Efficacy of fipronil against *Amblyomma hebraeum* ticks on boer goats and detection of *Ehrlichia ruminantium* in ticks in Limpopo Province, South Africa

by

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Declaration

I, Johan Gerhard Nel, hereby declare that this dissertation, which I hereby submit for the Master of Science degree in the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, to be my own work and has not been previously submitted by me for degree purposes at another tertiary institution.

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Johan Gerhard Nel

31 October 2018

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List of abbreviations

µl	microliter
Bp	Base pair
CG	control group
D0	day 0 of trial
D4	day 4 of trial
D7	day 7 of trial
DAFF	Department of Agriculture Forestry and Fisheries
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DoH	Department of Health
FTA cards	Flinders Technology Associates cards
GABA	gamma amino butyric acid
H_2O	chemical formula for water
kg	kilogram
map1	major antigenic protein 1
mg	milligram
ml	milliliter
MLST	multi-locus sequence typing
ORFs	open reading frames
PCR	Polymerase chain reaction
SNPs	Single Nucleotide Polymorphisms
TG	treatment group
Th 1	T- helper cells 1
VCC	Veterinary Clinical Committee
WAAVP	World Association for the Advancement of Veterinary Parasitology

Summary

Efficacy of fipronil against *Amblyomma hebraeum* ticks on boer goats and detection of *Ehrlichia ruminantium* in ticks in Limpopo Province, South Africa

by

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Promoter: **Prof Frans Jongejan**

Degree: **MSc (Tropical Animal Health)**

Department: **Veterinary Tropical Diseases**

Heartwater, caused by *Ehrlichia ruminantium,* causes significant economic losses to commercial small stock farmers, including commercial boer goat farmers in the Limpopo Province of South Africa. Heartwater is endemic in this region where *Amblyomma hebraeum*, the South African bont tick and the vector for this disease occurs. Due to their long mouthparts and specific attachment sites on goats, *A. hebraeum* is also associated with wounds, secondary abscess formation and lameness. Small stock farmers in the Limpopo Province employ various methods to control *A. hebraeum* and the negative effects associated with this tick, including heartwater.

Two approaches where followed to focus on the control of *A. hebraeum* on the one hand and the detection and cryo-preservation of *E. ruminantium* from *A. hebraeum* on the other hand. The aim of this study was therefore twofold. By firstly determining the efficacy of a 1.0% fipronil pour-on solution against *A. hebraeum* by performing a therapeutic- and persistent efficacy trial and secondly to detect and cryo-preserve *E. ruminantium* from *A. hebraeum* ticks from a heartwater endemic region in the Limpopo province of South Africa.

It was demonstrated that a 1.0% fipronil pour-on solution is 100% effective and has a persistent efficacy of seven days against *A. hebraeum,* but only if a targeted treatment approach is followed whereby the pour-on solution is applied to the predilection sites of *A. hebraeum*. A 1.0% fipronil pour-on solution can therefore be used by boer goat farmers to control *A. hebraeum* and the negative effects associated with this tick.

In order to detect and cryo-preserve *E. ruminantium* from *A. hebraeum* ticks, ground-up-tick-supernatant samples were prepared in the laboratory from *A. hebraeum* ticks collected from goats and cattle at specified time intervals. To determine whether *E. ruminantium* was present in *A. hebraeum* ticks, DNA extraction, nested PCR of the pCS20 region of the *E. ruminantium* genome and demonstration of the amplified DNA by agarose gel electrophoresis was performed. The results demonstrated that *E. ruminantium* was indeed present in eight out of eight ground-up-tick-supernatant samples that were cryo-preserved. In future research, these samples may become very useful for isolating current strains of heartwater, which will ultimately lead to a better understanding of the disease and to facilitate the implementation of novel control methods.

Introduction

Heartwater, caused by *Ehrlichia ruminantium,* causes significant economic losses to commercial small stock farmers, including commercial boer goat farmers in the Limpopo Province of South Africa. Heartwater is endemic in this region where *Amblyomma hebraeum*, the South African bont tick, and the vector for this disease occurs (Jongejan & Uilenberg 2018). *Amlyomma hebraeum* prefer to attach at specific sites on the body of its hosts. Since these ticks have long mouthparts, their attachment results in severe tissue damage, wounds and secondary abscess formation (Jongejan & Uilenberg 2004). The attachment of *A. hebraeum* to the interdigital region and feet of its hosts, results in severe pain and lameness which leads to reduced mobility, reduced browsing activity, reduced feed intake of the affected animal which ultimately leads to reduced productivity and production losses to the small stock farmer.

