UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA

UFS·UV
NATURAL AND AGRICULTURAL SCIENCES
NATUUR- EN LANDBOUWETENSKAPPE
CHEMISTRY
CHEMIE

Resistance of the African blue tick (*Rhipicephalus* (*Boophilus*) *decoloratus*) to Macrocyclic Lactones in the Eastern Cape, South Africa.

By

Setjhaba Kenneth Lesenyeho

(2010035356)

Submitted in fulfillment of the requirements for the Degree of Magister Scientiae in Department of Zoology and Entomology in the Faculty of Natural and Agricultural Sciences at the University of the Free State Bloemfontein, South Africa

Date: 31 January 2019

Supervisor: Mrs. E.M.S.P. van Dalen

The financial assistance of the National Research Foundation (NRF) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the author and are not necessarily to be attributed to the NRF

Table of Content

List of Tables

List of Figures

List of Appendices

UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA

UFS·UV
NATURAL AND AGRICULTURAL SCIENCES
NATUUR- EN LANDBOUWETENSKAPPE
CHEMISTRY
CHEMIE

Declaration

I, Setjhaba Kenneth Lesenyeho, declare that the Master's Degree research dissertation that I herewith submit for the Master's Degree qualification in Zoology at the University of the Free State is my independent work, and that I have not previously submitted it for a qualification at another institution of higher education.

31 January 2019

Setjhaba Kenneth Lesenyeho Date

Ethical Statement

The organisms that were tested on in this study, the adult blue ticks were removed from their natural host, the cattle. Ticks are ectoparasites and thus their removal did not have a negative effect on the ecosystem and was of an advantage to the cattle and the producer. This study was non-invasive and did not involve physical harm to the cattle. The study collections were conducted during routine farming practices in a familiar environment. The producer and farm workers were present at the collections in order to create a familiar environment. Minimal contact was made with the cattle and collections occurred as quickly as possible. Any animal which exhibited excessive physical distress, was released from the race that was used to isolate the animals, and not used in the study.

Ethical clearance was obtained from the Animal Ethics Committee of the University of the Free State. **Student project number: UFS-AED2016/0123**

Acknowledgements

Firstly, I would like to thank my parents for always supporting and helping me get through the stress and allowing me to use their van to do fieldwork in the Eastern Cape. They have never questioned my career choices and rather encouraged me to pursue my dreams and work hard at all times.

I would like to thank my supervisor Mrs Ellie van Dalen for mentoring me and always looking out for me. I honestly didn't know what on Earth I was doing, but she was able to guide me in the right direction. She took the time to get to know me and her other students and was able to understand us and treat us accordingly which I think is a good characteristic of an amazing supervisor. She is definitely one of my biggest supporters and contributors to my research and I thank her for that.

I would like to thank Michelle Pottinger, Abre Marais and Cwayita Duma who are students of my supervisor. They helped me to collect my samples in the Eastern Cape and also helped with identification of ticks and resistance testing.

I would like to thank Gernus Terblanche, a Medical Virology student at the University of the Free State, and also one of my best friends, who also helped me with my fieldwork, especially to collect blood samples from the cattle. Fieldwork can be quite frustrating and he helped a lot with emotional support just by making crazy and exaggerated jokes. His foot almost got crushed by an oxen a few times so I thank him for his sacrifice. I will always be grateful for his hard work and his friendship.

I would also like to thank the producers that allowed me to use their farms and animals for my research. I especially like to thank Glyn Dixon for allowing me to stay at his family town hall and use it as a lab, and the delicious meals we had at his house. I will forever cherish the great memories and support.

Thank you to the Department of Zoology & Entomology, University of the Free State for allowing me to use their facilities and for all the support.

I would like to thank the National Research Foundation (NRF) for funding me for the past two years of my masters. The money really helped me especially because both of my sisters were still studying, so this lessened the stress on my parents as they didn't have to worry about me too much. I will forever be grateful for their help, they have no idea how much they helped me.

Summary

Macrocyclic Lactones (MLs) are anti-parasitic drugs used to control blue ticks, mites and endoparasites. Resistance development of the Asiatic blue tick (*Rhipicephalus* (*Boophilus*) *microplus*) to Ivermectin (IVM) (product of MLs) was reported in Brazil, Uruguay, Mexico and Australia due to the frequent and misuse of this product. There has not been any incidents of tick populations resistant to MLs treatment that were reported up to now in South Africa for the Asiatic blue tick or African blue tick (*Rhipicephalus* (*Boophilus*) *decoloratus*) species although an increase in the use of MLs for tick control was also inevitable. However, pharmaceutical company agents are receiving a rising number of complaints from Eastern Cape producers concerning inadequate control of blue ticks by MLs. Therefore, a methodology had to be established to confirm MLs resistance of South African blue tick strains. This entailed comparing two Shaw Larval Immersion Tests (SLIT), the test-tube and pie-plate SLIT, determining a suitable diluent, TritonX/Ethanol vs. twice-distilled water and a post-exposure timeframe for mortality determination after 24, 48 and 72 hours, to detect resistance and prevent tick death from sources other than the exposure to the chemical. It was determined that the pie-plate SLIT was the most suitable methodology to determine MLs resistance as it was more efficient, less time consuming and caused less mechanical death to the tick larvae than the test-tube SLIT. Twice-distilled water and evaluation of mortality 24 hours post-exposure, were the most suitable diluent and post-exposure time, respectively, for the pie-plate SLIT. Reference strains, of both blue tick species, not previously exposed to MLs were obtained from ClinVet International. These reference strains were used to determine lethal concentrations (LC 50 and LC99) by means of Probit (Polo Suite) analysis. The reference strains of both blue tick species were found to be more susceptible to MLs than blue ticks in Brazil, Australia and Mexico. Blue ticks collected from farms in the Eastern Cape were divided into two groups, ticks that were previously exposed to MLs in the past five years, and those that have not been exposed to MLs in the last five years. The LCs and Confidence Intervals 95% (CI95%) of the field strains were calculated to determine the Factor of Resistance (FR) and resistance levels according to an established range. Strains not exposed to IVM in the past five years were confirmed to be susceptible to IVM, while strains suspected of being resistant to IVM due to complaints of poor to moderate results after treating with IVM also fitted into these ranges to be classified as resistant. More research on these ranges is needed in South Africa to determine when a classification of emerging resistance is valid and when a population can be classified as resistant as this range could not accommodate all the strains. More extensive sampling over different periods and comparing different generations will also be needed to confirm resistance on some of the farms.

Key words: *Macrocylic Lactones, Ivermectin, Rhipicephalus (Boophilus) decoloratus, Rhipicephalus (Boophilus) microplus, Eastern Cape, Shaw Larval Immersion Test.*

List of Abbreviations

AChE	Acetylcholinesterase
AIT	Adult Immersion Test
BHC	Benzene Hexachloride
CI	Chloride ionophore
CI95%	Confidence Interval 95%
DDT	Dichloro-diphenyl-trichloro-ethane
DW	Distilled water
CV	ClinVet
FAO	Food and Agricultural Organiation of the United Nations
FR	Factor of Resistance
GABA	Gamma-aminobutyric Acid
IVM	Ivermectin
LC	Lethal Concentration
LPT	Larval Packet Test
LTT	Larval Tarsal Test
MLs	Macrocyclic Lactones
OPs	Oganophosphates
PHPZ	Phenylpyrazoles
PRTF	Pesticide Resistance Testing Facility
SLIT	Shaw Larval Immersion Test
SPs	Synthetic Pyrethroids
SIT	Syringe Immersion Test
TXE	Triton X/Ethanol

Chapter 1

General introduction and literature review

1.0 Introduction

Ticks are obligate haematophagous ectoparasites of vertebrates such as mammals, birds and sometimes reptiles and amphibians (Rajbut *et al*. 2006). They belong to the phylum Arthropoda, order Acarina and are divided into two family groups, Ixodidae (hard-bodied ticks) and soft-bodied ticks (Argasidae) (Rajbut *et al.* 2006). Ticks from the family Ixodidae make up most of the tick population that parasitise vertebrates (Rajbut *et al.* 2006) and cause serious problems worldwide due to their ability to transmit a variety of pathogenic microorganisms to both humans and animals (Pérez-Cogollo *et al.* 2010; Rodríguez-Vivas *et al.* 2014b; Matysiak *et al.* 2016, Rodríguez-Vivas *et al.* 2018). Large infestations of ticks on a host can cause drastic physical damage such as creating lesions that can cause secondary infestation, blood loss and drastic weight loss (Matysiak *et al*. 2016). The pathogens they transmit can cause even more damage to the host animals, as they frequently result in death accompanied by huge economic losses for both commercial and communal farming systems (Matysiak *et al*. 2016). Ticks and the pathogens that they are associated with, affect 80% of cattle populations around the world (Amritha *et al.* 2015).

In Africa alone there are 40 tick species able to affect the health of domestic animals such as cattle, goats, and horses (Matysiak *et al.* 2016). In South Africa, there are an estimated 11 to 14 tick species of veterinary importance (Walker e*t al*. 2003) and it has been estimated that losses in the livestock industry in South Africa, amounts to between R70 - R200 million per year due to tick damage and the pathogens they transmit (Budeli *et al.* 2009; Spickett *et al.* 2011).

Worldwide, producers struggle to control ticks and different methods for tick control have been developed. These methods include the selection of resistant cattle breeds, culling of susceptible

breeds and allowing a certain number of ticks on cattle to build up resistance (tick challenge) (Rajbut *et al.* 2006). Modifying the environments that cattle graze in by burning pastures (Trollope 2011), using biological control such as chickens or parasitoid wasps, as well as the use of acaricides are also useful tools in controlling tick populations (Rajbut *et al.* 2006).

The main control method used by producers boil down to chemical control where acaricides such as amidines, synthetic pyrethroids, arsenicals, organochlorides, carbamates, organophosphates, phenylpyrazoles, insect growth regulators, and macrocyclic lactones (MLs) are used (Pérez-Cogollo *et al.* 2010; Rodríguez-Vivas *et al.* 2014a; Rodríguez-Vivas *et al.* 2014b; Castro-Janer *et al.* 2015). Initially, each of these acaricides was adequate to control ticks, however, over time, tick resistance developed to these acaricides due to overuse and misuse practices (Rodríguez-Vivas *et al.* 2014a; Rodríguez-Vivas *et al.* 2014b).

Resistance is defined as the capacity of a specific parasite strain to endure despite being treated with a specific chemical control substance at a concentration where most of the normal population would have died. This resistance can also be transmitted to the rest of the population over time (Abbas *et al.* 2014). Acaricide resistance has been shown to be more evident in single-host ticks than multi-host ticks (Mekennon *et al*. 2002). According to Kunz & Kemp (1994) this is due to the fact that multi-host ticks spend most of the time off the host, they have longer life cycles, they change hosts in between their different life stages and they have wider host ranges. Thus, the development of resistance in multi-host ticks would be much slower than in single-host ticks. Single-host ticks spend three of the four life stages on one host and are more exposed to acaricidal treatment than multi-host ticks (Kunz & Kemp 1994; Mekonnen *et al.* 2002).

Although resistance development of blue tick strains has been reported for most acaricides currently used in South Africa (Mekonnen *et al.* 2002; Mekonnen *et al*. 2003; Ntondini *et al*. 2008; Lovis *et al.* 2013), resistance against MLs and growth regulators are not yet demonstrated. Serious breakdown in control of MLs has however been reported for the Asiatic blue tick, *Rhipicephalus (Boophilus) microplus* in Brazil, Mexico and Australia (Sabatini *et al* 2001; Klafke *et al* 2010). Methods to determine the extent of resistance development were tested and employed for use in these areas (Klafke *et al.* 2010).

Rhipicephalus (*Boophilus*) *microplus* and *R. (B.) decoloratus* are the two blue tick species found in South Africa and recently an increasing number of complaints of insufficient control of blue ticks by MLs from producers in the Eastern Cape Province were reported to agents of pharmaceutical companies. Although this can be due to incorrect treatment practices, the possibility of resistance development to MLs needs to be investigated.

1.1 Resistance development

Resistance can be experienced in different forms in the field, namely, as required resistance, cross-resistance and multiple-resistance. The following are descriptions of each form:

Acquired resistance results from a decrease in susceptibility to control measures such as drugs/chemicals over time, which can be passed from generation to generation. If many generations are continuously exposed to a certain drug dose, it will allow for the selection of a resistant mutant strain. This can then be transmitted to the rest of the population and passed on to the next generation over time (Abbas *et al.* 2014).

Cross-resistance refers to ticks that are resistant to different acaricides that have a similar mode of action. Different acaricides can, for instance, attach to the same target site within an invertebrate. This type of resistance usually involves acaricides that are closely related. A good example is resistance of *R. (B.) microplus* to organophosphates and carbamates, which are closely related. These acaricides both target acetylcholinesterase (AChE), an enzyme that is important in functioning of the nervous system of invertebrates. The decrease in the sensitivity of AChE to organophosphates and carbamates is of importance for resistance development. Another example includes cross-resistance between fipronil and cyclodienes (dieldrin and lindane), both block chloride ion channels controlled by gamma-aminobutyric acid (GABA) that occur in the central neurons of the nervous system of arthropods (Abbas *et al.* 2014; Castro-Janer *et al.* 2015).

Multiple resistance refers to the development of tick resistance to many acaricides, regardless of different modes of action. This is mainly due to intensive use and misuse of acaricides. Cattle trading can also introduce resistant tick strains to other populations and lead to the spread and development of resistance to acaricides. An example of multiple resistance involves *R (B.) microplus* in Mexico and in Brazil that have been shown to have developed multiple resistance to different acaricide such as organophosphates, synthetic pyrethroids, chlorinated

hydrocarbons and formamidines (amitraz) (Pérez-Cogollo *et al*. 2010; Rodríguez-Vivas *et al.* 2014a; Rodríguez-Vivas *et al.*2014b). It was found that the most common mechanisms for resistance in Mexico was due to a mutation of the target site, inherited from generation to generation to expand to the whole population over time (Abbas *et al.* 2014).

When assessing resistance, one must take the following factors into account: the number of genes that are involved, the occurrences of resistant genes, dominance of resistant alleles, genetic diversity of the tick population, the overall fitness of resistant organisms and chances for genetic recombination (Abbas *et al.* 2014). In many cases, genes responsible for establishing resistance occur at very low levels in tick populations before the introduction of a new acaricide. This can also explain ticks that are resistant to multiple acaricides without prior exposure and regardless of the mode of action. The overuse of acaricides can also increase the chances of more resistant alleles occurring and can thus be strengthened in the next generation (Abbas *et al.* 2014).

The length of time that a resistant allele takes to establish as well as the rate in which the ticks break down the chemicals depend on five essential factors. Firstly, the number of occurrences of the initial mutation in the population before the use of treatment. Secondly, the dominance of the allele, which can either be dominant, recessive or it may be co-dominant with another gene. Thirdly, the frequency in which the acaricide is used. Fourthly, the concentration that was used to treat the tick population. Lastly, the number of members of the population that were not exposed to the treatment (Abbas *et al.* 2014).

1.2 Blue tick species

1.2.1 African blue tick (*Rhipicephalus* (*Boophilus*) *decoloratus*) (Koch, 1844)

The African blue tick belongs to the kingdom Animalia, phylum Arthropoda, class, Arachnida, subclass Acari, superorder Parasitiformes, order Ixodida, family Ixodidae, genus *Rhipicephalus*, subgenus *Boophilus* and species *decoloratus*. The African blue tick is a one-host tick that is endemic to and the most widely spread Ixodid tick species in Africa (Fig. 1) (Walker *et al*. 2003; Tønneson *et al*. 2004). These ticks can be found in areas with savannas and temperate climates, grasslands and wooded areas where cattle or suitable hosts are present (Walker *et al.* 2003; Zeman & Lynen 2010). They are usually absent in dry areas or areas with less vegetation cover. The African blue tick mostly feeds on cattle; however, they also feed on

other animals such as wild ungulates, sheep, goats, donkeys and horses. The feeding sites of these ticks are usually on the upper legs, back, belly, shoulders and dewlap of cattle (Walker *et al.* 2003).

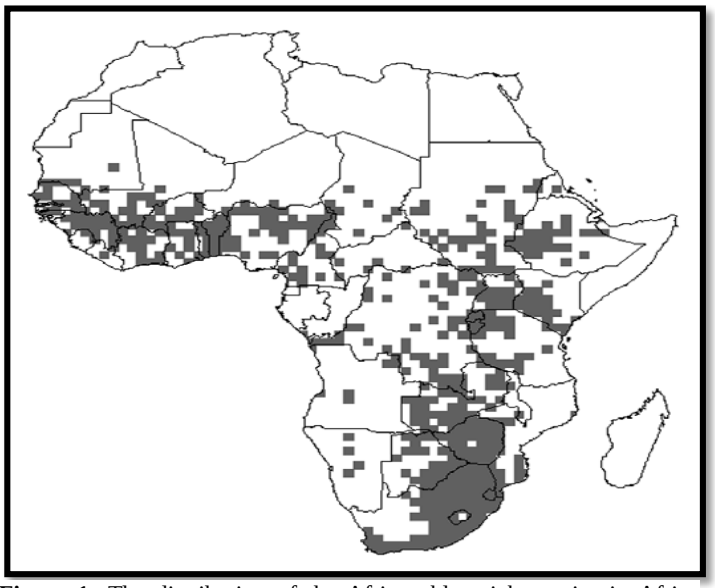


Figure 1: The distribution of the African blue tick species in Africa (Walker *et al.* 2003).

These one-host ticks complete their whole parasitic life cycle on one host. The engorged female can lay 1 000 to 2 500 eggs within a week to 14 days after dropping from the host, with egg hatch occurring within the next four weeks. The larvae then climb up onto the surrounding vegetation and wait until a host comes in close proximity to attach to it. Once the larvae have grabbed on to the host, they search for a suitable feeding site, feed until they are fully engorged, and then moult into a nymph on the host. The nymphs feed on the same host until fully engorged and then moult to an adult tick. The adults will feed until partially engorge and after mating, the female continues feeding until fully engorged. The engorged female tick then drops off the host and seeks a sheltered environment to lay eggs. This whole process takes up to two months to complete (Walker *et al.* 2003).

The African blue tick is a vector of the *Babesia bigemina* pathogen that causes African red water in cattle, which leads to severe fever and drastic weight loss (Walker *et al.* 2003; Tønneson *et al.* 2004; de Clercq *et al.* 2012). These ticks can also transmit other pathogens such as *Anaplasma marginale* which causes gall sickness, and *Borrelia theileri* which causes spirochaetosis in cattle, goats, horses and sheep (Walker *et al.* 2003; Tønneson *et al.* 2004; de Clercq *et al.* 2012). It can furthermore cause direct damage to cattle hides, a decrease in the quality and quantity of milk and meat as well as significant weight loss and prevention of weight gain. Blood loss, malnutrition, general stress,

irritation and death are also caused by this tick species (Walker *et al.* 2003; Tønneson *et al.* 2004; de Clercq *et al.* 2012).

1.2.2 Asiatic blue tick (*Rhipicephalis* (*Boophilus*) *microplus*) (Canestrini, 1888)

The Asiatic blue tick (*R.* (*B.*) *microplus*) is also a one-host tick originally from South-East Asia. These ticks were then spread to other parts of the world such as South America, Central America, North America, Australia, Madagascar and South Africa through cattle trading (Madder *et al*. 2011). This species occurs in savanna climate areas and wooded grasslands where cattle normally graze. In South Africa, these ticks were recorded in the Western Cape, Eastern Cape and KwaZulu-Natal Provinces (Fig. 2) (Walker *et al.* 2003). They feed primarily on cattle, however, they have been found to infest other livestock or wildlife in the absence of cattle (Tonetti *et al.* 2009; Horak *et al*. 2015; Matysiak *et al*. 2016). When they feed on cattle, their feeding sites are normally on the belly, shoulders, the sides and the dewlap (Walker *et al.* 2003).

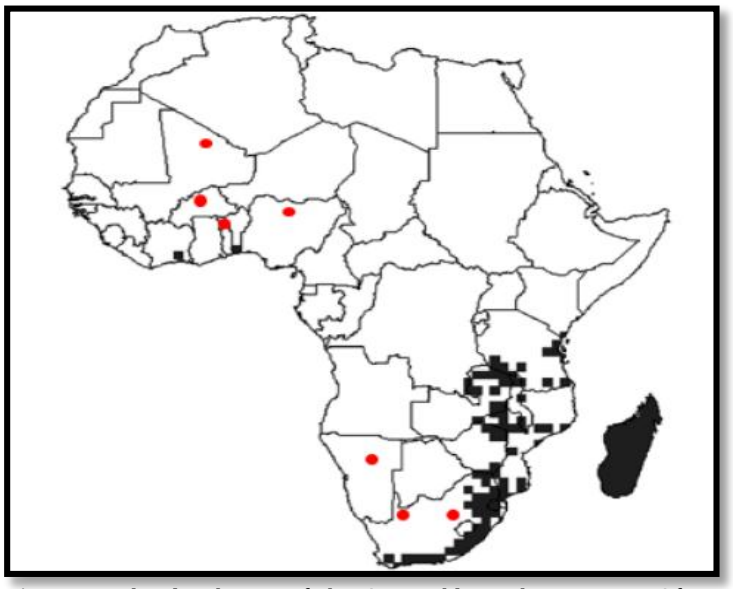


Figure 4: The distribution of the Asiatic blue tick species in Africa (Nyangiwe *et al.* 2017).

Rhipicephalus (*B.) decoloratus* and *R. (B.) microplus* look very similar to each other in terms of shape, form and colour and they can even occur in the same areas as is found in the Eastern Cape Province (Walker *et al.* 2003). To differentiate between the two species, one has to examine the mouth-parts, specifically the tube-like hypostome. The hypostome have teeth-like structures used to anchor the tick to the skin of the host. *Rhipicephalus (B.) decoloratus* has

two pairs of three vertical rows of teeth on the hypostome, whereas *R. (B.) microplus* has two pairs of four vertical rows of teeth on each hypostome (Fig. 3). Displacement of the African blue tick by the Asiatic blue tick was reported in different areas in South Africa (Tønneson *et al*. 2004, Nyangiwe *et al.* 2013). This is attributed partly to a somewhat shorter reproductive cycle of the Asiatic blue tick compared to the African blue tick as well as the fact that Asiatic blue tick females may produce up to 500 more eggs in a shorter period compared to African blue tick females (Walker *et al.* 2003).

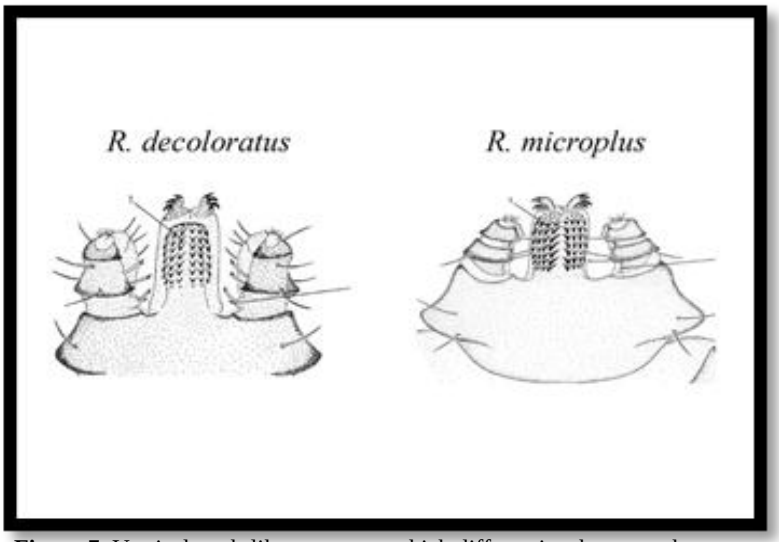


Figure 7: Vertical teeth-like structures which differentiate between the two Blue tick species (Walker *et al.* 2003).

