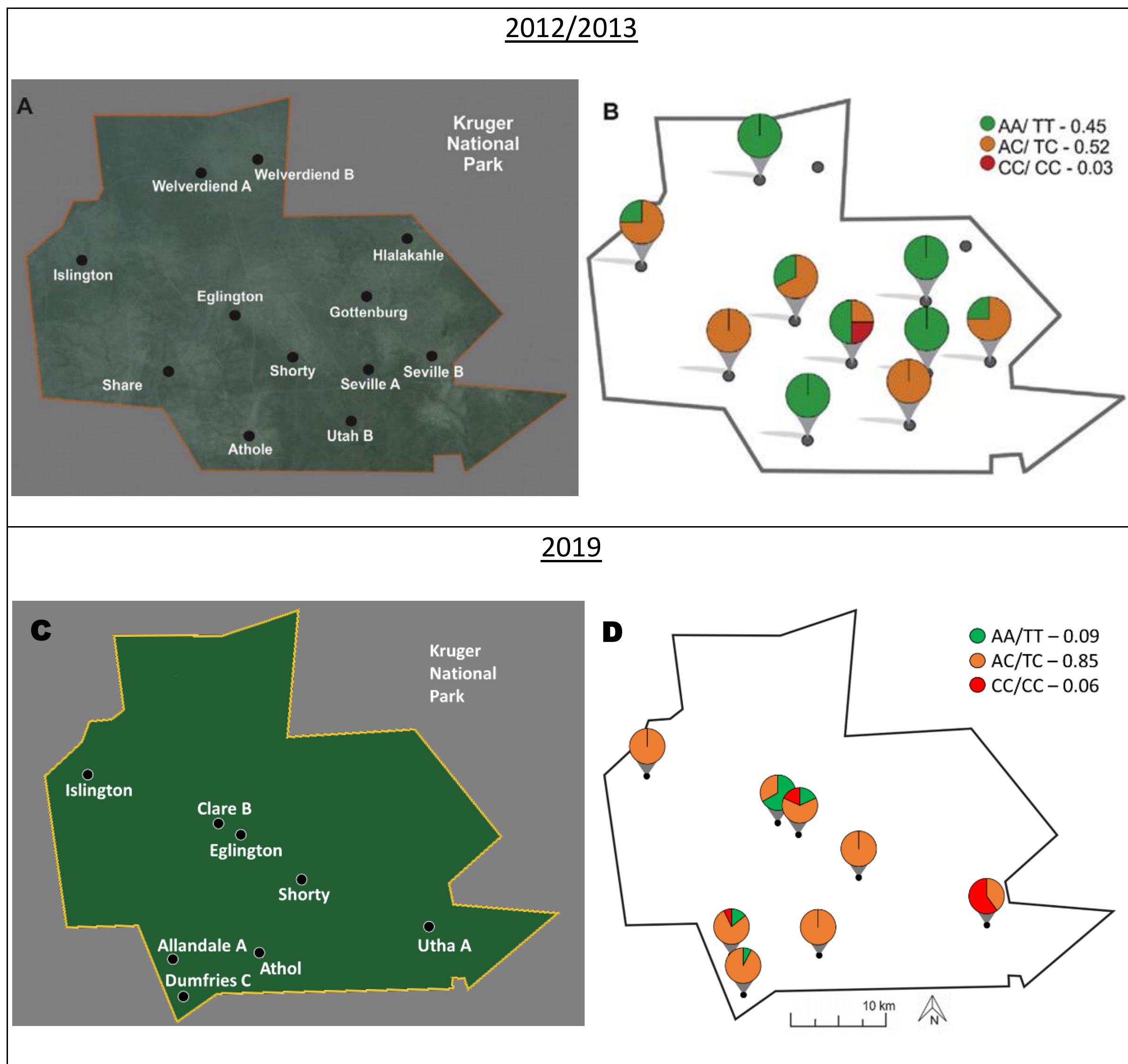


Figure 11: The distribution of cattle dip stations in the Mnisi communal area and their related amitraz resistance alleles. (A) Topographical map of the Mnisi communal area with dip stations sampled in 2012-2013. (B) Genotypic frequencies and distribution of the OCT/Tyr amitraz resistance alleles in 2012-2013. (C) Topographical map of the Mnisi communal area with dip stations sampled in 2019. (D) Genotypic frequencies and distribution of the OCT/Tyr amitraz resistance alleles in 2019.

The frequency of the homozygous susceptible genotype in the overall population has largely decreased over the past 6 years whilst the heterozygous genotype has largely increased. This is similar to what was observed at the four dip stations (Islington, Athol, Eglington and Shorty) that were sampled both in 2012/2013 and 2019, where a decrease in the number of susceptible samples can be observed as well an increase in the number of heterozygous samples. Only a slight increase (3%) was observed for the homozygous resistant genotype, although the 2019 frequency was double the 2012/2013 frequency.

For the ticks sampled in 2019, the heterozygous genotype was most prevalent across all dip stations, except for Clare B which was predominantly homozygous susceptible and Utah A which was predominantly homozygous resistant (Figure 11D). However, it should be noted that only 4, 3 and 4 samples were analysed for resistance at Islington, Clare B and Utah A, respectively, and that the genotype frequencies may differ if a larger number of samples were processed. A summary of the samples analysed per dip station in 2012/2013 and 2019 is summarized in Table 3.

Table 3: A table summarizing the *R. microplus* tick samples processed and analysed per dip station in the Mnisi communal area in 2012/2013 by Robbertse *et al.* (2016) and in 2019 (this study). The numbers in brackets indicate the number of homozygous susceptible (AA/TT), heterozygous (AC/TC) and homozygous resistant (CC/CC) samples. The common dip stations that were sampled are indicated in blue. NS= Not Sampled.

Dip station	**Number of samples processed**		**OCT/Tyr genotypes**	
	2012/2013	**2019**	**2012/2013**	**2019**
Islington	6	4	4 (2; 2; 0)	4 (0; 4; 0)
Athol	5	18	3 (1; 2; 0)	16 (0; 16; 0)
Eglington	6	14	5 (2; 3; 0)	11 (2; 7; 2)
Shorty	6	20	4 (2; 1; 1)	20 (0; 20; 0)
Utha A	NS	4	-	4 (0; 2; 2)
Clare B	NS	4	-	3 (2; 1; 0)
Allandale A	NS	14	-	14 (2; 11; 1)
Dumfries C	NS	13	-	10 (1; 9; 0)
Welverdiend B	4	Sampled in October 2020 and will be analysed in 2021	0 (0; 0; 0)	-
Welverdiend A	3		1 (1; 0; 0)	-
Share	6		3 (0; 3; 0)	-
Hlalakahle	7		0 (0; 0; 0)	-
Gottenburg	6		3 (3; 0; 0)	-
Seville B	4		4 (1; 3; 0)	-
Seville A	5		2 (2; 0; 0)	-
Utha B	6		2 (0; 2; 0)	-
Total	**64**	**91**	**31 (14; 16; 1)**	**82 (7; 70; 5)**

5. Discussion

Amitraz resistance status of *R. microplus* ticks in the Mnisi communal area

The 2019 sampling and investigation conducted in this study provides novel insights into the current amitraz resistance status of *R. microplus* ticks in the Mnisi communal area at selected dip stations. This study determined the genotype frequencies of the amitraz resistance-associated SNPs in the OCT/Tyr gene of *R. microplus* ticks using PCR and DNA sequencing, where it was found that for the *R. microplus* population that was investigated, 7% was susceptible to amitraz, 87% was heterozygous and 6% was resistant.

The sequence alignments of the OCT/Tyr gene fragment also identified several other nucleotide substitutions in addition to the two validated amitraz resistance-associated SNPs. However, the presence of additional mutations in the OCT/Tyr gene was as expected as these findings are consistent with those of previous studies. For instance, Baron *et al.* (2015) identified multiple mutations occurring in the OCT/Tyr gene and described certain nucleotide substitution patterns occurring in either the resistant or susceptible strains. It has previously been hypothesized that these additional mutations may be necessary for amitraz resistance to occur in the tick depending on whether or not the tick is under selection pressure by means of exposure to amitraz treatment (Reinecke 2015). However, these mutations have not previously been validated or linked to being associated with amitraz resistance. There is the possibility that these mutations could potentially increase the fitness of *R. microplus* ticks, but this remains to be investigated. For the purposes of this study these additional nucleotide substitutions were thus not investigated further, although their possible role in amitraz resistance is currently being investigated in a separate project currently being conducted by a student in our research group.

It should also be noted that there are three different classes of octopamine receptors that have been identified in arthropods which could serve as binding sites for amitraz in *R. microplus* ticks, including the α-adrenergic-like octopamine receptor (αAOR) the OCT/Tyr and the β-adrenergic like octopamine receptor (βAOR). This study only focused on the two validated SNPs in the OCT/Tyr gene that have previously been linked to amitraz resistance (Baron et al. 2015), but there is also a SNP that can occur in the βAOR gene that has been linked to amitraz resistance in *R. microplus* ticks (Corley *et al*. 2013). This mutation occurs at nucleotide position 181 where adenine is substituted by thymine, which results in an I61F substitution in the first transmembrane domain of the receptor, which is a highly conserved region (Corley *et al*. 2013). However, to date this mutation has only been linked to

resistance in the amitraz resistant Ultimo strain of *R. microplus* from central Queensland, Australia, and was not found in North and Southeast regions of Australia (Corley *et al*. 2013). A review paper by Jonsson *et al*. (2018) also described the presence of the I61F mutation in amitraz resistant South American isolates of *R. microplus*, although the primary research article has not yet been published and as such these results cannot be verified. Analysis of the amitraz resistance-associated SNP in the βAOR gene was therefore not included in this study, but PCR and sequencing of this gene should be conducted in future studies to confirm whether it is present in South African *R. microplus* populations.

Jonsson *et al*. (2018) also identified seven other SNPs in the βAOR gene of *R. microplus* ticks from multiple countries, including Brazil, Mexico, Australia, Thailand and South Africa. However, the association of these SNPs with amitraz resistance can only be verified after the publication of these results. Once association with resistance has been validated, these SNPs could also potentially be used as additional molecular markers in the future molecular diagnosis of amitraz resistance in *R. microplus* populations.

Amitraz resistance: a potential fitness cost for *R. microplus* ticks

This study investigated the change in amitraz resistance over time in the Mnisi communal area from ticks sampled in 2012/2013 to 2019. The results show a 38% decrease in susceptibility, a 35% increase in heterozygosity and a 3% increase in resistance over 6 years from the *R. microplus* population that was investigated in 2012/2013 (Robbertse *et al*. 2016). The amitraz resistance-associated genotype frequencies found in this study support the hypothesis of Robbertse *et al*. (2016) that the weekly dipping regime at the Mnisi communal area dip stations likely serves as continued selection pressure to maintain the amitraz resistance-associated alleles in a heterozygous state. The 3% increase in the homozygous resistant genotype and the 35% increase in the heterozygous genotype could be indicative that amitraz resistance is emerging as the number of resistance alleles in the population is increasing. However, this would not explain why the homozygous resistant genotype did not exhibit a larger increase in frequency over the past 6 years, as would be expected when a mutant allele increases the fitness of individuals in a population (Fisher 1930; Williams 1966). Considering that a loss of variation did not occur, it is likely that directional selection is not taking place in this population (Kaplan *et al.* 1989). The abundance of heterozygosity in the population is thus more likely indicative that positive balancing selection is taking place, where the SNPs in the OCT/Tyr gene are being maintained (Nordburg and Innan 2002).

It is therefore possible that the homozygous resistant genotype does not increase the relative fitness of individuals in a population and that it actually poses a fitness cost to *R. microplus* ticks. This fitness

cost may be associated with a significant disadvantage in comparison to heterozygous or homozygous susceptible individuals in a population, as has been reported for insects (Kliot and Ghanim 2012), which would explain the abundance of heterozygous individuals observed at both time points. This has previously been suggested to be the case for *R. microplus* ticks, but limited studies have been conducted to confirm this where there were inconclusive results (Davey *et al*. 2006).

Based on the results of this study and the observed change in amitraz resistance over time, it is hypothesized that the heterozygous genotype increases the relative fitness of *R. microplus* ticks, i.e., has a positive fitness cost, as it allows for increased genetic diversity (Reed and Frankham 2003). The heterozygous genotype could potentially confer partial resistance to amitraz but also maintain adequate reproductive fitness, which could explain why the majority of ticks remain heterozygous and do not become homozygous resistant. If this hypothesis is true, this could potentially explain why only a slight increase in the resistant genotype was observed over 6 years when major changes were observed in the susceptible and heterozygous genotypes between 2012/2013 and 2019 after a continued selection was applied by weekly dipping.

Amitraz for the future control of *R. microplus* ticks in the Mnisi communal area

The findings of this study can be used to advise farmers of how best to proceed with acaricide dipping regimes at cattle dip stations in the Mnisi communal area. Based on the results of this study, amitraz may still be viable for inclusion as part of an integrated tick control strategy for *R. microplus* as the ticks are not becoming homozygous resistant. The acaricide currently being used at dip stations in the Mnisi communal area (Delete® ALL) contains amitraz, but it also contains synthetic pyrethroids (SPs). A study conducted in parallel to this study by Smit (2021) found the frequency of SP resistance to be 100% in all of the same *R. microplus* ticks that were screened for amitraz resistance in this study. This is indicative that the SPs are not suitable for tick control in the Mnisi communal area, and that perhaps a different dip should be utilized that is either purely amitraz-based or utilizes a different class of acaricides such as macrocyclic lactones (MLs), although ML's are notably more expensive and may not be suitable for small-scale farmers.

Thereafter, a level of susceptibility can be maintained by rotating two or more acaricides that have different modes of action and no potential for cross-resistance, which will consequently reduce the selection pressure to any specific class of acaricides (Abbas *et al*. 2014; Rodriguez-Vivas *et al*. 2014). Synergists can also be used in combination with acaricides to potentiate their effects (Li *et al.* 2007),

although this may not be viable in the Mnisi communal area due to increased costs. It is also essential that the resistance status of tick populations continue to undergo regular monitoring from a diagnostics laboratory to allow farmers to implement well-informed tick control strategies on an ongoing basis.

Study limitations and future prospects

The time comparison in this study is limited and can merely serve as a rough indication of the change in amitraz resistance over time. The 2019 sampling process was limited by the availability of *R. microplus* ticks at the respective dip stations at the time that sampling took place. There were only four common dip stations that were sampled in both 2012/2013 and 2019. A more accurate indication of the change in amitraz resistance could be obtained if ticks are screened from all of the same dip stations that were sampled in 2012/2013. Additional samples were thus collected in 2020 from the dip stations that could not be sampled in 2019, and future studies will include this data to provide a more comprehensive understanding of the change in amitraz resistance over time. In addition, some of the dip stations that were sampled in 2019 did not have a representative enough sample to infer a resistance status for that population, for instance Islington, Utha A and Clare B only had four, four and three samples respectively. A more accurate representation of the amitraz resistance genotype frequencies could be obtained if a larger number of ticks were included in the study. Future studies will therefore include the PCR and sequencing of additional samples from these dip stations to obtain OCT/Tyr gene sequences for a minimum of 10 samples per dip station.

There are also a number of other factors which should be incorporated into future studies to better evaluate amitraz resistance in *R. microplus* populations. Firstly, the complex interaction between the continuous presence of amitraz selection pressure and the proposed positive fitness cost associated with heterozygosity could be further investigated if the functional mechanism of the resistance-associated SNPs in the OCT/Tyr gene is elucidated. Secondly, ticks could be subjected to bioassays, such as Larval Packet Tests (LPTs), to confirm the level of their phenotypic resistance in relation to their resistance genotype. Thirdly, synergistic assays could also be employed in conjunction with LPTs to detect the involvement of metabolic enzymes in acaricide resistance. These enzymes may induce/enable acaricide resistance/detoxification both through the increased levels of their expression and/or potentially through mutations.

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Chapter 3

Towards the development of a TaqMan SNP genotyping assay for the detection of amitraz resistance in *Rhipicephalus microplus*

1. Abstract

Amitraz resistance has been reported in *Rhipicephalus microplus* populations around the world. Conventional methods to detect amitraz resistance involves the use of bioassays, which are time consuming and provide no information on the resistance genotype of ticks. More recently, PCR-based assays have been employed to detect resistance-associated SNPs and to genotype the resistance of *R. microplus* populations. However, PCR-based assays are also associated with challenges and time constraints, where diagnostics usually takes two days per sample. TaqMan SNP (single nucleotide polymorphism) genotyping assays could prove to be a promising way forwards for the detection of acaricide resistance-associated mutations in *R. microplus* ticks. These assays could largely improve the turnover time of genetic testing, where hundreds of samples could be screened for all genetic markers within a few hours. A TaqMan SNP genotyping assay has previously been utilised for the diagnostic detection of a SNP in the para-sodium channel gene that is associated with resistance to synthetic pyrethroid acaricides in *R. microplus* ticks. To date, no TaqMan SNP genotyping assays have been developed for the detection of the amitraz-resistance associated SNPs in the octopamine/tyramine receptor (OCT/Tyr) gene. The aim of this study was therefore to design and test TaqMan SNP genotyping assays to detect the two SNPs T8P and L22S in the OCT/Tyr gene that have previously been linked to amitraz resistance. Findings indicate that one of the assays (Oct 2_v2) has the potential for the detection of the L22S amitraz resistance-linked SNP, where an overall rate of correct calling was calculated at 73.33% from 15 *R. microplus* field samples. Further optimization is however required. A potential species identification assay was also designed, which proved successful in identifying *R. decoloratus* ticks but not *R. microplus* ticks. As such, a new ITS2 TaqMan SNP genotyping assay may need to be developed for improved discrimination between *R. decoloratus* and *R. microplus* ticks.

2. Introduction

Rhipicephalus microplus, also referred to as the southern cattle fever tick, is the most economically important global ectoparasite of cattle (Cunha *et al.* 2012). Currently, the most common method of tick control is the use of chemical acaricides (Novato *et al.* 2018; Rodriguez-Vivas *et al.* 2018). However, acaricide resistance has been reported in tick populations around the world for all major classes of chemical acaricides (Rodriguez-Vivas *et al*. 2013; Rodriguez-Vivas *et al.* 2006; Rodriguez-Vivas *et al.* 2012; Li *et al.* 2003; Miller *et al.* 2005). Current methods to detect acaricide resistance include *in vitro* bioassays as well as PCR-based assays, where both have associated advantages and disadvantages.

In vitro bioassays are the most widely used method to detect acaricide resistance and generally involve the evaluation of dose responses (Rodriguez-Vivas *et al*. 2018). Briefly, ticks are exposed to serial dilutions of an acaricide and the lethal concentration (LC) is determined, whereafter the LC is utilized to calculate a resistance factor (Robertson *et al.* 2007). Examples of *in vitro* bioassays are the larval packet test (LPT) (Stone and Haydock 1962), larval immersion test (LIT) (Shaw 1966), adult immersion test (AIT) (Drummond *et al.* 1973) and larval tarsal test (LTT) (Lovies *et al.* 2011). The advantages of these bioassays are that they are portable, low cost and they are able to determine the resistance phenotype of ticks. The disadvantages are that bioassays are labour-intensive, live ticks are required, it can take up to six weeks to obtain results (Guerrero *et al.* 2014), large differences are observed in LC values with wide confidence intervals (Jonsson *et al.* 2018) and, lastly, they provide no information about the resistance genotype of ticks.

PCR-based assays offer several advantages over *in vitro* bioassays in detecting acaricide resistance. Examples of PCR-based assays include allele-specific PCR (AS-PCR), restriction fragment length polymorphisms (RFLP) (Baron *et al*. 2015; Robbertse *et al*. 2016 Sungirai *et al.* 2018), quantitative PCR (qPCR) utilizing TaqMan probes (Kim *et al*. 2007) and high-resolution melt (HRM) analysis (Klafke *et al.* 2019) as well as TaqMan SNP genotyping assays (Morgan *et al.* 2009). PCR-based assays are more rapid and accurate than bioassays, they can be performed from a single tick, live ticks are not required, resistance-associated mutations can be detected and resistance genotypes can consequently be determined (Kim *et al.* 2007; Morgan *et al.* 2009; Guerrero and Pruett 2003; Carvalho *et al*. 2013; Millán Leiva *et al.* 2018).