Strategic tick control with an effective acaricide can be used to ensure that *A. hebraeum* tick numbers remain sufficiently low to limit the negative effects on small stock associated with this tick (Jongejan & Uilenberg 2004). Commercial goat farmers currently employ various tick control methods to reduce tick burdens and the associated negative effects of high tick burdens. These methods include plunge dipping, hand spraying, belly baths, foot baths and targeted treatment using pour-on acaricides. Pour-on acaricides are normally used by first determining the total dosage required at a dosage of 1 ml per 5 kg of body weight and dividing the total dosage into five equal parts. The divided total dosage of the pour-on acaricide is then applied to the axillary region, inguinal region and perineal region. Should ticks be present on the feet and the interdigital region, the pour-on acaricide will be applied to these sites as well. Various studies have demonstrated the use of fipronil as an effective acaricide on companion animals and cattle (Fisher, Heidmann, Faria, Rizzi, Bragalia & Nascimento 2013; Davey, Ahrens, George, Hunter & Jeannin 1998). No studies have been performed to determine the efficacy of fipronil as an acaricide on goats.

The aim of this study was to determine the efficacy of a topical treatment with a 1.0% fipronil pour-on solution to reduce *A. hebraeum* tick numbers by 95% by performing a therapeutic- and persistent efficacy trial. Should a 1.0% fipronil pour-on solution be effective as an acaricide against *A. hebraeum* in order to reduce the tick burden and the negative effects associated with this tick, including heartwater, commercial boer goat farmers can be advised to implement the use thereof in a sustainable tick control program. The use of an effective acaricide as a pour-on formulation may also have significant practical benefits for farmers.

It is known that *E. ruminantium* can be isolated from *A. hebraeum* ticks (Theiler & Du Toit 1928; Alexander 1931). It is also known that genetic variability of *E. ruminantium* exist and various genotypes or strains of *E. ruminantium* are present in the field (Allsopp 2015). Another aim of this study was to detect and cryo-preserve *E. ruminantium* from *A. hebraeum* ticks from a heartwater endemic region in the Limpopo province of South Africa, by preparing ground-up-tick-supernatant samples. In order to detect *E. ruminantium*, DNA extraction, nested PCR of the pCS20 region of the *E. ruminantium* genome and demonstration of the amplified DNA by agarose gel electrophoresis was performed.

The benefit of the research project is therefore twofold. Firstly the research will give an indication of whether a 1.0% fipronil pour-on solution can be used as an effective acaricide, to be used by boer goat farmers to control *A. hebraeum* ticks through the use of an effective tick control program to reduce tick burdens and the negative consequence associated with a high tick burden, including heartwater. Secondly, the research will also help to demonstrate that *E. ruminantium* can be detected and cryo-preserved from ground-up-tick-supernatant samples prepared from *A. hebraeum* ticks on specific farms in the Limpopo Province of South Africa where heartwater is endemic. These cryo-preserved samples may be useful to isolate current strains of *E. ruminantium*, which in turn can be used in future research studies and may lead to the implementation of novel control methods to assist farmers to control heartwater.

Indirectly it will provide an opportunity to study the dynamics of *A. hebraeum* tick populations on boer goats in the Limpopo province as well as to advise commercial boer goat farmers on the implementation of a sustainable tick control program using a 1.0% fipronil pour-on solution.

Hypotheses

1. Topical treatment with a 1.0% fipronil pour-on solution will reduce *A. hebraeum* tick numbers by 95% on boer goats in the treatment group compared to the control group.
2. *Ehrlichia ruminantium* can be detected and cryo-preserved from infected *A. hebraeum* ticks.

Ethical approval

Approval from the Animal Ethics Committee (AEC) of the University of Pretoria (Project number V109-17) was obtained for this study and the certificate can be found in Appendix 1. Permission to conduct research in terms of section 20 of the Animal Diseases Act (Act 35 of 1984) from the Department of Agriculture Forestry and Fisheries (DAFF) was obtained and the certificate can be found in Appendix 2. Approval from the Veterinary Clinical Committee (VCC) of the Department of Health (DoH) was obtained for the research trial to be conducted and to use an unregistered veterinary pharmaceutical product. The certificate can be found in Appendix 3. Permission to import and use an unregistered veterinary pharmaceutical product in terms of the provisions under section 21 of Act 101 of 1965 was obtained from the Medicines Control Council (Permit reference number 26/2/2 VCT/01/2018). The permit can be found in Appendix 4.