Rhipicephalis (*Boophilus*) *microplus* is a major economic threat to the cattle industry in tropical, subtropical and temperate regions of the world. They are vectors for both *B. bovis* and *B. bigemina* causing bovine babesiosis in cattle, with *B. bovis* leading to a more severe type of red water causing death much quicker (Walker *et al.* 2003; Zeman & Lynen 2010; Madder *et al.* 2011; de Clercq *et al.* 2012). *Babesia bovis* is attained by adult ticks and transmitted transovarially by the larvae. Just like the African blue tick, this species can also transmit *A. marginale* and *B. theileri* (Zeman & Lynen 2010; de Clercq *et al.* 2012). High infestations can cause direct damage to the animals hides through feeding sites on the skin, which makes it vulnerable to secondary infections that can also induce blood loss (Zeman & Lynen 2010). According to studies done in Australia, for each *R. (B.) microplus* female that fully engorges, there will be a loss of about 0.6 grams of potential weight gain of the host animal (Walker *et al.* 2003; Matysiak *et al*. 2016).

1.3. Acaricide control and resistance development

1.3.1 Acaricide control history of *R. (B.) microplus* globally

The first acaricide used to control the Asiatic blue tick was a deep immersion version of an arsenic compound developed in 1896. It was initially used in Australia and later adopted for use by countries such as South Africa, some parts of North America and South America. Due to its short persistence on the animals, it had to be applied very frequently. The first report of resistance development of the Asiatic blue tick to arsenic was in 1939, about 40 years after its initial use (Yessinou *et al*. 2016).

Organochlorines were then introduced for tick control in 1939 (Yessinou *et al*. 2016). Organochlorines had a longer persistency, were more efficient, had a broader range of action than arsenic compounds and was cheaper and less harmful than arsenic compounds. These acaricides involve the micro-toxin binding to the gamma aminobutyric acid (GABA) chloride ionophore (CI) complex and inhibits CI−flux into the nervous system. The inhibition of the GABA functioning in the neurons, leads to hyper-excitation resulting in tick death (Abbas *et al.* 2014). By 1952, it was discovered that the Asiatic blue tick in Brazil developed resistance to organochlorides. A total ban of organochloride use followed in 1962, due to persistent residues in the milk and meat of the animals treated. The product was later banned as it was not biodegradable and negatively affected the environment (Yessinou *et al*. 2016).

Organophosphates (OPs) were consequently introduced in the mid-1950s for tick control (Yessinou *et al*. 2016). OPs were again less persistent, so more frequent use was necessary. OPs inhibit the functioning of AChE by preventing AChE from breaking down acetylcholine at the post-synaptic membrane. This build-up of acetylcholine then results in neuromuscular paralysis and even death (Abbas *et al.* 2014). The first report of resistance to these acaricides was in the early 1960's (Yessinou *et al*. 2016). Since then, ticks and mites have shown resistance to over 30 OPs in 40 countries (Yessinou *et al*. 2016) and resistance also spread over different continents. In the mid-1960s, organophosphate-resistant *R. (B.) microplus* strains were also found in Australia (Rodríguez-Vivas *et al.* 2014a; Yessinou *et al*. 2016). The common cause for resistance involved target-site susceptibility in ticks. Geneticists have found many point mutations that are involved in the development of resistance to organophosphates, especially by the Asiatic blue tick (Abbas *et al.* 2014; Rodríguez-Vivas *et al.* 2014a).

In 1970, amidines were introduced. The main active ingredient of amidines is amitraz which is widely used around the world to control ticks (Rodríguez-Vivas *et al*. 2014a; Yessinou *et al*. 2016). Amitraz targets octopamine receptors in ticks which causes neural hyper-excitability and even death. The process involves the overstimulation of the octopamine synapses in the central nervous system leading to tremors and spasms in immature and adult stages. Amitraz can also affect egg-laying, feeding behaviours, as well as the elevation of excitatory state of the ticks (Abbas *et al* 2014). Four to 10 years after its first use to control ticks, it was discovered that Asiatic blue tick strains developed resistance to amitraz in Australia by1980 (Yessinou *et al*. 2016). Resistance development was found to be due to altered target sites; however, they do not have much information about the precise mechanisms of this type of resistance (Abbas *et al.* 2014; Rodríguez-Vivas *et al.* 2014a). Amitraz is still used today to control ticks on cattle in many parts of the world, especially in countries such as Australia, South America and Southern Africa (Rodríguez-Vivas *et. al*. 2014a; Yessinou *et al*. 2016).

Pyrethroids were introduced in 1977 (de Oliveira *et al.* 2012). Pyrethroids pose a powerful neurotoxin to arthropods that targets the sodium ion channels causing it to stay open by preventing their deactivation and stabilisation. This results in nerve excitation due to the changes in the nerve membrane absorbency to sodium and potassium ions (Abbas *et al*. 2014) in the muscles, nerve and other excitable cells. Two groups of pyrethroids can be distinguished based on chemical structure, the poisoning symptoms, persistence, as well the effects to the nerve preparations of the invertebrates. These groups are called type I and type II pyrethroids. What makes these groups unique is that type I can cause multiple discharges as a reaction to a single stimulus, while type II can lead to the depolarisation of the membrane. Examples of Synthetic Pyrethroids that have been widely used worldwide to control ticks are cypermethrin, cyhalothrin and deltamethrin (Abbas *et al*. 2014; Rodríguez-Vivas1 e*t al.* 2014a).

Synthetic pyrethroids are more stable versions of the naturally occurring compounds specifically made to stay active for a longer period to kill more ticks compared to natural pyrethroids (Abbas *et al*. 2014). A little over 10 years after their first use, *R. (B.) microplus* strains in Mexico and Brazil were found to have developed resistance to pyrethroids (Guerrero *et al*. 2012; Higa *et al.* 2015).

Macrocyclic Lactones (MLs), introduced in 1979, were initially developed to control endoparasites such as nematodes. These MLs are anti-parasitic drugs that have a wide range of

activity (Borges *et al*. 2008; Pohl *et al*. 2011; Fernández-Salas *et al*. 2012; Lopes *et al*. 2013; Lopes *et al.* 2014). Due to their high affinity for adipose tissue, MLs are able to persist in animals for long periods of time (Pohl *et al*. 2011; Lopes *et al.* 2013). MLs are absorbed by the animals at low concentrations, or concentrations that are not harmful to the animals. MLs differ from conventional acaricides in that it can be applied to the host via an injectable route, causing the ticks to ingest the drug through the blood meal versus a pour on where the skin of the ticks is exposed to the chemical (Rodríguez-Vivas *et al.* 2018). MLs can be divided into two groups, avermectins and milbemycins (Pohl *et al.* 2011; Fernandez-Salas *et al.* 2012). Avermectins include ivermectin, abamectin, doramectin, selamectin and eprinomectin (Pohl *et al*. 2011; Fernández-Salas *et al.* 2012). These avermectins are products of the fermented soil bacterium *Streptomyces avermitilis*, whereas milbemycins are fermented products of *S. cyaneogriseus* and *S. hygroscopicus* (Pohl *et al*. 2011; Fernández-Salas *et al.* 2012). In 1981, it was discovered that MLs is also effective in the control of both blue tick species and therefore ivermectin (IVM), abamectins, doramectins and moxidectins are currently used by producers to control *R. (B.) microplus*, *R. (B.) decoloratus,* mites and endoparasitic nematodes (Pohl *et al*. 2011; Feránndez-Salas *et al.* 2012; Abbas *et al* 2014). Avermectins are used more often than milbemycins as they seem to be more reliable for control especially against blue ticks (Pohl *et al*. 2011).

Ivermectins, have a particularly high affinity to glutamate and gamma-aminobutyric acid receptors which control chloride ion channels found in the muscle and nerve cells of invertebrates (Klafke *et al*. 2006; Fernández-Salas *et al.* 2012; Geary & Moreno 2012). IVM can thus activate glutamate-gated chloride ion channels which will lead to peripheral motor function paralysis and death (Klafke *et al*. 2010; Fernández-Salas *et al.* 2012). Since the start of its use for tick control in 1981, it seemed to have become less effective partly due to their frequent use per year, as well as the lack of early detection of resistant individuals. The under- or overdose of IVM is also a problem as producers do not administer the treatment according to recommended dosage based on weight and size (Pérez-Cogollo *et al.* 2010; Abbas *et al.* 2014). Another factor that can play a part in the development of resistance is the management of acaricide application to complement these drugs with other acaricides. Producers might be using two different acaricides with a similar modes of action as in the case of MLs and Growth regulators which block GABA-gated chloride channels and glutamate-gated chloride (Castro-Janer *et al.* 2011; Rodríguez-Vivas *et al*. 2018), respectively. As a result, resistance to these

drugs developed in *R. (B.) microplus* in Brazil, Mexico, Uruguay and Australia (Pérez-Cogollo *et al*. 2010; Klafke *et al*. 2010; Fernández-Salas *et al.* 2012; Rodríguez-Vivas *et al*. 2018).

The first detection of *R. (B.) microplus* resistance to MLs was reported in 2001 in Rio Grande do Sul, southern Brazil (Klafke *et al.* 2010; Abbas *et al* 2014). Initial assessments at field conditions revealed a population of the cattle-tick (Sa˜o Gabriel strain) was not successfully controlled after treating with MLs such as doramectin. Using the Adult Immersion Test (AIT), the resistant strain was isolated according to the methodology used by Sabatini *et al*. (2001). It was found that ticks of the Sa˜o Gabriel strain were able to endure and yield viable eggs after using the immersion treatment with 200 to 1000 ppm of ivermectin, while the susceptible strain (Porto Alegre) showed a 100% mortality with those same concentrations. The AIT was found to showed too many variations between the tests and have not been used for many years. The other problem was that using adults for resistance testing was already difficult due to stronger immunity development with age, preventing reliable results in terms of treatment efficacy. A more reliable test called the Shaw Larval Immersion Test (SLIT), originally developed by Shaw (1966), was then used to test larvae for resistance (Klafke *et al.* 2006).

In 2004, Doramectin, Ivermectin and Moxidectin were tested for resistance. The findings show that these products were less effective at controlling the Sa˜o Gabriel strain. Experiments were conducted in the eastern part of the state of Sao Paulo in the Vale do Para´ıba region, where *R. (B.) microplus* is widespread with severe acaricide resistance problems (Klafke *et al.* 2010). This research led to the first *in vitro* detection of an ivermectin-resistant population of *R. (B.) microplus* by using the SLIT technique (Sabatini *et al*. 2001; Klafke *et al.* 2010). This study successfully distinguished the Brazilian susceptible reference strain (Porto Alegre) from the population suspected of resistance (Barra Alegre) (Klafke *et al.* 2010). The Barra Alegre population was acquired from a property in the municipality of Piquete-SP which had been using ivermectin for at least 10 years for tick control and was shown to have a factor of resistance of 3.78 to ivermectin when compared to a susceptible laboratory-reared strain (Porto Alegre) (Klafke *et al.* 2010).

Other acaricides that are currently in use for tick control are Fipronil and Fluazuron. Fipronil, a product of phenylpyrazoles (PHPZ-broad range insecticides) was introduced in the mid-1990s (Yessinou *et al*. 2016). Following pour-on application, the acaricide is effective for up to five weeks. The efficacy of Fipronil is much longer than MLs (Lopes *et al*. 2014). Its mode of action involves inhibiting the activation of GABA on the pre- and post-synaptic channel in

the neurons of the central nervous system which results in the blockage of the chloride channels and leads to the death of ticks (Castro-Janer *et al*. 2010; Lopes *et al*. 2014). In 2007, it was first reported that some *R. (B.) microplus* strains had developed resistance to fipronil in Brazil and Uruguay (Castro-Janer *et al*. 2010).

Fluazuron, also known as a growth regulator, was introduced in 1994 in Australia (Yessinou *et al*. 2016). There haven't been many reports of resistance to these growth regulators, yet. Fluazuron does not directly kill the ticks, but instead inhibits the process of ecdysis, by affecting the metabolism of chitin or by inhibiting the production of the hormones involved in ecdysis. This, in turn, inhibits development and growth of the ticks (de Oliveira *et al.* 2014). Situations where resistance development was suspected, turned out to be more a case of under-dosing rather than resistance problems (de Oliveira *et al*. 2012; Yessinou *et al*. 2016). So far these growth regulators look the most promising and one of the few acaricides that are still working well around the world (de Oliveira *et al.* 2012).

1.3.2 Acaricide control history of *R. (B.) decoloratus* in South Africa

The earliest reports of African blue tick resistance to acaricides in South Africa were in 1940 in the East London area (Du Toit *et al*. 1941) where sodium arsenate dipping solutions were commonly used to control ticks. Gamma benzene hexachloride (BHC) was later introduced and initially proved to be rather effective. However, after 18 months of use, a breakdown of control was detected. The first signs of BHC-resistance was reported in the East London area where the same blue tick strains developed resistance against arsenite a few years earlier. At this stage, the AIT was used to indicate the resistance of these tick species to both BHC and arsenite. Eighteen months after the first use of BHC, resistance development of the African blue tick against BHC was also reported around the Pretoria area, these strains had not shown any previous resistance to arsenic control (Whitehead 1973). In the areas where the African blue ticks showed resistance to BHC and arsenic, Dichloro-diphenyl-trichloro-ethane (DDT) (Organochlorines) was then used and perceived to be effective for over five years. However, laboratory tests using DDT to test control efficacy showed it to be effective against larvae, but not against adult *R. (B.) decoloratus* (Whitehead 1956).

In terms of OPs, SPs and Amitraz, research articles do not describe when these chemicals were first introduced to South Africa, so it is assumed they were introduced roughly the same time

globally. OPs were introduced in South Africa in the mid-1950s (Yessinou *et al*. 2016). Shaw *et al.* (1967) discovered that a specific strain of *R (B.) decoloratus*, the Berlin strain, developed resistance to OPs in some parts of the Eastern Cape Province. The Berlin strain was also found in some parts of KwaZulu-Natal and the Free State with emergence of resistance to OPs in the latter (Baker *et al*. 1978; Fourie *et al.* 2013). It is presumed that amidines were introduced to South Africa around the 1970s (Yessinou *et al*. 2016). Twenty-five years after amidines were first used in South Africa (1995), it was discovered that strains of *R. (B.) decoloratus* that had developed resistance in the East London area (Fourie *et al.* 2013). After SPs were introduced during the late 1970's (Yessinou *et al*. 2016), Coetzee *et al*. 1987 applied Shaw Larvel Immersion Test (SLIT) to tests for resistance develoment in KwaZulu-Natal. The authors confirmed that the particular strain of the African blue tick, namely the Braemar strain, did indeed develop resistance to SPs in the province (Coetzee *et al.* 1987; Fourie *at el.* 2013).

1.4 Rationale for this study

In South Africa, currently, acaricides that are most commonly used are OPs, SPs and amitraz. Resistance development of both blue tick species has been found against these chemicals in some parts of South Africa, such as in the Eastern Cape areas (Mekonnen *et al.* 2002; Ntondini *et al*. 2008). One of the more recent acaricides that has been developed to control blue ticks is MLs. Tick resistance development against MLs by the *R. (B.) microplus* has been reported in Brazil, some parts of North America and Australia (Rodríguez-Vivas *et al*. 2018). In some parts of South Africa such as the Eastern Cape, there has been an increase in the use of MLs such as Ivermectin. Agents from pharmaceutical companies recently started getting increasing number of complaints of insufficient control of blue ticks by Ivermectin from producers in the Eastern Cape. This can be due to the incorrect treatment or frequent exposure to MLs. To investigate this problem, a method to determine blue tick resistance against MLs is needed for South African strains to either confirm or deny this suspicion of chemical resistance.

Known methodologies used to detect resistance first needed to be tested for South African conditions and tick species before it can be used for local resistance detection. Different variations of methodologies are used globally to detect resistance development, however, a standardised method to use for testing of South African strains has not yet been established.

Previous studies have investigated on *R. (B.) microplus* for MLs resistance in South Africa (Lovis *et al.* 2013), but no research has been done on the African blue tick, *R. (B.) decoloratus*, and the potential MLs resistance development (Rodríquez-Vivas *et al.* 2018).

This study aimed to compare methodologies used for the detection of MLs resistance in Brazil and Australia for *R. (B.) microplus* populations, for efficacy in detecting resistance development of both *R. (B.) microplus* and *R. (B.) decoloratus* in South Africa. This also included determining the Lethal Concentrations/dosages at 50- and 99 % of MLs for both *R. (B.) microplus* and *R. (B.) decoloratus* reference strains in South Africa.

1.5 Objectives

1. To compare methodologies used for the detection of MLs resistance of *R. (B.) microplus* populations in Brazil and Australia for efficacy in detecting resistance development of *R. (B.) decoloratus* in South Africa.

2. To determine the lethal dosages at 50% and 99% for both *R. (B).microplus* and *R. (B.) decoloratus* in South Africa.

3. To investigate the perceived resistance of blue ticks to Ivermectin that has been experienced by some of the producers in the Eastern Cape Province by using the applicable test determined in objective 1.

4. To compare results obtained in objective 3 with results from farms where Ivermectin is not used for tick control.

1.6 References

ABBAS, R.Z., ZAMAN, M.A., COLWELL, D.D., GILLEARD, J. & RAJBUT, ZI. 2014. Acaricide resistance in cattle ticks and approaches to its management: The state of play. *Veterinary Parasitology* **203**: 6-20.

AMRITHA, A., DEEPA, A.V., SHALINI, K., BHARADWAJ, T., RAVINDRAN, R., PULIKKAN, J., SHAMJAA, U., RUMESH, K.N. & GRACE, T. 2015. Nuances of transcriptomics in understanding acaricide resistance in ticks. *Journal of Agricultural and Veterinary Science* **8**: 15-21.

BAKER, J.A.F., MILES, J.O., ROBERTSON, W.D., STANDFORD, G.D. & TAYLOR, R.J. 1978. The current status of resistance to organophosphorus ixodicides by the blue tick, *Boophilus decoloratus* (Koch) in the Republic of South Africa and Transkei. *Journal of the South African Veterinary Association* **49**: 327-333.

BORGES, F.A., SILVA, H.C., BUZZULINI, C., SOARES, V.E., SANTOS, E. OLIVEIRA, G.P. & COSTA, A.J. 2008. Endectocide activity of new long-action formulation containing 2.25% ivermectin, + 1,25 % abamectin in cattle. *Veterinary Parasitology* **155**: 299-307.

BUDELI, M.A., NEPHAWE, K.A., NORRIS, D., SELAPA, N.W., BERGH, L. & MAIWASHE, A. 2009. Genetic parameters estimates for tick resistance in Binsmara cattle. *South African Journal of Animal Science* **39**: 321-327.

CASTRO-JANER, E., KLAFKE, G.M., CAPURRO, M.L. & SCHUMAKER, T.T.S. 2015. Cross-resistance between fipronil and lindane in *Rhipicephalus* (*Boophilus*) *microplus. Veterinary Parasitology* **210**: 77-83.

CASTRO-JANER, E., MARTINS, J.R., MENDES, M.C., NAMINDOME, A.B, KLAFKE, G.M. & SCHUMAKER, T.T.S 2010. Diagnoses of fipronil resistance in Brazilian cattle ticks (*Rhipicephalus* (*Boophilus*) *microplus*) using in vitro larval bioassays. *Veterinary Parasitology* **173**: 300-306.

CASTRO-JANER, E., RIFRAN, L., GONZĀLEZ, P., NEILL, C., PIAGGIO, J., GIL, A. & SCHUMAKER, T.T.S. 2011. Determination of the susceptibility of *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae) to ivermectin and fipronil by larval immersion test (LIT) in Uruguay. *Veterinary Parasitology* **178**: 148-155.

COETZEE, B.B., STANDFORD, G.D, & DAVIS, D.A.T. 1987. Resistance by the blue tick (*Boophilus decoloratus*) to the synthetic pyrethroid, fenvalerate. *Onderstepoort Journal of Veterinary Research* **54**: 83-86.

DE CLERCQ, E.M., VANWAMBEKE, S.O., SUNGIRAI, M., ADEHAN, S., LOKOSSOU, R. & MADDER, M. 2012. Geographic distribution of invasive cattle tick *Rhipicephalus microplus*, a country-wide survey in Benin. *Experimental and Applied Acarology* **58**: 441-452.

HIGA, L.O.S., GARCIA, M.V., BARROS, J.C., KOLLER, W.W. & ANDREOTTI, R. 2015. Acaricide resistance status of the *Rhipicephalus microplus* in Brazil: a literature overview. *Medical Chemistry* **5**: 326-333.

DE OLIVEIRA, P.R., CALLIGARIS, I.B., NUNES, P.H., BECHARA, G.H. & CAMARGO-MATHIAS, M.I. 2014. Fluazuron-induced morphological changes in *Rhipicephalus sanguineus* Latreille, 1806 (Acari: Ixodidae) nymphs: An ultra-structural evaluation of the cuticle formation and digestive processes. *Acta Tropica* **133**: 45-55.

DE OLIVEIRA, P.R., CALLIGARIS, I.B., ROMA, G.C., BECHARA, G.H., PIZANO, M.A. & MATHIAS, M.I.C. 2012. Potential of the insect growth regulator, fluazuron, in the control of *Rhipicephalus sanguineus* nymphs (Latreille, 1806) (Acari: Ixodidae): Determination of the LD95 and LD50. *Experimental Parasitology* **121**: 35-39.

DU TOIT, R., GRAF, H. & BEKKER, P.M. 1941. Resistance to arsenic as displayed by the single host blue tick *Boophilus decoloratus* (Koch) in a localised area of the Union of South Africa: Preliminary report. *Student American Veterinary Medical Association* **12**: 50-58.

FERNĀNDEZ-SALAS, A., RODRÍQUEZ-VIVAS, R.I., ALANSO-DIAZ, A.A. & BASURTO-CAMBERAS, H. 2012. Ivermectin resistance status and factors associated in *Rhipicephalus microplus* (Acari: Ixodidae) populations from Veracruz, Mexico. *Veterinary Parasitology* **190**: 210-215.

FOURIE, J.J, LIEBENBERG, J.E., NYANGIWE, N., AUSTIN, C., HORAK, I.G. & BHUSHAN, C. 2013. The Effects of a pour-on formulation of fluazuron 2.5 % and flumethrin 1 % on populations of *Rhipicephalus decoloratus* and *Rhipicephalus microplus* both on and off bovine (Bonsmara breed) hosts. *Parasitology Research* **112**: 67-79.