The main disadvantage of PCR-based assays is that more advanced equipment is required to conduct testing, while the others come with their own potential disadvantages. For instance, HRM analysis may not be suitable for high-throughput screening as differences between the curves of only 2°C were observed (Klafke *et al.* 2019), which makes accurate and consistent genotyping difficult. In addition,

AS-PCR and RFLP takes two days per sample for diagnostics (Baron *et al*. 2015), where PCR from field samples (often degraded) and sequencing of resistance target genes has proven to be challenging in the past (Reinecke 2015).

TaqMan SNP genotyping assays could prove to be a viable and feasible way forward for the detection of SNPs associated with acaricide resistance in *R. microplus* ticks. For instance, a TaqMan SNP genotyping assay has previously been utilised for the detection of resistance-associated SNPs in *R. microplus*, where Morgan *et al.* (2009) developed a TaqMan SNP genotyping assay to detect a SNP in the para-sodium channel gene that is associated with resistance to synthetic pyrethroid acaricides. These assays offer several advantages over conventional PCR and sequencing. For instance, TaqMan SNP genotyping assays have the potential to largely improve the turnover time of resistance screening in ticks, where hundreds of individual samples can be rapidly genotyped for resistance-linked SNPs simultaneously due to the allele-specific discrimination abilities of the assays. In addition, TaqMan SNP genotyping assays are conducted directly from genomic DNA (gDNA) utilising automated qPCR and post-PCR methodologies are not required, such as gel electrophoresis, PCR clean-up, sequencing and sequence analysis, which reduces the cost and time requirements of diagnostic screening. The only limitation is that TaqMan SNP genotyping assays can only provide information on targeted SNPs and additional sequence variation will not be detected as with conventional PCR and sequencing.

TaqMan technology utilizes allele-specific probes for the quick and reliable genotyping of known polymorphic sites, including SNPs, insertions/deletions and the presence/absence of variants (Woodward 2014). TaqMan SNP genotyping is useful for the detection and screening of SNPs in hundreds of individual samples (Heissl *et al.* 2016), where the alleles of the SNPs define genotypes that are of interest in a population (Walker *et al.* 2007; De la Vega *et al.* 2005; Callegaro *et al.* 2006). TaqMan SNP genotyping assays designed to detect a single bi-allelic polymorphism involve the qPCR amplification of a target sequence utilising specific forward and reverse primers and the hybridization of fluorescently labelled TaqMan probes to detect the alleles of a SNP within the amplicon (Walker *et al*. 2007). The assay contains two probes that are distinctly labelled with fluorophores/reporter dyes (e.g. VIC or FAM) at the 5' end (Woodward 2014), which are designed to hybridize to the different alleles of a SNP operating at the same locus. Hybridization will result in fluorescence of either one or both of the fluorophores which allows for genotyping of the alleles of interest (Walker *et al*. 2007). TaqMan SNP genotyping assays are able to reliably distinguish between two alleles differing at only one position, because a single base mismatch in the short TaqMan probe is sufficient to influence the hybridization of the probe (Heissl *et al.* 2016).

Each TaqMan probe also has a MGB (minor-groove binder) as well as a nonfluorescent quencher at the 3’ end (Hartsthorne 2013). The MGB binds to the minor groove of the DNA molecule which enhances the probe hybridization efficiency (de Kok *et al.* 2002; Latif *et al.* 2006) by stabilizing the binding of the probe to the template DNA. This consequently increases the probe melting temperature (T_m) and allows for the design of shorter probes in TaqMan SNP genotyping assays. The TaqMan probes can therefore be subjected to higher annealing temperatures, which increases the specificity of probe hybridization as well as the annealing of the primers to the target DNA sequence during PCR amplification (Hartsthorne 2013). The quencher serves to absorb the light emission of the fluorophore on the 5’ end of the TaqMan probe as the absorption spectrum of the quencher overlaps with the emission spectrum of the reporter dye (Heissl *et al.* 2016). The quencher thus prevents the fluorescence of the fluorophore when the TaqMan probe is intact, i.e. prior to PCR amplification (Hartsthorne 2013). In addition, any mismatched probes will be unstable and will not be cleaved by the 5’-3’ exonuclease activity of the *Taq* polymerase, which prevents the release of fluorescence due to the 3’ quencher (Heissl *et al.* 2016). The shorter probes also promote a better quenching effect on the 5’ fluorophore and high background noise can be avoided in the qPCR results (Reynisson *et al.* 2006).

During PCR amplification, the complementary TaqMan probe will hybridize to the target DNA sequence and the fluorophore will be cleaved from the probe by the 5’-3’ exonuclease activity of the *Taq* polymerase (Hartsthorne 2013; Walker *et al.* 2007; ThermoFisher Scientific 2017). The separation of the fluorophore from the quencher results in the fluorescence of the fluorophore, which will increase as PCR product accumulates in the qPCR reaction (Walker *et al.* 2007; ThermoFisher Scientific 2017). Upon completion of the qPCR reaction, the emission intensity of each fluorophore is measured and the alleles at the site of interest can be determined (Woodward 2014). The amount of fluorescence of one fluorophore relative to the other becomes an X, Y bivariate, of which the values indicate the likelihood of one or both of the genotypes being present in the sample (De la Vega *et al.* 2005; Callegaro *et al.* 2006). Statistical algorithms classify the fluorescence values to the correct allele utilising cluster analysis and maximum likelihood estimation, allowing for the genotype "calling" of the alleles (Callegaro *et al.* 2006). This ultimately allows for allele-specific discrimination of a single base pair (Hawthorne 2013; Callegaro *et al.* 2006).

When designing and testing a TaqMan SNP genotyping assay there are several factors to consider. Firstly, specific primers and probes need to be designed. Highly conserved areas for probe and primer design can be identified from multiple sequence alignments of the DNA target region (Bass *et al*. 2008). Heissl *et al.* (2016) makes the following recommendations for probe design: the SNP should be as central as possible; the 5’ base must not be a G nucleotide, as a G can possibly quench the fluorophore

even after the fluorophore has been cleaved from the probe; the GC content of the probe should be between 40 and 60 % and should contain more Cs than Gs. Species-specific factors also need to be taken into consideration in probe and primer design. For instance, probe and primer design may be challenging for target DNA sequences from ticks given the repetitive nature of their genomes, where it was estimated that the *R. microplus* genome may contain up to 70% repetitive DNA (Barrero *et al*. 2017). TaqMan probe design is very difficult in repetitive sequences or for SNPs which are surrounded by mono-, di-, and trinucleotides (Heissl *et al.* 2016). It is therefore essential to carefully design the primers and probes to be as robust as possible to reduce assay optimization and to save costs and time to redesign (Heissl *et al*. 2016). The primers and probes should also be assessed for self-complementarity and a primer BLAST search should be conducted to ensure that the primer is specific to the target sequence and does not bind to any other site (Western and Surani 2002).

Prior to large-scale screening of samples, TaqMan SNP genotyping assays should be optimized utilizing appropriate controls, where the specificity and sensitivity of the assays need to be determined. Previously it was recommended that the primers and probes should be optimized separately (Heissl *et al.* 2016), but in more recent years TaqMan SNP genotyping assays have been made available for custom order as pre-optimized single tube assays which contain both primers and probes (ThermoFisher Scientific 2017) which greatly reduces the time for setup and optimization. The specificity and efficiency of the single-tube assays can be determined by assessing their allelic discrimination abilities (Heissl *et al.* 2016). Suitable controls that have been successfully utilised to test the specificity and efficiency of TaqMan SNP genotyping assays in previous studies include DNA samples of known genotype, plasmid DNA controls that contain a PCR amplicon of the target sequence of known genotype and no-template controls (Walker *et al.* 2007; Bass *et a*l. 2008; Heissl *et al.* 2016). These controls will aid in evaluating the genotyping accuracy of the probe (Heissl *et al.* 2016). To determine the sensitivity of the TaqMan SNP genotyping assays, a dilution series of gDNA samples is typically conducted (Bass *et al.* 2008; Brocannello *et al*. 2018). This allows for the limit of detection (LOD) of the assays to be determined as well as the optimal template concentration.

Following optimization, a larger number of samples can be screened utilising the TaqMan SNP genotyping assay to determine the rate of correct calling, i.e. the percentage of genotyping results that are correctly determined. This is important to assess the accuracy of the assay to determine whether it will be suitable as a high-throughput SNP detection tool. This can be done by screening DNA samples of known genotype with the TaqMan SNP genotyping assay, for instance DNA samples which have previously undergone conventional PCR and sequencing (Walker *et al.* 2007). The genotyping results of the TaqMan assays can then be compared to the known genotypes of the samples to obtain a percentage of correct genotype calls. For an assay to be considered suitable as a

high-throughput detection tool it should have a genotyping accuracy of 95% and above (McGuigan and Ralston, 2002).

To date, no TaqMan SNP genotyping assays have been developed for the detection of amitraz resistance-associated SNPs in ticks or any other parasite to our knowledge. Three validated SNPs have been linked to amitraz resistance in *R. microplus* ticks, two in the octopamine/tyramine receptor (OCT/Tyr) gene (Baron *et al.* 2015; Chen 2007) and one in the β-adrenergic octopamine receptor (βAOR) (Corley *et al*. 2013). The SNPs in the OCT/Tyr gene occur at nucleotide position 157 where adenine is substituted by cytosine and at position 200 where thymine is substituted by cytosine, resulting in amino acid substitutions, T8P and L22S respectively, both occurring in loops between transmembrane domains (Chen *et al*. 2007). The SNP in the βAOR at nucleotide position 181 where adenine is substituted by thymine, resulting in an I61F substitution in the first transmembrane domain of the receptor, which is a highly conserved region (Corley *et al*. 2013). The OCT/Tyr SNPs have been identified in resistant *R. microplus* populations around the world, including South Africa (Baron *et al.* 2015; Robbertse *et al.* 2016), whilst the βAOR SNP has only been linked to resistance in the amitraz resistant Ultimo strain of *R. microplus* from central Queensland, Australia (Corley *et al.* 2013).

The aim of this study was therefore to establish a rapid, high-throughput screening test for the detection of amitraz resistance-associated SNPs in *R. microplus* ticks. TaqMan SNP genotyping assays were designed to detect the two SNPs T8P and L22S in the OCT/Tyr gene. Different TaqMan SNP genotyping assays were tested on positive controls to determine the specificity and efficiency of the probes. The most optimal assay was subjected to a dilution series to determine the LOD of the assay as well as the optimal template concentration. *Rhipicephalus microplus* field samples of known amitraz-resistance genotype (as detected in field samples from the Mnisi area, Chapter 2) were subjected to genotyping utilising the TaqMan SNP genotyping assay (referred to as Oct 2) to determine the rate of correct calling of the assay. A potential species identification TaqMan SNP genotyping assay was also designed and tested utilizing a highly conserved point mutation in the ITS2 gene in an attempt to rapidly differentiate between *R. microplus* and R*. decoloratus* ticks in the screening process.

3. Methodology

3.1. Collection of *R. microplus* samples

Permission under Section 20 of the Animal Disease Act, 1984 (Act number 35 of 19841) was obtained from the Department of Agriculture, Forestry and Fisheries (DAFF) for tick collection in the Mnisi area during 2019. *Rhipicephalus microplus* gDNA samples of known amitraz resistance status were available for Zimbabwe and the Eastern Cape in South Africa, where collections were done by state

veterinarians, Drs Marvellous Sungirai and Dr Jason Zanga, under section 20 approval granted to Dr Jannie Crafford (Department of Tropical Veterinary Medicine). These were utilized for the generation of positive controls (as detailed in Table 3). *Rhipicephalus microplus* gDNA samples of known amitraz resistance collected from the Mnisi communal area (as described in chapter 2 of this study) were utilized for testing of field samples.

3.2. Design of TaqMan SNP genotyping assays for the OCT/Tyr gene

Sequences of the octopamine/tyramine receptor were obtained from previous amitraz resistance studies in South Africa (Baron *et al.* 2015; Robbertse *et al*. 2016) and Zimbabwe (Sungirai *et al.* 2018). Sequences were curated and aligned using CLC Main Workbench version 20.02.2., where sequences were manually curated to determine the reliability of base calling and areas of low confidence were removed. The OCT/Tyr gene fragments were compared via sequence alignment to GenBank sequence entries for the resistant Santa Luiza *R. microplus* strain (GenBank Accession EF490688) and the Gonzalez susceptible *R. microplus* strain (GenBank Accession: EF490687.1). The amitraz resistance-associated SNPs in the OCT/Tyr gene were identified and the resistance genotype of each sample was determined.

The TaqMan genotyping assays were designed to detect the two SNPs in the *R. microplus* OCT/Tyr gene associated with amitraz resistance using the ThermoFisher Scientific online design tool for TaqMan SNP genotyping assays (https://www.thermofisher.com/order/custom-genomic-products/tools/genotyping/). One assay named “Oct 1” was designed to detect the T8P SNP at nucleotide position 157 which was input as [A/C], and a second assay called “Oct 2” was designed to detect the L22S SNP at nucleotide position 200 which was input as [T/C]. The point mutations identified in the sequence alignments were masked in the design process by inputting a “N” into the sequence. All of the assays were ordered via the Custom TaqMan® Assay Design Tool in single tubes (40× assay mix concentration), preoptimized reactions from Thermo Fisher Scientific, USA. Both a version 1 (Oct 1_v1 and Oct 2_v1) and version 2 (Oct 1_v2 and Oct2_v2) set of assays were designed to be evaluated in this study (Table 1).

A “catch-all” probe was also designed in a highly conserved area of the OCT/Tyr gene sequence to be used as a control to ensure that the correct target gene is amplifying (Table 1). The catch-all probe, named “Oct CAP”, was ordered as a TaqMan gene expression assay on the ThermoFisher Scientific online design tool (https://www.thermofisher.com/order/custom-genomic-products/tools/gene-expression/).

Table 1: Summary of versions 1 and 2 of the TaqMan SNP genotyping assays designed to detect the amitraz resistance associated SNPs T8P and L22S in the *R. microplus* OCT/Tyr gene. F= forward, R= reverse, v= version, Oct= OCT/Tyr gene, CAP= catch-all probe, Tm= melting temperature, N/A= not applicable.					
Primers and probes	**Sequence 5'-3'**	**Base pairs**	**Tm (°C)**	**Dye label**	**Dye colour**
Oct TaqMan SNP genotyping assays version 1					
Oct primers v1					
Oct F primer	ATCCCGCGAACAACATGGTGAGAC	24	64.3	N/A	N/A
Oct R primer	GGTAGGCTTCCACCACATCATCCA	24	63.4	N/A	N/A
Oct 1 probes v1 (to detect the T8P SNP): Oct 1_v1					
Oct 1 resistant probe	ACGAGACCGATGG	13	44.5	VIC	Green
Oct 1 susceptible probe	ACGAGAACGATGG	13	41.4	FAM	Blue
Oct 2 probes v1 (to detect the L22S SNP): Oct 2_v1					
Oct 2 resistant probe	CGCCCTCGCCCAG	13	53.9	VIC	Green
Oct 2 susceptible probe	CGCCCTTGCCCAG	13	50.8	FAM	Blue
CAP control					
Oct CAP	GGTCATTTCCGAAGCGG	17	55.1	FAM	Blue
Oct TaqMan SNP genotyping assays version 2					
Oct primers v2					
Oct 1 F primer	AACAACATGGTGAGACCCCA	20	57.3	N/A	N/A
Oct 2 F primer	GGTGAGACCCCACACGAG	18	60.7	N/A	N/A
Oct 1 & 2 R primer	ACGTATTAACCGCTTCGGAAATGA	24	59.3	N/A	N/A
Oct 1 probes v2 (to detect the T8P SNP): Oct 1_v2					
Oct 1 resistant probe	CCTCCATCGGTCTCGTG	17	57.6	FAM	Blue
Oct 1 susceptible probe	CCTCCATCGTTCTCGTG	17	55.2	VIC	Green
Oct 2 probes v2 (to detect the L22S SNP): Oct 2_v2					
Oct 2 resistant probe	CGCCCTCGCCCAGC	14	58.0	FAM	Blue
Oct 2 susceptible probe	CGCCCTTGCCCAGC	14	55.1	VIC	Green

3.3. Design of TaqMan SNP genotyping assays for the ITS2 gene

An ITS2 species identification assay named "ITS2 Rm/Rd" was designed to allow for the rapid discrimination between *R. microplus* and *R. decoloratus*, as morphologically these species are extremely similar. ITS2 sequences from a previous study (Baron *et al.* 2015) were included in all alignments. Additional ITS2 data was obtained using samples from the Eastern Cape and Zimbabwe (collected by Drs Zunguria and Moregood) for PCR amplification and DNA sequencing. Sequences were curated and aligned using CLC Main Workbench version 20.02.2., manually curated and areas of low confidence removed. GenBank sequence entries for *R. microplus* (GenBank Accession: U97715.1) and *R. decoloratus* (GenBank Accession: U97716.1) were used as reference sequences.

Conserved SNPs between *R. microplus* and *R. decoloratus* was identified and used for TaqMan probe and primer using the ThermoFisher Scientific online design tool for TaqMan SNP genotyping assays (https://www.thermofisher.com/order/custom-genomic-products/tools/genotyping/). The sequences and assay conditions are summarised in Table 2.

Table 2: Summary of the ITS2 Rm/Rd species identification assay designed to discriminate between *R. microplus* and *R. decoloratus* ticks. F= forward, R= reverse, ITS2= internal transcribed spacer region 2, Rm= *R. microplus,* Rd= *R. decoloratus,* Tm= melting temperature, N/A= not applicable.					
Primers and probes:	**Sequence 5'-3'**	**Base pairs**	**Tm (°C)**	**Dye label**	**Dye colour**
ITS2 TaqMan SNP genotyping assay: ITS2 Rm/Rd					
ITS2 Rm/Rd F primer	CGGCCAGCAAAGCTTCTC	18	58.2	N/A	N/A
ITS2 Rm/Rd R primer	CGAGCGCCCGGTCT	14	55.0	N/A	N/A
Rm probe	CGTGCGCAAAGTG	13	44.5	VIC	Green
Rd probe	CGTGCGCGAAGTG	13	47.7	FAM	Blue

3.4. Primer BLAST search for the identification of possible off-target binding sites

A nucleotide BLAST search of the designed primers (Table 1 and 2) was conducted against the GenBank nucleotide database (BLASTn) to verify their sequence specificity. The following format was used: Forward-sequence-(5'-3')-(20 Ns)-Reverse-sequence-(5'-3'). The algorithm parameters were set as: max target sequences: 1000; automatically adjust parameters for short input sequences; expect threshold=1000; word size=7; word/mismatch scores: 1,-3; gap costs: existence: 5 extension: 2; filters and masking: all options deselected.

A BLAST search of the designed primers (Table 1 and 2) was also conducted against the Bovine Genome Database (https://bovinegenome.elsiklab.missouri.edu/), including: Bos taurus UMD3.1 chromosome assembly and Bos_taurus_ARS-UCD1.2_chromosomes_with_Y_from_Btau_5.0.1.fa. The following advanced parameters were specified: -word_size 7 -evalue 10.

3.5. Generation of positive controls for the various TaqMan assays

OCT/Tyr gene

gDNA samples of known amitraz resistance genotype from the Eastern Cape (EC) were selected to generate positive controls for the Oct 1 and Oct 2 TaqMan SNP genotyping assays. One gDNA sample of homozygous susceptible genotype (AA/TT) and one sample of homozygous resistant genotype (CC/CC) were selected. The selected samples were subjected to PCR amplification. Published primers were used for PCR amplification of a 417 bp fragment of the OCT/Tyr gene: forward primer 5' GGT TCA CCC AAC CTC ATC TCT GAA 3' and reverse primer 5' GCA GAT GAC CAG CAC GTT ACC G 3' (Chen *et al*. 2007). The PCR amplification of the OCT/Tyr gene was performed as described by Baron *et al*. (2015). PCR amplification was performed in 25 µl reactions containing 10 pmol of each primer and 200

ng of gDNA using One*Taq* Quick-Load 2X Master Mix with Standard Buffer New England Biolabs (undiluted master mix contents include 20 mM Tris-HCl; 22 mM KCl; 22 mM NH_4Cl; 1.8 mM $MgCl_2$; 5% Glycerol; 0.05% Tween 20; 0.06% IGEPAL® CA-630; 0.2 mM dNTPs; 25 units/ml One*Taq*® DNA Polymerase; pH 8.9 at 25°C). The PCR cycling parameters were as follows: 94°C for 30 seconds, 40 cycles of 94°C for 30 seconds, 55°C for 30 sec and 68°C for 1 minute with a final extension at 68°C for 5 minutes. PCR products were analysed using agarose electrophoresis, purified and used to create plasmid constructs (details below). In summary three controls for the OCT/Tyr gene was created, namely: gDNA from the selected samples, purified PCR products and plasmids.