Literature review

Amblyomma hebraeum

Amblyomma hebraeum, known as the South African bont tick, is a three host ixodid tick, and is the main vector for *E. ruminantium* that is the cause of heartwater in cattle, sheep and goats in South Africa. *Amblyomma hebraeum* has a specific distribution in South Africa that extends from the Limpopo Province through areas of the North West Province, Mpumalanga Province and the coastal regions of the Kwa-Zulu Natal Province and the Eastern Cape Province. Climate plays an important role in the seasonal occurrence of *A. hebraeum* in these various distribution ranges and higher numbers of adult ticks are found during the warm wet summer, whilst larvae are more numerous during the colder dry late autumn and nymphs during the winter and spring (Jongejan & Uilenberg 2018). Londt, Horak and De Villiers (1979) demonstrated that peak seasonal occurrence of adult *A. hebraeum* on cattle in the Limpopo Province is mainly during the spring and summer between August and February. The predilection site on domestic livestock include the axillary region, perineal region and inguinal region. The larval stages are found on the feet, legs and muzzle of their hosts, while the nymphs attach on the feet, legs, groin, sternum and neck (Jongejan & Uilenberg 2018; Walker, Bouattour, Camicas, Estrada, Horak, Latif, Pegram & Preston 2014).

Due to their long mouthparts, *A. hebraeum* ticks are capable of causing severe tissue damage in the areas where they attach on the host. This damage may result in secondary infection and abscess formation (Jongejan & Uilenberg 2004). In cattle the resulting wounds may also predispose towards secondary blowfly infection which further aggravate tissue damage (Walker *et al.* 2014). In sheep and goats, these ticks mostly attach to the interdigital region and at the back of the feet between the claws where they tend to cluster. The attachment of the ticks at these sites often result in secondary bacterial infection, abscess formation and severe lameness. A study by MacIvor and Horak (1987) demonstrated that there is a significant relationship between the occurrence of foot abscess in goats and the presence of adult *A. hebraeum* and other ticks including *Rhipicephalus glabroscutatum*. Figure 1 demonstrates the attachment of *A. hebraeum* ticks

to the interdigital region which will eventually lead to tissue damage, abscess formation and lameness.

Figure 1 A cluster of adult male and female *Amblyomma hebraeum* ticks attached in the interdigital region of a goat

Because of damage caused to the host, *Amblyomma* ticks need to be controlled, but sustained intensive tick control may lead to animals becoming susceptible to heartwater (Bath, Van Wyk & Pettey 2005). Baker and DuCasse (1968) advised the use of either local spraying, hand dressing of the ears or upper perineum or walk-through trough as adequate means of effectively controlling ticks on goats.

Amblyomma hebraeum prefers cattle as hosts and the numbers of adult *A. hebraeum* on goats may be relatively low in some instances compared to numbers of adult *A. hebraeum* found on cattle. Nyangiwe and Horak (2007) reviewed results from various studies performed in various regions of South Africa which indicated that in regions where cattle and goats were evaluated in the same localities, goats harbored less adult *A. hebraeum*

ticks than cattle. Ngumi, Rumberia, Williamson, Sumtion, Lesan and Kariuki (1997) also found that sheep and goats in parts of Kenia yielded very few *Amblyomma* ticks, even when no tick control was implemented and when other tick species were numerous. Baker and DuCasse (1968) found relatively low numbers of adult *A. hebraeum* on goats in regions of Kwa-Zulu Natal in South Africa compared to very high numbers of larvae and nymphs of *A. hebraeum* and other ticks including *Riphicephalus evertsi*, *Rhipicephalus appendiculatus* and *Rhipicephalus (Boophilus) decoloratus*. During a study to determine Ixodid ticks that infests domestic goats in communal areas of Zimbabwe, Hove, Mukandi, Horak and Latif (2008) did not encounter large numbers of *A. hebraeum* ticks on these goats. They however found that wherever these ticks attached which included the interdigital space, lower posterior abdomen and udder, it was associated with lesions. Rechav and De Jager (1991) also found that mean numbers of *A. hebraeum* found on goats in the Limpopo Province of South Africa is relatively low in comparison to cattle.