GEARY, I.G. & MORENO, Y. 2012. Macrocylytic lactones anthelmintics: spectrum of activity and mechanism of action. *Current Pharmaceutical Biotechnology* **13**: 866-872.

GUERRERO, F.D., LOUIS, L. & MARTINS, J.R. 2012. Acaricide resistance mechanisms in *Rhipicephalus (Boophilus*) *microplus*. *The Brazillian Journal of Veterinary Par*asitology **21**: 1-6.

HORAK, I.G., JORDAAN, A.J., VAN HEERDEN, J., HEYNE, H. & VAN DALEN, E.M. 2015. Distribution of endemic and introduced tick species in Free State province, South Africa. *Journal of South African Veterinary Association* **86**: a1255.

KLAFKE, G.M., DE ALBUQUERQUE, T.A., MILLER, R.J., SCHUMAKER, T.T.S. 2010. Selection of ivermectin-resistant strain of *Rhipicephalus microplus* (Acari: Ixodidae) in Brazil. *Veterinary Parasitology* **168**: 97-104.

KLAFKE, G.M., SABATINI, G., DE ALBUQUERQUE, T.A., MARTINS, J.R., KEMP, D.H., MILLER, R.J. & SCHUMAKER, T.T.S. 2006. Larval Immersion Tests with ivermectin in populations of the cattle tick *Rhipicephalus* (*Boophilus*) *microplus* (Acari: Ixodidae) from State of Sao Paulo, Brazil. *Veterinary Parasitology* **142**: 386-390.

KUNZ, S.E. & KEMP, D.H. 1994. Insecticides and acaricides: resistance and environmental impact. *Revue Scientifique Et Technique (International Office of Epizootics)* **13**: 1249-1286.

LOPES, W.D.Z., CRUZ, B.C., TEIXEIRA, W.F.P., FELIPPELLI, G., MACIEL, W.G., BUZZULINI, G., GOMES, L.V.C., FAVERO, F., SOARES, V.E., BICHUETTE, M.A., DE OLIVEIRA, G.P. & DA COSTA, A.J. 2014. Efficacy of fipronil (1.0 mg/kg) against *Rhipicephalus* (*Boophilus*) *microplus* strains resistant to ivermectin (0.63 mg/kg). *Preventative Veterinary Medicine* **115**: 88-93.

LOPES, W.D.Z., TEIXEIRA, W.F., DE MATES, L.V., FELIPELLI, G., CRUZ, B.C., MACIEL, W.G., BUZZULINI, C. FAVERO, F.C., SOARES, V.E., DE OLIVEIRA, G.P., DA COSTA, A.J. 2013. Effects of macrocyclic lactones on the reproductive parameters of engorged *Rhipicephalus* (*Boophilus*) *microplus* females detached from experimentally infested cattle. *Experimental Parasitology* **135**: 72-78.

LOVIS, L., REGGI, J., BERGGOETZ, M., BETSCHART, B., & SAGER, H. 2013. Determination of acaricide resistance in *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae) field populations of Argentina, South Africa, and Australia with the larval tarsal test. *Journal of Medical Entomology* **50**: 326-334.

MADDER, M., THYS, E., ACHI, L., TOURÉ, A. & DE DEKEN, R. 2011. *Rhipicephalus* (*Boophilus*) *microplus*: a most successful invasive tick species in West-Africa. *Experimental and Applied Acarology* **53**:139-145.

MATYSIAK, A., DUBKO, P., DUDEK, K.J, JUNKUSZEW, A. & TRYJANOWSKI, P. 2016. The occurrence of pathogens in *Rhipicephalus microplus* ticks from cattle in Madagascar. *Veterinarni Medicina* **61**: 516-523.

MEKONNEN, S., BRYSON, N.R., FOURIE, L.J., PETER, R.J., SPICKETT, A.M., TAYLOR, R.J., STRYDOM, T. & HORAK, I.G. 2002. Acaricide resistance profiles of single- and multi- host ticks from communal and commercial farming areas in the Eastern Cape and North-West Provinces of South Africa. *Onderstepoort Journal of Veterinary Research* **69**: 99-105.

MEKONNEN, S., BRYSON, N.R., FOURIE, L.J., PETER, R.J., SPICKETT, A.M., TAYLOR, R.J., STRYDOM, T., KEMP, D.H. & HORAK, I.G. 2003. Comparison of 3 tests to detect acaricide resistance in *Boophilus decoloratus* on dairy farms in the Eastern Cape Province, South Africa. *Journal of the South African Veterinary Association* **74**: 41-44.

NYANGIWE, N., HARRISON, A. & HORAK, I.G. 2013. Displacement of *Rhipicephalus decoloratus* by *Rhipicephalus microplus* (Acari: Ixodidae) in the Eastern Cape Province, South Africa. *Experimental and Applied Acarology* **61**: 371-382.

NYANGIWE, N., YAWO, M. & MUCHENJE, V. 2017. Driving forces for changes in geographic range of cattle ticks (Acari: Ixodidae) in Africa: A review. *South African Journal of Animal Science* **48**: 829-841.

NTONDINI, Z., VAN DALEN, E.M.S.P. & HORAK, I.G. 2008. The extent of acaricide resistance in 1-, 2- and 3-host ticks on communally grazed cattle in the eastern region of the Eastern Cape Province, South Africa. *Journal of the South African Veterinary Association* **79**: 130-135.

PÉREZ-COGOLLO, L.C., RODRÍGUEZ-VIVAS, R.I., RAMIREZ-CRUZ, G.I. & MILLER, R.J. 2010. First report of the cattle tick, *Rhipicephalus microplus* resistant to ivermectin in Mexico. *Veterniary Parasitology* **168**: 165-169.

POHL, P.C., KLAFKE, G.M., CARVELHO, D.D., MARTINS, J.R., DAFFRE, S., DA SILVA VAS, I. & MASUDA, A. 2011. ABC transporter efflux pumps: defense mechanisms against ivermectin in *Rhipicephalus* (*Boophilus*) *microplus. International Journal for Parasitology* **41**: 1323-1333

RAJBUT, I.Z., HUA, S.H., CHEN, W.J., ARIJO, A.G. & XIQO, C.W. 2006. Importance of ticks and their chemical and immunological control in livestock. *Journal of Zhejiang University Science B* **7**: 912-921.

RODRÍGUEZ-VIVAS, R.I., JONSSON, N.N. & BHUSHAN, C. 2018. Strategies for the control of *Rhipicephalus microplus* ticks in a world of conventional acaricide and macrocyclic lactone resistance. *Parasitology Research* **117**: 3-19.

RODRÍGUEZ-VIVAS, R.I, PÉREZ-COGOLLO, L.C., ROSADO-AGUILAR, J.A., OJEDA-CHI, M.M., TRINIDAD-MARTINEZ, I.C., MILLER, R.J., LI, A.Y., DE LEÓN, A.P., GUERRERO, F. & KLAFKE, G. 2014a. *Rhipicephalus* (*Boophilus*) *microplus* resistant to acaricides and ivermectin in cattle farms of Mexico. *Brazillian Journal of Veterinary Parasitology* **23**: 113-122.

RODRÍGUEZ-VIVAS, R.I. MILLER, R.J., OJEDA-CHI, M.M., ROSADO-AGUILAR, J.A., TRINIDAD-MARTINEZ, I.C. & DE LEÓN, A.P. 2014b. Acaricide and ivermectin resistance in a field population of *Rhipicephalus microplus* (Acari: Ixodidae) collected from red deer (*Cervus elaphus*) in Mexican tropics. *Veterinary Parasitology* **200**: 179-188.

SABATINI, G.A., KEMP, D.H., HUGHES, S., NARI, A. & HANSEN, J. 2001. Tests to determine LC50 and discriminating doses for macrocyclic lactones against the cattle tick, *Boophilus microplus*. *Veterinary Parasitology* **95**: 53-62.

SHAW, R.D. 1966. Culture of an organophosphorous-resistant strain of *Boophilus microplus* (Can.) and an assessment of its resistance spectrum. *Bulletin of entomological research* **56**: 389-409.

SHAW, R.D., THOMPSON, G. & BAKER, J.A.F. 1967. Resistance to cholinesterase- inhibitors in the blue tick, *Boophilus decoloratus*, in South Africa. *Veterinary Research* **81**: 548.

SPICKETT, A.M., HEYNE. I.H. & WILLIAMS, R. 2011. Survey of the livestock ticks of North-West province, South Africa. *Onderstepoort Journal of Veterinary Research* **78**: 305

TAYLOR, R. J. & OBEREM, P. 1995. Some characteristics of an amitraz resistant strain of *B. decoloratus* originating from South Africa. in: Proceedings of the second international conference on tick-borne pathogens at the host-vector Interface. P. 62, August 28 to September 1, 1995. Kruger National Park, South Africa.

TØNNESEN, M.H., PENZHORN, B.L., BRYSON. N.R., STOLTSZ, W.H. & MASIBIGIRI, T. 2004. Displacement of *Boophilus decoloratus* by *Boophilus mocroplus* in the Soutpansberg region, Limpopo Province, South Africa. *Experimental and Applied Acarology* **32**: 199-208.

TONETTI, N., BERGGOETZ, M., RÜHLE, C., PRETORIUS, A.M. & GERN, L., 2009, Ticks and tick-borne pathogens from wildlife in the Free State Province, South Africa. *Journal of Wildlife Diseases* **45**: 437-446.

TROLLOPE, W.S.W. 2011. Personal perspectives on commercial versus communal African fire paradigms when using fire to manage rangelands for domestic livestock and wildlife in southern and east African ecosystems. *Fire Ecology* **7**: 57-73.

WALKER, A.R., BOUATTOUR, A., CAMICAS, J.-L., ESTRADA-PEÑA, A., HORAK, I.G., LATI, A.A., PEGRAM, R.G. & PRESTON, P.M. 2003. *Ticks of domestic animals in Africa: a guide to identification of species. Edinburgh*: Bioscience reports.

WHITEHEAD, G.B. 1956. DDT resistance in the blue tick, *Boophilus decoloratu~* Koch. *Student American Veterinary Medical Association* **27**: 117-24.

WHITEHEAD, G.B. 1973. Resistance to acaricides in tick in the Eastern Cape Province. *South African Medical Journal* **47**: 342-344.

YESSINOU, R.E., AKPO, Y., ADOLOGBE, C., ADINEI, J., ASSOGBA, M.N., KOUTINHOUIN, B., KARIM, I.Y.A. & FAROUGON, S. 2016. Resistance of tick *Rhipicephalus microplus* to acaricides and control strategies. *Journal of Entomology and Zoology Studies* **3**: 408-414.

ZEMAN, P. & LYNEN, G. 2010. Conditions for stable parapatric coexistence between *Boophilus decoloratus* and *B. microplus* ticks: a simulation study using the competitive lotka-volterra model. *Experimental and Applied Acarology* **52**: 409-426.

Chapter 2

Method validation to determine tick resistance to macrocyclic lactones

2.0 Introduction

Since the 1981 discovery that MLs were effective in the control of blue tick species, it is currently widely used by producers to control *R. (B.) microplus*, *R. (B.) decoloratus*, mites and endoparasitic nematodes (Pohl *et al*. 2011; Fernández-Salas *et al.* 2012; Abbas *et al* 2014). In South Africa, a possible breakdown of control was, however, recently perceived by producers in the Eastern Cape Province as reported by Novartis agents, selling acaricides in South Africa. (Freven 2018: personal communication). Researchers from many different countries such as Brazil, Mexico and Australia have however also reported the development of resistance of blue ticks (Sabatini *et al.* 2001; Klafke *et al.* 2006; Pérez-Cogollo *et al*. 2010). Methodologies that can detect tick resistance are therefore; very important tools in the management of tick resistance. It can enable producers to be informed on the resistance status of the ticks on their farms, as well as to assess the potential resistance of new acaricides being developed (Sabatini *et al*. 2001).

The Shaw Larval Immersion Test (SLIT) is one of the methods used to detect development of resistance in ticks against conventionally used acaricides such as amidines, synthetic pyrethroids and organophosphates. This method, first developed by Shaw (1966) and later modified by Sabatini *et al*. (2001) by adapting the method for micro-centrifuge tubes (Santos *et al.* 2013). Although it is not the method recommended by the Food and Agriculture Organisation of the United Nations (FAO) (FAO 1984), it is a standardised method that many scientists around the world use to detect resistance development in ticks such as the Asiatic blue ticks (*R. (B.) microplus)* (Sabatini *et al*. 2001; Klafke *et al*. 2010). The advantage of SLIT is that the acaricidal effects of the chemical can be observed at low concentrations due to direct exposure of the larvae to the acaricides, with the added advantage that commercial formulations can be used to test resistance (Sindhu *et al*. 2012).

There are various methods to assess tick resistance, all entailing exposure of different life stages of ticks to acaricides, either directly or indirectly. Larval Packet Test (LPT) entails larvae that are exposed indirectly by placing the larvae into acaricide impregnated envelopes. This method was designed to simulate the larvae coming in contact with the animal's integument that has been treated with acaricides, but not with the blood of the animal. (Sabatini *et al*. 2001; Santos *et al.* 2013), Adult Immersion Test (AIT) involves fully engorged female ticks, immersed in the acaricide (Sabatini *et al.* 2001) soon after dropping from the host. The number of eggs laid by the females after immersion would indicate the effectiveness of the acaricide. This methodology simulates the adults ticks exposed to the acaricide (pour-ons and sprays) while on the host (Sabatini *et al.* 2001). The disadvantage of this method is that the time between collection and transferring to the laboratory may allow a false resistant result. The Syringe Immersion Test (SIT) where larvae are immersed in the chemical within a syringe which allows the solution to flow out over time was designed to simulate larvae coming into direct contact with the chemical that "decreases in the blood of the host" over time. This is a fairly new methodology not used by many researchers. The original developer of this test found many errors and limitations to determine the factor of resistance of most of their samples (Santos *et al.* 2013). The Larval Tarsal Test (LTT) involving the use of microplates that are pre-treated with the chemical in which eggs are distributed and the number larvae hatching was evaluated (Lovis *et al*. 2011; Lovis *et al.* 2013). According to Lovis *et al.* 2011, this method is less time consuming and easier to use than the previously mentioned methodologies. It also uses some attributes from the other tests. The disadvantage of this tests is that the sample size is much smaller than the SLIT. The actual procedure may be faster, but preparation beforehand is more time consuming. Not many researches have used this test either, therefore; comparisons cannot be made.

Resistance testing for amidine, synthetic pyrethroid and organophosphates was done at the Pesticide Resistance Testing Facility (PRTF) at the Zoology & Entomology Department of the University of the Free State for the past 12 years by making use of the pie-plate SLIT. It was found to be more sensitive as the recommended FAO LPT (FAO 1984) for resistance testing and are therefore mostly used for contact acaricides tested in South Africa (Mekonnen *et al.* 2003; Ntondini *et al*. 2008). Sabatini *et al.* (2001) compared three different methodologies to detect MLs resistance in *R. (B) microplus* during their survey; The Shaw Larval Immersion Test (SLIT), Larval packet test (LPT) and the Adult immersion test (AIT). They also found the SLIT to be the most suitable methodology to detect ML resistance at low concentrations but

made use of a test-tube version of the test. This version was also used by Klafke *et al.* (2006) to determine resistance in Brazil as well as Pérez-Cogollo *et al.* (2010) in Mexico, and these authors obtained similar results. The test-tube SLIT was preferred as was it was designed to be a more systemic type of method to simulate larvae coming in contact with the chemical through the ingestion of the chemical within the blood meal. This method however caused the larvae to be totally submerged in the chemical and not just exposed to a diluted concentration found in the blood after a blood meal. The pie-plate SLIT was designed to simulate larvae coming into contact with the chemical on the skin of the host through spraying or using a pour-on application. MLs are, however, mostly administered via injection and this poses the question to be asked if the pie plate SLIT will not be a better representation of larvae exposed to MLs via filter paper soaked with MLs.

An outbreak of resistance was documented in countries such as Brazil, Mexico and Australia and was eventually confirmed by using Sabatini's modified SLIT (Sabatini *et al.* 2001; Pérez-Cogollo *et al*. 2010; Klafke *et al.* 2012). Emerging resistance of Asiatic blue ticks was reported in South Africa by Lovis *et al.* (2013) using the LTT, however at present, African blue tick resistance against MLs has not reported in South Africa. However, the findings of Lovis *et al*. (2013) will not be compared due a lack of research using the LTT. To be prepared for the detection of resistance development against MLs in South Africa it is important to already establish and standardise the correct methodology to be used to test for ML resistance in South African blue tick species. For this purpose, two variations of the SLIT method, the test-tube method, modified by Sabatini *et al.* (2001) for exposure of larvae to the acaricides in test tubes, in contrast with the pie-plate method which was originally developed by Shaw (1966), were chosen for this study. Currently, the pie-plate SLIT is mostly used by many South African researchers such as Ntondini *et al.* (2008) for testing of conventional acaricides resistance to Amidines, OPs and SPs (Mekonnen *et al.* 2003; Ntondini *et al*. 2008).

The main objective of this chapter was to compare methods used for detecting ML resistance in Brazil and Australia for *R. (B.) microplus* populations for efficacy in detecting resistance development of both *R. (B.) microplus* and *R. (B.) decoloratus* in South Africa. A further objective was to determining the Lethal Concentrations/dosages at 50- and 99 % of MLs for both *R. (B.) microplus* and *R. (B.) decoloratus* reference strains in South Africa.

2.1 Methods and Materials

2.1.1. Tick sample acquisition and handling

Susceptible reference strains were used for comparison of the two test methodologies as well as for test methodology development. These strains, which were obtained from ClinVet International, Bloemfontein; South Africa, are frequently used at the Pesticide Resistance Testing Facility (PRTF) situated in the Zoology & Entomology Department of the University of the Free State, Bloemfontein; South Africa, for resistance testing for acaricides.

2.1.1.1 *Rhipicephalis* (*Boophilus) decoloratus*

The *R. (B.) decoloratus* strain destined for cycling at ClinVet was acquired from Malalane research unit in March 2015. The immature stages of ticks in the breeding program are fed on cattle, not treated with any acaricides.

2.1.1.2 *Rhipicephalus (Boophilus) microplus*

A *R. (B.) microplus* Malelane strain used at ClinVet for cycling as a reference strain was acquired from Malelane research unit, South Africa in January 2011 and was then maintained and cycled on cattle, not previously treated with acaricides at ClinVet from then onwards.

2.1.1.3 Handling of ticks

Upon receipt at the lab, each collection was allocated an identification number and documented on an accountability form (Appendix 2: PRTF M01).

Fully engorged female blue ticks from each collection were placed on a sieve, rinsed with water and allowed to dry on paper towels. Species identification of individual ticks was confirmed using the identification keys described in 'Ticks of Domestic Animals in Africa: a Guide to Identification of Species' by Walker *et al.* (2003). Not more than 25 engorged female ticks were placed in glass Erlenmeyer flasks with a cotton stopper to prevent escape and to allow air flow. The flasks were incubated in a humidity container at 25 – 29 degrees centigrade and >70% RH for oviposition (more or less 6-21 days post collection) and egg hatching (ca. 42 post collection).

Thirty days after collection, the flasks were observed daily for egg hatching. The hatch date was considered to be the day when an estimated 70% of the larvae hatched. Testing for resistance and method evaluation was done between 16 and 21 days after the determined hatch date.

Superfluous larvae were destroyed by drenching dead females and larvae in acetone and left to stand overnight. The flasks were then filled with boiling water and left overnight again. The water was drained out of the flasks and the ticks and larvae were discarded by pouring the contents into a funnel with filter paper that was discarded in a waste container labeled as "Biological waste".

2.1.2 Method development

2.1.2.1 Preparation of test chemicals

2.1.2.1.1 Macrocyclic Lactones

Ecomectin is marketed by Afrivet (Bach number 508041, Expiry date: July 2018). Ivermectin is an active ingredient of Ecomectin, chosen as it is one of the most commonly used IVM product in the Eastern Cape to control blue ticks (personal communication, Novartis agent). Ivomectin is also commonly used by producers in the Eastern Cape, however, it is similar to Ecomectin and was not used in this study.

Ecomectin, an injectable form of MLs, containing 1% mass/volume IVM, was used for method development. The product was securely stored in its original container in the laboratory in a refrigerator below 6 ºC. Care was taken not to expose it to direct light.

A stock solution of 0.1 % was prepared by adding two ml of the commercial remedy to 18 ml twice-distilled water and TXE solution, respectively.

The LC 50 for IVM determined for the *R. (B.) microplus* reference strains by Sabatini *et al.* (2001) and Klafke *et al*. (2006) in Australia and Brazil, respectively, were 0.0004%. This concentration was therefore chosen to be used in method comparison in this study. An IVM concentration of 0.0004% was prepared from each stock solution by adding 0.4 ml of the stock solutions two 99.6 ml of each of the two diluents.

2.1.2.1.2 Diluents

Three diluents for chemical preparations were initially compared for solubility of the test chemical, Ecomectin, to decide on the appropriate diluents used for test comparisons. The chosen diluents were also used as control solutions for each test series.

The diluents consisted of; twice distilled water, Triton X/Ethanol solution and normal tap water. Twice distilled water is commonly used at the Pesticide Resistance Testing Facility (PRTF) situated in the Zoology & Entomology Department of the University of the Free State for resistance testing on Ops, SPs and Amidines. Triton X/Ethanol solution (TXE) is used by many researches doing similar studies (Sabatini *et a*l. 2001, Klafke *et al*. 2006; Pérez-Cogollo *et al*. 2010). In the case of normal tap water, it has not been used in any research articles doing similar studies. For this purpose, these solutions will be compared to determine which one is more suitable for resistance detection. Two ml of Ecomectin were diluted into 18 ml of each diluent to produce a 0.1% Ivermectin stock solution. After mixing on a magnetic stirrer, each of the solutions was poured into a test tube and a photo was taken for comparison purposes. The solutions were then vortexed for 15 seconds to ensure a thorough mix of the Ecomectin with each of the diluents. A photograph was then taken two, five and 10 minutes after vortexing and again after 20 hours to observe the stabilisation of the mixtures of each of the solutions to indicated solubility.

Two diluents were decided upon for method comparison. The first diluent consisted of a stock solution of 98 ml absolute ethanol (99,9% ethanol) mixed with two ml of Triton X-100 to give a 97,9% ethanol, 2% Triton X solution. From this stock solution, a working solution was prepared by mixing four ml with 396 ml of distilled water giving a final concentration of 0,979% ethanol and 0, 02% Triton X. The second diluent was twice distilled water.

2.1.2.2 Safety measures

Both tests were done in an allocated area surrounded by double-sided tape to prevent larvae from escaping. A stainless-steel tray was placed on the allocated area. The edges of the tray was also safe guarded by double sided tape. The Erienmeyer-flask containing the larvae for testing was placed within the allocated area on a petri-dish with water and double-sided tape fixed on the outer side of the petri dish and around the neck of the flask.

All material that came in contact with larvae during testing was discarded in a dustbin containing a plastic bag with double-sided tape at the top to prevent any surviving larvae escaping.

2.1.2.3 Test methodologies

Two variations of the SLIT methodology described in literature were compared; the pie-plate (Shaw, 1966) and test-tube tests (Sabatini *et al.* 2001). Both make use of a direct exposure of the larvae to the acaricides.