ITS2 gene

Five gDNA samples of each species were also selected for use as positive controls, including *R. microplus* and *R. decoloratus*. For *R. microplus*, field samples from the Mnisi area were used which have previously been subjected to PCR and sequencing of ITS2 (Chapter 2). For *R. decoloratus*, stored gDNA samples from the PhD studies of Samantha Baron were utilized (Baron *et al.* 2018). Genomic DNA samples of known species were selected for the generation of positive controls for the ITS2 species identification assay. One *R. microplus* gDNA sample and one *R. decoloratus* gDNA sample were selected for subjection to PCR. Published primers were used to amplify the internal transcribed spacer 2 (ITS2) gene from gDNA samples for species identification: forward primer 5' GCC-GTC-GAC-TCG-TTT-TGA 3' and reverse primer 5' TCC GAA CAG TTG CGT GAT AAA 3' (Lempereur *et al*. 2010). The PCR amplification of ITS2 was adapted from Baron *et al*. (2015). PCR amplification was performed in 25 µl reactions containing 10 pmol of each primer and 200 ng of gDNA using One*Taq* Quick-Load 2X Master Mix with Standard Buffer New England Biolabs (undiluted master mix contents include 20 mM Tris-HCl; 22 mM KCl; 22 mM NH_4Cl; 1.8 mM $MgCl_2$; 5% Glycerol; 0.05% Tween 20; 0.06% IGEPAL® CA-630; 0.2 mM dNTPs; 25 units/ml One*Taq*® DNA Polymerase; pH 8.9 at 25°C). The PCR cycling parameters were as follows: 94°C for 30 seconds, 30 cycles of 94°C for 30 seconds, 58°C for 30 seconds and 68°C for 1 minute with a final extension at 68°C for 5 minutes. All PCR reactions were performed in a GeneAmp 9700 thermocycler (PE Applied Biosystems, USA). PCR products were analysed using agarose electrophoresis, purified and used to create plasmid constructs (details below). In summary three controls for the ITS2 gene was created, namely: gDNA from the selected samples, purified PCR products and plasmids.

3.6. Agarose gel electrophoresis

PCR amplified DNA fragments were analysed using 2% agarose gel electrophoresis with TAE as electrophoresis buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.0) containing ethidium bromide (1 µg/ml). Electrophoresis was conducted in a Mini ReadySub-Cell™ GT Cell (Bio-Rad) with TAE as electrophoresis buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.0) at 10 V/cm. DNA was visualized using the Gel Doc™ XR+ System (BioRad, USA).

3.7. Purification of PCR products

PCR products were purified using the QIAquick® PCR Purification Kit (Qiagen) according to the manufacturer's protocols. Briefly, the principle of this kit entails the binding of nucleic acids bind to a silica membrane in the presence of chaotropic salts flowed by washing and elution of purified DNA. To ensure binding to the silica membrane,200 µl binding buffer (PB) was added to the PCR reaction before loading it onto a QIAquick column and centrifugation at 17900 x*g* for 1 minute. Wash buffer PE (750 µl) was added to the column and centrifuged at 17900 x*g* for 1 minute. The flow-through was discarded. A final centrifugation step was done before placing the column in a clean 2 ml collection tube. DNA was eluted by adding 30 µl (37°C) ddH20 and centrifugation. The DNA concentrations were determined using a Nanodrop Spectrophotometer (ThermoFisher Scientific).

3.8. Big Dye sequencing reaction and clean-up

The purified PCR products were prepared for sequencing with an adapted Big Dye v3.1 reaction. For each gene, one reaction was performed with the forward primer and one reaction was performed with the reverse primer to allow for sequencing in both directions. The Big Dye reaction was prepared as follows: 0.5 µl Big Dye v3.1, 2.4 µl 5X sequencing buffer (undiluted buffer contains 5X solution of Tris-HCl, pH 9.0 and $MgCl_2$), 10 pmol primer, 100 ng DNA and ddH_2O up to a total volume of 12 µl. The PCR cycling parameters were as follows: 96°C for 1 minute and 25 cycles of 96°C for 10 seconds, the annealing temperature of the primer for 5 seconds and 60°C for 4 minutes.

The Big Dye sequencing reaction was cleaned-up using a standard sodium acetate/ethanol precipitation method. Master mix A was prepared with 3 µl of 3 M NaOAc (pH 4.6), 62.5 µl 100% ethanol and ddH20 to a total volume of 80 µl. Master mix B was prepared by adding ddH_20 to the Big Dye reaction (12 µl) to a total volume of 20 µl. Master mix B was mixed by pipetting up and down and then added to master mix A. The combined mix was centrifuged for 45 minutes at 4°C at 15,600 *xg*.

The supernatant was then removed, 250 µl of ice-cold 70% ethanol was added, and the tube was centrifuged at 15,600 *xg* for 5 minutes. The 70% ethanol wash step was repeated, and the tube was centrifuged for an additional 5 minutes at 15,600 *xg*. The supernatant was removed and the ethanol was evaporated.

3.9. DNA sequencing and analysis

DNA Sanger sequencing was performed at the University of Pretoria on an ABI 3500xL Genetic Analyser (ThermoFisher Scientific). The sequences were analysed and aligned using CLC Main Workbench version 20.02.2., manually curated and areas of low confidence removed. The previously described GenBank sequence entries for ITS2 and the OCT/Tyr gene were utilized as reference sequences in the alignments and to check for correctness of curation. The ITS2 sequence alignment was utilised to conduct species identification. The OCT/Tyr sequence alignment was utilised to identify the amitraz resistance-associated SNPs in the OCT/Tyr gene and to determine the resistance genotype of each sample.

3.10. Ligation of PCR products

The OCT/Tyr PCR products from *R. microplus* as well as the *R. microplus* and *R. decoloratus* ITS2 PCR products were ligated into a pJET1.2/blunt cloning vector using the CloneJET PCR Cloning Kit (ThermoFisher Scientific) according to the manufacturer's protocol. To comply with the blunt end cloning requirements of the system, the 3'-dA overhangs generated during PCR by the *Taq* DNA polymerase needed to be removed. The blunting reaction was set up on ice and contained: 10 µl 2 x Reaction Buffer, 0.15 pmol purified PCR product, 1 µl Blunting Enzyme (units unspecified) and ddH$_2$O up to a total volume of 18 µl. The reaction was briefly vortexed, centrifuged for 5 seconds and incubated at 70°C for 5 min and then chilled on ice. The ligation reaction was set up on ice, where 0.05 pmol of pJET1.2/blunt cloning vector (50 ng/µl) and 1 µl of T4 DNA ligase (5 units/ µl) was added to the blunting reaction to a total volume of 20 µl. The reaction was vortexed briefly, centrifuged and incubated overnight at 16°C. A positive control ligation reaction was also set up using the control PCR product (976 bp with 3'-dA overhangs) from the CloneJET PCR Cloning Kit (ThermoFisher Scientific) following the same blunt end cloning protocol.

3.11. Preparation of calcium chloride competent DH5-α *Escherichia coli* cells

DH5-α *E. coli* cells were inoculated into 3 ml of Luria-Bertani (LB) broth (1% NaCl, 1% tryptone, 0.5% yeast extract, in ddH20, pH 7.5) grown overnight at 37°C with shaking at 250 rpm followed by dilution of 1 ml overnight culture into 150 ml pre-warmed LB Broth in a 250 ml Erlenmeyer flask. The flask was incubated at 37°C with shaking at 250 rpm until an OD_{600nm} reading of approximately 0.4 was reached. The culture was equally divided into five Falcon tubes (30 ml per tube) that were pre-cooled on ice and then centrifuged at 10000 x*g* for 10 minutes at 4°C. The supernatant was discarded, and the cell pellet was resuspended in 15 ml ice-cold filter-sterilized 50 mM $CaCl_2$ and centrifuged at 10000 x*g* for 5 minutes at 4°C. The cell pellets were resuspended in 1.5 ml ice-cold filter-sterilized 50 mM $CaCl_2$ and incubated on ice for 1 hour before aliquots of 100 µl were made and stored at -80°C until use.

3.12. Heat shock transformation of DH5-α *Escherichia coli* cells

The ligation reaction was used directly for the transformation of calcium chloride competent *E. coli* using heat shock transformation. Once the cells were thawed, the ligation reaction (10 µl) was added to 100 µl competent cells and incubated on ice for 30 min. The cells were then heat shocked at 42°C for 90 sec, immediately incubated on ice for 2 min after which 900 µl pre-warmed LB broth containing 50 mM D-glucose was added. The cells were incubated at 37°C with shaking at 250 rpm for one hour before plating onto LB-Amp plates (2% agar with 100 µg/ml ampicillin) and subsequent overnight incubation at 37°C in a stationary incubator. Colonies were picked, inoculated into 5 ml LB-Amp (50 µg/mL ampicillin) and grown overnight at 37°C with shaking at 250 rpm. Cells were used immediately for plasmid isolation and for creating stocks which were stored at -80°C until use.

3.13. Plasmid isolation and sequencing

Plasmids were isolated using the Zyppy™ Plasmid Miniprep Kit (Zymo Research) according to the manufacturer's instructions. Firstly, 1.5 ml cell culture was placed in a 1.5 ml Eppendorf tube and centrifuged for 30 seconds at 15,600 x*g*. The supernatant was discarded, and the cell pellet was resuspended in 600 µl ddH20 and 100 µl of 7x Lysis Buffer. The solution was mixed by gently inverting the tube several times with subsequent incubated at room temperature for 3 minutes until the solution changed from opaque to clear blue. Following the colour change, 350 µl ice-cold Neutralization Buffer was added and the solution was mixed by inverting the tube until the sample turned yellow with a yellow precipitate. The tube was centrifuged for 4 minutes at 15,600 x*g*. The

supernatant was transferred to a Zymo-spin IIN column and centrifuged for 15 seconds at 15,600 x*g*. The flow-through was discarded, 200 µl Endo-wash buffer added and again centrifuged 45 seconds. Zyppy Wash Buffer (400 µl) was added to the column and centrifuged for 4 minutes at 15,600 x*g* before plasmid DNA was eluted in 30 µl pre-warmed ddH_20 (55°C). The concentration of the isolated plasmid was determined on the Nanodrop Spectrophotometer.

The isolated plasmids were submitted for sequencing at the University of Pretoria sequencing facility to confirm the cloned OCT/Tyr and ITS2 sequences. The respective plasmid forward and reverse primers provided by the manufacturers were utilised for sequencing to confirm the presence of the ligated genes (pJET1.2/blunt cloning vector forward and reverse primers from the CloneJET PCR Cloning Kit from ThermoFisher Scientific). The ligated OCT/Tyr gene fragments were compared via sequence alignment to GenBank sequence entries for the resistant Santa Luiza *R. microplus* strain (GenBank Accession EF490688) and the Gonzalez susceptible *R. microplus* strain (GenBank Accession: EF490687.1) and the genotypes of the amitraz resistance-associated SNPs in the OCT/Tyr gene were confirmed (as described previously). The sequenced plasmids of known resistance genotype and known species were used as positive controls for downstream TaqMan SNP genotyping assays.

3.14. TaqMan™ SNP genotyping assays

The TaqMan SNP genotyping assays were performed using the TaqPath™ ProAmp™ Master Mix (ThermoFisher Scientific) according to the manufacturer's protocol. PCR reactions of 10 µl for genotyping experiments were set up on a MicroAmp™ Optical 384-Well Reaction Plate (ThermoFisher Scientific) as follows: 5.0 µl TaqPath™ ProAmp™ Master Mix, 0.5 µl TaqMan SNP Genotyping Assay, gDNA or no template control (NTC) up to a maximum volume of 4.5 µl (concentration of 0.2 ng DNA) and SABAX H_2O up to a final volume of 10 µl. A mastermix for each assay was set up in 1.5 ml Eppendorf tubes containing: the TaqPath™ ProAmp™ Master Mix (ThermoFisher Scientific), the TaqMan SNP genotyping assay and SABAX H_2O. Sequenced plasmids and gDNA of known amitraz resistance status and/or species were used as positive control templates and a no template control was included. Bovine gDNA (obtained from Dr Carina Visser from the University of Pretoria Animal Sciences department) was also included as a negative control for the Oct 1 and Oct 2 TaqMan SNP genotyping assays (for both versions 1 and 2) to detect any possible host DNA interference. The Mastermix was aliquoted into a 96-well plate on ice and the template DNA was aliquoted into the 96-well plate per respective assay. The 96-well plate was sealed with foil and briefly mixed and centrifuged. The samples were transferred to a MicroAmp™ Optical 384-Well Reaction Plate (ThermoFisher Scientific) on ice, where each reaction was aliquoted into three technical replicates. The plate was sealed with an optical

adhesive cover, briefly mixed by manual shaking, centrifuged at 3,100 x*g* for one minute and loaded into the QuantStudio® 12K Flex Real-Time PCR system (ThermoFisher Scientific). The PCR thermal cycling conditions were set as follows: 60°C for 30 seconds, 95°C for 5 minutes, 40 cycles of 95°C for 15 seconds and 60 °C for 60 seconds, and then 60°C for 30 seconds. Genotyping was selected as the experiment type and the ROX passive reference dye was selected.

The QuantStudio® 12K Flex Software v1.3.0 (Applied Biosystems by Life Technologies™) was used to design the plate layout of the 384-well plates, to assign sample names and to match the corresponding assay to the sample being tested. Amplification plots were appraised to confirm amplification and multicomponent plots were utilised for manual genotyping calling where an "undetermined" genotype result was obtained. The TaqMan® Genotyper Software v1.5.0 (Applied Biosystems by Life Technologies™) was used to produce allelic discrimination plots which allows for automatic genotyping.

3.15. Sensitivity test

A 20-fold dilution series was conducted from gDNA using various DNA input ranging from 100 to 0.1 ng DNA per reaction (Brocannello *et al.* 2018). A total of ten gDNA samples were selected, including five *R. microplus* gDNA samples of known wild-type (homozygous susceptible) genotype and five samples of known heterozygous genotype. The TaqMan SNP genotyping assays were set up and performed as described previously. A no template control was included. The LOD was evaluated using the normalized fluorescence levels (ΔRn values) obtained for allele 1 and allele 2 of the assay reported by the QuantStudio® 12K Flex Software v1.3.0 (Applied Biosystems by Life Technologies™) at cycle 40 of the qPCR reaction. An average ΔRn was calculated for each allele at each concentration utilizing the five biological repeats for each genotype. The average ΔRn values were then plotted on a graph against the gDNA input concentration and a trend line was constructed for each allele of each genotype. The optimal gDNA concentration for each genotype was then determined as well as the limit of detection (LOD) of the assay below which confident genotyping cannot be performed.

3.16. Rate of correct calling

A total of 15 gDNA samples, five of each genotype (homozygous susceptible, heterozygous and homozygous resistant), were subjected to genotyping using the validated SNP genotyping assay as described previously. The optimal gDNA concentration determined previously was utilized for each

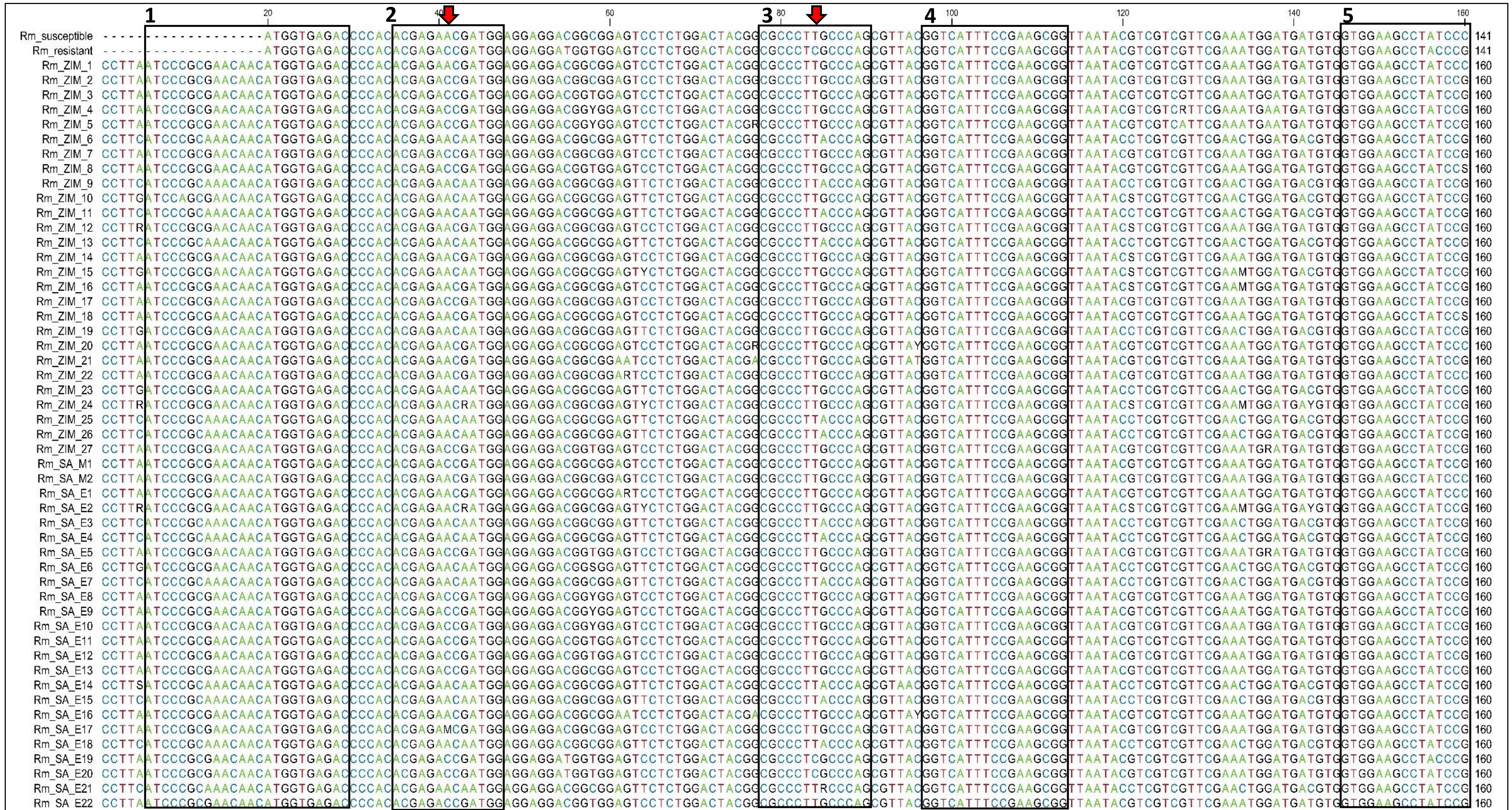
sample. An NTC was included in triplicate. The rate of correct calling was determined as a percentage for each genotype as well as overall for all samples.

4. Results

4.1. Design of version 1 of the Oct 1 and Oct 2 TaqMan SNP genotyping assays

A total of 128 OCT/Tyr gene fragment sequences from previous amitraz resistance studies in the Mnisi communal area in South Africa (Baron *et al.* 2015; Robbertse *et al*. 2016) (42 sequences), the Eastern Cape (Dr Jason Spargo) (32 sequences) and Zimbabwe (Sungirai *et al*. 2018) (54 sequences) were curated and aligned to identify conserved areas for probe and primer design (Figure 1). The two amitraz resistance-associated SNPs T8P and L22S (Baron *et al*. 2015; Chen *et al*. 2007) are indicated as well as the areas selected for probe design. In addition to the latter, it is evident that there is additional variation between the sequences, where there are additional nucleotide substitutions, some of which are degenerative as indicated by the "M" and "R" bases in the alignment. This variation was as expected based on the findings of Baron *et al.* (2015) and Sungirai *et al.* (2018). The same nucleotide substitutions were found to occur in both the South African and Zimbabwean sequences, which indicates that there is not large geographic variation in the OCT/Tyr gene between these *R. microplus* populations.