Amblyomma hebraeum is the vector for the transmission of *E. ruminantium* to livestock in South Africa. *Ehrlichia ruminantium* is found in the gut epithelial cells, salivary glands and haemolymph of *A. hebraeum* ticks and transmission of *E. ruminantium* to the vertebrate host is through the saliva of the tick while feeding (Kocan & Bezuidenhout 1987). After an infective blood meal is taken by the tick, replication of *E. ruminantium* takes place in the gut epithelium after which the salivary glands of the tick eventually become parasitized (Hart, Kocan, Bezuidenhout & Prozesky 1991 cited by Allsopp *et al.* 2004). The minimum time period required for *E. ruminantium* to be transmitted after ticks have attached to a susceptible animal is between 27 and 38 hours in nymphs and between 51 and 75 hours in adults (Bezuidenhout 1988 cited by Allsopp *et al.* 2004). Transstadial transmission of *E. ruminantium* occurs between larval, nymphal and adult ticks (Uilenberg 1983 cited by Howard & Norval 1989). Both male and female *A. hebraeum* ticks are able to transmit *E. ruminantium* and intrastadial transmission in male *A. hebraeum* ticks may play an important role in the epidemiology of heartwater (Howard & Norval 1989).

The prevalence of infection of *A. hebraeum* ticks varies and the accuracy of determining the infection rates depends on the detection methods used. Peter, Perry, O'Callaghan, Medley, Mlambo, Barbet and Mahan (1999) determined the prevalence of *E. ruminantium*

in adult and nymph stages of *A. hebraeum*, at two study sites in Zimbabwe, using PCR as a method of detection. The overall prevalence of infection of *E. ruminantium* in adult *A. hebraeum* ticks was 11.2% and 10.2% at the two respective study sites while the prevalence of infection of *E. ruminantium* in nymph stages of *A. hebraeum* was 8.5% at one of the study sites. The prevalence of *E. ruminantium* infection in nymphal *A. hebraeum* ticks collected from goats in the Mnisi area of the Mpumalanga Province of South Africa ranged between 11.8% and 23.5% as determined by PCR (Jongejan *et al.* in press).

Fipronil

Fipronil is classified as a phenylpyrazole agent and is commonly used on companion animals as a spot-on treatment for tick and flea control. Fipronil pour-on solutions are also used on cattle as an effective acaricide. Fipronil is absorbed percutaneously after which it spreads and sequesters in the lipids of the skin and hair follicles (Anadon & Gupta 2012). It then distributes back to skin appendages, primarily the pilo-sebaceous units and accumulates in the sebaceous glands, which in turn act as a reservoir for further continuous release via follicular ducts in cutaneous oils onto the skin (Breyden, Oudot & Baird 2010). It then spreads by passive diffusion to all body areas. Cochet, Birckel, Bromet-Petit, Bromet and Weil (1997) demonstrated the distribution of fipronil, after topical application at a dosage rate of 10 mg/kg, from the application site across the whole skin of the dog and the persistence of radiolabeled compound in the *stratum corneum* for up to 56 days post treatment. Fipronil inhibits gamma amino butyric acid (GABA) - regulated chloride influx by binding to GABA and glutamate gated receptor sites of the insect nervous system and inhibiting the influx of chloride into nerve cells which results in hyper-excitability and death (Anadon & Gupta 2012).

Fisher *et al.* (2013) demonstrated a high therapeutic and persistent efficacy of 90.35%, 42 days after the application of a fipronil spot-on solution at a dosage rate of 6.4 mg/kg against *Rhipicephalus sanguineus* in dogs. A study to determine the therapeutic efficacy and persistent efficacy of fipronil in cattle demonstrated that a 1.0% fipronil pour-on solution was 99.7% effective against the one host tick *Rhipicephalus* (*Boophilus) microplus*,