2.1.2.3.1 Pie-plate method

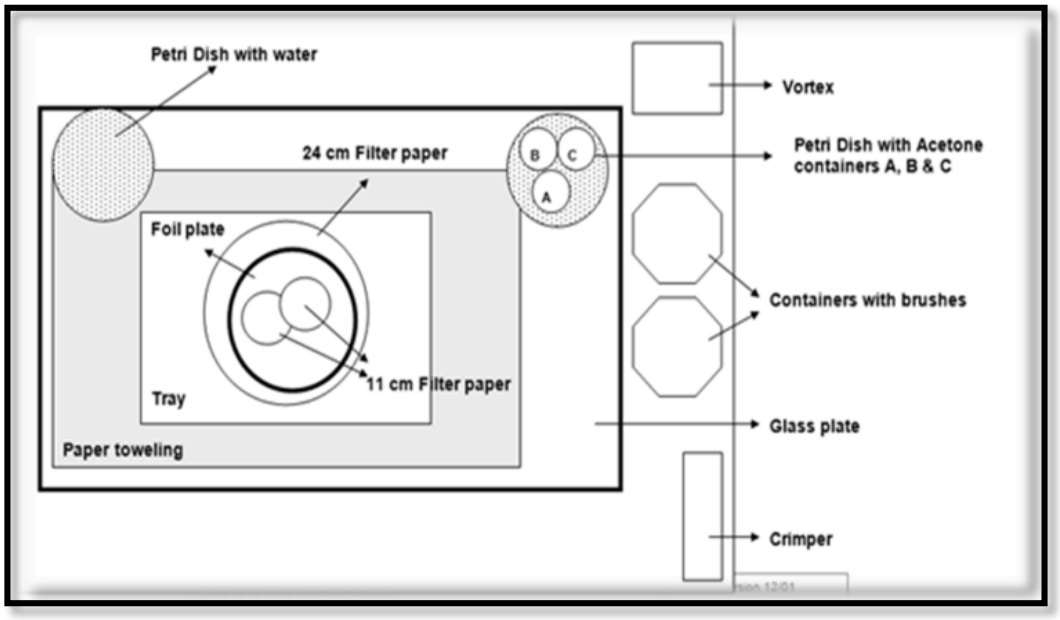


Figure 4: Standard layout of the pie-plate SLIT developed by Shaw (1966). Adapted from the PRTF SOP.

The pre-exposure setup for the pie plate immersion method is indicated in Figure 4. A round filter paper with a 24 cm diameter was placed on a stainless steel tray to soak up any drops of water or liquid which may spill during the procedure. A foil pie-plate containing two round Whatman no. one filter papers with a diameter of 11cm was placed on the 24 cm filter paper. The Ehrienmeyer-flask containing larvae was positioned in the petri-dish with water on the left hand top side. Double-sided tape was placed around the neck of the flask to trap any straying larvae from the flask. The cotton wool plug from the flask in which the larvae were kept was removed with forceps and placed on the side in the foil pie-plate. A fine uncontaminated (never in contact with chemical solution) brush was used to pick up roughly 500 larvae from the flask.

The larvae were brushed onto one filter paper in the tin foil pie-plate. The second filter paper was placed over the first containing the larvae, to form a sandwich. The cotton wool stopper was placed back into the neck of the flask using forceps, and the flask was placed back in the petri-dish to prevent the larvae escaping. The forceps were rinsed in Acetone in Acetone tube "A" and left on the paper toweling to be ready for handling the next test concentration. The brush that was used to transfer larvae onto clean filter paper was placed in an Acetone tube "B" to kill any remaining larvae.

Ten ml of the control solution was mixed on a vortex for five seconds and poured in a zig-zag pattern over the filter paper sandwich until it was drenched with the solution. The stopwatch was started simultaneously and the treated pie-plate was placed next to the work tray. The next pie-plate was placed on the filter paper tray and the same procedure was followed to make a larval filter paper sandwich using a new clean brush. Ten ml of the test solution was poured in the same manner onto the filter paper sandwich. This was repeated at 60-second intervals for all the test solutions and the pie-plates were placed in a row on the side of the work tray after each treatment in the same order it was treated. Using a 24 cm filter paper, the tray was wiped down at the end of the batch of treatment exposures to mop up any stray larvae and drops of liquid that might have spilled and thrown away in the allocated dustbin. The larvae were exposed to the test solution in the pie-plates for 10 minutes. A new sheet of 24 cm filter paper was placed on the tray. After 10 minutes of exposure, using the forceps, the filter paper sandwich from the first plate (control solution) was picked up from the pie-plate and placed on the 24 cm filter paper to drain excess solution. The foil plate was thrown away into an allocated dustbin.

The sandwich was opened using forceps and the two 11 cm filter papers were placed next to each other on the dry part of the 24 cm filter paper to absorb most of the acaricide. The forceps were rinsed in Acetone tube "A" between each use. Roughly a 100 larvae were brushed from each filter paper into each of two dry filter paper envelope using brushes that were designated as "Contaminated" (brushes that had come in contact with the chemical when brushing the larvae into the envelopes). The brushes were placed into Acetone tube "C" and kept separate from the "uncontaminated brushes". The envelopes were closed by crimping and further sealed with masking tape to prevent larvae escaping during incubation. The control samples were placed in separate incubation boxes than the treated envelopes to prevent contamination. The envelopes were then incubated for 24 / 48 / 72 hours at a relative humidity of >75%.

Used brushes were cleaned by transferring "clean" and "contaminated" brushes in separate containers filled with soap water and marked as "soap clean" and "soap contaminated". After five minutes, brushes were rinsed with water until the soap was completely rinsed out. The brushes were placed into separate glass beakers filled with acetone and marked as "acetone clean" and "acetone contaminated". The brushes were left in the beakers until all the tick larvae had been removed. The brushes were then dried with clean absorbent paper.

2.1.2.3.2 Test – tube method:

Eppendorf-tubes (1.5ml) were placed into an Eppendorf-test-tube rack. Each tube was filled with 500 microliters of the control or test solution. Using an uncontaminated brush, roughly 500 larvae were transferred from the conical larval incubation flask to each tube after which each tube was closed. The clean brushes where transferred to Acetone tube B. Due to difficulty in submerging the larvae into the test solution the method was modified by centrifugation for 15-seconds to ensure sinking of the larvae to the bottom of the tube so as to be fully immersed into the chemical/control solution. After 10 minutes of immersion, the larvae were tipped from the tube onto a clean piece of filter paper. Separate clean contaminated brushes were used for each tube to brush out the larvae that were stuck inside the tubes and then placed into Acetone tube C designated for the contaminated brushes. The larvae were then dried via air and transferred into two dry filter paper packets and incubated as described for the pie-plate method (Sabatini *et al*. 2001; Klafke *et al.* 2006).

2.1.2.4 Method comparison

A comparison of the two methods was done by investigating the following aspects for each method: Type of diluent, the method of determining resistance and post-exposure incubation times.

Larvae from a susceptible reference strain from both blue tick species, at age of 16 – 21 days after hatching, were used for method development. Execution of the tests was done as summarized in Table 2.1. The two methodologies, pie-plate SLIT and test-tube SLIT, were performed alternately in triplicate starting with the pie-plate method and hourly intervals for starting each consecutive exposure on one day. For each test, larvae were exposed in triplicate

to each of the two diluents as control exposures as well as the 0.0004% Ecomectin concentration prepared in twice distilled water or TXE solution. This produced six incubation envelopes for each test, at each time interval, to be able to evaluate larval survival at three different post exposure times of 24, 48 and 72 hours.

Table 2.1: Schedule for exposure of the reference strain to different solutions to provide for evaluation of survival at 24h, 48h and 72h post-exposure for both methodologies. The schedule followed at 9:00 were followed for all the consecutive timeslots.

Day 16 -21 post hatching				
		Incubation time post exposure to acaricide		
Test intervals	**Solutions**	**24h**	**48h**	**72h**
09:00	**Pie Plate SLIT**			
	H_2O	1 (x2)	1 (x2)	1 (x2)
	0.0004% IVM Solution(Diluted with distilled water)	1 (x2)	1 (x2)	1 (x2)
	Diluent 1	1 (x2)	1 (x2)	1 (x2)
	0.0004% IVM Solution (Diluted with TritonX/Ethanol)	1 (x2)	1 (x2)	1 (x2)
10:00	**Test-tube SLIT**			
11:00	**Pie plate SLIT**			
12:00	**Test tube SLIT**			
13:00	**Pie plate SLIT**			
14:00	**Test tube SLIT**			

2.1.3 Formulas and statistical analysis

SLIT

After post-exposure incubation to the chemical (24 or 48 or 72 hours), enclosed envelopes were opened and larvae that were found to be alive or died were counted for each envelope. Only ticks that were able to walk were considered as being alive. They were killed by pressing on them with a spatula while counting. The following formula developed by Abbot (1925) was used to calculate corrected mortality:

$$\textbf{Corrected Mortality (CM)}\ \% = \frac{(\%\ \text{mortality sample} - \%\ \text{mortality of control sample})}{(100 - \%\ \text{mortality of control sample})} \times 100$$

P-value

In the control diluent comparison section of the results, the p-value was calculated by determining the z –value (standard normal probability) to determine if the larvae mortality rates from the two diluents is significantly different or not. The test level of significance used was 5% ($\alpha = 0.05$).

2.1.4 Lethal concentration determination

Comparison of the Pie-plate and Test-tube methods formed the basis to establish the development of a suitable method for resistance testing of *R. (B.) decoloratus* and *R. (B.) microplus* against MLs for South African strains. The LC50 of 0.00004% obtained by Sabatini *et al*. (2001) and Klafke *et al*. (2006), on *R. (B.) microplus* seemed to be much higher than for South African strains used in this study. A suitable LC50 concentration that was applicable to South African strains and conditions needed to be established to be able to test for MLs resistance of blue tick species in South Africa. For this purpose, the susceptible reference strain of *R. (B.) decoloratus* was exposed to a MLs concentration range, starting at 0.01% and diluted by a factor of 2 up to 0.0001562% as indicated in Table 2.2. This was done by making use of distilled water as a diluent, the pie plate SLIT and a post-incubation time of 24 hours.

Table 2.2: Seven concentrations of IVM were prepared according to the method described in stage one.

Dilution	Concentration
Control	-
1	0,01%
2	0,005%
3	0,0025%
4	0,00125%
5	0,000625%
6	0,0003125%
7	0,0001562%

The LC50 and LC99 were then calculated using the software program Polo Suite. This program uses log dose probit to determine the lethal concentration necessary to kill a certain percentage of a population (in this case 50% and 99% of the population) and to establish 95% confidence intervals (CI) (Miller *et al.* 2010).

The concentrations used for LC50 and LC99, as set out in Table 2.2, was found to be too high to determine LC50 and LC99 of South African *R. (B.) microplus* reference strains. To determine the lethal concentrations, the concentration range was reduced and the tests were repeated for *R. (B.) microplus* reference strain. The concentrations ranged between 0.005 – 0.0000049% as shown in Table 2.3.

Table 2.3: The lowered IVM concentration ranged to determine lethal concentrations for R. *(B.) microplus*.

Dilution	Concentration
Control	-
1	0,005%
2	0,00125%
3	0,000313%
4	0,000078%
5	0,0000195 %
6	0,0000098%
7	0,0000048%

2.2 Results

2.2.1. Control solutions comparison

2.2.1.1 Solubility Ivermectin into solvents

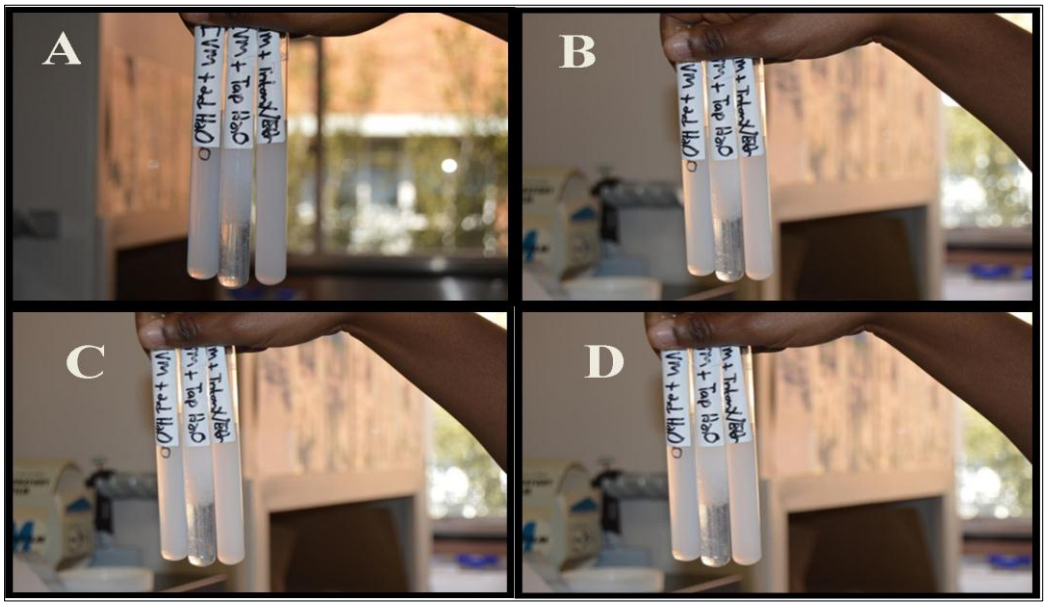


Figure 5: 0.1% IVM diluted into twice distilled water, tap water and the TritonX/Ethanol solution. **A**: Appearance of the dilutions before vortexing (mixing). B: Diluents left for two minutes after vortexing. C: Diluents five minutes after vortexing. D: Diluents 10 minutes after vortexing.

Results obtained from the solubility testing are indicated in Fig. 5. The IVM diluted in the TXE and twice distilled water (DW) mixed well, but the solvent did not properly dissolve in tap water as seen in Fig. 5 B**,** D and C even after mixing on a Vortex. The solvent dissolved into the DW and TXE and stayed in solution up to 10 minutes after mixing. The last observation made after 20 hours also showed no difference in solubility between the TXE-IVM and DW-IVM solutions, however, in the tap water-IVM solution a visual separation between the tap water and IVM solution was observed (Fig. 6).

Figure 6: Diluents left for 20 hours without being vortexed.

These results indicated that twice-distilled water and TXE solutions could be used as a diluent in the preparation of IVM dilutions but, tap water provided unreliable results.

2.2.2. SLIT comparison

2.2.2.1 Control diluent comparison

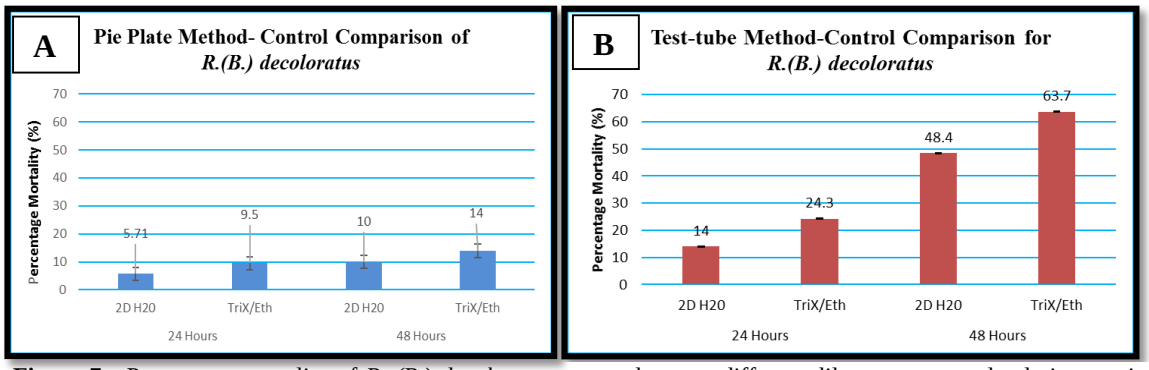


Figure 7: Percentage mortality of *R. (B.) decoloratus* exposed to two different diluents as control solutions, twice-distilled water and TXE, by means of the pie plate (A) and Test tube (B) SLIT as determined at 24 and 48 hours post exposure incubation times

The normal percentage of mortality still acceptable as a control sample is 10% (Abbot 1925). The mortality rate of the *R. (B.) decoloratus* larvae for both control solutions used in the pie-plate SLIT was below 10% at 24 hours post exposure (Fig. 7A). The distilled water showed a statistically significant (p=0.0004) lower mortality rate at 5.71% than the TXE diluent at 9.5%. At 48 hours post-exposure, the larval mortality rate for the distilled water solution was still below 10%, with the TXE solution at 14% and no statistical difference between them (p=0.89). In the case of the test-tube method (Fig. 7B), both solutions at both the post-exposure times showed a higher than 10% larval mortality. The distilled water and TXE solution showed a 14% and 24% mortality, respectively, at 24 hours with no statistical difference (p=0.53). After 48 hours, the larvae mortality rate of the distilled water and TXE were found to be 48.4% and 63.7% mortality, respectively, much higher than the values of the pie-plate SLIT at both post-exposure evaluation times. The mortality rate of the two solutions after 48 hours post-exposure

as not statistically different to each other (p=0.96). All values were higher than 10% and thus not acceptable as a control value for resistance testing.

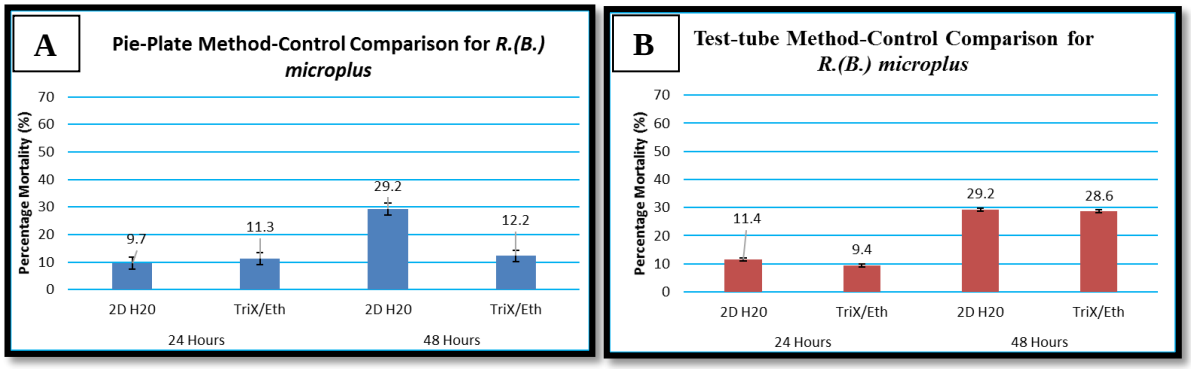


Figure 8: Percentage mortality of *R. (B.) microplus* exposed to two different diluents as control solutions, twice distilled water and TXE by means of the pie-plate (A) and the test-tube (B) SLIT as determined at 24 and 48 hours post exposure incubation times.

In Fig. 8A, the mortality rates of the *R. (B.) microplus* larvae for the distilled water solutions used in the pie-plate test was below 10%, at 9.7% after 24 hours exposure. The mortality rate of the TXE showed a slightly higher value at 11.3%. It was found that TXE was not statistically different from the distilled water control (p=0.99). The high mortality value was not acceptable as a normal control value for testing mortality. At 48 hours post-exposure, the larval mortality for both distilled water and TXE solutions were unacceptably high at 29.2% and 12.2%, respectively, and was found to be not statistically different to each other (p=0.09).

The larvae mortality rates for distilled water by means of the test-tube SLIT (Fig. 8B) was slightly higher than 10% at 11.4%, 24 hours post-exposure and the TXE showed a lower mortality rate of 9.4% which is acceptable as a control solution. These mortality rates of the two diluents 24 hours post-exposure, were found to be not statistically different (p=0.36). At 48 hours post-exposure, the larval mortality for both distilled water and TXE solutions were unacceptable as their mortality rates were above 10% at 29.2% and 28.6%, respectively. The mortality rates of the two diluents were, however, shown not to be statistically different (p=0.49).

2.2.2.2 Comparison of test-tube vs. pie-plate methods for Ivermectin exposure

The LC 50 concentration of 0,0004% determined by Sabatini *et al*. (2001) and Klafke *et al.* (2006) killed all the larvae for both blue ticks species at all post-exposure times and both test methodologies as seen in Table 2.4. The only exception was the 24 hour post exposure

evaluation of mortality for *R. (B.) decoloratus* larvae exposed to 0.0004% Ivermectin diluted in distilled water and making use of the pie-plate SLIT where the larvae had a mean mortality of 96.1 %.

Table 1.4: The mean percentage mortality of the 0,0004 % IVM LC 50 determined by Sabatini *et al.* (2001) and Klafke *et al.* (2006) for both *R. (B) decoloratus* and *R. (B.) microplus* **and** SLIT methodologies

Solutions	Mean % Mortality at Post exposure time (Hours)					
	R.(B.) decoloratus			*R.(B.) microplus*		
	24 H	48 H	72 H	24 H	48 H	72 H
Pie-plate						
0,0004 IVM - DW	96.1%	100 %	100 %	100 %	100 %	100 %
0,0004 IVM-TXE	100 %	100 %	100 %	100 %	100 %	100 %
Test-tube						
0,0004 IVM - DW	100 %	100 %	100 %	100 %	100 %	100 %
0,0004 IVM-TXE	100 %	100 %	100 %	100 %	100 %	100

2.2.3 Lethal concentrations for South African blue tick species

Table 2.5 indicates results obtained from concentration ranges shown in Table 2.2 for *R. (B.) decoloratus* and Table 2.3 for *R. (B.) microplus* to determine applicable LC50 concentrations for susceptible strains of both blue tick species found in South Africa.

Table 2.5: The LC50 and LC99 of the African blue tick (*R. (B.) decoloratus*) and Asiatic blue tick (*R.(B.) microplus*) reference strains

Species	Reference	LC50 (%)	CI95 (%)	LC99 (%)	CI95 (%)
R.(B.) decoloratus	CV 17/01	0.00003	0.000002-0.0001	0.04	0.02-0.2
R.(B.) microplus	Mean of strains	0,00001	0.000003-0.00001	0.2	0.004-0.2

LC: Lethal Concentration **CI95%:** Confidence Interval 95%

The LC 50 and 99 values for the ClinVet reference strains were obtained by means of the pie-plate method for Ivermectin resistance determination with the use of water as diluent and control. The only *R. (B.) decoloratus* reference strain collection (CV 17/01) that could be

obtained from Clinvet International during 2017 was found to have a LC 50 value of 0.00003% which is 10-fold lower than the LC 50 value (0.0004 %) determined by Sabatini *et al.* (2001) and Klafke *et al.* (2006). The LC 99 value of this strain was found to be 0.04 %.

The mean of the three generations of the susceptible *R. (B.) microplus* strain was calculated to give a mean LC 50 of 0.00001 %, which is lower than that of both the Sabatini *et al*. (2001) and *R .(B.) decoloratus* susceptible strains. However, the LC99 of this strain was found to be 0.21, which is higher than concentration for the African blue tick.