Two separate TaqMan SNP genotyping assays were designed to detect the T8P and L22S SNPs in the OCT/Tyr gene, which were named "Oct 1" and "Oct 2" respectively (Table 1). The catch-all probe (CAP) was designed in a highly conserved area of the OCT/Tyr gene spanning from nucleotide 97 to 113 (Figure 1, Table 1).



<u>Figure 1:</u> Nucleotide sequence alignment of a 160 bp region of the OCT/Tyr gene fragment containing the two amitraz resistance associated SNPs. The two amitraz-resistance associated SNPs T8P and L22S are indicated with red arrows. The resistant Santa Luiza *R. microplus* strain (GenBank Accession EF490688) and the Gonzalez susceptible *R. microplus* strain (GenBank Accession: EF490687.1) were included as reference sequences. Box 1 indicates the position of the Oct F primer, box 2 indicates the Oct 1_v1 probe (SNP T8P: A=susceptible allele; C=resistant allele), box 3 indicates the Oct 2_v1 probe (SNP L22S: T=susceptible allele; C= resistant allele), box 4 indicates the CAP and box 5 indicates the Oct R primer. Sequence names: Rm= *Rhipicephalus microplus*; Zim= Zimbabwe; SA= South Africa; E= Eastern Cape; M= Mnisi; number= individual tick. "M" = A or C and "R" = A or G nucleotide bases.

A nucleotide BLAST search of the Oct primers v1, Oct F (forward) and R (reverse) primers (Table 1) was conducted against both the GenBank and the Bovine Genome Database. Multiple significant hits for the OCT/Tyr gene were found across the various *R. microplus* sequences on the NCBI (Figure 2), and no significant off-target hits (results not shown). It is evident that both primers share 100% identity with the *R. microplus* OCT/Tyr gene. No hits were found against the Bovine Genome Database and no significant hits were found against the GenBank database for *Babesia* or *Anaplasma* species, which indicated that the primers should not amplify any fragments from possible contaminating DNA in the *R. microplus* gDNA samples.

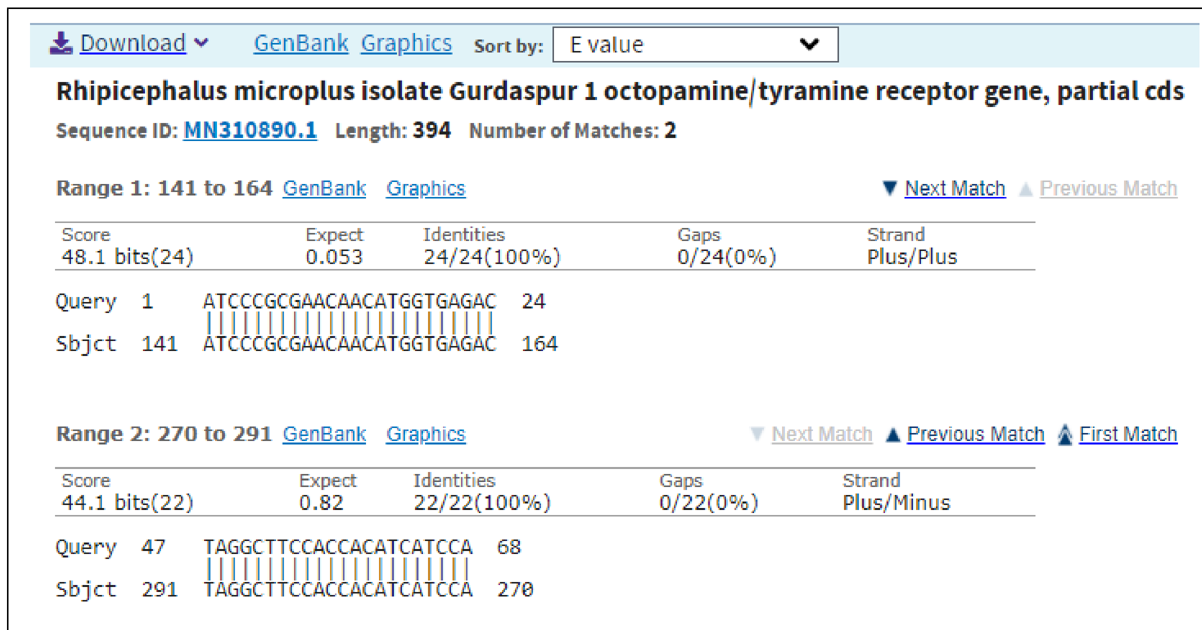
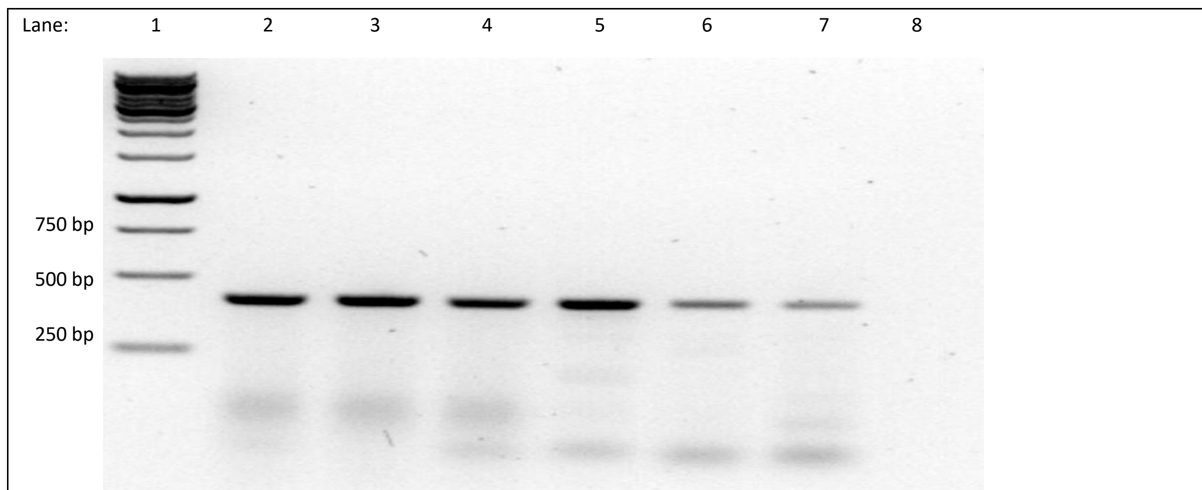


Figure 2: Example of nucleotide BLAST results of the Oct primers version 1. The top outputs correspond to the Oct F primer results and the Oct R primer results are shown below. Complete identity of the forward (top) and reverse (bottom) primers to the Gurdaspur *R. microplus* isolate (MN310890.1) is evident.

4.2. Positive controls for the Oct 1 and 2 TaqMan SNP genotyping assays

Two gDNA samples that were confirmed as *R. microplus* and of known amitraz-resistance genotype were selected for the generation of positive controls for the Oct 1 and Oct 2 TaqMan SNP genotyping assays. One of the samples was homozygous susceptible (EC 24) at both SNPs (T8P and L22S) and the other was homozygous resistant (EC 25) at both SNPs (Table 3). For both gDNA samples, the OCT/Tyr gene was PCR amplified in triplicate and products of the expected size of 417 bp amplified (Figure 3). Primer dimers are visible on the gel, but were removed during downstream DNA purification steps.



<u>Figure 3:</u> A 2% agarose gel showing the PCR amplification of the OCT/Tyr gene fragment from susceptible and resistant gDNA samples. Lanes correspond to (1) 1 Kb molecular marker, (2-4) OCT/Tyr gene fragment amplified from homozygous susceptible gDNA sample EC 24, (5-7) OCT/Tyr gene fragment amplified from homozygous resistant gDNA sample EC 25 and (8) no template control.

The products from the triplicate reactions were pooled, purified and submitted for DNA sequencing. Sequence analysis confirmed the resistance genotypes of both samples (results not shown). The purified OCT/Tyr PCR products from the homozygous resistant and homozygous susceptible samples were subsequently cloned into a pJET1.2/ blunt linearized plasmid vector (ThermoFisher Scientific). A nucleotide BLAST search was conducted using the TaqMan primer sequences and the plasmid sequence to ensure that were no off-target sites. None were found.

Plasmids were purified and submitted for sequencing, which confirmed the correct OCT/Tyr gene sequence was ligated into the plasmids. The gDNA, purified PCR product and plasmids were used as positive controls during the optimisation of the TaqMan SNP genotyping assays. A summary of the positive controls is shown in Table 3.

Table 3: Positive controls generated for use in the Oct 1 and 2 (versions 1 and 2) TaqMan SNP genotyping assays.
EC= Eastern Cape. gDNA= genomic DNA. SNP= single nucleotide polymorphism.

Positive controls	Genotype *	Description	Source
gDNA	Susceptible	EC 24 gDNA- susceptible at T8P and L22S.	Stored gDNA sample of known genotype received from EC.
	Resistant	EC 25 gDNA- resistant at T8P and L22S.	Stored gDNA sample of known genotype received from EC.
	Heterozygous	EC 21 gDNA- heterozygous at T8P. EC 23 gDNA- heterozygous at L22S.	Stored gDNA samples of known genotype received from EC.
Purified PCR product	Susceptible	OCT/Tyr sequence amplified via PCR cleaned using QIAquick PCR purification kit (Qiagen). Susceptible at T8P and L22S.	EC sample 24 (susceptible at T8P and L22S)
	Resistant	Oct/Tyr sequence amplified via PCR and cleaned using QIAquick PCR purification kit (Qiagen). Resistant at T8P and L22S.	EC sample 25 (resistant at T8P and L22S).
Plasmid	Susceptible	OCT/Tyr sequence cloned into pJET1.2/ blunt linearized plasmid vector (ThermoFisher Scientific). Susceptible at T8P and L22S.	EC sample 24 (susceptible at T8P and L22S).
	Resistant	OCT/Tyr sequence cloned into pGEM T-Easy plasmid vector (Promega). Resistant at T8P and L22S.	EC sample 25 (resistant at T8P and L22S).
	Heterozygous	Pseudo-heterozygous plasmid. Heterozygous at T8P and L22S..	EC 24 + EC 25 plasmids combined in equal concentrations to produce a pseudo-heterozygous plasmid.

*For sequence information please refer to Figure 1.

4.3. Pilot run of version 1 of the Oct 1 and Oct 2 TaqMan™ SNP genotyping assays

The Oct 1_v1 and Oct 2_v1 TaqMan SNP genotyping assays were tested in a pilot run utilizing the positive controls of susceptible, resistant and heterozygous genotypes (Table 3). For the Oct 1_v1 assay (Figure 4A), only the one gDNA control was correctly genotyped as susceptible whilst none of the plasmid and PCR product controls were correctly genotyped.

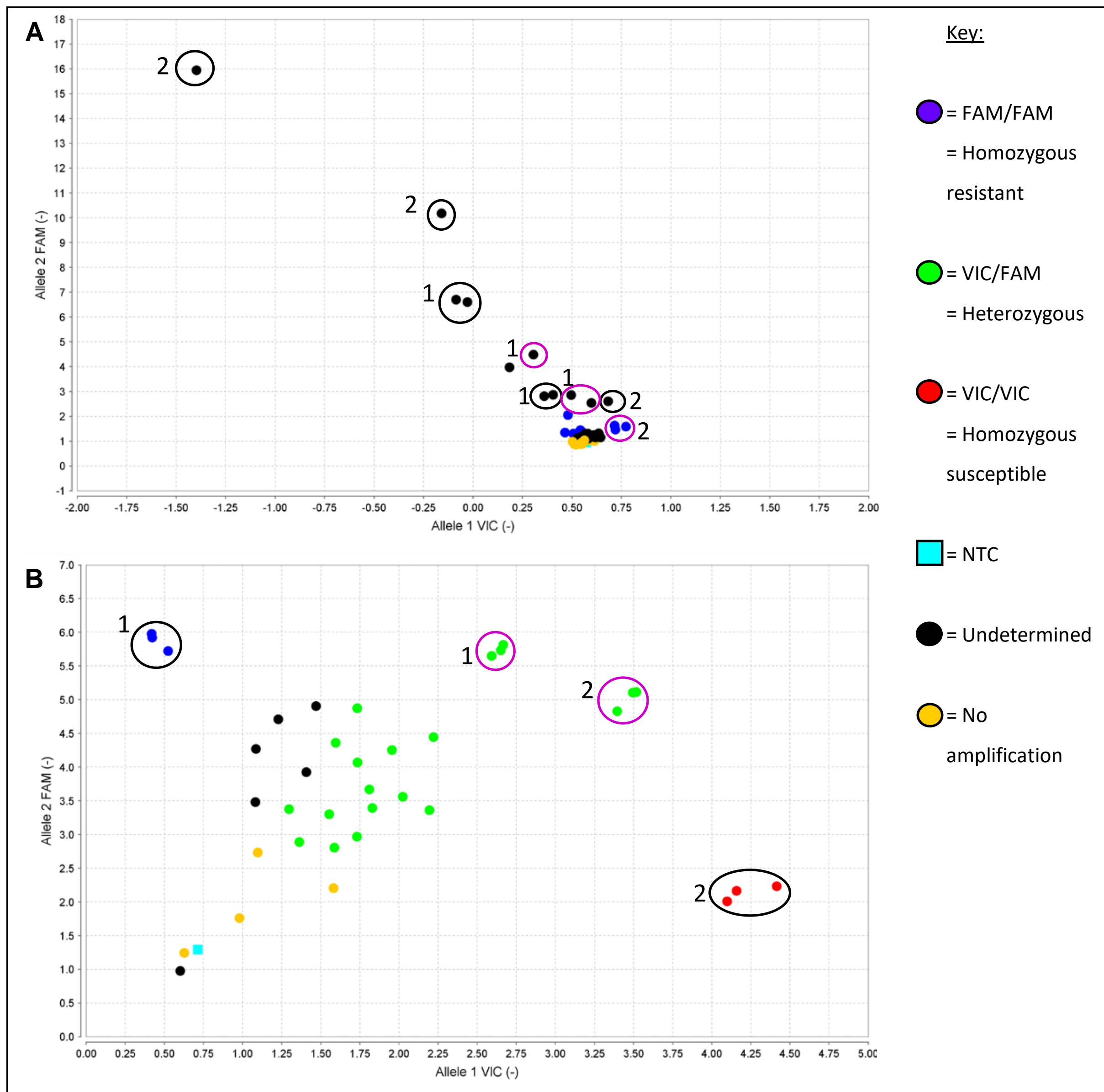


Figure 4: An allelic discrimination plot of the pilot run of the (A) Oct 1_v1 and (B) Oct 2_v1 TaqMan SNP genotyping assays for the positive controls. The black circles indicate the plasmid controls while the purple circles indicate the PCR product controls. The unselected samples correspond to the gDNA controls. 1 and 2 indicate the expected susceptible and resistant controls, respectively.

The susceptible plasmid and PCR product controls were genotyped as undetermined. Identically, the resistant plasmid was also undetermined. The resistant PCR product control was incorrectly genotyped as susceptible (Figure 4A). Analysis of the FAM and VIC signals via manual genotype calling revealed that only the susceptible probe was binding and as such this assay could only detect the susceptible genotype. No fluorescent signal was observed for the resistant probe in the genotyping reactions, indicating that the resistant probe was not binding and the resistant genotype could thus not be detected. Furthermore, the incorrect binding of the susceptible probe to all of the controls could be indicative that the affinity of the susceptible probe was much higher than the resistant probe.

Although this is unlikely as the probes only differed at one nucleotide, where the resistant probe had a "C" and the susceptible probe had a "A" nucleotide base. The possibility that there was an error in the design and/or manufacturing of the assay also cannot be excluded. It is possible that both probes were accidentally labelled with the same reporter dye or that only one of the probes was included in the assay. Overall, these results indicated that the Oct 1_v1 assay was unsuitable for the genotyping the T8P SNP in the OCT/Tyr gene.

For the Oct 2 assay (Figure 4B), the plasmid controls were correctly genotyped but the PCR product and the majority of the gDNA controls were incorrectly genotyped. Both the susceptible and resistant PCR product controls were incorrectly genotyped as heterozygous. For the gDNA controls, all replicates of the susceptible gDNA control were undetermined, all replicates of the heterozygous gDNA were correctly genotyped and all replicates of the resistant gDNA controls were incorrectly genotyped as heterozygous. Analysis via manual genotype calling revealed that both probes were binding with equal affinity in the amplification of the PCR product and gDNA controls. This demonstrated that the binding of the probes was not specific enough to correctly determine the genotypes of the PCR product and gDNA controls. The Oct 2_v1 assay was therefore not suitable for this study and was discarded.

The plasmid and PCR product controls indicated that the problems that were observed in the TaqMan SNP genotyping assays were due to the design of the probes and primers. Firstly, the Oct CAP assay was included in the pilot run as a control in both the Oct 1_v1 and Oct 2_v1 TaqMan SNP genotyping assays to ensure that the OCT/Tyr target gene was indeed being amplified from the respective templates. Amplification was observed for all reactions which demonstrates that the OCT/Tyr gene was indeed amplifying in all reactions (Figure 5). The plasmid and PCR controls start to amplify earlier (cycle 15) than the gDNA controls (cycle 35/36), which is likely due to the target template constituting a greater proportion of the total DNA input. Secondly, no amplification was observed for any of the NTCs which indicated that where was no contamination for any of the assays.

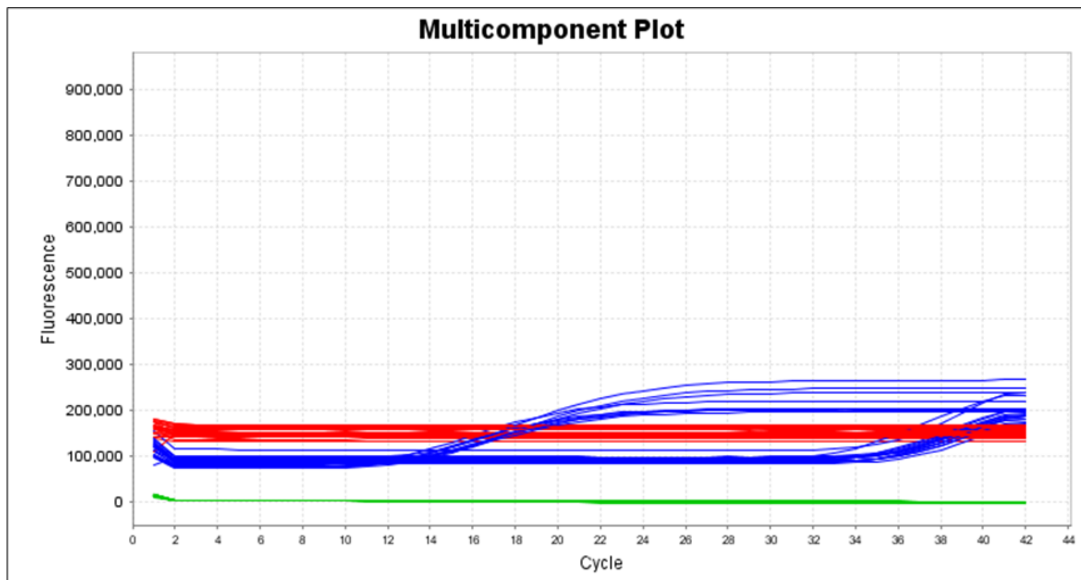


Figure 5: A multicomponent plot showing the amplification of the CAP assay from all control samples tested. The samples amplifying at cycle 15 are the plasmid and PCR product controls, while the samples amplifying at cycle 35/36 correspond to the gDNA controls. Blue lines= gene expression (FAM-labelled); red lines= ROX passive reference dye; green lines =typical VIC baseline (not present in this reaction).