prevented larval re-infestation for 6-8 weeks and had an effect on engorgement weight, egg mass weight and egg hatchability on female ticks that survived treatment (Davey *et al.* 1998). Studies performed under field conditions to evaluate a 1.0% fipronil pour-on solution against *Rhipicephalus (Boophilus) annulatus* on cattle demonstrated that repeated applications at various intervals resulted in a drastic reduction of the tick population found on pastures (Davey, George, Hunter & Jeannin 1999). Lopez, Cruz, Teixeira, Felippelli, Maciel, Buzzulini, Costa Gomez, Favero, Soares, Bichuette, Pereira de Oliveira and José da Costa (2014) demonstrated that the acaricidal efficacy of a fipronil pour-on solution, administered at a dosage rate of 1.0 mg/kg on cattle was ≥ 90%, 35 days after treatment, against ivermectin resistant strains of *R. (B) microplus*. The therapeutic and persistent efficacy of fipronil are dose related and are increased by repeat treatments (Davey *et al.* 1998; Jongejan & Uilenberg 2018). Fipronil can be considered an important alternative to control acaricide resistant ticks (Castro-Janer, Martins, Mendes, Namindome, Klafke & Schumaker 2010). However, resistance against fipronil has been demonstrated in ticks which may be attributed to the use of agricultural fipronil on animals (Castro-Janer, Rifran, Gonzales, Piaggio, Gil & Schumaker 2010; Castro-Janer *et al.* 2010). Responsible use of fipronil should therefore be advocated to ensure that it remains an effective acaricide to be used for the control of ticks on livestock.

Heartwater

Heartwater caused by the intracellular rickettsial pathogen *E. ruminantium,* is an important limiting factor for profitable commercial small stock production in the Limpopo province of South Africa. Typically the disease is characterized by high fever, nervous signs, hydropericardium, hydrothorax and oedema of the lungs and brain (Allsopp, Bezuidenhout & Prozesky 2004).

The incubation period varies between five and 35 days, and in the majority of cases, signs of disease are noticed between seven and 14 days, with an average of nine days, after intravenous inoculation (Alexander 1931). Experimental infection of goats, with a quantified amount of viable elementary bodies of *E. ruminantium*, induced a mean

incubation period of approximately 7-13 days with death occurring around 12-20 days after infection (Vachiéry, Lefrançois, Esteves, Molia, Sheikboudou, Kandasamy & Martinez 2006). Mortality rates in naïve animals introduced to a heartwater endemic area may range from 67% in cattle to 90% in sheep and 84% in goats (Mahan, Smith, Kumbula, Burridge & Barbet 2001).

Alexander (1931) differentiated between different forms of heartwater, which depends on the susceptibility of the animals, the duration of the disease and the virulence of the organism. These different forms of heartwater include a per-acute form, an acute form, a sub-acute or chronic form and a mild abortive or heartwater fever form, and was described separately by Alexander (1931) and summarized below.

The per-acute form is characterized by sudden death with the presence of clinical symptoms for periods less than 36 hours. The acute form, which may have a duration of between 3-6 days, is the most common form seen in susceptible animals and clinical signs may initially be less prominent and include pyrexia with normal feeding and rumination which gradually progresses to inappetence, rumen stasis and a tucked up and anxious staring appearance. This is followed by tachypnoea, tachycardia and nervous symptoms with progression of the disease. In sheep and goats progressive unsteadiness of the gait, with legs apart, head bent downwards and dropped ears is noticed. Lateral recumbence with legs stretched out with continuous galloping movements of the legs, masticating movements of the jaw, frothing at the mouth with continual licking of the lips, the head thrown back with strabismus are further classical signs seen in sheep and goats. In cattle these nervous signs are more pronounced with cattle often wandering aimlessly in circles and bumping against objects appearing blind. The sub-acute or chronic form may have a duration of 10 days or more and signs may resemble the acute form but less pronounced. The mild abortive form is characterized by an absence of all the clinical symptoms except for the hyperthermia. This form is seen in animals that have natural resistance against the disease or animals that have acquired immunity after recovering from the disease.

Livestock owners should be advised to closely observe the goat flock on a regular basis and to look out for these typical signs of heartwater. It should also be advised for a livestock owner to always have a thermometer close at hand in order to evaluate the temperature of an animal which is suspected to be sick. This will ensure that a case of heartwater is detected early on in the course of the disease.

Treatment consist of timely administration of an oxytetracycline injectable solution via intravenous or intramuscular route at a dosage of 10-20 mg/kg or a doxycycline injectable solution via the intravenous route at a dosage of 2 mg/kg.

Typical macropathological signs noticed on post mortem examination include hydropericardium, hydrothorax and ascites characterized by a clear yellow transudate in the pericardial sac, thoracic- and abdominal cavity which is slightly turbid and which coagulate upon exposure to air, pulmonary oedema characterized by frothy oedematous fluid in the trachea and bronchi which oozes from the cut surface of the lungs (Allsopp *et al.* 2004). Brain oedema is characterized by swollen gyri of the cerebrum, with varying degrees of congestion and oedema of the meninges. Petechiae and ecchymoses are also sometimes noted in the brain (Allsopp *et al.* 2004). The hydropericarium, hydrothorax, ascites and brain oedema which develop in a heartwater case is the result of the vascular leakage caused by the *E. ruminantium* organisms situated in the endothelial cells of the capillaries. The nervous signs seen in clinical cases of heartwater is due to the severe brain oedema which develops.