2.3 Discussion

The first detection of *R. (B.) microplus* resistance to MLs was reported in 2001 in Rio Grande do Sul, southern Brazil (Klafke *et al.* 2010; Abbas *et al* 2014). Methodology to be able to confirm the presence of tick resistance to MLs was investigated for the sensitivity of *in vitro* detection and confirmation of ivermectin-resistant populations of *R. (B.) microplus* by using the SLIT technique by Sabatini *et al*. (2001) and Klafke *et al.* (2010)

Tick species from different continents can have different sensitivities for the same acaricides depending on different farming management as seen in the findings of Sabatini *et al*. (2001) in Australia, Klafke *et al.* (2006) in Brazil and Fernández-Salas *et al*. (2012) in Mexico where *R.* (*B.*) *microplus* strains previously exposed to IVM had varying resistance levels. It was therefore; necessary to establish a methodology for *in vitro* testing of tick resistance to MLs, by validating methodology used elsewhere for South African blue tick strains. Sensitivity to MLs was also not previously tested for the African blue tick (*R. (B.) decoloratus*) and needed to be established. The current study firstly indicated that the LC50 concentration of 0.0004% determined by Sabatini *et al*. (2001) and Klafke *et al.* (2006) for Australia and Brazil were higher than the LC 50 concentrations for susceptible strains of both *R. (B.) microplus* and, *R. (B.) decoloratus* in South Africa. The LC 50 for *R. (B) microplus* was found to be 0.00001% and for *R. (B.) decoloratus* it was found to be higher with an LC50 of 0.00003% and an LC99 concentration of 0.21% and 0.04% for the two species respectively. This also indicated that both blue tick species currently found in South Africa (Eastern Cape Province) are more

susceptible to MLs compared to *R. (B.) microplus* in Australia and Brazil. This is true at least for strains that have not been exposed to MLs in South Africa.

.

In this study the pie-plate and test-tube SLIT methodologies were compared to determine which method will be best suited for resistance testing of South African blue tick species to MLs. Different aspects, the two methodologies have in common were compared to determine the best outcome. This entailed comparisons of different diluents for use in preparation of the test concentrations of the test chemical, the use of these diluents as control samples and the most reliable timeframe for post-exposure evaluation of tick death or survival for both test methodologies.

The solubility of the test solution into the diluent is important for the uniform distribution of the test chemical in the test mixture to assure equal exposure of all larvae to the acaricides during testing. It is also important for the diluent to have a low enough concentration that will not have a detrimental effect on the larvae when used as a control solution. Mortality of less than 10 % of larvae exposed to a control diluent is considered to be acceptable if Abbot's formula is used to then correct mortalities of larvae exposed to the test chemical as well (Abbot 1925).

Many researchers used either twice distilled water (Mekonnen *et al.* 2003) or a Triton X/ethanol diluent (Sabatini *et al.* 2001; Fernández-Salas *et al*. 2012; Pérez-Cogollo *et al*. 2010) as diluents for the test chemical. Sabatini *et al*. (2001) tested both solutions using the AIT and found similar results in terms of the lethal concentration, however, this needed to be confirmed for SLIT as well.

The Triton X/Ethanol diluent is a very popular solution and is recommended by Sabatini *et al*. (2001). According to them, the Triton X/ethanol diluent prevents the larvae from floating on the surface and cause larvae to become fully submerged into the chemical. In the current study, distilled water seemed to be equally if not more suitable than the Triton X/Ethanol diluent as it was found to be just as effective mixing with MLs to form a uniform mixture of the test chemical. For both diluents thorough mixing on a vortex before exposure was done to further ensure a uniform solution. As the larvae are trapped between two filter paper layers in the pie-plate SLIT, submersion into the solutions is also ensured. Distilled water is also more readily available, cheaper and less labour intensive to prepare than Triton X/Ethanol diluent. Distilled water was therefore; chosen as the preferred diluent for this study. Although tap water is often

used to dilute chemicals in the field, it did not mix well with the solvent and was not previously used and tested globally. This might be explained by the fact that different continents have water with different additives and using tap water may compromise the results.

Distilled water as control solution at 5.78% mortality (for pie plate SLIT) and 14% mortality (for test tube SLIT) performed better than Triton X/Ethanol at 9.5% (for pie plate SLIT) and 24.3% (for test tube SLIT) for both the pie plate and the test tube SLITs performed on *R.(B.) decoloratus* at 24 hour post-exposure. Forty-eight hour post exposure produced results much higher than the allowed 10% mortality for a control substance except for the pie-plate distilled water control. For *R. (B.) microplus* distilled water performed similar to Triton X/Ethanol for both types of tests, but 48 hours post exposure for both tests and control substances produced mortalities much higher than the allowed 10%. These findings therefore support the decision that the pie-plate SLIT with twice-distilled water as diluent and control solution and a post-exposure time of 24 hours was the most suitable method to determine sensitivity of blue tick larvae to MLs.

Similar studies used different post-exposure incubation times before mortality of the tests is determined. Sabatini *et al.* (2001), Klafke *et al.* (2006) and Pérez-Cogollo *et al*. (2010) made use of a 24 hours post-exposure time before mortality was determined. For the other chemicals such as OPs, SP and Amidines, 72 hours post exposure mortality counts were used (Mekonnen *et al.* 2003). These acaricides are, however, contact chemicals and absorbed by the tick's integument compared to MLs that is normally taken in during a blood meal. For this purpose, the post-exposure incubation times were tested to determine their influence on the mortality of the larvae.

In the current study the longer the larvae were exposed to the control dilutions, the higher the mortality rate. High mortality rates by means of the test-tube SLIT, might have been due to full submersion of the larvae with the Triton X/Ethanol solution and the fact that tubes needed to be vortexed to obtain this full larval immersion into the chemical. This may lead to mechanical death of some of the larvae. In the pie-plate SLIT, the larvae are transferred from the incubation flask and put on top of a filter paper without further mechanical handling, other than placing a second filter paper on top prior to immersion into the solution.

The initial expectation of testing both methodologies while larvae are exposed to 0.0004% MLs, the LC50 value determined by Sabatini *et al*. (2001) and Klafke *et al.* (2006), diluted in both distilled water and Triton X/Ethanol, provided results of 100% mortality for both tick

species, both diluents and for both methodologies tested. The only exception was for larvae exposed to MLs diluted in water after 24 hours exposure where a mean of 96.1% mortality was found. This made it impossible to distinguish between the positive or negative influences of the different variables, but made it necessary to establish new LC50 values for both blue tick species found in South Africa.

Comparing the test methodologies with each other also elucidated the following aspects with regard to the ease of implementing each method. The test-tube SLIT was more time-consuming and efforts to get enough larvae into the Eppendorf-tube caused many larvae to escape. This placed further time constraints on the time repeatability of the tests due to time spent preventing ticks escaping and recapturing escapees. All larvae were not decanted with a single attempt onto the filter paper after exposure, causing more time lost in efforts to brush all larvae from the tubes onto the filter paper as well resulted in inconsistent exposure timeframes for each repetition.

In the case of the pie-plate SLIT, larvae were transferred with more ease from the incubation flask onto the filter paper and escapees were at a minimum and easy to capture. After immersion, the exposed filter paper sandwich was drained from excess chemical and transferred and separated onto a larger drier filter paper to limit further exposure to the chemical. The two parts of the filter paper sandwich with fairly equal numbers of larvae on each also provided a duplicate for larvae to be transferred into two filter paper envelopes without much mechanical handling. The larvae were broadly distributed on both small filter papers and each one represented a duplicate. Chances of contamination are also less as the brushes used for larval transfer had less contact with the chemical than with the test-tube SLIT. All these factors also made the pie-plate SLIT a more user-friendly test to conduct and the method of choice for further resistance testing conducted and described in the next chapter.

It is important that further susceptible strains should be obtained to confirm if South African blue ticks are indeed less susceptible than Asiatic blue ticks to IVM.

In conclusion, LC50 values of 0.00001% and 0.00003% were set for South African strains of *R. (B.) microplus* and *R. (B.) decoloratus,* respectively. These values can be used as reference values for tick resistance testing in South Africa although it is recommended that susceptible strains from different areas in South Africa should also be tested. This will help to confirm these values as well as the fact that South African blue tick strains seemed to be more susceptible to MLs than the Brazilian and Australian strains.

Tick resistance testing by making use of the pie-plate SLIT was found to be most effective method to determine resistance for both blue tick species, with twice distilled water as diluent for MLs as well as for use as a control. Post exposure time was set to be 24 hours post-exposure to the MLs as this proved to be the most reliable timeframe to detect resistance and prevent tick death from sources other than exposure to the chemical.

In the next chapter this LC values determined for South African blue tick species in this study as well as the methodology previously described, will be used to determine tick resistance to MLs on different farms in the Eastern Cape Province.

2.4 References

ABBAS, R.Z., ZAMAN, M.A., COLWELL, D.D., GILLEARD, J. & RAJBUT, Z.I. 2014. Acaricide resistance in cattle ticks and approaches to its management: The state of play. *Veterinary Parasitology* **203**: 6-20.

ABBOT, W.S. 1925. A method of computing the effectiveness of an insecticides. *Journal of Economic Entomology* **18**: 264 - 267.

FAO (Food and Agricultural Organization of the United Nations). 1984. *Ticks and tick borne disease control, a practical field manual*, Vol. I.

FERNĀNDEZ-SALAS, A., RODRÍQUEZ-VIVAS, R.I., ALANSO-DÍAZ, M.M. & BASURTO-CAMBERAS, H. 2012. Ivermectin resistance status and factors associated in *Rhipicephalus microplus* (Acari: Ixodidae) populations from Veracruz, Mexico. *Veterinary Parasitology* **190**: 210-215.

FREVER, R. 2016. (Pharmacutical salesman, Norvatis pharmaceutical, Kempton Park, Gauteng)

KLAFKE, G.M., SABATINI, G.A, DE ALBUQUERQUE, T.A., MARTINS, J.R., KEMP, D.H., MILLER, R.J. & SCHUMAKER, T.T.S. 2006. Larval immersion tests with ivermectin in populations of the cattle tick *Rhipicephalus* (*Boophilus*) *microplus* (Acari: Ixodidae) from State of Sao Paulo, Brazil. *Veterinary Parasitology* **142**: 386-390.

KLAFKE, G.M., DE ALBUQUEQUE, T.A., MILLER, R.J. & SCHUMAKER, T.T.S. 2010. Selection of an ivermectin-resistant strain *of Rhipicephalus microplus* (Acari: Ixodidae) in Brazil. *Veterinary Parasitology* **168**: 97-104.

KLAFKE, G.M., CASTRO–JANER, E., MENDES, M.C., NAMINDOME, A., SCHUMAKER, T.T.S. 2012. Applicability of *in vitro* bioassays for the diagnosis of ivermectin resistance in *Rhipicephalus microplus* (Acari:Ixodidae). *Veterinary Parasitology* **184**: 212-220.

LOVIS, L., PERRET, J., BOUVIER, J., FELLAY, J.-M, KAMINSKY, R., BETSCHART, B. & SAGER, H. 2011. A new *in vitro* test to evaluate the resistance level against acaricides of the cattle tick, *Rhipicephalus (Boophilus) microplus. Veterinary Parasitology* **182**: 269-280.

LOVIS, L., REGGI, J., BERGGOETZ, M., BETSCHART, B., & SAGER, H. 2013. Determination of acaricide resistance in *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae) field populations of Argentina, South Africa, and Australia with the larval tarsal test. *Journal of Medical Entomology* **50**: 326-334.

MEKONNEN, S., BRYSON, N.R., FOURIE, L.J., PETER, R.J., SPICKETT, A.M., TAYLOR, R.J., STRYDOM, T., KEMP, D.H. & HORAK, I.G. 2003. Comparison of 3 tests to detect acaricide resistance in *Boophilus decoloratus* on dairy farms in the Eastern Cape Province, South Africa. *Journal of the South African Veterinary Association* **74**: 41-44.

MILLER, A.L.E., TINDOL, K. & LEONARD, B.R. 2010. Bioassays for monitoring insecticide resistance. *Journal of Visualized Experiments* **46**: 1-5.

NTONDINI, Z., VAN DALEN, E.M.S.P. & HORAK, I.G. 2008. The extent of acaricide resistance in 1-, 2- and 3-host ticks on communally grazed cattle in the eastern region of the Eastern Cape Province, South Africa. *Journal of the South African Veterinary Association* **79**: 130-135.

PÉREZ-COGOLLO, L.C., RODRÍGUEZ-VIVAS, R.I., RAMIREZ-CRUZ, G.T. & MILLER, R.J. 2010. First report of the cattle tick, *Rhipicephalus microplus* resistant to ivermectin in Mexico. *Veterniary Parasitology* **168**: 165-169.

POHL, P.C., KLAFKE, G.M., CARVALHO, D.D., MARTINS, J.R., DAFFRE, S., DA SILVA VAZ, I. Jr & MASUDA, A. 2011. ABC transporter efflux pumps: defense mechanisms against ivermectin in *Rhipicephalus* (*Boophilus*) *microplus. International Journal for Parasitology* **41**: 1323-1333.

RAJBUT, I.Z., HUA, S.H., CHEN, W.J., ARIJO, A.G. & XIQO, C.W. 2006. Importance of ticks and their chemical and immunological control in livestock. *Journal of Zhejiang University Science B* **7**: 912-921.

RODRÍQUEZ-VIVAS, R.I., JONSSON, N.N. & BHUSHAN, C. 2018. Strategies for the control of *Rhipicephalus microplus* ticks in a world of conventional acaricide and macrocyclic lactone resistance. *Parasitology Research* **117**: 3-19.

SABATINI, G.A., KEMP, D.H., HUGHES, S., NARI, A. & HANSEN, J. 2001. Tests to determine LC_{50} and discriminating doses for macrocyclic lactones against the cattle tick, *Boophilus microplus*. *Veterinary Parasitology* **95**: 53-62.

SANTOS, T.R.B., KLAFKE, G.M., PAPPEN, F.G., NIZOLI, L.Q., BIELGELMEYER, P. & FARIAS, N.A.R. 2013. Comparison of three larval bioassays to evaluate susceptibility of *Rhipicephalus (Boophilus) microplus* to amitraz. *Revista Brazileira de Parasitologia Veterinaria* **22**: 495-501.

SHAW, R.D. 1966. Culture of an organophosphorus resistant strain of *Boophilus microplus* and an assessment of its resistance spectrum. *Bulletin of Entomological Research* **56**: 389-405.

SINDHU, Z.P., JONSSON, N.N. & RAJBUT, Z.I. 2012. Syringe test (modified larval immersion test): A new bioassay for testing acaricidal activity of plant extracts against *Rhipicephalus microplus*. *Veterinary Parasitology* **188**: 362-367.

WALKER, A.R., BOUATTOUR, A., CAMICAS, J.-L., ESTRADA-PEÑA, A., HORAK, I.G., LATI, A.A., PEGRAM, R.G. & PRESTON, P.M. 2003. *Ticks of domestic animals in Africa: a guide to identification of species. Edinburgh*: Bioscience reports.

Chapter 3

Resistance determination of African blue ticks collected from farms in the Eastern Cape, South Africa

3.0 Introduction

The African blue tick (*R.* (*B.*) *decoloratus*), which is indigenous to the continent of African, is the most widespread Ixodid tick species in Africa (Nyangiwe *et al*. 2017). It can cause severe direct and indirect damage to cattle with great economic losses for both commercial and communal producers (Walker *et al.* 2003; Tønnesen *et al.* 2004; de Clercq *et al*. 2012). *Rhipicephalus (B.) decoloratus* has been shown to develop resistance against acaricides most commonly used in Africa and in South Africa, namely, Ops, SP and Amitraz. In South Africa, some parts of the Eastern Cape, KwaZulu-Natal and North West Province were found to have resistant tick populations (Mekonnen *et al.* 2003; Fourie *et al.* 2013). As early as 1967, resistance to OPs was reported for some parts of the Eastern Cape and KwaZulu-Natal (Baker *et al*. 1978; Fourie *et al.* 2013) followed by reports of tick populations resistant to synthetic pyrethroids emerging in some parts of KwaZulu-Natal during 1987 (Coetzee *et al.* 1987; Fourie *et al.* 2013) and Amitraz resistance development in some parts of the Eastern Cape, close to East London in 1995 (Fourie *et al*. 2013). Further studies have shown that resistance to the three main acaricides in those areas is prevalent for both the African blue tick, and Asiatic blue tick in South Africa (Mekonnen *et al.* 2002; Ntondini *et al*. 2008). Although many blue tick populations have developed resistance to many of these acaricides, they can still be used to control blue ticks, depending on how producers implement them. The need for more effective acaricides as well as more information on the current tick resistance situation to available acaricides is becoming increasingly more important as tick resistance is keeping pace with the development of new acaricides.

The breakdown of control with more conventional acaricides simulated exploring new avenues of chemical control. New products such as MLs were produced and used to combat blue ticks (Abbas *et al.* 2014). Originally intended to control nematodes infecting livestock it was later discovered to be effective against blue ticks and have been used as such for over 20 years worldwide (Pohl *et al.*

2011; Fernández-Salas *et al.* 2012 ; Abbas *et al.* 2014). The most widely used MLs is Ivermectin (IVM). This acaricide has a very high affinity to glutamate and gamma-aminobutyric acid (GABA) receptors which control the chloride ion-channels found in the muscle and nerve cells of invertebrates (Klafke *et al.* 2010; Fernández-Salas *et al.* 2012; Geary & Moreno 2012). As a result it can activate glutamate-gated chloride ion channels which will then cause peripheral motor function paralysis and death (Klafke *et al.* 2010; Fernández-Salas *et al.* 2012). Due to its overuse and misuse, IVM has become less effective which has led to the development of resistance in blue ticks (Pérez-Cogollo *et al.* 2010; Abbas *et al.* 2014). In 2001, it was discovered that *R. (B.) microplus* was developing resistance to IVM in Rio Grande do Sul, in southern Brazil. Thereafter, reports of the *R. (B.) microplus* populations developing resistance to IVM Australia, Mexico, some parts of the USA and Uruguay started to emerge (Klafke *et al.* 2010; Abbas *et al* 2014).

Resistance to IVM treatment has not been reported in South Africa for *R. (B.) decoloratus* species (Rodríguez-Vivas *et al.* 2018), although an increase in the use of MLs for tick control was also inevitable. Recently, agents from pharmaceutical companies have been receiving numerous complaints of inadequate control of blue ticks by MLs from producers in the Eastern Cape. It was not specified which species of blue ticks were suspected of developing resistance. However, there was a report of emerging resistance of *R. (B.) microplus* species by Lovis *et al.* (2013) from two communal farms and one commercial farm in the Western Cape Province, South Africa. However, a different method called the Larval Tarsal Test (LTT), which is a fairly new method which w=has not been used by many researches, was used to detect emerging resistance.

Along with the worldwide reports of the Asiatic blue tick developing resistance to MLs, this forms the basis for this study. Therefore; the aim of this study was to use the tools developed in Chapter 2 to investigate the perceived resistance that has been experienced by some of the producers in the Eastern Cape Province and to develop criteria to be used to determine the presence of resistant individuals in a tick population. For this purpose, the pie-plate SLIT methodology was used to determine IVM resistance in *R. (B.) decoloratus.* At present, no information on resistance of African blue tick species to IVMs is available as most studies in South Africa focus on the Asiatic blue tick. Commercial farms used for tick collections in this study also did not show significant Asiatic blue tick invasions to make it possible to test for *R. (B.) microplus* resistance on these farms. A few *R. (B.) microplus* collections, obtained from communal areas in the Eastern Cape were included to also address possible resistant populations of this species.

3.1. Methods and Materials

3.1.1 Study areas

Farms with treatment practices that include and exclude the use of IVM were investigated.

3.1.1.1 Farms with animals previously exposed to IVM in the last five years

- **Hereford**

The farm Hereford is situated 60 km outside of the Great Kei Municipality, East London, Eastern Cape (32˚64'76" S, 27˚97', 18"E). It comprises of lush grass with patches of trees. The farm has multiple camps that allow frequent rotation of cattle (Fig. 9). The farm was situated on the eastern side of a hill near a stream often visited by the cattle. The producer farms extensively with beef and sheep and the cattle consisted mostly of mixed breeds of Bonsmara and Nguni (Fig. 10). The producer has been using MLs for blue tick control for over five years and injects his animals on a yearly basis. Treatments are administered at the end of autumn and twice in early spring. The producer suspects that MLs do not work to control blue ticks on his animals anymore, therefore the strain of blue ticks from this farms was considered as a potential resistant strain.

Ticks were collected from cattle, grazing in a single camp for a two-week period. After tick collections, the animals were treated with Ecomectin ad returned to the same camp.

Figure 9: The general landscape of Hereford farm, Mooiplaas, East London, Eastern Cape, South Africa

Figure 10: Mixed breeds of Bonsmara and Nguni cattle on Hereford farm, Mooiplaas, East London, Eastern Cape, South Africa.

- **Sandhurst**

The farm Sandhurst is situated in the Cefani Mouth area, 50 km outside of East London, Eastern Cape (32 ˚80', 89"S, 28 ˚13', 33"E). This farm has no fenced areas and cattle are allowed to graze freely in a landscape with long grasses, thorn bushes and some trees (Fig. 11). There is a gated area used to gather the cattle and guide them into a race for treatment against tick infestations (Fig. 12). This particular area of the farm is not maintained and has long grasses and an abundance of weeds. According to the producer, this area has the highest occurrence of bont-ticks (*Amblyomma* spp.). This farm is situated near the coast where the cattle would also visit. The producer mostly farmed beef extensively which consisted of a mixture of Bonsmara and Nguni breeds, that he has been crossbreeding for over 20 years, but the animals were however dominantly Bonsmara. Animals are treated with IVM three times a year, though moderate persistence of blue ticks has been noted following IVM treatment. Based on this, the development of tick resistance is suspected at Sandhurst farm.

Figure 11: The gated area where the cattle were kept in when treating Sandhurst farm, Cefani Mouth, East London, Eastern Cape, South Africa

Figure 12: Dominantly Bonsmara cattle from Sandhurst Farm, Cefani Mouth, East London, Eastern Cape, South Africa.

The farm Claypits is situated 20-30 km outside of Makhanda (Grahamstown), Eastern Cape, in the Coombs area (33 ˚ 31', 06" S, 26 ˚ 52', 56" E). The farm has 40 camps and animals are regularly rotated between different camps. Most of the camps were at a low altitude and mostly consisted of short grass, thorn bushes and thorn trees. The lower altitudes consisted of sweet grasses, whereas the higher altitudes consisted of sour grasses (Fig. 13). Animals spend most of their time in the camps in the lower altitudes areas. The producer only farms with Bonsmara cattle (Fig. 14). The producer has been using IVM for the last ten years. He treats his animals with IVM twice a year and additionally treats individual animals when heavy tick loads are experienced.

Ticks were collected from animals in three different camps on Claypits: Milkcow, Lonweni and Sheds. At least 50 ticks were collected from 10 animals in each camp and considered as three separate strains to determine difference in tick resistance to IVM obtained from each camp. The producer also couriered fourth collection of blue ticks from the Gavinhill camp to the PTRF laboratory for sampling, where tick resistance development to IVM were suspected.