4.4. Design of version 2 of the Oct 1 and Oct 2 TaqMan SNP genotyping assays

Upon failure of the Oct 1_v1 and Oct 2_v1 TaqMan SNP genotyping assays to correctly and reliably genotype the control samples of known resistance genotype, a decision was made to design and order new TaqMan SNP genotyping assays, Oct 1_v2 and Oct 2_v2. The new probe and primer target areas for Oct 1_v2 and Oct 2_v2 are shown in Figure 6 and their characteristics summarized in Table 1. The version 2 probes for both assays were longer and had higher Tm's in an attempt to increase their binding specificity.

The version 2 forward and reverse primers were subjected to a nucleotide BLAST search against both the GenBank and the Bovine Genome Database. Multiple significant hits with 100% identity between the query and database was observed for the OCT/Tyr gene were found across the available *R. microplus* sequences (Figure 7). No hits were found against the Bovine Genome Database and no significant hits were found against the GenBank database for *Babesia* or *Anaplasma* species, which indicated that the primers should not amplify any fragments from possible contaminating DNA in the *R. microplus* gDNA samples.

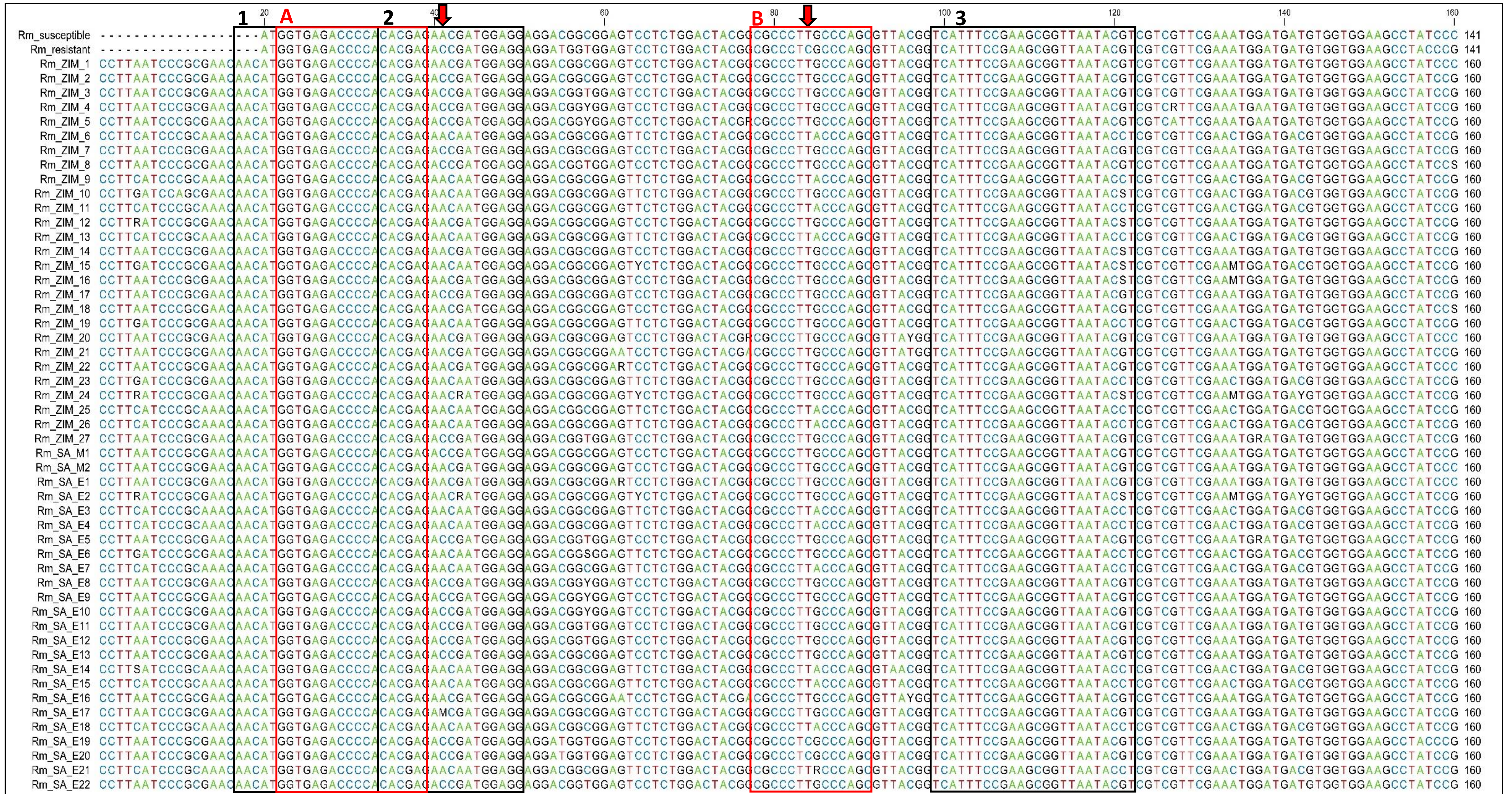


Figure 6: Nucleotide sequence alignment of a 160 bp region of the OCT/Tyr gene fragment containing the two amitraz resistance associated SNPs. The two amitraz-resistance associated SNPs T8P and L22S are indicated with red arrows. The resistant Santa Luiza *R. microplus* strain (GenBank Accession EF490688) and the Gonzalez susceptible *R. microplus* strain (GenBank Accession: EF490687.1) were included as reference sequences. Box 1 indicates the position of the Oct 1 F primer, box 2 indicates the Oct 1_v2 probe (SNP T8P: A=susceptible allele; C=resistant allele) and box 3 indicates the Oct 1&2 R primer. Box A indicates the Oct 2 F primer and Box B indicates the Oct 2_v2 probe (SNP L22S: T=susceptible allele; C= resistant allele). Sequence names: Rm= *Rhipicephalus microplus*; Zim= Zimbabwe; SA= South Africa; E= Eastern Cape; M= Mnisi; number= individual tick. “M” = A or C and “R” = A or G nucleotide bases.

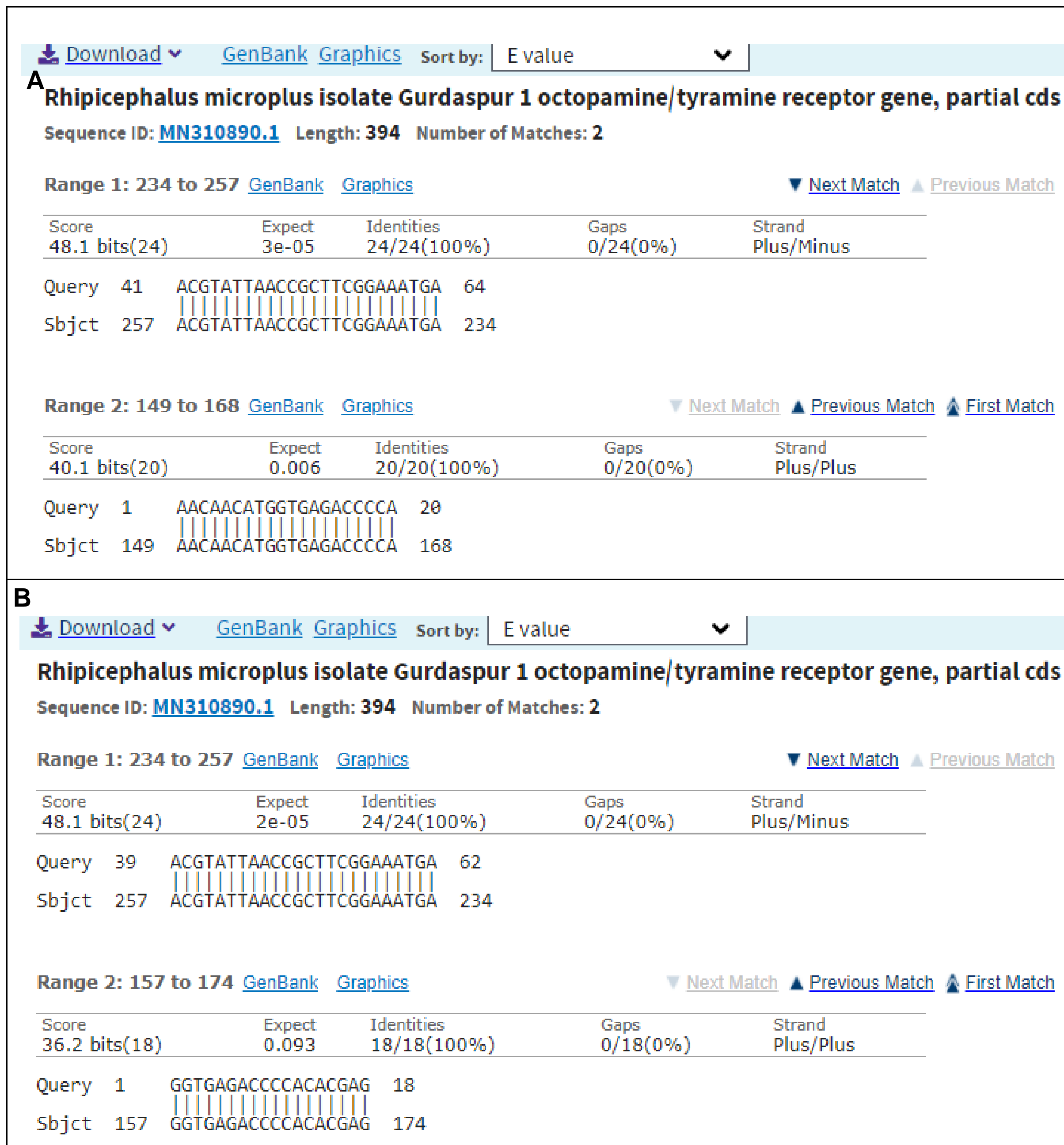


Figure 7: Example of nucleotide BLAST results of the Oct primers v2. Figures correspond to (A) Oct 1 F and Oct 1 &2 R primer and (B) Oct 2 F and Oct 1&2 R primer. Complete identity of the forward (top) and reverse (bottom) primers to the Gurdaspur *R. microplus* isolate (MN310890.1) is evident for both (A) and (B).

4.5. Pilot run of version 2 of the Oct 1 and Oct 2 TaqMan™ SNP genotyping assays

Oct 1_v2 assay

The second version of the Oct 1 TaqMan SNP genotyping assay, Oct 1_v2, (Table 1) was tested in a pilot run using the plasmid and gDNA positive controls of susceptible, resistant and heterozygous genotype (Table 3). From the data it is evident that the plasmids proved to be the most effective positive controls as they amplify efficiently (Figure 5) and are more likely to be correctly genotyped

than PCR product controls (Figure 4B). The plasmid controls were therefore used for all subsequent experiments. It should be noted that this data only corresponds to testing of the positive controls (limited sample size), and as such proper clustering is not observed as would be expected for allelic discrimination plots.

For the Oct 1_v2 TaqMan SNP genotyping assay, the susceptible controls were incorrectly genotyped as heterozygous (Figure 8A), whilst the resistant and heterozygous controls were correctly genotyped (Figure 8B and C). Manual genotyping revealed that both the susceptible and resistant probes were binding to the susceptible controls in the TaqMan SNP genotyping reactions, and the susceptible genotype thus could not be determined. The Oct 1_v2 assay probes likely do not have a high enough specificity to allow for the differentiation between alleles at a single nucleotide base. These results indicate that the Oct 1_v2 assay may suitable to genotype homozygous resistant samples, but it is not suitable to genotype homozygous susceptible or heterozygous alleles at the T8P SNP as samples of susceptible genotype could not be distinguished from samples of heterozygous genotype.

Bovine gDNA was also included in triplicate as a negative control, for which no amplification was observed (Figure 8D). This confirms the results of the BLAST search and indicates that there is no species cross-reactivity of the Oct 1_v2 assay with the bovine host DNA. In addition, the no template controls indicate that there was no contamination of the Oct 1_v2 TaqMan SNP genotyping assay.

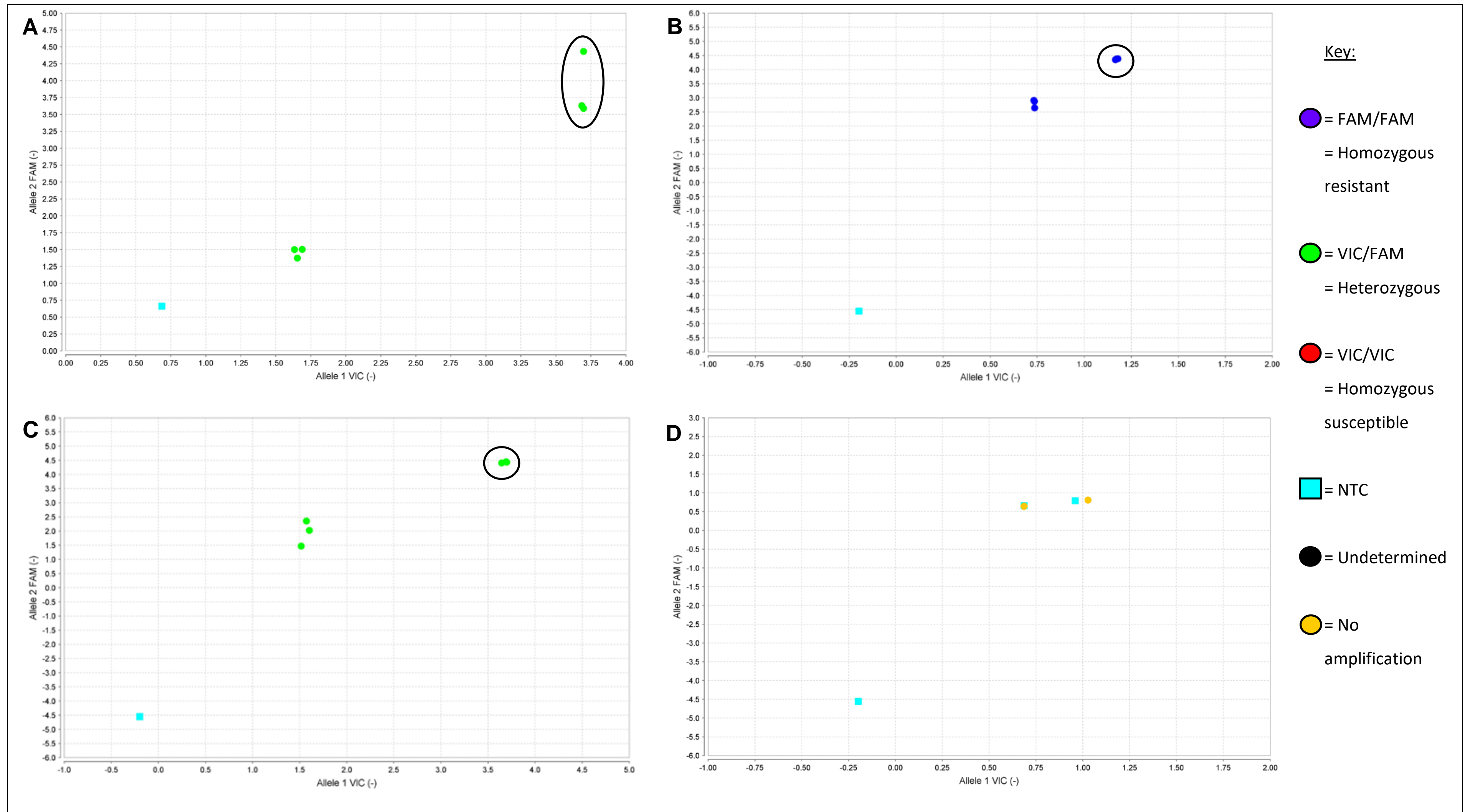


Figure 8: An allelic discrimination plot of the Oct 1_v2 assay for (A) the susceptible, (B) resistant, (C) heterozygous and (D) negative controls. The circled samples correspond to the plasmid control constructs, while the unselected samples are gDNA controls. In blue the NTC is shown. The yellow shows no amplification of the bovine gDNA.

Oct 2_v2 assay

The second version of the Oct 2 TaqMan SNP genotyping assay, Oct 2_v2, (Table 1) was tested in a pilot run using the plasmid and gDNA positive controls of susceptible, resistant and heterozygous genotype (Table 3). Both the susceptible and resistant controls were correctly genotyped (Figure 9A and B respectively). The pseudo-heterozygous plasmid control (Table 3) was correctly genotyped, but the heterozygous gDNA was incorrectly genotyped as susceptible (Figure 9C). Manual genotyping showed that in one of the heterozygous gDNA replicates, fluorescence levels above the ROX passive reference dye were observed for both probes with a lower level of fluorescence observed for the resistant probe (Figure 10). In the other two replicates, the levels of fluorescence for the resistant probe were not above the ROX passive reference dye but there was a slight increase in fluorescence at cycle 38 (Figure 10). This resulted in the incorrect calling of a susceptible genotype.

However, the fluorescence levels overall were low, where amplification of the gDNA controls only started at cycle 36 or higher (Figure 10). This is of concern as the low levels of fluorescence could lead to inaccurate genotyping calls, as evidenced by the incorrect genotyping call of the heterozygous gDNA control. This observation could be due to insufficient template, which evidences the need for the optimum input DNA concentration to be determined. The fluorescence levels could also potentially be increased if there are more amplification cycles and this should be investigated in future studies.

Bovine gDNA was also included in triplicate as a negative control, for which no amplification was observed (Figure 9D). This confirms the results of the BLAST search and indicates that there is no species cross-reactivity of the Oct 2_v2 assay with the bovine host DNA. In addition, the no template controls indicate that there was no contamination of the Oct 2_v2 TaqMan SNP genotyping assay.

In summary, the Oct2_v2 assay could potentially be suitable for genotyping the L22S SNP but additional optimisation is required and larger sets of gDNA samples need to be tested and analysed.

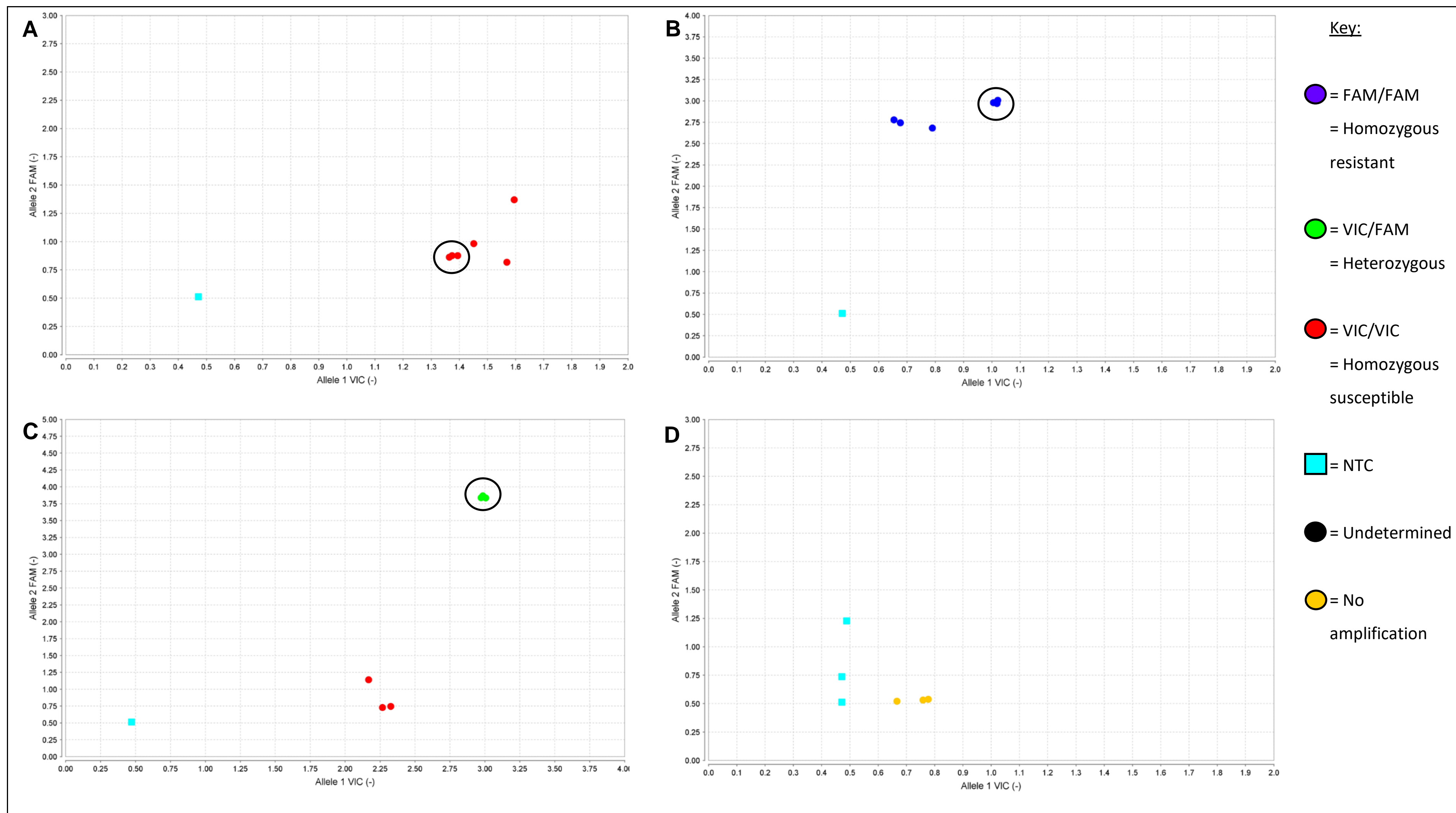


Figure 9: An allelic discrimination plot of the Oct 2_v2 assay for (A) the susceptible, (B) resistant, (C) heterozygous and (D) negative controls. The circled samples correspond to the plasmid control constructs, while the unselected samples are gDNA controls. In blue the NTC is shown. The yellow shows no amplification of the bovine gDNA.