Heartwater is typically diagnosed on post mortem by means of a Diff Quick or Giemsa stained brain smear, made from the hippocampus, and identifying the *E. ruminantium* colonies in the endothelial cells of the capillaries under light microscopy (Allsopp 2015).

Control of heartwater

Commercial farmers in the Limpopo province employ various methods to control heartwater. Strategic tick control have been advocated as one of the control methods for heartwater (Alsopp *et al.* 2004). Strategic tick control, which entails acaricide treatment every seven to 28 days, depending on the tick burden, forms part of the heartwater control strategy on most farms in the Limpopo Province. By applying strategic tick control, tick numbers are sufficiently controlled to prevent damage that may result from high tick burdens, whilst at the same time ensuring natural infection and immunity in order to create an endemically stable situation with respect to heartwater (Allsopp 2015).

A popular method to control heartwater is the prophylactic antibiotic treatment method which comprises of the administration of an injectable oxytetracycline solution at regular time intervals. This method is recommended when susceptible animals are introduced into a heartwater endemic region. Allsopp *et al.* (2004) described how this method should be employed. In goats, a short acting oxytetracycline injectable solution is administered at a dosage rate of 3 mg/kg on day 10, 20, 30, 45 and 60 after introduction to a heartwater endemic region. In cattle, an oxytetracycline injectable solution is administered at a dosage rate of 10-20 mg/kg on day 7, 14, and 21 or day 7, 12 and 17 or on day 7 and 14 after introduction to a heartwater endemic region. The theory behind this control method is for the animals to become infected during the period that they are protected by the oxytetracycline antibiotic. Tick control should therefore not be applied during this period to ensure that the animals are exposed to *A. hebraeum* ticks and naturally infected with *E. ruminantium*.

Another method that is available to control heartwater is the infection-and-treatment method. This is the only commercially available vaccination method used in South Africa for decades, and consists of inoculating the Ball 3 strain of *E. ruminantium* intravenously and then subsequently treating with tetracycline (Adakal, Stachurski, Konkobo, Zoungrana, Meyer, Pinarello, Aprelon, Marcelino, Alves, Martinez, Lefrançois & Vachiery 2010). Vaccinated animals are treated with an oxytetracycline after the development of a temperature reaction towards the vaccine have been detected using a rectal thermometer

(Allsopp 2015). It is important for the livestock producer to closely monitor these temperature reactions and administer the oxytetracycline treatment at the correct time period. Should the oxytetracycline be administered too early, before a temperature reaction has developed, immunity may not develop (Allsopp *et al.* 2004). Should the oxytetracycline not be administered or administered too late, the vaccinated animal may succumb to heartwater caused by the Ball 3 vaccine strain (Allsopp *et al.* 2004). It is recommended that calves should be vaccinated at 3-4 weeks of age and goat kids and sheep lambs at seven days of age in order to take advantage of the age related innate resistance of these young animals (Allsopp *et al.* 2004). It is generally not necessary to treat these young animals with an oxytetracycline after vaccination.

Disadvantages of this immunization procedure include the necessity for chemotherapeutic intervention after immunization, the need to maintain a cold chain for the distribution of the vaccine, and the requirement for the vaccine to be injected intravenously, which is very inconvenient especially when large number of animals have to be injected (Zweygarth, Josemans & Steyn 2008). Many livestock producers find it difficult to administer the vaccine intravenously and a veterinarian is therefore called upon to perform the vaccination, which increases the cost of using this method of control. Failure of the Ball 3 blood-based "vaccine" strain of heartwater to confer cross protection to immunologically different strains has been demonstrated (Jongejan, Thielemans, Brière & Uilenberg 1991).

Heartwater vaccines

Extensive research has been performed and is still ongoing in order to develop an effective vaccine against heartwater. Allsopp (2015) discussed the current state of development of the three types of heartwater vaccines namely, inactivated vaccines, attenuated vaccines and recombinant vaccines. Various studies have been performed to demonstrate the ability of these vaccines to protect ruminants against virulent *E. ruminantium* challenge and field tick challenge.