Figure 13: The general landscape of the area these animals grazed in on the Claypits farm, Coombs, Grahamstown, Eastern Cape, South Africa.

Figure 14: Bonsmara cattle from all three groups, Claypits farm, Coombs, Grahamstown Eastern Cape, South Africa.

- **Forest View**

This farm is also situated 20-30 km outside of Makhanda (Grahamstown), Eastern Cape, in the Coombs area (33 ˚ 31', 06" S, 26 ˚ 52', 56" E) and is situated 3-5 km away from Claypits. The farm mostly consisted of open fields of short grasses and is surrounded by forest (Fig.15). The producer farmed extensively with oxen that were mixed breeds of Nguni and other cattle breeds (Fig. 16). The animals from each camp were also regularly moved between camps.

Ticks were collected from animals that had been kept in a camp for a week. This camp mostly consisted of open fields of grass and mostly had trees at higher altitudes. The camp also had a small dam where the cattle mostly gathered, and the dam is where the cattle feed containers were situated. The producer uses IVM once or twice a year and has not experienced problems with blue ticks on his animals after injecting his animals with IVM.

Figure 15: The general landscape of Forest View Farm, Coombs, Grahamstown, Eastern Cape, South Africa.

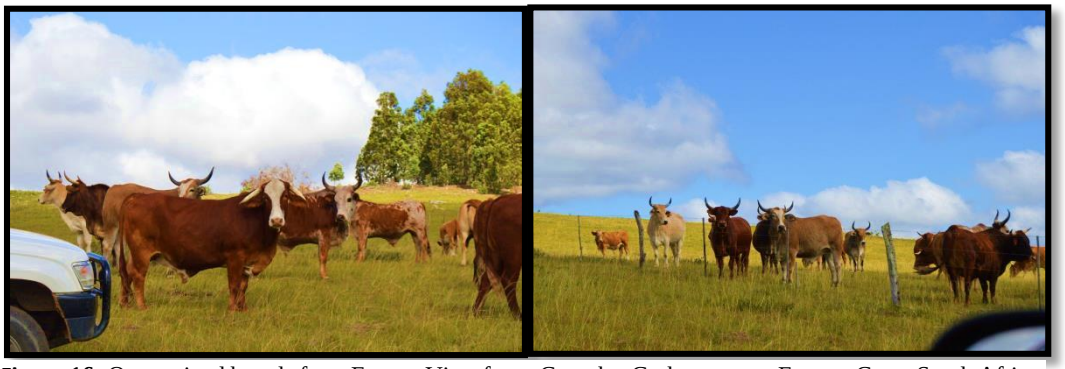


Figure 16: Oxen mixed breeds from Forrest View farm, Coombs, Grahamstown, Eastern Cape, South Africa.

- **Gilead**

This farm is also situated 20-30 km outside of Makhanda (Grahamstown), Eastern Cape, in the Coombs area (33 ˚ 31', 06" S, 26 ˚ 52', 56" E), 5km away from the Claypits. The group sampled was situated in a camp near the N2 highway, towards Port Alfred. The camps were also at high altitudes. The general landscapes of the camp consisted of open fields, short grasses, as well as many patches of thorn trees and bushes (Fig. 17). The producer farms with beef which consisted of mixed breeds of Bonsmara and Nguni cattle (Fig. 18). According to the producer, animals have been treated with IVM for over five years. Treatments are administered at least three to four times a year or when heavy tick loads were experienced.

Figure 17: The general landscape of the N2 camp at Gilead farm, Coombs, Grahamstown, Eastern Cape, South Africa.

Figure 18: Bonsmara and mixed breed cattle from Gilead farm, Coombs, Grahamstown, Eastern Cape, South Africa.

3.1.1.2 Farms with animals not exposed to IVM for the past five years

- **Hillside**

The farm Hillside is situated 5 km outside of the town of Hogsback (32˚59', 52" S, 26˚93'. 23" E). The general landscape of the grazing area consisted of open land with short grass, and was surrounded by many different aggregated trees, as well as many hills (Fig. 19). The farm was not divided into camps and the animals were allowed to graze throughout the whole area. The producer farms both dairy and beef animals which consisted mostly of Nguni cattle and mixed breeds (Fig. 20). He has not treated his animals with IVM for over five years and has not experienced heavy tick loads on his animals.

Figure 19: General landscape of Hillside farm, Hogsback, Eastern Cape, South Africa.

Figure 20: Nguni and mixed breeds from Hillside farm, Hogsback, Eastern Cape, South Africa.

- **Doringhoek**

This farm is situated 62 km from Queenstown (31˚91', 27" S, 26˚95', 97" E). The producer farms with beef (Nguni mixed breeds) and pork. The producer is relatively new to farming that has never treated his animals with IVM. He also has not experienced problems with heavy blue tick loads on his animals. The producer supplied us with fully engorged blue ticks and were therefore; the farm itself was not visited for tick collections.

- **Communal farms**

Dr. Nkululeko Nyangiwe (2018: personal communication) from the Doline Agricultural Development Institute provided two different *R. (B.) microplus* strains collected from a private farm (Mqombothi farm – NK 18/01 and a communal farm Sotho Village farm – NK 18/02) in the East London region (32˚64'76" S, 27˚97', 18"E). These strains were tested for resistance to Amidines, SPs and OPs and were found to be susceptible to these acaricides. According to Dr. Nyangiwe, there is no history of MLs use in these areas as they are not affordable for the owners of the animals.

3.1.2 Study methods

3.1.2.1 Field collection

3.1.2.1.1 Tick collection

Tick collections were done before application of any acaricide or MLs. A minimum of 20 ticks from at least 10 different animals were collected on each farm. These collections were considered to be Day 0 collections on farms where the animals were treated with MLs after collections were done. Follow up collection or evaluation of blue tick presence on the hosts were made seven days after treatment to determine if treatment had been successful as the ML product peaks in the blood of the animal during this period (Herd *et al.* 1999). For this purpose, animals from which the ticks were collected were identified by coloured /numbered ear tags or spray tags and photographic of identifiable markings or horn shapes were taken in cases where ear tags and spray tags were absent. Animals used for sampling were isolated from the herd until follow up tick collections. Follow up visits were not made to farms where MLs were not used.

Collected ticks were placed inside a plastic collection bottle, containing a paper towel to absorb any moisture and to prevent any damage while traveling. The lid of the bottle had small punctures to allow air exchange, but small enough not to allow ticks escaping. The collection bottles were labeled with the following information: the name of the farm, the collection date, the camp of collection (if applicable) and the breed of cattle.

A collection form (Appendix 3.1: PRTF M01) was completed for each sample and filed in the study file. Ticks were washed and identified at the temporary field laboratory using keys and descriptions (Walker *et al.* 2003). After identification, ca. 20 fully engorged female *R. (B).decoloratus* of each collection were placed in a conical incubation flask with a cotton wool stopper, marked with the information of the collection site as described on the collection bottles as well as the estimated hatch date (ca. 42 days after collection). The conical flasks were then placed in an incubation box which contained water and salt and was also lined with wet tissue paper to create a higher humidity in the container until they were transferred to incubation cabinets in the laboratory. The ticks that were not used for the study were identified and stored in politop vials filled with 70% ethanol. Unidentified collections were kept in a refrigerator in the temporary laboratory to prevent egg laying and identified at a later stage.

Upon return to the PRTF, the flasks with engorged female ticks, whose progeny was destined to be used for resistance testing, were transferred and then incubated in a humidity container at 25–29 degrees centigrade and >70% RH for oviposition (more or less 16-21 days post collection) and egg hatching (ca.42 post collection).

3.1.2.2 Laboratory handling of ticks

30 days after collection, the flasks were observed daily for egg hatching. The hatch date was considered to be the day when an estimated 70% of the larvae had hatched. Testing for resistance and method evaluation was done 16 and 21 days after the determined hatch date.

3.1.2.3 Shaw Larval Immersion Test (SLIT)

3.1.2.3.1 Preparation of test chemicals

Both Ecomectin and Ivomec Gold were used by producers in the Eastern Cape Province. Ecomectin was chosen for testing in the laboratory since both products have the same formulation and mode of action.

Ecomectin, a commercially available injectable containing 1% m/v IVM 1%, marketed by Agrivet was used for resistance testing using the pie plate method.

A stock solution of 0.1 % was prepared by adding 2 ml of the commercial remedy into 18 ml of twice-distilled water.

Seven concentrations (Table 2.2) ranging between 0.001-0. 0001562% were serially diluted from the stock solution to determine the mortality rates of the larvae to IVM as well as to be used to determine the Lethal Concentrations at 50% and 99 %.

3.1.2.3.2 SLIT – pie-plate method

The test methodology chosen for testing tick resistance to MLs in Chapter 2 was used to determine the presence or absence of resistance in the tick collections obtained from the different farms. Therefore the pie-plate SLIT methodology as described in 2.1.2.2.1 was used with twice-distilled water as diluent for the IVM dilutions and as control sample. Mortality was evaluated 24 hours after exposure.

3.1.2.3.3 Formulas and statistical analysis

- **Shaw Larval Immersion Test: pie-plate method**

After +24 hours, the enclosed envelopes were opened and ticks that were alive and died were counted. Only ticks that were able to walk were counted as being alive. The ticks that were alive were killed by pressing on them with a spatula while counting. The percentage mortality was then calculated according to Abbot's formula by making use of the % mortality of the water control to calculate corrected mortality and thereby take into account larvae that died due to factors other than chemical exposure:

$$\textbf{Corrected Mortality (CM)}\ \% = \frac{(\%\ \text{mortality sample} - \%\ \text{mortality of control sample})}{(100 - \%\ \text{mortality of control sample})} \times 100$$

- **Lethal concentration determination**

The Lethal concentration (LC) at 50% and 99 % was calculated using a software program called Polo Suite. This program uses the log dose probit analysis to determine the lethal concentration necessary to kill a certain percentage of a population (in this case 50 and 99% of the population) and to establish 95% confidence intervals (CI 95%) (Miller *et al.* 2010).

The LC50s and LC99s obtained from susceptible reference strains as shown in Table 2.5 were used to compare with the results found in this chapter.

- **Factor of Resistance (FR)**

The determination of resistance in a population needs to be measured against a LC50 values set, to indicate susceptibility, resistance and the emergence of resistance. The range that can be used to evaluate resistance in a population was set by comparing the factor of resistance (FR50) determined by dividing the LC 50 of the field sample with the LC 50 of the susceptible reference strain. It then gives an indication of the magnitude of the difference between the reference strain and the collected field strain. This value was combined with the inclusion or exclusion of the Confidence Interval 95% (CI95%) value range of the test collection into the CI95% range of the reference strain. The difference between the two strains was considered to be significant when the CI95% of the field strain was not included in the CI95% of the reference strain (Fernāndez-Salas *et al*. 2012). If the LC50 (CI95%) of a tick strain was found not to be statistical different from the reference strain, it was considered to be susceptible. Statistical

difference of the LC50 value (CI95%) of a tested strain from the reference strain was classified as emergence of resistance when the FR50 was <2 and resistant when the FR50 was ≥ 2 when using the categories proposed by Castro-Janer *et al.* (2011).

3.2 Results

3.2.1 Field collections

3.2.1.1 Tick collections

Ticks collected from cattle grazing on fields of the different farms are indicated in Table 3.1. Enough engorged female ticks (*R. (B.) decoloratus)* for testing could be collected from all the farms classified as farms with animals previously exposed to IVM except on the Hereford and Forest View farms. Although fewer than 10 adult ticks were found on cattle on Hereford farm, immature ticks could be felt on the skin of the animals when searching for adults. Cattle on Forest View farm only produced two ticks from all the animals investigated. Sandhurst produced 27, while the groups of cattle on Milkcow, Sheds and Loweni, produced more than 50. The other group, Gavinhill, was collected by the producer and delivered to the PRTF laboratory. More than 100 engorged females was collected from this group. Gilead produced 25 engorged females. Only African blue ticks females were collected from these farms and these collections took place shortly before the next treatment with IVM.

On the second visit to these farms, seven days after treatment with IVM, the number of engorged female ticks increased from fewer than 10 to 23 for Hereford, decreased from 27 to 18 on Sandhurst. The number of ticks collected from the Claypits camps decreased from more than 50 to two on Milkcow, four on Sheds and two on Lolweni. From animals on Forest View, engorged tick were not found. The Gilead producer failed to treat his animals after initial collections, hence 22 engorged female were collected. Farms not previously exposed to IVM for five years were only visited once. 24 engorged female *R. (B.) decoloratus* were collected on Hillside, and 55 on Doringhoek.

The only *R. (B.) microplus* females received for testing were from the Mqombothi and Sotho Village farms located in communal areas and was treated as animals not previously exposed to IVM for five years. More than 100 ticks were received from each of the two areas for IVM resistance testing.

Table 3.2: Number of ticks collected on animals before and after treatment with Ivermectin from Eastern Cape farms.

Farms with animals previously exposed to IVM				
Farms	**Before treatment**		**+7 Days after treatment**	
	***N* ticks collected**	**Camp**	***N* ticks collected**	**Camp**
Hereford	<10	*	23	Same camp
Sandhurst	27	-	18	-
Claypits	>50	Milkcow	2	Milkcow
	>50	Sheds	4	Sheds
	>50	Lonweni	2	Lonweni
	>100	Gavinhill	>100	Gavinhill
Forest view	2	*	0	Same camp
Gilead	25	-	22	-
Farms with animals not previously exposed to IVM for the last 5 years				
Hillside	24	-	NA	-
Doringhoek	55	-	NA	-
Mqombothi	>100	-	NA	-
Sotho Village	>100	-	NA	-

No camp name: * No camps: - NA: Not applicable

3.2.2 SLIT: Resistance testing

IVM resistance testing was performed on all tick collections where more than 15 engorged females were collected except for the farm Gilead as IVM treatment had not been executed after the first collection on this farm. Where more than one collection was made the mean of

the two results was calculated. Forest View was the only farm where on both occasion too few tick were found to be tested and was therefore assumed to be susceptible to IVM**.**

3.2.1.2 Lethal concentrations and factor of resistance

Figure 3.2: The Lethal concentrations of the field samples to Ivermectin for both blue tick species. The Factor of Resistance of the lethal concentration was calculated to determine the status of resistance of Eastern Cape field strains.

Previously in contact with IVM					
Strain	**LC50 (%)**	**CI95 (%)**	**LC99 (%)**	**CI95 (%)**	**FR50**
***R. (B.) dec*. Ref Strain**	**0.00003**	**0.000002-0.0001**	**0.4**	**0.02-0.2**	
Hereford	0.0001	00.00001-0.0003	0.001	0.0004-0.003	3.3
Sandhurst	0.0001	0.00004-0.0002	0.051	0.02-0.4	3.3
CP-Milkcow	0.00013	0.0001-0.0002	0.003	0.001-0.01	4.3
CP- Sheds	0.0002	0.0001-0.0003	0.005	0.002-0.04	7
CP-Lonweni	0.00013	0.0001-0.0002	0.002	0.001-0.003	4.3
CP-Gavinhill	0.00005	0.00002-0.0001	0.007	0.003-0.04	1.7
Forest View	-	-	-	-	-
Gilead	0.0001	0.00002-0.0001	0.04	0.01-0.04	3.3
Not exposed to IVM for at least 5 years					
Hillside	0.00003	0.000002-0.00006	0.001	0.0005-0.0042	1
Doringhoek	0.000003	0.000001-0.00001	0.02	0.0003-0.002	0.11
***R. (B.) mic*. Ref Strain**	**0.00001**	**0.000003-00001**	**0.2**	0.004-0.2	
NK 18/01	0.0001	0.0001-0.001	0.04	0.01-0.05	10
NK 18/02	0.00002	0.000004-0.0001	0.03	0.001-0.02	2

LC: Lethal Concentration FR: Factor of Resistance CP: Claypits CI95%: Confidence Interval 95%

Investigation of mortality counts of *R. (B.) decoloratus* showed that ticks from the farms not previously exposed to IVM; Doringhoek, and Hillside strains, showed a LC50 of 0.000003 (LC99: 0.02%), and 0.00003 (LC99: 0001%) respectively, (Table 3.2). These values were

equal to the LC50 of the susceptible reference strain in the case of Hillside and even lower in the case of Doringhoek and produced a FR50 of 1 (FR99:0.50) and 0.11 (FR99: 0.03) respectively.

Rhipicephalus (B.) decoloratus collections from farms previously exposed to IVM, produced varying results from an LC50 of 0.00005% for the strain GavinHill with a FR50 of 1.7, to 0.0001% for the strains Hereford, Sandhurst and Gilead, producing a FR50 of 3.3. (Table 3.2). The different camps investigated on the farm Claypits had tick collections with even higher LC50 values with Milkcow and Lolweni at 0.00013% producing a FR50 of 4.3 and the camp Sheds with an LC50 value of 0.0002% and a FR50 of as high as 7.

Table 3.2 also shows the lethal concentrations and factor of resistance of untreated field *R. (B.) microplus* strains from two communal farms in the Eastern Cape region. The NK 18/01 strain had a high LC50 of 0.0001% (LC99: 0.04) and FR50 of 10 (FR99: 0.2). The NK 18/02 strain had a lower LC 50 value of 0.00002 % (LC99: 0.04) and a FR50 of 2.

3.2.1.3 Factor of resistance range determination

3.2.1.3.1 *Rhipicephalus (Boophilus) decoloratus*

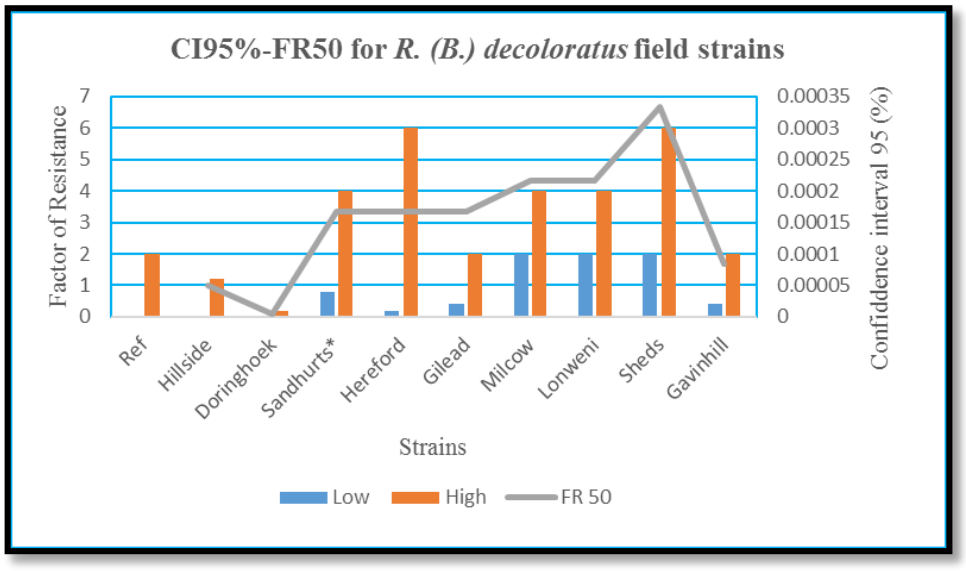


Figure 21: An overlap of the LC50 (CI95%) and FR50 of all the Eastern Cape strains collected for this study to establish a range of susceptibility and resistance for *R. (B.) decoloratus* strains.

Ranges for resistance development, as defined by Castro-Janer *et al.* (2011), were set to be susceptible if the LC50 (CL95%) of a tick strain was found not to be statistically different from

the reference strain. Therefore, Doringhoek (FR: 0.1), Hillside (FR: 1) and Gavinhill (FR: 1.7) can be considered to be susceptible to IVM. Statistical difference of the LC50 value (CI95%) of a tested strain from the reference strain with a FR50 value of <2 were classified as the emergence of resistance. Using this classification ranges none of the strains tested showed signs of development of emerging resistance. When there is a statistical difference of the LC50 value (CI95%) of a tested strain from the reference strain with a FR50 value of ≥ 2, it was classified as resistant. This placed, Sandhurst (FR: 3.3), Hereford (FR: 3.3), Milkcow (FR. 4.3), Lonweni (FR: 4.3) and Sheds (FR: 7) in the category classified as resistant to IVM.

3.2.1.3.2 *Rhipicephalus (Boophilus) microplus*

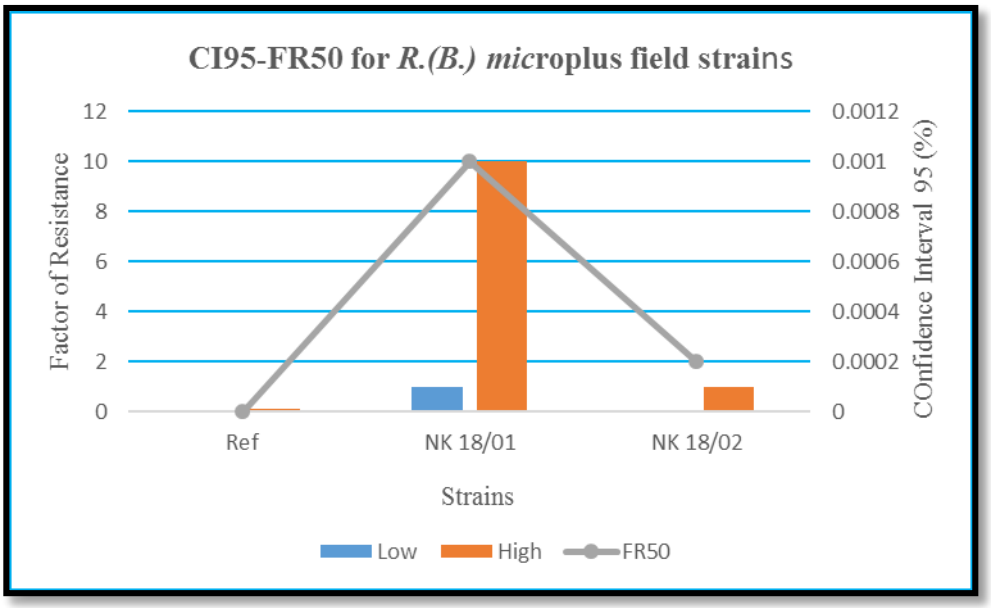


Figure 22: An overlap of the LC50 (CI95%) and FR50 of all the Eastern Cape communal farm strains collected for this study to establish a range of susceptibility and resistance for *R. (B.) microplus* strains.

According to the classification ranges defined by Castro-Janer *et al.* (2011) both NK 18/02 (Sotho Village) strain (FR: 2) and NK 18/01 (Mqombothi) strain (FR: 10) can be considered to be resistant to IVM).