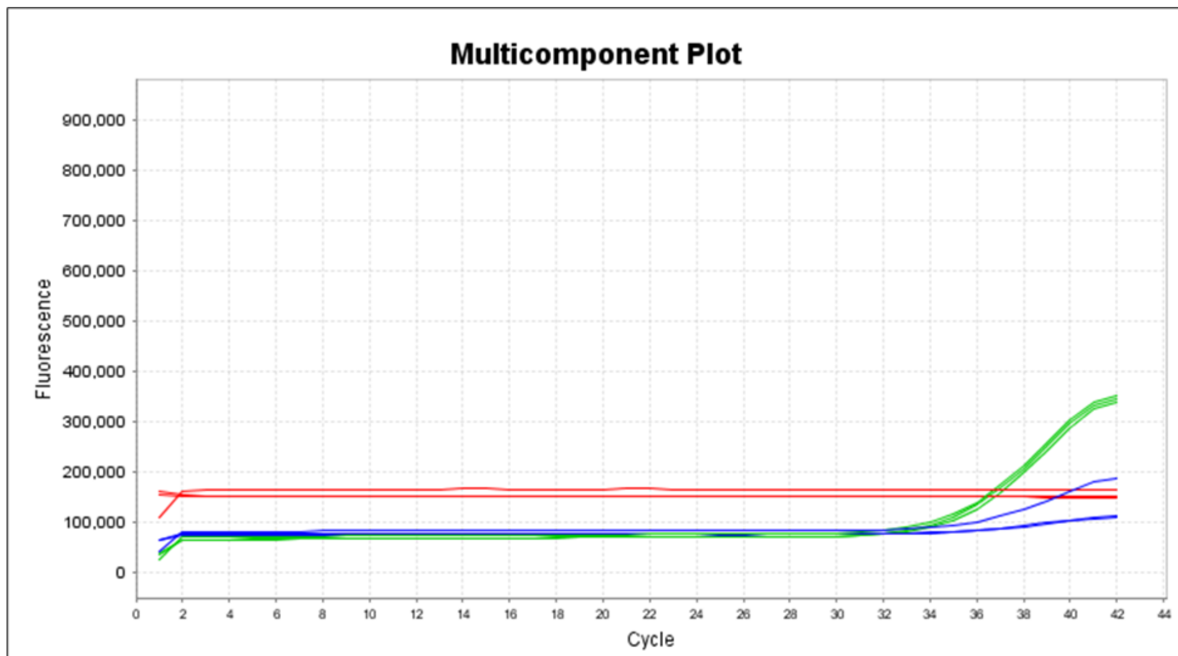


Figure 10: Multicomponent plot showing the fluorescence levels of the Oct 2_v2 assay to genotype heterozygous gDNA controls. Green lines= resistant probe; Blue lines= susceptible probe. Red= ROX passive reference dye.

Considering the results that were obtained for the testing of the Oct 1_v2 and Oct 2_V2 TaqMan SNP genotyping assays, only the Oct 2_v2 assay was selected for further testing as it was able to correctly genotype the susceptible and resistant controls at the L22S SNP. The Oct 1_v2 assay was not selected for further testing due to its inability to distinguish between susceptible and heterozygous genotypes. Optimization of the Oct 1_v2 assay was not undertaken due to time and resource constraints but this could be a possibility for future studies, or alternatively an additional assay may have to be designed to detect the T8P SNP.

4.6. Evaluation of the sensitivity of the Oct 2_v2 TaqMan™ SNP genotyping assay

A 20-fold dilution series of gDNA was conducted for the Oct 2_v2 TaqMan SNP genotyping assay to determine the LOD and optimal gDNA concentration for downstream genotyping experiments using field samples. Wild-type and heterozygous gDNA samples were selected for the dilution series, because for wild-type samples the one allele is expected to be present at a higher frequency in comparison to the heterozygous genotype, where the frequency of the alleles is expected to be 50% for allele 1 and 50 % for allele two. This could therefore potentially affect the normalized fluorescence levels observed and ultimately the LOD of the assay depending on the genotype of the sample. The average ΔRn values for allele 1 and allele 2 of the susceptible and heterozygous gDNA samples are

detailed in Table 4 and graphed in Figure 11. On average the ΔRn values were higher for allele 1 than 2 for the wild-type samples as expected, but allele 2 was higher than allele 1 for the heterozygous samples which was not expected. This may be indicative that the resistant probe binds with a higher affinity in the heterozygous samples.

Table 4: Average normalized fluorescence levels (ΔRn values) obtained for the L22S SNP detected by the Oct 2_v2 TaqMan SNP genotyping assay from a 20-fold dilution series.

DNA input (ng)	ΔRn for susceptible gDNA samples		ΔRn for heterozygous gDNA samples	
	Allele 1	Allele 2	Allele 1	Allele 2
100	1.7	0.6	1	3
90	1.6	0.6	1	2.8
80	1.6	0.7	0.9	2.8
70	1.6	0.5	0.6	2.7
60	1.5	0.7	1	2.7
50	1.5	0.5	1	2.7
40	1.6	0.7	1	2.8
30	1.6	0.6	0.9	2.7
20	1.5	0.6	0.9	2.7
10	1.4	0.8	0.9	2.9
1	0.7	0.7	0.5	1.4
0.9	0.8	0.9	0.6	1.7
0.8	0.8	0.6	0.6	1.3
0.7	0.8	0.8	0.5	1.6
0.6	0.7	0.8	0.6	1.4
0.5	0.8	0.7	0.6	1.2
0.4	0.8	0.9	0.5	1.5
0.3	0.7	0.7	0.5	1.5
0.2	0.7	0.9	0.5	1.2
0.1	0.6	1	0.4	1.4

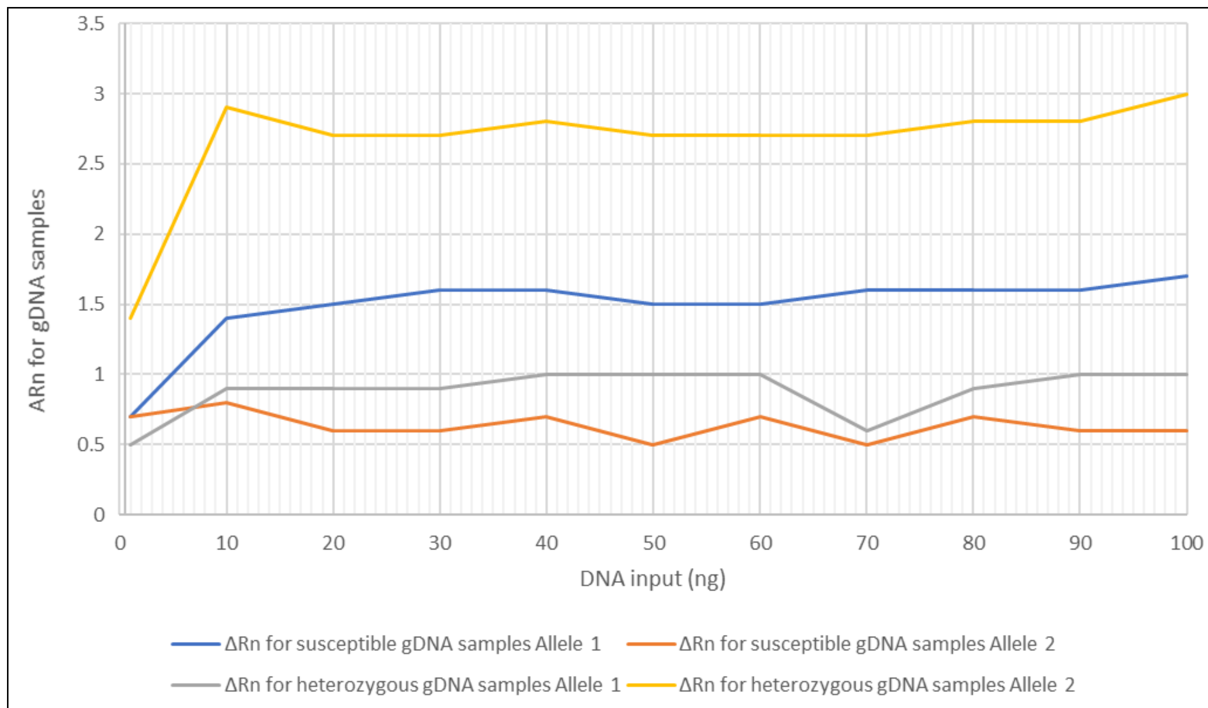
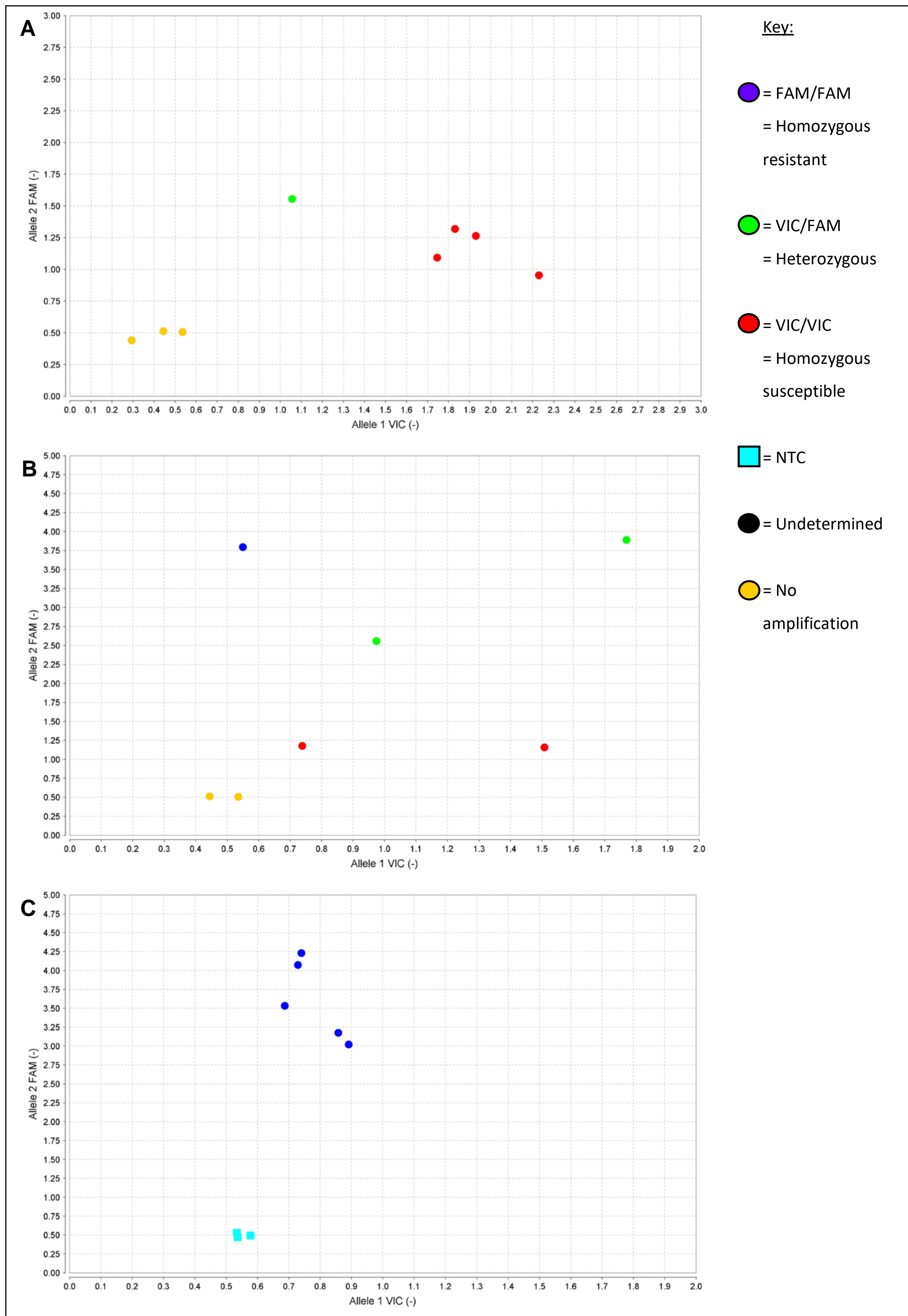


Figure 11: Average ΔRn values obtained for the L22S SNP detected by the Oct 2_v2 TaqMan SNP genotyping assay from a 20-fold dilution series.

From the results, it is evident that the Oct 2_v2 assay was able to amplify from as little as 0.1 ng of DNA per reaction. At an input DNA concentration of 0.1 ng, 90% of samples tested could be automatically genotyped and 100% could be manually genotyped. Optimal levels of fluorescence were observed for an input of 10 ng of DNA per reaction for both wild-type and heterozygous genotypes (Table 4), where an increased frequency of correct genotyping calls was observed for reactions where 10 ng of DNA or more was tested.

4.7. The rate of correct calling of the Oct 2_v2 TaqMan™ SNP genotyping assay

Based on these results of the dilution series, 10 ng of DNA input per reaction was selected to proceed with for genotyping of *R. microplus* field samples of known genotype using the Oct 2_v2 assay to ensure that reliable genotyping calls can be made. The rate of correct calling for gDNA field samples was 80% for the susceptible genotype (Figure 12A), 40% for the heterozygous genotype (Figure 12B) and 100% for the resistant genotype (Figure 12C). The overall rate of correct calling for the Oct 2_v2 assay was calculated at 73.33%.



<u>Figure 12:</u> An allelic discrimination plot of the Oct 2_v2 assay for (A) susceptible (B) heterozygous and (C) resistant *R. microplus* gDNA field samples of known genotype. In blue the NTC is shown. The yellow shows no amplification of samples.

The inaccurate calling of the heterozygous genotype from gDNA is consistent with the results that were obtained in the test run (Figure 9C). Increasing the DNA input per reaction to the optimally determined concentration (10 ng) did not improve the rate of calling for gDNA samples of heterozygous genotype. These results are indicative that genotyping heterozygous individuals from *R. microplus* field populations is likely not viable using this assay. However, the rate of correct calling for susceptible and resistant genotypes is high, where the Oct 2_v2 assay can potentially serve as a tool for detecting amitraz resistance as heterozygous individuals are still susceptible to the acaricide during conventional bioassays.

4.8. Towards the design of a potential ITS2 species identification assay

An additional TaqMan SNP genotyping assay was designed to differentiate between *R. microplus* and *R. decoloratus* ticks, called ITS2 Rm/Rd (Table 2). This assay was designed utilizing a conserved point mutation between the two tick species on the ITS2 gene, which has previously been used for species identification of *Rhipicephalus* ticks in PCR-RFLP assays (Lempereur *et al.* 2010; Robbertse *et al.* 2016).

4.9. PCR and sequencing of the ITS2 gene

A total of 63 *Rhipicephalus* gDNA samples were received from Zimbabwe (Zim) and 36 from the Eastern Cape (EC). Seven EC and seven Zim gDNA samples were subjected to PCR and sequencing of ITS2 to validate that the gDNA samples received were from *R. microplus* ticks as well as to include in a sequence alignment with previously obtained ITS2 sequences from *R. microplus* ticks from Mpumalanga (Mnisi area) (Reinecke 2015). It is evident that ITS2 was successfully amplified from the EC and Zim gDNA samples at an expected size of 700- 850 bp (Lempereur *et al*. 2010) (Figure 13).

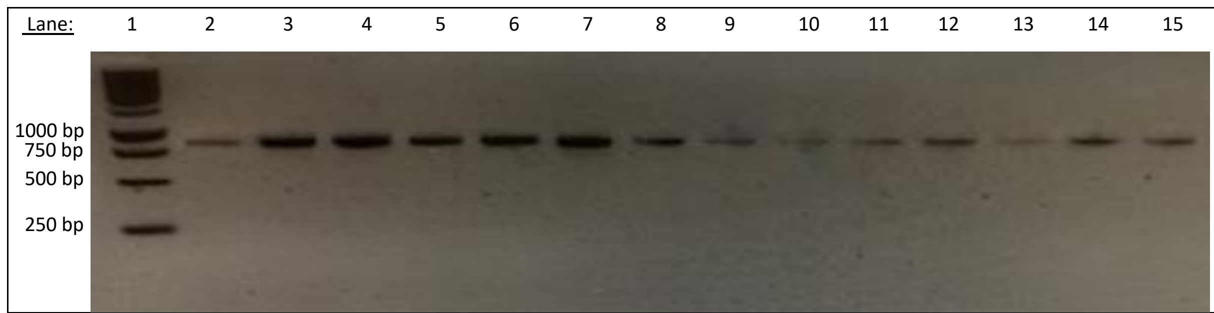


Figure 13: A 2% agarose gel showing electrophoresis of ITS2 PCR products amplified from *R. microplus* gDNA samples. Lanes correspond to (1) 1 Kb molecular marker, (2-8) ITS2 PCR product amplified from EC gDNA samples (9-15) ITS2 PCR product amplified from Zim gDNA samples.

PCR products were subsequently for sequencing. Sequencing of the ITS2 PCR products from the EC and Zim gDNA samples confirmed all samples as *R. microplus*, including the samples that were utilized to generate positive controls for the TaqMan SNP genotyping assays designed to detect the T8P and L22S SNPs in the OCT/Tyr gene (Table 3). These sequences were included in ITS2 sequence alignment for the design of the ITS2 Rm/Rd assay (figure 15).

4.10. Design of the ITS2 Rm/Rd assay

An ITS2 sequence alignment of 95 ITS2 sequences was constructed, including 37 *R. decoloratus* and 58 *R. microplus* sequences*.* The seven EC and seven ZIM ITS2 sequences obtained in this study for *R. microplus* were included as well as sequences from a previous study, including ITS2 sequences from 44 *R. microplus* ticks collected from the Mnisi area in Mpumalanga, South Africa (Baron *et al*. 2015) and 58 *R. decoloratus* ticks from South Africa (Baron *et al*. 2018). The inclusion of ITS2 sequences from different geographic locations allowed for the identification of areas of variation and conservation. Appropriate areas for probe and primer design were then identified for the ITS2 Rm/Rd assay (Figure 14; Table 2)

A highly conserved point mutation was identified between *R. microplus* and *R. decoloratus* ticks to discriminate between the two species indicated at nucleotide position 40 of the alignment (Figure 14). Numerous additional point mutations were identified between the ITS2 sequences of the two tick species, which can be considered for future assay design. For instance, conserved point mutations are shown in the alignment (Figure 14) at nucleotide positions 1, 50, 58, 63, 65, 72, 73, 80, 83, 92, 97, 98, 102, 125, 126, 130, 132 and 139. The point mutation at nucleotide position 40 was selected for probe design as it was conserved amongst all 95 sequences included in the alignment.