Inactivated heartwater vaccines

An inactivated vaccine, which can be administered subcutaneously, is prepared by using elementary bodies of *E. ruminantium* which are partially purified from bovine endothelial cell culture and chemically inactivated and formulated with a suitable adjuvant (Allsopp 2015).

Martinez, Maillard, Coisne, Sheikboudou and Bensaid (1994) demonstrated successful protection of goats against heartwater after immunization with antigens consisting of inactivated elementary bodies of *E. ruminantium*. This immunization however did not render 100% protection against challenge with a high dose of *E. ruminantium* culture supernatant. Mahan, Kumbula, Burridge and Barbet (1998) demonstrated that an inactivated *E. ruminantium* vaccine protected sheep against lethal intravenous challenge, laboratory raised infected ticks, heterologous intravenous challenge with *E. ruminantium* strains from diverse geographical locations and against heterologous natural tick challenge in the field. These authors also suggested that local *E. ruminantium* strains can be isolated and incorporated into vaccines.

Vachiéry *et al.* (2006) optimized the dose of an inactivated heartwater vaccine and demonstrated that there was successful protection of between 71 and 100% against experimental virulent infection, even when using a minimum dose of antigen. Adakal *et al.* (2010) demonstrated that an inactivated vaccine may be protective against a field challenge of heartwater with protection varying from 50%-89%. These authors also evaluated comparative protective effects of two different strains (Gardel & Welgevonden) of *E. ruminantium* against local strains in order to choose appropriate region specific vaccine strains. Mahan *et al.* (2001) demonstrated that an inactivated heartwater vaccine significantly protects sheep, goats and cattle against tick challenge compared to unvaccinated control animals, although protection was not 100% but varied between a minimum of 26% to a maximum of 81%. The results of a study by Faburay, Geysen, Ceesay, Marcelino, Alves, Taoutik, Postigo, Bell-Sakyi and Jongejan (2007) demonstrated that an inactivated *E. ruminantium* vaccine (Gardel) provided non-significant, partial protection of 42% against a heterologous *E. ruminantium* needle challenge.

Advantages of an inactivated vaccine include the ability to be multivalent, by the pooling of different antigenic strains, which may enable region specific vaccination strategies and more robust storage requirements which are more compatible to be used in the field (Vachiéry *et al.* 2006). Inactivated vaccine can also be employed in non-endemic regions. It is however necessary to provide booster vaccinations for inactivated vaccines and inactivated vaccines induced higher morbidity rates with a longer period of hyperthermia compared to attenuated vaccines (Adakal *et al.* 2010).

Attenuated heartwater vaccines

An attenuated vaccine is prepared by using an avirulent live organism, which will not cause clinical disease, but which is still able to stimulate immunity against virulent forms of the organism (Allsopp 2015). Attenuated heartwater vaccines are prepared by the attenuation of virulent *E. ruminantium* through serial passage of these organisms through cell cultures like bovine umbilical endothelial cells.

Jongejan (1991) was the first to demonstrate that, after administration of an attenuated isolate of *E. ruminantium* originating from Senegal, goats and sheep were fully protected against homologous challenge with a virulent *E. ruminantium* blood stabilate. Subsequently, it has been demonstrated that, when sheep and boer goats were immunized with an attenuated (Welgevonden stock) vaccine, the sheep were protected against homologous and heterologous (Ball 3, Mara 87/7, Blaauwkrans & Gardel) virulent challenge and that boer goats were protected against homologous virulent challenge (Zweygarth, Josemans, Van Stijp, Lopez-Rebollar, Van Kleef & Allsopp 2005).

An attenuated hearwater vaccine trial, wherein sheep were immunized with an attenuated *E. ruminantium* (Senegal) vaccine, 100% protection was achieved against a heterologous needle challenge with a virulent *E. ruminantium* (Kerr Seringe) strain and 75% protection was achieved when exposed to a natural tick challenge (Faburay *et al.* 2007). Zweygarth *et al.* (2008) demonstrated that the administration of an attenuated *E. ruminantium* (Welgevonden) vaccine by intra-muscular and subcutaneous route protects Merino sheep against homologous virulent heartwater challenge and that the immunity which develops

6-12 months after vaccination is highly satisfactory.

Zweygarth *et al.* (2008) also demonstrated that it is possible for *A. hebraeum* ticks, which were fed on an animal that was immunized with an attenuated *E. ruminantium* (Welgevonden stock) vaccine, to transmit this attenuated *E. ruminantium* organism. They also demonstrated that sheep, on which ticks infected with the attenuated *E. ruminantium* fed, seroconverted and were subsequently protected against a virulent homologous challenge, suggesting that these sheep have been immunized by the ticks as a result of the transmission of these attenuated *E. ruminantium* organisms.