3.3 Discussion

Resistance development of the *R. (B.) microplus* to MLs was reported in Brazil, Uruguay, Mexico and Australia (Sabatini *et al.* 2001; Klafke *et al.* 2006; Pérez-Cogollo *et al.* 2010). Possible factors responsible for resistance development to MLs can be one or a combination of

many aspects. The frequent use of MLs (Klafke *et al*. 2006; Castro-Janer *et al*. 2011; Klafke *et al.* 2012) which causes an overexposure of the chemical to the ticks over time can allow for evolutionary changes. Indiscriminate treatment by not weighing animals to be treated, according to weight and size as recommended, could also lead to overdosing or under dosing with the development of resistance (Abbas *et al*. 2014) as an outcome in the long run. Exposure to MLs as a secondary target organism during treatments for endoparasitic nematodes, as well as a lack of rotation with other acaricides with different modes of action to reduce the selection pressure for resistance, are also possible factors. The use of certain cattle breeds with animals that are less resistant to ticks, can lead to heavy tick loads and a greater exposure of tick population to the acaricides (Yessinou *et al.* 2016). Lack of integration of other methodologies such as rotation of the host's grazing areas or by keeping grazing areas free of cattle until the larvae starve and die (Abbas *et al*. 2014; Yessinou *et al.* 2016) can also have an influence.

All of these factors are also valid for South African producers, especially from the Eastern Cape Province who are perceiving a breakdown of control of blue tick species when treated with MLs. This perceived tick resistance development could not be confirmed up to now and therefore this study embarked on the journey to investigate the possible resistance of blue tick species to MLs on some farms in the Eastern Cape Province.

For this purpose, methodology was developed and LC50 and LC99 values were determined for susceptible reference strains of both *R. (B.) microplus* and *R. (B) decoloratus*, as described in Chapter two by using the pie-plate SLIT. Results of testing of tick collections from the different farms, were difficult to classify into the different resistant and susceptible groups as described by Castro-Janer *et al.* (2011). In the current study cattle from some of the farms were also followed up after 7 days to investigate if the IVM treatment was successful or not in lowering tick numbers.

Rhipicephalus (B.) decoloratus collected from two farms, Doringhoek and Hillside, not exposed to MLs for the last five years, were confirmed to be susceptible to IVM when compared to the reference strain with a RF50 of 1 and 0.1 respectively; and CI95% values that were statistically similar to those of the reference strains. On these farms, there were no selection pressures for resistance development to IVM, but there are always some members in a tick population with a higher tolerance to IVM that can build up tolerance during the period when they were exposed to IVM. However, these individuals are less prominent due to their lower numbers (Abbas *et al*. 2014; Yessinon *et al.* 2016). Over many generations the selection

for resistance to IVM will slowly decrease if IVM is still not used to control blue ticks (Abbas *et al.* 2014).

Field tick collections from farms near East London, Makhanda (Grahamstown), Hogsback and Queenstown were obtained. All of these commercial producers, except for the producers on the Hillside and Doringhoek farms, treated their animals with MLs two to three times a year depending on weather conditions such as the amount of rain and droughts. The producers tend not to use MLs or other acaricides when it does not rain for long periods. MLs were usually applied to their animals in March and April and then again between October and December to account for periods when blue ticks are prevalent. Short spells of rain during these periods will cause blue tick eggs to hatch and larvae to emerge on grasses to seek a host to feed on (Walker *et al*. 2003).

For endo-parasite control, the producer from Forest View farm treats animals with IVM (Ivermec Gold – effective for 75 days) once, or if necessary twice a year. This is in agreement with the advice given by his consultant on tick control due to high cost involved with treatment. Only a few partly engorged female ticks were found on his animals, though ticks were absent seven days after treatment. This caused no larvae to be available for testing but subjectively this could indicate that the blue ticks are still susceptible to IVM on his farm. Susceptibility can be confirmed if fully engorged ticks are available in future. Possible reasons for absence of resistance development to IVM on his farm, can be the low usage of IVM and the fact that the producer farms extensively with oxen consisting of Nguni mixed breeds. They are considered to be hosts resistant to blue ticks due to their short and smooth coats not protecting the ticks from ultra-violet rays. This makes it difficult for ticks to attach. Low cutaneous basophil and mast cell infiltrations (Marufu 2013) as well as frequent grooming, can lead to tick rejection.

Gilead farm and Gavinhill camp at Claypits farm were not followed up after treatment and the second tick collections were only tested for resistance. The producer from Gilead decided not to treat his cattle after tick collections was made. A FR 50 value of was 3.3, with no statistical difference to the reference strain (CI95%) were found when the *R. (B.) decoloratus* strain were tested. The same results were obtained from a collection made seven days later from the same 10 animals, indicating the repeatability of the results on two consecutive collections without treatment. The results were difficult to interpret as the classification of Castro-Janer *et al.* (2011) did not make provision for this combination of FR50 and similarity.

The tick collection from Gavinhill, was submitted by the producer after discovering a high tick load before treatment. Once tested, this strain presented with a FR50 of 1.7 and statistically the same as the reference strain (CI95%) that can be classified as susceptible. The seven day post-treatment visual evaluation done by the producer, still indicated a heavy tick load but the producer could not confirm that it was from the same animals from which the initial ticks were collected. This producer also tends to only treat individual animals with heavy tick burdens with IVM and not the whole herd each time heavy tick burdens are experienced. This might explain the lack of current resistance development in this strain together with the fact that this group of animals was also rotated between Gavinhill and two other camps depending on availability of water and food. The rotation might cause IVM emerging resistant larvae to starve due to a periodic lack of hosts. Resistance to OPs, Amitraz and SPs were proved in a master's study currently being conducted by Pottinger (2018: personal communication) due to exposure to these acaricides for a long-periods and frequent dipping.

Five collection areas were tested for IVM resistance and followed up seven days after treatment for visual inspection of tick presence and a repeat collection for resistance testing. The two strains that were suspected of being resistant to IVM was the Hereford and Sandhurst strains, as the producers complained of poor to moderate results after treating with IVM. Numbers of adult ticks on the selected animals were relatively low as on the rest of the cattle population. The fact that the animals were Nguni mix breeds might have helped in keeping the numbers low. The FR 50 was found to be 3.3 and the CI95% was statistically different to the reference strain for both strains, which indicated resistance to IVM. On Hereford this was also confirmed by the visual inspection 7 days after treatment. Less than 10 fully engorged females could be collected from the Hereford herd before treatment but after treatment 23 were found. This indicated that the larvae and nymphs present on the animals during treatment were still able to feed and molt into adults, able to further engorge to adults that are able to reproduce. Enough larvae were produced from these collected adults to be able to do resistance testing. Davey *et al.* (2010) stated that treatment with IVM lowers the index of fecundity as well as the engorgement weight and egg mass of treated ticks. This study did not determine the engorgement weight or the egg mass of collected ticks, however, visually it seemed to be similar to the reference strain but this must be further investigated in follow-up studies. The Sandhurst strain also fit the resistant result as there was no dramatic decline of ticks on the 7 day visual inspection and collection.

The producer from Claypits where three camps were investigated for tick resistance to IVM indicated that satisfactory results were found after treatment with IVM. The strains from; Milkcow, Lonweni and Sheds, were classified as resistant according to the classification ranges of Castro-Janer *et al.* (2011). All three strains displayed a CI95% that was statistically different to that of the reference strain and with a FR50 value of above two. Both Milkcow and Lonweni strains had a FR 50 value of 4.3, while the Sheds strain had a FR 50 value of seven. The visual inspection of all three cattle groups seven days after treatment, however, showed a decrease in tick numbers from >50 ticks collected to two in the case of Milkcow and Lonweni and four in the case of the Sheds camp. This visual observation contradict the resistance finding through testing results and needs to be explained and further investigated.

The crush used for spray dipping and IVM treatment is situated in one corner of the Sheds camp with the implication that cattle from all over the farm are gathered in this camp every two to three weeks for dipping. The cattle herds are usually kept in this camp on dipping days for anything from one to 8 hours. Drop off of both resistant and susceptible engorged female populations in this camp, originating from other camps is therefore possible. At the same time pick up of both susceptible and resistant larval populations is also possible. The Sheds camp might thus present with a mixture of individuals with different resistance levels to IVM. In the case of the Milkcow and Lonweni camps, resistant populations may have been picked up three weeks prior to the collection in Sheds area during dipping day that presented with the FR50 of above two when collected and tested. The ticks that were picked up seven days later while back in their grazing camps could then have been sensitive to IVM resulting in low numbers of ticks found a week after treatment. Animals kept in Sheds as their grazing camp were also exposed in the same way to both susceptible and resistant populations.

More samples will have to be collected as well, and importantly, a Factor of resistance range will have to be investigated for South African blue tick strains to accommodate such results.

Rhipicephalus. (B.) microplus collected from a private and communal farm near the East London area, namely, Mqombothi (NK 18/01) and Sotho Village (NK 18/02), respectively. Unexpectedly both presented with populations that could be classified as resistant with a FR50 value of 10 (Mqombothi) and 2 (Sotho Village) and with a CI95% that is statistically different to the reference strain. On the communal farm, samples were collected from different animals from different owners at a communal dip tank. Both strains were said to have never been exposed to IVM by their current owners as these chemicals are expensive according to

Nyangiwe (2018: personal communication). Communal producers typically use one or two acaricides that the whole group uses to treat their animals for ticks (Lovis *et al.* 2013). Although the current owners of the animals may not use IVM to treat their animals, many factors could have contributed to the development of resistance. Cattle could have been acquired by the current owners from commercial producers that may have used IVM frequently and they could also have been sold to the new owners with blue tick larvae or adults, resistant to IVM already on them. These strains were then able to reproduce and spread in these areas. Another reason could be that these animals might have been grazing in areas near to, or wandered off into nearby commercial farms which use IVM and larvae from those areas might have climbed onto these animals. Since the current owners do not use IVM to treat their animals for blue ticks or even endo parasites, over time resistance to IVM should decrease as there will be no selection pressure for resistance. Both the private and communal farms need to be investigated and more information should be gathered from the current owners and workers to be able determine where the cattle come from or where the animals generally graze to get a better idea of the situation.

In conclusion, this study indicated that by testing tick populations, making use of the pie plate SLIT, strains not exposed to IVM in the past five years, could be confirmed to be susceptible to IVM by making use of the classification ranges established by Castro-Janer *et al.* (2011). The two strains that were suspected of being resistant to IVM due to complaints of poor to moderate results after treating with IVM also fitted into these ranges to be classified as resistant although the overall tick counts on these herds were low, probably due to the Nguni breed that was used as hosts. Three of the tick strains collected from groups where the perception of good control was indicated and very low tick numbers were found after IVM treatment, however, also presented as resistant if measured against the criteria set by Castro-Janer *et al.* (2011). One farm presented with a set of criteria not classified by these authors.

As far as could be established, available literature provides no indication to why Castro-Janer *et al.* (2011) decided on a FR50 of 2 and higher to be an implication of resistance. It might be that in the case of the current study a FR50 of higher than 2 up to a yet undetermined value might actually only be considered to be classified as emergence of resistance. No other studies to our knowledge followed up on what happens after treatment to populations classified as resistant using these authors ranges.

More research on these ranges is needed in South Africa to determine when a classification of emerging resistance is valid and when a population can be classified as resistant. This study lay the foundation to enable further investigations into this critical important aspect to enable South African producers to be aware of when to explore alternative options in the event of tick resistance development to IVM on their farms.

3.4 References

ABBAS, R.Z., ZAMAN, M.A., COLWELL, D.A., GILLEARD, J. & RAJBUT, Z.I. 2014. Acaricide resistance in cattle ticks and approaches to its management: the state of play. *Veterinary Parasitology* **203**: 6-20.

BAKER, J.A.F., MILES, J.O., ROBERTSON, W.P., STANFORD, G.D. & TAYLOR, R.J. 1978. The current status of resistance to organophosphorus ixodicides by the blue tick, *Boophilus decoloratus* (Koch) in the Republic of South Africa and Transkei. *Journal of the South African Veterinary Association* **49**: 327-333.

CASTRO-JANER, E., RIFRAN, L., GONZĀLEZ, P., NEILL, C., PIAGGIO, J., GIL, A. & SCHUMAKER, T.T.S. 2011. Determination of the susceptibility of *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae) to ivermectin and fipronil by larval immersion test (LIT) in Uruguay. *Veterinary Parasitology* **178**: 148-155.

COETZEE, B.B., STANFORD, G.D, & DAVIS, D.A.T. 1987. Resistance by the blue tick (*Boophilus decoloratus*) to the synthetic pyrethroid, fenvalerate. *The Onderstepoort Journal of Veterinary Research* **54**: 83-86.

DAVEY, R.B., POUND, J.M., MILLER, J.A. & KLAVONS, J.A. 2010. Therapeutic and persistent efficacy of a long-acting (LA) formulation of ivermectin against *Rhipicephalu*s (*Boophilus*) *microplus* (Acari: Ixodidae) and sera concentration through time in treated cattle. *Veterinary Parasitology* **169**: 149-156.

DE CLERCQ, E.M., VANWAMBEKE, S.O., SUNGIRAI, M., ADEHAN, S., LOKOSSOU, R. & MADDER, M. 2012. Geographic distribution of the invasive cattle tick *Rhipicephalus microplus*, a country-wide survey in Benin. *Experimental and Applied Acarology* **58**: 441-452.

FOURIE, J.J., LIEBENBERG, J.E., NYANGIWE, N., AUSTIN, C., HORAK, I.G. & BHUSHAN, C. 2013. The Effects of a pour-on formulation of fluazuron 2.5 % and flumethrin 1 % on populations of *Rhipicephalus decoloratus* and *Rhipicephalus microplus* both on and off bovine (Bonsmara breed) hosts. *Parasitology Research* **112**: 67-79.

FERNĀNDEZ-SALAS, A., RODRÍQUEZ-VIVAS, R.I., ALANSO-DÍAZ, A.A. & BASURTO-CAMBEROS, H. 2012. Ivermectin resistance status and factors associated in

Rhipicephalus microplus (Acari: Ixodidae) populations from Veracruz, Mexico. *Veterinary Parasitology* **190**: 210-215.

GEARY, I.G. & MORENO, Y. 2012. Macrocylytic lactones anthelmintics: spectrum of activity and mechanism of action. *Current Pharmaceutical Biotechnology* **13**: 866-872.

HERD, P.P., SAMS, R.A. & ASCHCRAFT. 1996. Persistence of ivermectin in plasma and faeces following treatment of cows with ivermectin sustained-release, pour-on or injectable formulations. *International Journal for Parasitology* **26**: 1087-1093.

KLAFKE, G.M., SABATINI, G., DE ALBUQUERQUE, T.A., MARTINS, J.R., KEMP, D.H., MILLER, R.J. & SCHUMAKER, T.T.S. 2006. Larval immersion tests with ivermectin in populations of the cattle tick *Rhipicephalus* (*Boophilus*) *microplus* (Acari: Ixodidae) from State of Sao Paulo, Brazil. *Veterinary Parasitology* **142**: 386-390.

KLAFKE, G.M., DE ALBUQUERQUE, T.A., MILLER, R.J., SCHUMAKER, T.T.S. 2010. Selection of ivermectin-resistant strain of *Rhipicephalus microplus* (Acari: Ixodidae) in Brazil. *Veterinary Parasitology* **168**: 97-104.

KLAFKE, G.M., CASTRO-JANER, E., MENDES, M.C., NAMINDOME, A. & SHUMAKER, T.T.S. 2012. Applicability of in vitro bioassays for the diagnosis of ivermectin resistance in *Rhipicephalus microplus* (Acari: Ixodidae). *Veterinary Parasitology* **184**: 212- 220.

LOVIS, L., REGGI, J., BERGGOETZ, M., BETSCHART, B., & SAGER, H. 2013. Determination of acaricide resistance in *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae) field populations of Argentina, South Africa, and Australia with the Larval Tarsal Test. *Journal of Medical Entomology* **50**: 326-334.

MARUGU, M.C. 2013. Mechanisms of resistance to *Rhipicephalus* ticks in Nguni cattle reared in the semiarid areas of South Africa. PhD thesis, University of KwaZulu-Natal, South Africa.

MEKONNEN, S., BRYSON, N.R., FOURIE, L.J., PETER, R.J., SPICKETT, A.M., TAYLOR, R.J., STRYDOM, T. & HORAK, I.G. 2002. Acaricide resistance profiles of single- and multi- host ticks from communal and commercial farming areas in the Eastern Cape and North-West Provinces of South Africa. *Onderstepoort Journal of Veterinary Research* **69**: 99-105.

MEKONNEN, S., BRYSON, N.R., FOURIE, L.J., PETER, R.J., SPICKETT, A.M., TAYLOR, R.J., STRYDOM, T., KEMP, D.H. & HORAK, I.G. 2003. Comparison of 3 tests to detect acaricide resistance in *Boophilus decoloratus* on dairy farms in the Eastern Cape Province, South Africa. *Journal of the South African Veterinary Association* **74**: 41-44.

NYANGIWE, N., YAWO, M. & MUCHENJE, V. 2017. Driving forces for changes in geographic range of cattle ticks (Acari: Ixodidae) in Africa: A review. *South African Journal of Animal Science* **48**: 829-841.

MILLER, A.L.E., TINDOL, K. & LEONARD, B.R. 2010. Bioassays for monitoring insecticide resistance. *Journal of Visualized Experiments* **46**: 1-5.

NTONDINI, Z., VAN DALEN, E.M.S.P. & HORAK, I.G. 2008. The extent of acaricide resistance in 1-, 2- and 3-host ticks on communally grazed cattle in the eastern region of the Eastern Cape Province, South Africa. *Tydskrif van die Suid-Afrikaanse Veterinere Vereniging* **79**: 130-135.

NYANGIWE, N. 2018. (Researcher, Doline Agricultural Development Institute, Steerheim, Eastern Cape).

PÉREZ-COGOLLO, L.C., RODRÍQUEZ-VIVAS, R.I., RAMIREZ-CRUZ, G.I. & MILLER, R.J. 2010. First report of the cattle tick, *Rhipicephalus microplus* resistant to ivermectin in Mexico. *Veterniary Parasitology* **168**: 165-169.

POHL, P.C., KLAFKE, G.M., CARVELHO, D.D., MARTINS, J.R., DAFFRE, S., DA SILVA VAZ, I Jr. & MASUDA, A. 2011. ABC transporter efflux pumps: defense mechanisms against ivermectin in *Rhipicephalus* (*Boophilus*) *microplus. International Journal for Parasitology* **41**: 1323-1333.

RODRÍGUEZ-VIVAS, R.I., JONSSON, N.N. & BHUSHAN, C. 2018. Strategies for the control of *Rhipicephalus microplus* ticks in a world of conventional acaricide and macrocyclic lactone resistance. *Parasitology Research* **117**: 3-19.

POTTINGER, M. 2018. (Masters student, University of the Free State, Bloemfontein, Free State).

SABATINI, G.A., KEMP, D.H., HUGHS, S., NARI, A. & HANSEN, J. 2001. Tests to determine LC50 and discriminating doses for macrocyclic lactones against the cattle tick, *Boophilus microplus*. *Veterinary Parasitology* **95**: 53-62.

TØNNESEN, M.H., PENZHORN, B.L., BRYSON. N.R., STOLTSZ, W.H. & MASIBIGIRI, T. 2004. Displacement of *Boophilus decoloratus* by *Boophilus microplus* in the Soutpansberg region, Limpopo Province, South Africa. *Experimental and Applied Acarology* **32**: 199-208

WALKER, A.R., BOUATTOUR, A., CAMICAS, J.-L., ESTRADA-PEÑA, A., HORAK, I.G., LATI, A.A., PEGRAM, R.G. & PRESTON, P.M. 2003. *Ticks of domestic animals in Africa: A guide to identification of species*. Edinburgh: Bioscience reports**.**

YESSINON, R.E., AKPO, Y., ADOLOGBE, C., ADINEI, J., ASSOGBA, M.N., KOUTINHOUIN, B., KARIM, I.Y.A. & FAROUGON, S. 2016. Resistance of tick *Rhipicephalus microplus* to acaricides and control strategies. *Journal of Entomology and Zoology Studies* **3**: 408-414.

ZEMAN, P. & LYNEN, G. 2010. Conditions for stable parapatric coexistence between *Boophilus decoloratus* and *B. microplus* ticks: A simulation study using the competitive Lotka-Volterra model. *Experimental and Applied Acarology* **52**:409-426.

Appendix 1: PTRF M03

Producer questionnaire

Resistance of the African blue tick (*Rhipicephalus* (*Boophilus*) *decoloratus*) to Macrocyclic lactones in the Eastern Cape, South Africa.

Student: Lesenyeho S.K. (2010035356): Cell no: 074 218 3719 Email: Kenny.lesenyeho@gmail.com

Supervisor: E van Dalen Cell no: 083 388 5539 Email: vdalenem@ufs.ac.za

Department of Zoology and Entomology, University of the Free State, P.O. Box 339, Bloemfontein 9300, South Africa

Good day

My name is Kenny Lesenyeho, a **Master's** student at the University of the Free State. I am currently working on African blue tick resistance to Macrocyclic lactones, specifically Ivermectin.

The **aim** of my study is to develop a method to detect resistance to Ivermectin in blue tick control as well as the influence of regular Ivermectin use on internal parasites.

For this purpose, I want to make use of questionnaires to identify:

- Four farms that frequently use Ivermectin for tick control (more than 5 times per year) or experience lack of tick control by Ivermectin treatment.
- Four farms that do not use Ivermectin for tick control, to act as control farms for the study and for this purpose cattle must not be treated with Ivermectin more than twice a year.

If your farm is **found to be suitable** for this study and you are **willing to participate**, the study will entails the following:

I will have to visit the farm twice, at a time convenient to you and organized with you in advance to

- Collect tick, blood and dung samples from at least 10 animals from each farm during a normal gathering up of your animals for dipping or treatment actions.
- A second collection of tick, blood and dung samples will then have to be collected 8 -10 days after the first collection from the **same ten** animals.

These samples will be used to **investigate the following** aspects:

- Testing of ticks for Ivermectin resistance as well as routine testing for resistance to three other acaricides (Amitraz, Pyrethroids and Organophosphates) to compile a resistance profile for the specific farm.
- Determination of Ivermectin concentration in blood and dung samples to determine the persistence of the drug in the blood and dung after treatment.

These tests are worth about R4 000 per farm and will be done at our expense. If you are willing to participate, the results will be made available to you free of charge. Results might be beneficial to you in planning your fight against tick resistance to chemical control

If you are interested in taking part in this study, please fill out the attached questionnaire and submit the completed document to my email address, so that we can determine if your farm qualifies. None of your information will be shared with anyone other than myself and my study supervisor, Me Ellie van Dalen.

If you know of any other producers in the Eastern Cape that might benefit from this study, please feel free to also forward the questionnaire to them.

I will get into contact with you early in 2017 to organize further actions if your farm is chosen.

I will really appreciate your participation and if you have any further questions with regards to the study, feel free to contact me or my supervisor and we will gladly provide you with whatever information you need.

Looking forward to hearing from you!