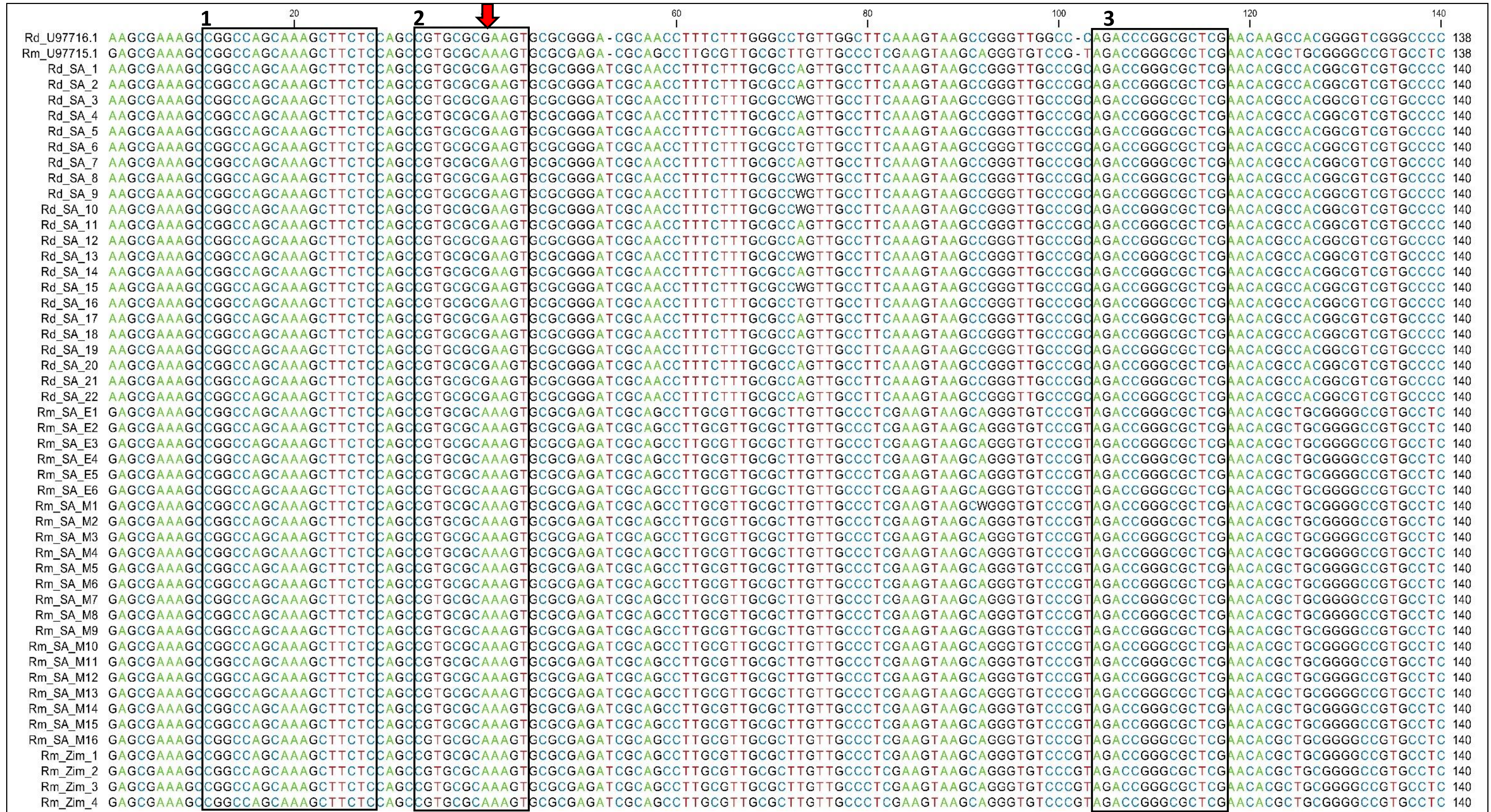


Figure 14: Nucleotide sequence alignment of a 140 bp region of the ITS2 gene from *R. microplus* and *R. decoloratus* ticks. GenBank sequence entries for *R. microplus* (GenBank Accession: U97715.1) and *R. decoloratus* (GenBank Accession: U97716.1) were included as reference sequences. The red arrow indicates the point mutation selected for probe design. Box 1 indicates the forward primer, box 2 indicates the Rm/Rd assay probe (A= *R. microplus;* G= *R. decoloratus)* and box 3 indicates the reverse primer. Sequence names: Rm= *Rhipicephalus microplus*; Rd= *Rhipicephalus decoloratus*; SA= South Africa; Zim= Zimbabwe; E= Eastern Cape; M= Mnisi; number= individual tick. “W” = A or T nucleotide bases.

A BLAST search of the ITS2 Rm/Rd primers (Table 2) was conducted against both GenBank (*Babesia* or *Anaplasma* species) and the Bovine Genome Database to ensure that the primers do not bind to off target sites. Multiple significant hits for the ITS2 gene were found against various *R. microplus* isolates (Figure 15) and that both primers have 100% identity with the *R. microplus* ITS2 gene. No hits were found against the Bovine Genome Database and no significant hits were found against the GenBank database for *Babesia* or *Anaplasma* species.

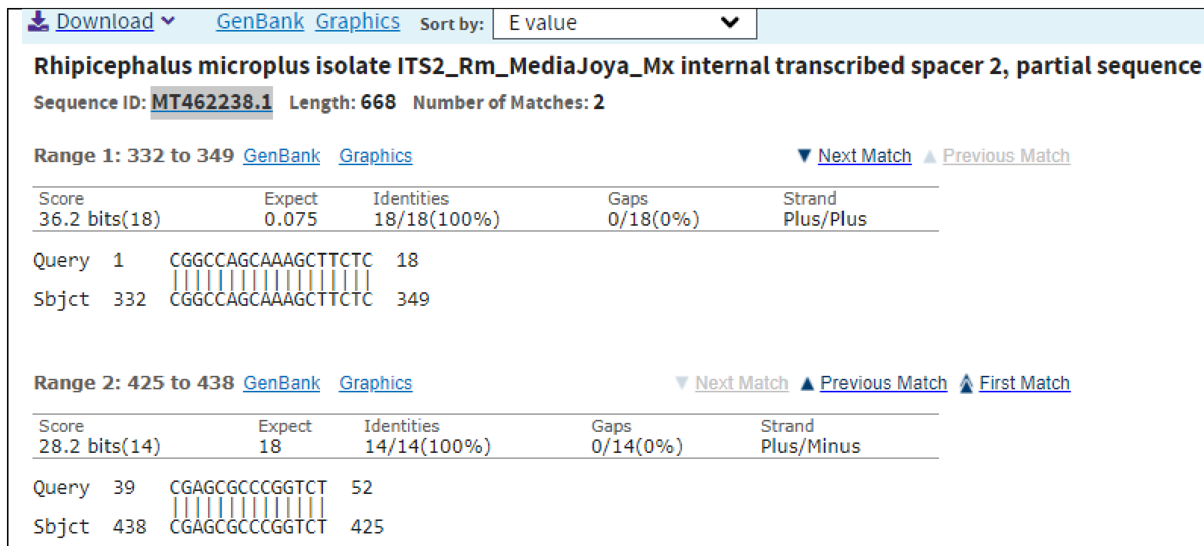


Figure 15: Example of nucleotide BLAST results of the ITS2 Rm/Rd primers. The best hit for the forward (top) and reverse primer (bottom) is shown, where complete identity of the primers to an *R. microplus* isolate (MT462238.1) is evident.

4.11. Positive controls for the ITS2 Rm/Rd assay

Five *R. microplus* and five *R. decoloratus* gDNA sample as of known species were selected as positive controls for the ITS2 Rm/Rd assay. In addition, plasmid controls were constructed. A gDNA sample (sample number 25 from the Eastern Cape) that was previously confirmed as *R. microplus* and gDNA samples (samples number 40.1 and 78.1) from South Africa that were previously confirmed as *R. decoloratus* (Baron *et al.* 2018) were selected to generate ITS2 plasmid controls. ITS2 purified PCR product amplified from the *R. microplus* gDNA sample previously in this study was subjected to cloning. For both of the *R. decoloratus* gDNA samples, ITS2 was amplified at an expected size of 700-850 bp (Lempereur *et al.* 2010) (Figure 16). One the ITS2 PCR products (Figure 16, lane 2) was selected for subsequent purification and cloning. Sequence analysis confirmed that the correct ITS2 amplicon for both *R. microplus* and *R. decoloratus* was ligated into the plasmids. Table 5 summarizes the positive controls for the ITS2 Rm/Rd assay.

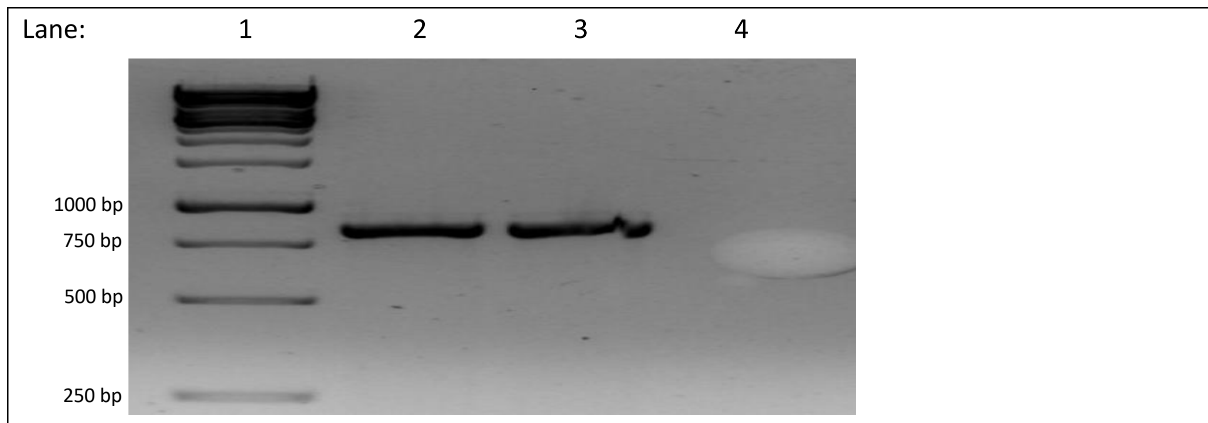


Figure 16: A 2% agarose gel of the PCR products amplified from *R. decoloratus* gDNA samples. Lanes correspond to (1) 1 Kb molecular marker, (3) ITS2 PCR product amplified from *R. decoloratus* gDNA samples (4) no template control.

Table 5: Plasmid controls generated for testing the ITS2 Rm/Rd assay. gDNA= genomic DNA. EC= Eastern Cape; SA= South Africa. M= Mnisi; EG, UTA and ALA= Eglington, Utah A and Allandale A cattle dip stations respectively; numbers= individual ticks.

Positive controls	Tick species	Description	Source
Plasmid	*R. microplus*	ITS2 sequence cloned into pJET1.2.	EC gDNA sample number 25.
	R. decoloratus	ITS2 sequence cloned into pJET1.2.	SA gDNA sample (number 40.1).
gDNA	*R. microplus*	SAMEG 21-8 gDNA SAMEG 21-9 gDNA SAMUTA P-1 gDNA SAMUTA P-4 gDNA SAMALA 3-1 gDNA	gDNA samples of known species (Chapter 2).
	R. decoloratus	Rd-1 gDNA Rd-2 gDNA Rd-3 gDNA Rd-4 gDNA Rd-5 gDNA	gDNA samples of known species (Baron *et al.* 2015).

4.12. Pilot run of the ITS2 Rm/Rd assay

The ITS2 Rm/Rd was tested for its species identification abilities utilizing the positive controls (Table 5). The optimal gDNA input concentration that was determined for the Oct 2_v2 assay (10 ng) was utilized for all gDNA samples that were subjected to testing. The input DNA for the plasmid controls was 0.2 ng which proved to be sufficient for the testing of the OCT/Tyr plasmid controls (Figure 9).

The results indicated that the *R. decoloratus* allele could be correctly genotyped from all plasmid controls as well as sequence-verified gDNA controls (Figure 17A). The *R. microplus* allele could be correctly genotyped from the plasmid control and from 40% of the gDNA controls (Figure 17B). For the 60% of the *R. microplus* gDNA samples that could not be correctly genotyped, they were called either as heterozygous or as undetermined.

Manual genotyping revealed that the fluorescence levels for both probes was relatively low in all *R. microplus* controls (Figure 18A) that were tested in comparison to the *R. decoloratus* controls (Figure 18B). These results could be due to both probes binding with equal affinity or both probes having a low affinity for binding to the ITS2 gene of *R. microplus* samples. For instance, the fluorescence levels for the *R. microplus* plasmid control (Figure 18B) were low in comparison to the *R. decoloratus* plasmid control (Figure 18A). This is likely indicative that the design of the probes is not suitable for binding to the *R. microplus* ITS2 gene and that this assay is likely only suitable for the identification *R. decoloratus* ticks. This is not ideal for the rapid species identification of *R. microplus* ticks when screening field samples for acaricide resistance and a new probe design should be considered for future studies.

The observed variation in fluorescence levels (Figure 18A and B) could also be due differences in the integrity of the gDNA samples that were tested. The *R. microplus* and *R. decoloratus* gDNA samples were sourced from different studies where different methods of gDNA isolation were performed. It is possible that the *R. microplus* samples were more degraded than the *R. decoloratus* samples, which could have resulted in lower fluorescence levels. This demonstrates the impact that the integrity of gDNA samples can potentially have on genotyping results. Additional gDNA samples isolated using the same technique should be tested in future studies to further evaluate this hypothesis.

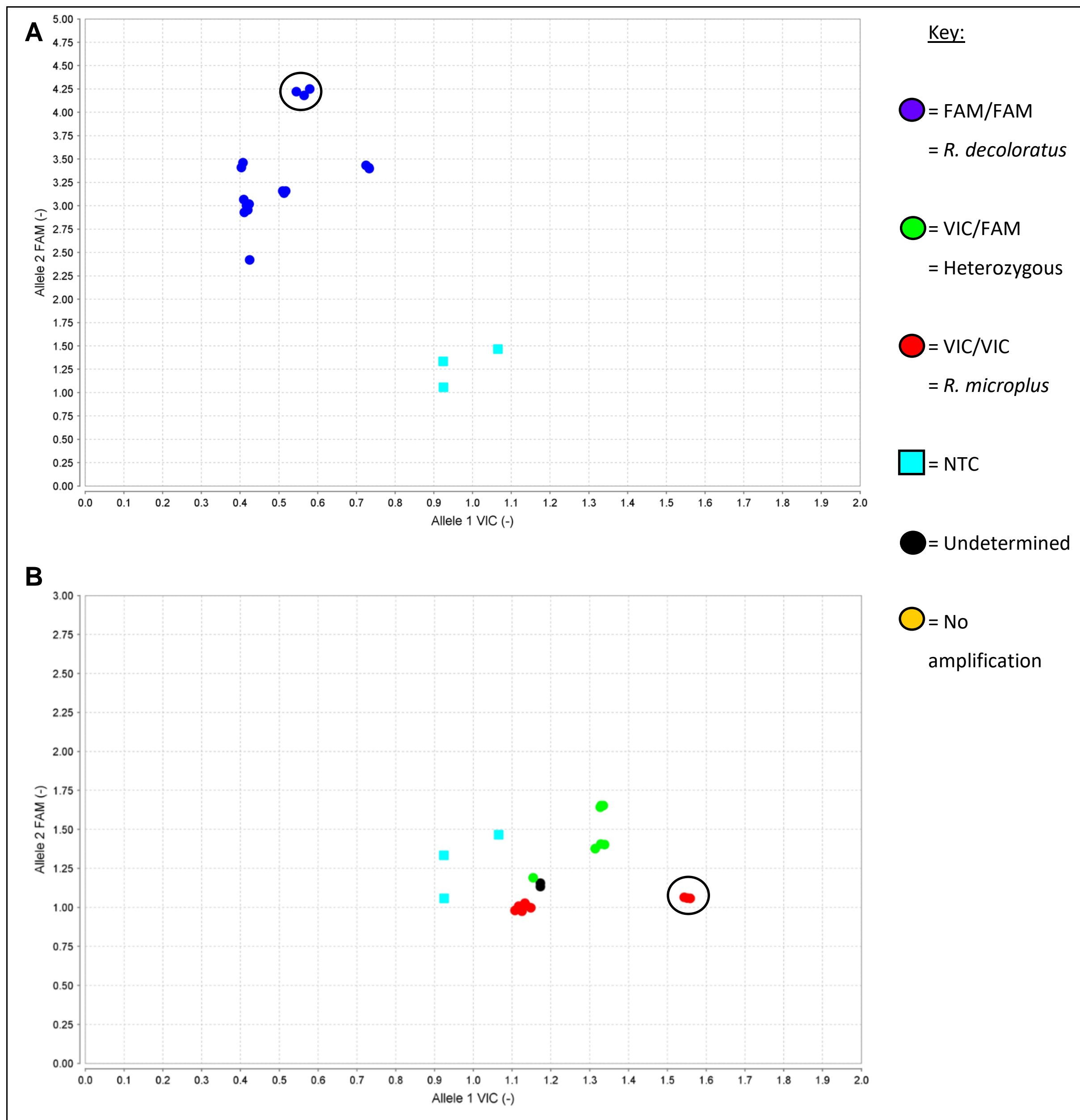


Figure 17: Allelic discrimination plots of the ITS2 Rm/Rd assay for (A) the *R. decoloratus* and (B) the *R. microplus* controls. The circles correspond to the plasmid constructs, while the unselected samples are gDNA controls. In blue the NTC is shown.

A

B

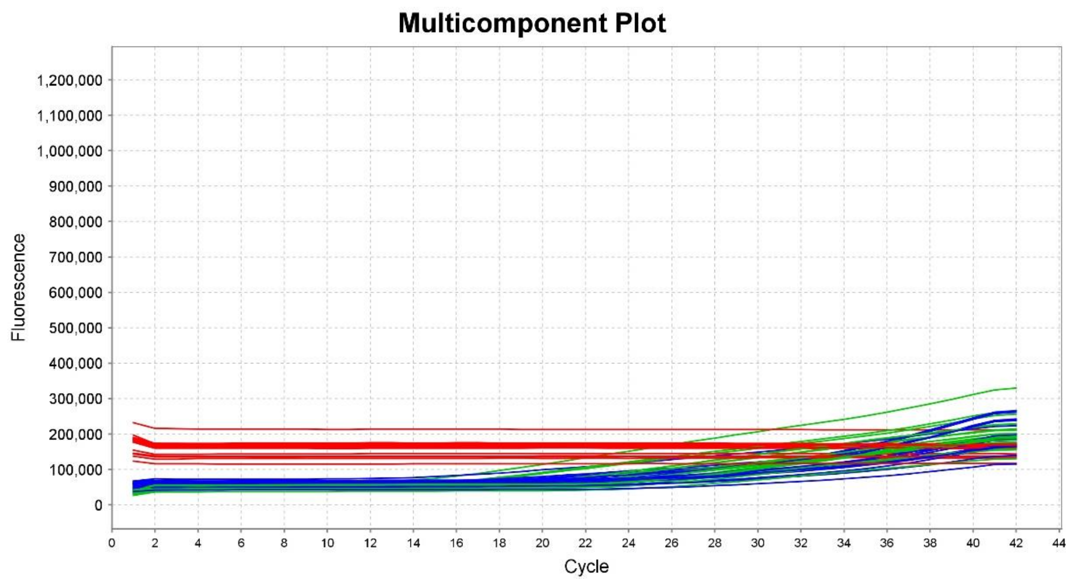


Figure 18: Multicomponent plot showing the fluorescence levels of the ITS2 Rm/Rd assay to detect the (A) the *R. decoloratus* and (B) the *R. microplus* controls. Green lines= *R. microplus* probe; blue lines= *R. decoloratus* probe. Red lines= ROX passive reference dye.

5. Discussion

TaqMan SNP genotyping assays to detect the T8P and L22S SNPs in the OCT/Tyr gene of *R. microplus* ticks

Both versions of the Oct 1 assays tested in this study, Oct 1_v1 and Oct 1_v2, did not prove to be suitable for genotyping the amitraz resistance-linked alleles at the T8P SNP in the OCT/Tyr gene of *R. microplus* ticks. The Oct 1_v2 assay was able to correctly genotype resistant samples, but it was not able to distinguish between susceptible and resistant genotypes. Future studies need to be conducted to further optimize the Oct 1 _v2 assay. For instance, this assay was not tested utilising the optimal DNA input concentration and by doing so the genotyping accuracy could potentially be improved. Alternatively, a new TaqMan SNP genotyping assay will need to be designed to detect the T8P SNP.