Regards

_____

Lesenyeho S.K. (Kenny) (2010035356): Cell no: 074 218 3719 Email: Kenny.lesenyeho@gmail.com

Supervisor:

_____

EMS van Dalen (Ellie)
Office no: 0514013271 Cell no: 0833885539 Email: vdalenem@ufs.ac.za

UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA

UFS·UV
NATURAL AND AGRICULTURAL SCIENCES
NATUUR- EN LANDBOUWETENSKAPPE
CHEMISTRY
CHEMIE

Questionnaire to determine suitability of farm for Ivermectin resistance study

Personal Information:
Name:
Farm name:
Telephone number:
Cell phone number:
Email address:
Address of farm:
Nearest town:
Type of farming (dairy/ beef, intensive/extensive):
Dipping history:
1) Present dip used:
2) Spray race or plunge dip:
3) Period of use:
4) Previous dips used (within last five years at least):
5) Approximate number of dip treatments per year:
6) Date of last dipping:
Pour – on history:
1) Present pour-on used:
2) Period of use:
3) Previous pour-on used (within last five years at least):
4) Approximate number of applications per year:
5) Date of last application:

Macrocyclic lactone history:
1) Present injectable used: (Ivomec, Ecomectin, Dectomax etc):
2) Period of use:
3) Previous injectables used (within last five years):
4) Number of applications per year:
5) Date of last application:
6) Present oral Macrocyclic lactones used for deworming (E.g. Ivomec SR Bolus):
7) Previous oral macrocyclic lactones used:
8) Date of last oral ivermectin dose:
Perceived effectiveness of tick treatment
1) After dipping, control of blue ticks is poor/moderate/excellent:
2) After dipping, control of multi host ticks is poor/moderate/excellent:
3) After pour-on application, control of blue ticks is poor/moderate/excellent:
4) After pour-on application, control of multi-host ticks is poor/moderate/excellent:
5) After injectable treatment, control of blue ticks is poor/moderate/excellent:
6) Incidence of tick borne disease is low/ high. (If high please state whether red water/heart water/gall sickness):

I _____hereby state that I would like to participate in the study "**Resistance of the African blue tick (*Rhipicephalus* (*Boophilus*) *decoloratus*) to Macrocyclic lactones in the Eastern Cape, South Africa."**

_____ _____

Sign **Date**

Please email the completed questionnaire to Kenny lesenyeho kennylesenyeho@gmail.com or Ellie van Dalen vdalenem@ufs.ac.za

Appendix 2: Producer consent - PRTF M04

Farms with animals previously exposed to IVM

Consent for from Dr Nolan Weyer (Hereford farm)

I, Dr N.R.Weyer hereby state that I would like to participate in the study "**Resistance of the African blue tick (*Rhipicephalus* (*Boophilus*) *decoloratus*) to Macrocyclic lactones in the Eastern Cape, South Africa.**"

N.R.Weyer — **26th October, 2017**

Sign — **Date**

Please email the completed questionnaire to Kenny lesenyeho kennylesenyeho@gmail.com or Ellie van Dalen vdalenem@ufs.ac.za

Consent form from Mr Charles Hartley (Sandhurst farm)

I CHARLES HARTLEY hereby state that I would like to participate in the study "**Resistance of the African blue tick (*Rhipicephalus* (*Boophilus*) *decoloratus*) to Macrocyclic lactones in the Eastern Cape, South Africa.**"

Sign — 24/10/2017 Date

Please email the completed questionnaire to Kenny lesenyeho kennylesenyeho@gmail.com or Ellie van Dalen vdalenem@ufs.ac.za

Consent form from Mr Glyn Dixon (Claypits farm)

I Glyn Lawrence Dixon hereby state that I would like to participate in the study "**Resistance of the African blue tick (*Rhipicephalus* (*Boophilus*) *decoloratus*) to Macrocyclic lactones in the Eastern Cape, South Africa.**"

Sign

21 March 2017.
Date

Please email the completed questionnaire to Kenny Iesenyeho kennyIesenyeho@gmail.com or Ellie van Dalen vdalenem@ufs.ac.za

Consent form from Mr Richard Muir (Forest View farm)

I Richard Muir hereby state that I would like to participate in the study "**Resistance of the African blue tick (*Rhipicephalus* (*Boophilus*) *decoloratus*) to Macrocyclic lactones in the Eastern Cape, South Africa.**"

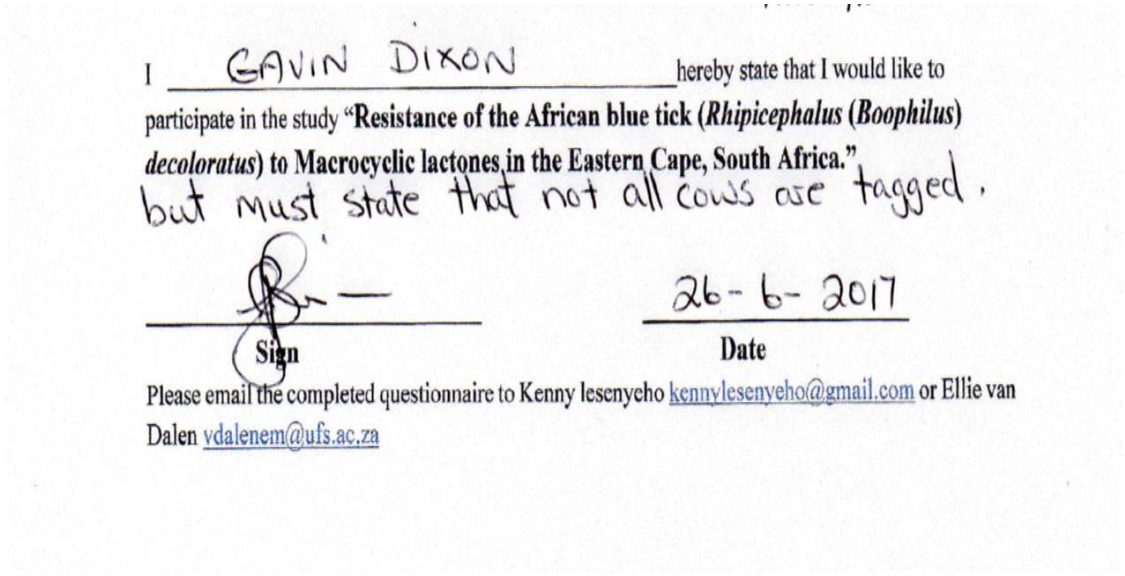
Sign

30.3.2017.
Date

Please email the completed questionnaire to Kenny Iesenyeho kennylesenyeho@gmail.com or Elli van Dalen vdalenem@ufs.ac.za

8.28 x 11.69 in

Consent form from Mr Gavin Dixon (Gilead farm)



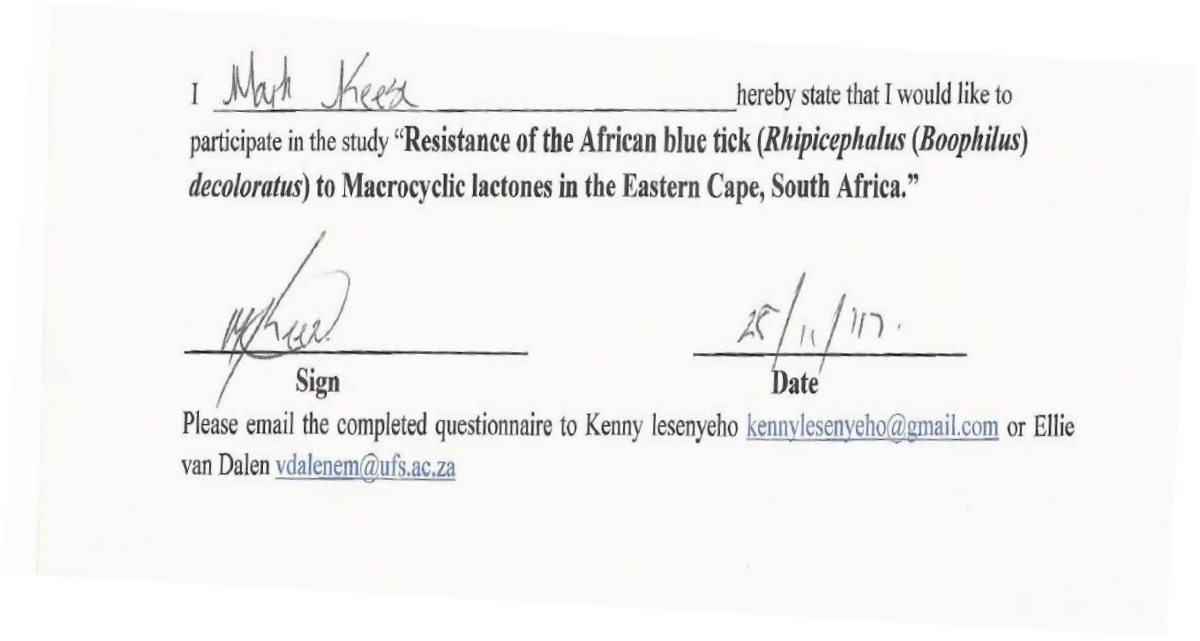
I GAVIN DIXON hereby state that I would like to participate in the study "**Resistance of the African blue tick (*Rhipicephalus* (*Boophilus*) *decoloratus*) to Macrocyclic lactones in the Eastern Cape, South Africa.**"
but must state that not all cows are tagged.

Sign 26-6-2017 Date

Please email the completed questionnaire to Kenny lesenyeho kennylesenyeho@gmail.com or Ellie van Dalen vdalenem@ufs.ac.za

Farms with animals not exposed to IVM for the past 5 years

Consent form from Mr Mark Keese (Hillside farm)



I Mark Keese hereby state that I would like to participate in the study "**Resistance of the African blue tick (*Rhipicephalus* (*Boophilus*) *decoloratus*) to Macrocyclic lactones in the Eastern Cape, South Africa.**"

Sign 25/11/17. Date

Please email the completed questionnaire to Kenny lesenyeho kennylesenyeho@gmail.com or Ellie van Dalen vdalenem@ufs.ac.za

Consent form from Mr Kevin Webster (Doringboom Farm)

I _____Kevin Webster_____ hereby state that I would like to participate in the study "Resistance of the African blue tick (*Rhipicephalus* (*Boophilus*) *decoloratus*) to Macrocyclic lactones in the Eastern Cape, South Africa."

16.02.017

_____ _____

Sign Date

Appendix 3: PTRF M01

Appendix Table 3.1*:* Accountibility Form

Tick Collection								
Reference Number	Collection Date	Signature	Farm	Estimated hatch date	Actual hatch date	Test Date	Ticks in ETOH

Appendix 4: Shaw Larval Immersion Test

Farms with animals previously exposed to IVM before treatment

Appendix Table 4. 1: Sandhurst

Acaricide	Remedy	Conc	Total	Alive	Dead	Total	Alive	Dead	Mortality	Corrected mortality *
Ivermectin	Ecomectin	0.01	134	0	134	82	0	82	**100.0**	100.0
Ivermectin	Ecomectin	0.005	86	4	82	91	3	88	**96.0**	95.6
Ivermectin	Ecomectin	0.0025	123	11	112	135	18	117	**88.8**	87.6
Ivermectin	Ecomectin	0.00125	146	23	123	181	35	146	**82.3**	80.4
Ivermectin	Ecomectin	0.000625	180	49	131	189	67	122	**68.6**	65.3
Ivermectin	Ecomectin	0.000313	181	68	113	239	74	165	**66.2**	66.2
Ivermectin	Ecomectin	0.000156	139	52	87	206	88	118	**59.4**	59.4
Control	dH2O		131	117	14	123	113	10	**9.4**	

Appendix Table 4. 2: Claypits - Milkcow

Acaricide	Remedy	Conc	Total	Alive	Dead	Total	Alive	Dead	Mortality	Corrected mortality *
Ivermectin	Ecomectin	0.01	127	0	127	162	0	162	**100.0**	100.0
Ivermectin	Ecomectin	0.005	134	0	134	157	0	157	**100.0**	100.0
Ivermectin	Ecomectin	0.0025	113	0	113	122	0	122	**100.0**	100.0
Ivermectin	Ecomectin	0.00125	142	0	142	133	2	131	**99.3**	99.2
Ivermectin	Ecomectin	0.000625	141	17	124	166	18	148	**88.6**	87.8
Ivermectin	Ecomectin	0.000313	128	17	111	163	51	112	**76.6**	76.6
Ivermectin	Ecomectin	0.000156	159	48	111	222	104	118	**60.1**	60.1
Control	dH2O		135	122	13	274	260	14	**6.6**	

Appendix Table 4.3: Claypits - Sheds

Acaricide	Remedy	Conc	Total	Alive	Dead	Total	Alive	Dead	Mortality	Corrected mortality *
Ivermectin	Ecomectin	0.01	132	0	132	171	0	171	**100.0**	100.0
Ivermectin	Ecomectin	0.005	134	0	134	155	0	155	**100.0**	100.0
Ivermectin	Ecomectin	0.0025	167	0	167	138	0	138	**100.0**	100.0
Ivermectin	Ecomectin	0.00125	145	3	142	117	0	117	**98.9**	98.8
Ivermectin	Ecomectin	0.000625	286	33	253	263	91	172	**77.4**	76.4
Ivermectin	Ecomectin	0.000313	292	148	144	257	92	165	**56.3**	56.3
Ivermectin	Ecomectin	0.000156	198	91	107	148	71	77	**53.2**	53.2
Control	dH2O		461	448	13	409	383	26	**4.5**	

Appendix Table 4.4: Claypits - Lonweni

Acaricide	Remedy	Conc	Total	Alive	Dead	Total	Alive	Dead	Mortality	Corrected mortality *
Ivermectin	Ecomectin	0.01	183	0	183	219	0	219	**100.0**	100.0
Ivermectin	Ecomectin	0.005	169	0	169	175	0	175	**100.0**	100.0
Ivermectin	Ecomectin	0.0025	142	0	142	133	0	133	**100.0**	100.0
Ivermectin	Ecomectin	0.00125	166	4	162	98	0	98	**98.5**	98.4
Ivermectin	Ecomectin	0.000625	96	9	87	111	6	105	**92.8**	92.2
Ivermectin	Ecomectin	0.000313	166	13	153	187	47	140	**83.0**	83.0
Ivermectin	Ecomectin	0.000156	194	71	123	141	68	73	**58.5**	58.5
Control	dH2O		185	172	13	125	116	9	**7.1**	

Appendix Table 4.5: Claypits -Gavenhill

Acaricide	Remedy	Conc	Total	Alive	Dead	Total	Alive	Dead	Mortality	Corrected mortality *
Ivermecti	Ecomectin	0.01	121	0	121	209	0	209	**100.0**	100.0
Ivermecti	Ecomectin	0.005	129	0	129	174	0	174	**100.0**	100.0
Ivermecti	Ecomectin	0.0025	113	0	113	187	4	183	**98.7**	98.6
Ivermecti	Ecomectin	0.00125	155	13	142	173	6	167	**94.2**	93.8
Ivermecti	Ecomectin	0.000625	175	13	162	195	26	169	**89.5**	88.6
Ivermecti	Ecomectin	0.000313	156	22	134	182	37	145	**82.5**	82.5
Ivermecti	Ecomectin	0.000156	196	42	154	145	32	113	**78.3**	78.3
Control	dH2O		465	436	29	262	239	23	**7.2**	

Appendix Table 4.6: Gilead 01

Acaricide	Remedy	Conc	Total	Alive	Dead	Total	Alive	Dead	Mortality	Corrected mortality *
Ivermecti	Ecomectin	0.01	129	2	127	96	4	92	**97.3**	97.0
Ivermecti	Ecomectin	0.005	144	17	127	128	6	122	**91.5**	90.5
Ivermecti	Ecomectin	0.0025	122	9	113	120	11	109	**91.7**	90.7
Ivermecti	Ecomectin	0.00125	123	14	109	115	23	92	**84.5**	82.6
Ivermecti	Ecomectin	0.000625	169	46	123	186	39	147	**76.1**	73.1
Ivermecti	Ecomectin	0.000313	121	31	90	163	44	119	**73.6**	73.6
Ivermecti	Ecomectin	0.000156	184	86	98	161	52	109	**60.0**	60.0
Control	dH2O		146	129	17	129	116	13	**10.9**	

Appendix Table 4.7: Gilead 02

Acaricide	Remedy	Conc	Total	Alive	Dead	Total	Alive	Dead	Mortality	Corrected mortality *
Ivermecti	Ecomectir	0.01	163	0	163	149	0	149	**100.0**	100.0
Ivermecti	Ecomectir	0.005	68	0	68	174	0	174	**100.0**	100.0
Ivermecti	Ecomectir	0.0025	81	0	81	167	1	166	**99.6**	99.6
Ivermecti	Ecomectir	0.00125	125	2	123	155	3	152	**98.2**	98.1
Ivermecti	Ecomectir	0.000625	156	19	137	195	37	158	**84.0**	82.8
Ivermecti	Ecomectir	0.000313	192	33	159	168	26	142	**83.6**	83.6
Ivermecti	Ecomectir	0.000156	241	54	187	180	37	143	**78.4**	78.4
Control	dH2O		412	381	31	459	427	32	**7.2**	

Farms with animals previously exposed to IVM after treatment

Appendix Table 4.8: Hereford

Acaricide	Remedy	Conc	Total	Alive	Dead	Total	Alive	Dead	Mortality	Corrected mortality *
Ivermecti	Ecomectir	0.01	77	0	77	134	0	134	**100.0**	100.0
Ivermecti	Ecomectir	0.005	92	0	92	117	0	117	**100.0**	100.0
Ivermecti	Ecomectir	0.0025	73	0	73	144	0	144	**100.0**	100.0
Ivermecti	Ecomectir	0.00125	97	0	97	112	0	112	**100.0**	100.0
Ivermecti	Ecomectir	0.000625	86	0	86	110	2	108	**99.0**	98.9
Ivermecti	Ecomectir	0.000313	164	18	146	188	40	148	**83.5**	83.5
Ivermecti	Ecomectir	0.000156	114	22	92	142	26	116	**81.3**	81.3
Control	dH2O		150	141	9	255	242	13	**5.4**	

Appendix Table 4.9: Sandhurst

Acaricide	Remedy	Conc	Total	Alive	Dead	Total	Alive	Dead	Mortality	Corrected mortality *
Ivermectii	Ecomectin	0.01	143	0	143	138	0	138	**100.0**	100.0
Ivermectii	Ecomectin	0.005	155	1	154	123	2	121	**98.9**	98.8
Ivermectii	Ecomectin	0.0025	137	3	134	134	7	127	**96.3**	96.0
Ivermectii	Ecomectin	0.00125	159	6	153	192	14	178	**94.3**	93.8
Ivermectii	Ecomectin	0.000625	162	28	134	174	33	141	**81.8**	80.3
Ivermectii	Ecomectin	0.000313	253	69	184	190	48	142	**73.6**	73.6
Ivermectii	Ecomectin	0.000156	176	55	121	218	67	151	**69.0**	69.0
Control	dH2O		266	248	18	123	111	12	**7.7**	

Appendix Table 4.10: Claypits - Gavinhill

Acaricide	Remedy	Conc	Total	Alive	Dead	Total	Alive	Dead	Mortality	Corrected mortality *
Ivermectii	Ecomectin	0.01	151	0	151	177	0	177	**100.0**	100.0
Ivermectii	Ecomectin	0.005	241	0	241	217	0	217	**100.0**	100.0
Ivermectii	Ecomectin	0.0025	168	0	168	134	0	134	**100.0**	100.0
Ivermectii	Ecomectin	0.00125	297	10	287	327	19	308	**95.4**	95.0
Ivermectii	Ecomectin	0.000625	128	3	125	119	6	113	**96.4**	96.4
Ivermectii	Ecomectin	0.000313	263	46	217	125	14	111	**84.5**	84.5
Ivermectii	Ecomectin	0.000156	394	82	312	328	74	254	**78.4**	78.4
Control	dH2O		469	439	30	211	189	22	**7.6**	

Farms with animals not exposed to IVM for the past 5 years

Appendix Table 4.11: Hillside

Acaricide	Remedy	Conc	Total	Alive	Dead	Total	Alive	Dead	Mortality	Corrected mortality *
Ivermecti	Ecomectir	0.01	117	0	117	141	0	141	**100.0**	100.0
Ivermecti	Ecomectir	0.005	103	0	103	122	0	122	**100.0**	100.0
Ivermecti	Ecomectir	0.0025	132	0	132	121	0	121	**100.0**	100.0
Ivermecti	Ecomectir	0.00125	142	0	142	86	0	86	**100.0**	100.0
Ivermecti	Ecomectir	0.000625	132	2	130	128	6	122	**96.9**	96.6
Ivermecti	Ecomectir	0.000313	127	9	118	115	3	112	**95.0**	95.0
Ivermecti	Ecomectir	0.000156	138	18	120	140	4	136	**92.1**	92.1
Control	dH2O		200	179	21	141	129	12	**9.7**	

Appendix Table 4.12: Doringhoek

Acaricide	Remedy	Conc	Total	Alive	Dead	Total	Alive	Dead	Mortality	Corrected mortality *
Ivermecti	Ecomectir	0.005	118	0	118	133	1	132	**99.6**	99.6
Ivermecti	Ecomectir	0.00125	256	35	221	195	43	152	**82.7**	80.7
Ivermecti	Ecomectir	0.000313	131	25	106	157	28	129	**81.6**	79.5
Ivermecti	Ecomectir	0.000078	231	66	165	181	45	136	**73.1**	70.0
Ivermecti	Ecomectir	0.0000195	129	34	95	234	88	146	**66.4**	66.4
Ivermecti	Ecomectir	0.0000098	170	68	102	142	44	98	**64.1**	64.1
Ivermecti	Ecomectir	0.0000049	151	68	83	126	44	82	**59.6**	59.6
Control	dH2O		208	187	21	181	162	19	**10.3**	

Appendix Table 4.13: Mqombothi NK 18/01

Acaricide	Remedy	Conc	Total	Alive	Dead	Total	Alive	Dead	Mortality	Corrected mortality *
Ivermecti	Ecomectin	0.005	237	18	219	217	24	193	**90.7**	90.0
Ivermecti	Ecomectin	0.00125	239	56	183	231	47	184	**78.1**	76.2
Ivermecti	Ecomectin	0.000313	172	47	125	181	51	130	**72.2**	69.9
Ivermecti	Ecomectin	0.000078	215	92	123	212	89	123	**57.6**	54.0
Ivermecti	Ecomectin	1.95E-05	495	368	127	347	209	138	**31.5**	#REF!
Ivermecti	Ecomectin	9.8E-06	536	374	162	517	438	79	**22.9**	#REF!
Ivermecti	Ecomectin	4.9E-06	394	336	58	652	551	101	**15.2**	#REF!
Control	dH2O		299	272	27	425	395	30	**7.9**	

Appendix Table 4.14: Sotho Village (NK 18/02)

Acaricide	Remedy	Conc	Total	Alive	Dead	Total	Alive	Dead	Mortality	Corrected mortality *
Ivermecti	Ecomectin	0.005	158	17	141	186	11	175	**91.9**	91.2
Ivermecti	Ecomectin	0.00125	149	15	134	145	31	114	**84.4**	83.0
Ivermecti	Ecomectin	0.000313	228	95	133	368	136	232	**61.2**	57.9
Ivermecti	Ecomectin	0.000078	241	112	129	145	55	90	**56.7**	53.0
Ivermecti	Ecomectin	1.95E-05	488	222	266	315	128	187	**56.4**	#REF!
Ivermecti	Ecomectin	9.8E-06	124	46	78	195	97	98	**55.2**	#REF!
Ivermecti	Ecomectin	4.9E-06	444	322	122	430	278	152	**31.4**	#REF!
Control	dH2O		355	337	18	287	254	33	**7.9**	