For the Oct 2 assays, the Oct 2_v2 assay showed large improvements in genotyping accuracy over the Oct 2 _v1 assay, where all plasmid controls were correctly genotyped and the overall rate of correct calling was found to be 73.33% for gDNA samples. Even though the Oct 2_v2 assay was able to correctly genotype 100% of the resistant controls, it was not able to correctly genotype all susceptible and heterozygous gDNA controls. The observed rate of correct calling is not sufficient to justify the Oct 2_v2 assay as a high-throughput screening tool to detect the L22S SNP (McGuigan and Ralston, 2002). However, the rate of correct calling could be improved via additional optimization. For instance, future studies can test the Oct 2_v2 assay with an increased annealing temperature to increase the binding specificity of the probes or the number of cycles could be increased to increase the levels of fluorescence to improve genotyping calls (Heissl *et al.* 2016). In addition, future studies should test a higher number of gDNA samples to obtain a more accurate rate of correct calling (Walker *et al.* 2007; Bass *et al*. 2008). There are 82 *R. microplus* gDNA samples of known genotype available from Chapter 2 of this study which can be tested with the Oct 2_v2 assay to compare the accuracy of the TaqMan SNP genotyping assay to conventional PCR and sequencing.

The results from chapter 2 indicate that all *R. microplus* samples that were screened were either homozygous or heterozygous at both the T8P and L22S SNPs, which is in agreement with previous studies conducted on samples from the Mnisi area (Baron *et al.* 2015; Robbertse *et al*. 2016). It could thus potentially be assumed if the resistant genotype is detected at the L22S SNP by the Oct 2_v2 assay then the resistant genotype is also present at the T8P SNP.

Insights into the LOD and optimal DNA input for TaqMan SNP genotyping assays

This study provides novel insights into the LOD of TaqMan SNP genotyping assays. It was found that the Oct 2_v2 assay could amplify from as little as 0.1 ng of DNA per reaction and accurately genotype 90% of samples tested at this concentration. These results are similar to the findings of Brocannello *et al.* (2018) where it was found that a SNP could amplify from as little as 0.2 ng of DNA using a TaqMan SNP genotyping assay. This emphasizes the advantages of TaqMan SNP genotyping assays over template dependant PCR amplifications. For instance, HRM analysis requires high quality and quantity of DNA template to produce clearly distinguishable melt-curves to genotype samples (Klafke *et al*. 2019). High quality and quantity of gDNA is not always easy to obtain, especially under conditions where ticks are collected in the field as gDNA may degrade. The ability of TaqMan SNP genotyping assays to perform genotyping from low concentrations will therefore be suitable for its use as a high-throughput screening tool.

In addition, this study also provided insight into the optimal template input concentration for TaqMan SNP genotyping assays. For instance, the manufacturer's recommendations for template input concentration for the TaqMan SNP genotyping assays is 0.2 ng, but this study found that optimal levels of fluorescence were only observed at an input of 10 ng of DNA or higher for both wild-type and heterozygous genotypes. This finding is consistent with Brocanello *et al.* (2018), who also found that 10 ng of DNA was the optimal input concentration for TaqMan SNP genotyping assays. Therefore, even though the LOD of TaqMan SNP genotyping assay may be low, genotyping accuracy from field samples could be improved if the optimal DNA input is utilised for genotyping reactions.

TaqMan SNP genotyping assays to discriminate between *R. microplus* and *R. decoloratus* ticks

The ITS2 Rm/Rd that was designed to discriminate between tick species based on variation in the ITS2 gene was only successful in detecting *R. decoloratus* ticks, whilst *R. microplus* ticks could not reliably be distinguished. An additional ITS2 species assay could be designed and tested in future studies utilizing the same methodology described in this study but for a different highly conserved point mutation that can be identified from the ITS2 sequence alignment. Considering the numerous conserved nucleotide differences identified between the two species, future studies could also consider designing a TaqMan SNP genotyping assay to detect more than one SNP. For instance, a TaqMan SNP genotyping assay was designed to distinguish *P. falciparum* from three other *Plasmodium*

species in the mosquito vector in a single reaction, where the design for the *P. falciparum* probe was based on two SNPs that are four nucleotide bases apart (Bass *et al*. 2008). Alternatively, it could also be possible to design a TaqMan qPCR assay that can detect more than just a single point mutation/SNP, i.e., multiple mutations could be included in the design. TaqMan qPCR assays have been employed for rapid diagnostics in other species, for instance for the diagnostic detection of *B. bovis* and *B. Bigemina* in bovine blood samples (Kim *et al*. 2007), for the quantification of *Trypanosoma cruzi* satellite DNA in human blood samples (Duffy *et al.* 2013) and for the identification of *Trypanosoma cruzi* Discrete Typing Units (DTCs) in biological and clinical samples (Cura *et al.* 2015). Although separate assays would likely need to be designed for each species, the TaqMan qPCR assays can be multiplexed (Cura *et al.* 2015; Duffy *et al.* 2013).

Summary and future prospects

This study is the first to design and test TaqMan SNP genotyping assays to detect the amitraz resistance-associated SNPs in the OCT/Tyr gene of *R. microplus* ticks. TaqMan SNP genotyping assays provide several advantages over other methods of detecting amitraz resistance, such as: no prior PCR or purification steps are required after DNA isolation, the assays are less expensive and more time efficient and resistance genotypes can be accurately detected from low quantities of DNA. This study provides a proof of concept for TaqMan SNP genotyping assays to detect amitraz resistance-linked alleles, although further optimization and testing is required before the assays can be utilised as a high-throughput resistance detection tool. Future studies could also expand on this research by designing a TaqMan SNP genotyping assay to detect the amitraz resistance-associated I61F SNP in the βAOR gene of *R. microplus* ticks. The concept of a TaqMan SNP genotyping assay as a rapid species identification tool based on the ITS2 gene may also hold potential and should be further investigated in future studies. In addition to *R. microplus* and *R. decoloratus*, this assay could also be expanded to distinguish between multiple *Rhipicephalus* species based on the ITS2 gene (Lempereur *et al.* 2010).

6. References

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Chapter 4

Amitraz resistance in *Rhipicephalus microplus* ticks: current status and future detection prospects

1. Amitraz resistance in the Mnisi communal area

This study evaluated the current amitraz resistance status (samples collected in 2019) in the Mnisi communal area as well as the change in amitraz resistance over a period of 6 years from ticks evaluated in 2012/2013 by a previous study (Robbertse et al. 2016), where novel insights were provided. For instance, four dip stations were tested that were not previously sampled in this area. In addition, this study allowed for investigation into the change in amitraz resistance over time in a controlled *R. microplus* population where a continued selection pressure is applied, where a comparison could be made between four dip stations that were sampled in both 2012/2013 and 2019.

However, there were also limitations to this study. Eight dip stations that were sampled in 2012/2013 could not be sampled in 2019 due to a lack of *R. microplus* ticks at some of the dip stations in this area at the time of collection as well as time constraints imposed on the field trip. For instance, no *R. microplus* ticks were found on cattle at the Welverdiend dip stations (A and B) or at Share. This is likely due to seasonal elements, where *R. microplus* ticks could have been at the egg or larval life stage, hindering tick collection. Additional ticks were therefore collected in 2020 from the dip stations that could not sampled in 2019 which will be subjected to PCR and sequencing of the OCT/Tyr gene in the near future. Furthermore, additional PCR and sequencing of samples collected in 2019 will be conducted, as there were a total of 2944 ticks that were collected and 810 gDNA isolations that were performed, where this study only selected 96 gDNA samples for analysis. Future research will focus on obtaining at least 10 sequences for the OCT/Tyr gene from individual ticks collected per dip station. This will provide a sufficient number of resistance alleles to conduct appropriate statistical calculations to better infer a resistance status to the population that was sampled, namely through exact binomial tests. Additional sequences from a higher number of samples and dip stations will allow for the more comprehensive evaluation of the change in amitraz resistance over time.

Based on the results of this study, it was hypothesized that the weekly dipping regime at the Mnisi communal area dip stations serves as a continued selection pressure to maintain both the resistance and wild-type alleles in the *R. microplus* tick population. This explains the observed abundance of

heterozygosity, which is indicative that balancing selection is taking place in the population. It is therefore likely that positive selection is not acting on the population, which has been reported to be the case for synthetic pyrethroids (MSc dissertation of Ms. Michaela Smit), where resistance alleles become fixed in the population. A possible explanation for this phenomenon could be that the amitraz resistance-linked alleles at the SNPs in the OCT/Tyr gene (T8P and L22S) pose a negative fitness cost on *R. microplus* ticks. In this case, the heterozygous genotype could be advantageous over the wild-type genotype as some resistance can be conferred, but the tick is not fully subjected to the fitness cost imposed by the resistant genotype. This would be in agreement with what has been observed in insect populations (Kliot and Ghanim 2012), and what has previously been suggested to be the case in *R. microplus* populations (Davey *et al*. 2006). However, there is insufficient evidence to support this hypothesis and further research needs to be conducted.

The results of this study still raise concern as the resistant genotype is double the frequency that was observed previously, where the overall frequency of resistance alleles has increased in the population (considering both the heterozygous and resistant genotypes) whilst the overall frequency of wild-type alleles has decreased. Despite the theory of balancing selection acting on the population, it is possible that these results are indicative that amitraz resistance is emerging *R. microplus* populations in the Mnisi area and will be further tested using the 2020 samples. This would be in agreement with what has been reported for amitraz resistance in other *R. microplus* populations. For instance, the RuVASA 2019 report stated that resistant *R. microplus* ticks have been detected in multiple provinces in South Africa and that amitraz resistance is emerging. In addition, levels of amitraz resistance in South American countries are considerably higher than the levels of resistance observed in the Mnisi communal area, where a study in Brazil showed that some *R. microplus* tick populations were up to 100% resistant to amitraz (Anredotti *et al*. 2011). This ultimately demonstrates the importance of regular monitoring of *R. microplus* tick populations to enable the implementation of appropriate knowledge-based tick control strategies.

However, it should be considered that the majority of reports of amitraz resistance in South Africa and in other countries are based on the results of bioassays. Bioassays have proven to be inaccurate due to large differences in LC (lethal concentration) values with wide confidence intervals (Jonsson *et al*. 2007) and they do not provide any information about the resistance mechanisms employed by ticks (Rodriguez-Vivas *et al.* 2018). This consequently does not allow for a distinction to be made between homozygous resistant and heterozygous genotypes, which is most valuable for long-term planning. To obtain a more holistic overview of the amitraz resistance status in the Mnisi communal area, it would be beneficial for the abundance of heterozygous tick populations to be subjected to bioassays to confirm the level of their phenotypic resistance. Synergistic assays could also be employed to detect

the involvement of metabolic resistance and its potential impact on the resistance phenotype in combination with target site resistance.

Lastly, it should also be taken into consideration that there is a complex interaction between the continuous presence of amitraz selection pressure and the resultant amitraz resistance status of different *R. microplus* populations. For instance, the *R. microplus* tick populations in the Mnisi communal area are subjected to unique selection pressures, where there is a continuous weekly dipping regime and the migration of ticks is limited due the restrictions placed on the movement of cattle (similar to a genetic island model). Different tick populations from different geographical locations will likely be subject to varying selection pressures as well as other influencing factors, such as operational factors and human error, which could potentially result in differences in the evolution of amitraz resistance.

The evolution of amitraz resistance could also be better understood if the mechanisms of amitraz resistance are further elucidated. For instance, the mechanism of resistance imposed by the two validated amitraz-resistance associated SNPs in the OCT/Tyr gene (Baron *et al.* 2015) is currently not known. It is also possible that additional SNPs in the OCT/Tyr gene play a role in influencing amitraz resistance. Baron *et al.* (2015) identified numerous SNPs in the OCT/Tyr gene, many of which remain to be further investigated for a potential association with amitraz resistance.

2. PCR and sequencing versus TaqMan SNP genotyping: considerations and future prospects

Various PCR-based assays have previously been employed to detect acaricide resistance-associated SNPs and to genotype the resistance of *R. microplus* ticks, but they have been associated with challenges and time constraints. PCR and sequencing of acaricide target genes, such as the OCT/Tyr gene, usually takes two days per sample. However, this study demonstrated that the PCR amplification and sequencing of the OCT/Tyr gene as well as the ITS2 gene used for species identification is not so straightforward. Despite the availability of PCR and DNA sequencing methodologies from previous publications such as Baron *et a*l. (2015) and Robbertse *et al.* (2016), several challenges were faced which resulted in a notable increase in the time and cost required to genotype the ticks collected in the Mnisi communal area by DNA sequence analysis. For instance, PCR of the ITS2 gene required additional optimization, several different polymerases had to be tested in an attempt to improve PCR reaction conditions for the target genes, different BigDye sequencing reactions were tested as well as various sequencing reaction precipitation methods (data not shown). For the PCR amplification of

both the OCT/Tyr and ITS2 genes it was evident that the concentrations of the PCR products produced was largely template dependant, where varying concentrations were observed for all samples processed. This can most likely be attributed to the degradation status of the sample as received from the field. Precipitation of these small gene fragments also proved to be extremely challenging, where many unsuccessful sequencing results were received as a result of insufficient template yield. As a result, sequencing was outsourced to Macrogen Europe B. V. (Amsterdam, Netherlands).

TaqMan SNP genotyping could potentially be a viable and beneficial way forwards for the detection of the amitraz resistance-associated SNPs in the OCT/Tyr gene as well as other acaricide resistance genes as TaqMan SNP genotyping assays pose several advantages over conventional PCR and DNA sequencing. This study aimed to design and test TaqMan SNP genotyping assays to detect the two SNPs in the OCT/Tyr gene that have previously been linked to amitraz resistance, T8P and L22S (Baron *et al.* 2015; Chen *et al.* 2007), to improve the turnover time of diagnostic testing. TaqMan SNP genotyping allows for 384 individual samples to be screened in four hours on a 384-well qPCR plate, which can also be upscaled to an open-array format for future commercial use where thousands of samples can be screened in one day. In addition, no sequence analysis is required as the genotypes of all samples can immediately be obtained via an allelic discrimination plot that is generated by the TaqMan Genotyping Software, which will further reduce time and labour requirements. The TaqMan cost-effective than conventional PCR and DNA sequencing, where there is a 10-fold difference in the price to genotype the same number of samples (Table 1). For PCR and DNA sequencing the total cost per 384 ticks is ZAR 33,448.77 versus ZAR 3414.32 for TaqMan SNP genotyping. Per sample, this translates to ZAR 87.11 versus ZAR 8.89 respectively.

Table 1: A cost-breakdown of PCR and DNA sequencing versus TaqMan SNP genotyping assays for the diagnostic detection of acaricide resistance-linked SNPs.					
Item Description:	**Supplier:**	**Catalogue number:**	**Full cost of reagent (including VAT) in ZAR:**	**Number of reactions that can be performed:**	**Cost per 384 ticks in ZAR:**
PCR and sequencing					
PCR amplification					
Primer pair	Inqaba Biotech	Custom Order	300.00	5000	23.04
OneTaq Mastermix	NEB	NEB M0486S	651.55	100	2,501.95
96 Well PCR plate	Inqaba Biotech	TRE 11204.9.01	1,939.71	50	155.17
PCR strip tube caps	Merck	BR781413-300EA	1,795.00	300	287.20
UltraPure™ DNase/RNase-Free Distilled Water (500 ml)	ThermoFisher Scientific	10977035	200.38	50000	1.54
		TOTAL	4,886.64		2,968.90
PCR product purification					
Ethanol (99.9%) (2.5L)	ChemLab Suppliers	LC30012	399.00	2500	61.23
Purification kit (250 preps)	Anatech	PRA9282	7,622.57	250	11,708.27
		TOTAL	8,021.57		11,769.50
DNA sequencing					
Sequencing service	Macrogen		4,567.01	96	18,268.00
96 Well PCR plate	Inqaba Biotech	TRE 11204.9.01	1,939.71	50	155.17
PCR strip tube caps	Merck	BR781413-300EA	1,795.00	300	287.20
		TOTAL	8,301.72		18,710.37
Total cost of PCR and sequencing			**21,209.93**		**33,448.77**
TaqMan SNP genotyping assays					
MicroAmp™ Optical 384-Well Reaction Plate with Barcode	ThermoFisher Scientific	4309849	4,528.13	50	90.56
MicroAmp™ Optical Adhesive Film	ThermoFisher Scientific	4360954	1,304.65	25	52.19
UltraPure™ DNase/RNase-Free Distilled Water (500 ml)	ThermoFisher Scientific	10977035	200.38	50000	1.54
96 Well PCR plate	Inqaba Biotech	TRE 11204.9.01	1,939.71	50	38.79
Foil seal	Inqaba Biotech	TRE 09597.9.01	1,122.00	100	11.22
10ul Filter tips	Lasec	P2TIP006C-000010ST	1,040.75	1000	399.65
100ul Filter tips	Lasec	P2TIP006C-000100ST	1,049.95	1000	806.36
TaqPathTM ProAmp™ Master Mix (10 ml)	ThermoFisher Scientific	A30866	7,546.19	10	754.62
CUST TQMN SNP ASSAYS, NON-HUMAN	ThermoFisher Scientific	4332077	2,623.73	800	1,259.39
Total cost of TaqMan SNP genotyping assays			**21,355.49**		**3,414.32**

The Oct 1_v2 TaqMan SNP genotyping assays that was designed may hold potential for detecting the T8P SNP upon further optimization, but it is likely that a new design will need to be considered to reliably distinguish between genotypes. The Oct 2_v2 TaqMan SNP genotyping assay that was designed proved to be suitable for genotyping both the homozygous susceptible and resistant genotypes but not heterozygous genotypes from gDNA. This assay thus shows potential for the detection of amitraz resistance in *R. microplus* field populations, although further optimization and testing is required to improve the rate of correct calling for heterozygous genotypes. This will be of particular importance for future studies and resistance monitoring involving the use of the Oct 2_v2 assay, especially in the Mnisi communal area where the ticks that were screened were found to be predominantly heterozygous (87%).

It was also found that the Oct 2_v2 assay was able to amplify from as little as 0.1 ng of DNA per reaction for 90% of the samples tested, which is advantageous over the template dependant PCR amplifications that were observed in this study. It should also be noted that even though the LOD of the TaqMan SNP genotyping assays is low, the dilution series revealed that for optimal amplification and correct genotyping calls to be made an input DNA concentration of 10 ng should be utilized. This is of particular value for any downstream TaqMan SNP genotyping assays, as this value is much higher than the DNA input of 0.2 ng recommended by the manufacturer (ThermoFisher Scientific) that should be utilized for future TaqMan SNP genotyping assays.

A novel ITS2 TaqMan SNP genotyping assay was also designed to perform rapid species identification. Species identification of *Rhipicephalus* ticks is usually conducted using morphology which require intact mouthparts for the ticks collected from the cattle. This is not always possible, as the mouthparts are frequently damaged during tick removal or they are covered in cement, preventing morphological identification. Alternatively, PCR in combination with either restriction fragment length polymorphism (RFLP) (Lempereur *et al.* 2010) or DNA sequencing (Robbertse *et al*. 2016) is used which is time consuming, costly and adds a new reaction to sample analysis (i.e., resistance and species analyses). By utilising a TaqMan SNP genotyping assay for species identification, a larger number of samples (384 or more) could be identified simultaneously in a rapid and accurate manner. There is also the potential that the ITS2 TaqMan SNP genotyping assay could be duplexed with the Oct 2_v2 TaqMan SNP genotyping assay in one reaction using different fluorescently labelled probes to determine both the tick species and the amitraz resistance status at the same time. However, the ITS2 TaqMan SNP genotyping assay that was designed and tested in this study proved to be suitable only for identifying *R. decoloratus* ticks and not *R. microplus* and is therefore likely not viable for distinguishing between the two species. The concept and design process may still hold potential and an additional assay

should be designed in future that is targeted at a different conserved point mutation or multiple mutations between the ITS2 sequences of *R. decoloratus* and *R. microplus* ticks.

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