An attenuated vaccine have several advantages over the Ball 3 blood vaccine. These include that treatment with a tetracycline is unnecessary, it is much cheaper to produce and the expected pattern of cross-protection is wider, while a disadvantages that this vaccine shares with the Ball 3 blood vaccine is the fact that both vaccines have to be distributed frozen due to the lability of the *E. ruminantium* organism (Zweygarth *et al.* 2005). It is not advisable to use attenuated vaccines in non-endemic areas due to fear of potential reversion to virulence.

Recombinant heartwater vaccines

Recombinant vaccines for heartwater is based on the principle that certain *E. ruminantium* cellular components have the ability to act as antigens and induce protective immunity. An example is the major antigenic protein 1 (map1), encoded by the map1 gene, which is an immunodominant surface protein of *E. ruminantium*.

DNA based vaccines are prepared by identifying specific genes from the *E. ruminantium* genome, which may have antigenic properties, and cloning these specific genes, referred to as open reading frames (ORFs), into a DNA vaccine vector which can be used to immunize animals in order to stimulate a protective immune response. Recombinant protein subunit vaccines are prepared by inserting these genes, encoding potential antigens, into an expression system, which may be bacterial or eukaryotic cells, in order to express and produce these antigens which can be purified and used to immunize animals. A DNA prime

and recombinant protein boost vaccination strategy entails an initial immunization with a DNA based vaccine and a subsequent immunization with a recombinant protein vaccine.

Collins, Pretorius, Van Kleef, Brayton, Allsopp, Zweygarth and Allsopp (2003) prepared and evaluated a DNA vaccine consisting of four immunizing plasmids containing open reading frames (ORFs). This study demonstrated that sheep immunized with the DNA vaccine containing the open reading frames (ORFs) were protected against homologous (Welgevonden) and heterologous (Blaauwkrans, Ball 3, Gardel, Kwanyanga & Mara 87/7) virulent *E. ruminantium* needle challenge. However, sheep that were immunized with the DNA vaccine, which have not been previously challenged with a virulent *E. ruminantium* needle challenge, did not have any protection against natural heartwater challenge.

Pretorius, Collins, Steyn, Van Strijp, Van Kleef and Allsopp (2007) demonstrated that by immunizing sheep with a DNA vaccine which contains four specific *E. ruminantium* open reading frames (ORFs), either as part of a combined cocktail or individually, protected sheep against lethal homologous heartwater challenge due to the specific antigenic properties of these open reading frames (ORFs).

Sebatjane, Pretorius, Liebenberg, Steyn and Van Kleef (2010) demonstrated that by inoculating sheep using a cocktail of five open reading frames (ORFs), coding for low molecular weight proteins of *E. ruminantium*, and given as a DNA prime-recombinant protein boost, only resulted in 25% protection against virulent *E. ruminantium* challenge.

Pretorius, Liebenberg, Louw, Collins and Allsopp (2010) evaluated the ability of the *E. ruminantium* polymorphic gene, Erum2510, to induce protection against virulent *E. ruminantium* (Welgevonden) needle challenge. Only 20% and 40% of animals survived the virulent needle challenge during two trials where the DNA only vaccination strategy, using the Erum2510 DNA construct was used. None of the animals survived that were inoculated with a DNA cocktail vaccine containing three open reading frames (ORFs) adjacent to ERum2510 in the genome. However, a 100% protection was induced in animals that were inoculated using a DNA prime and recombinant protein boost strategy.

For the latest in recombinant vaccine development, Faburay, McGill and Jongejan (2017) evaluated a glycosylated recombinant subunit vaccine consisting of *E. ruminantium* major antigenic protein 1 (map1). According to these authors the glycosylated proteins and glycans contained in the antigenic protein are important epitope determinants and will stimulate a more robust immune response that will protect against virulent *E. ruminantium* challenge. It was demonstrated in their study that this glycosylated recombinant subunit vaccine induced *E. ruminantium* specific humoral and T-helper 1 (Th 1) cell responses in sheep. This recombinant vaccine shows promise to be evaluated in a virulent *E. ruminantium* needle challenge and field challenge study.

Genetic variability and recombination of *Ehrlichia ruminantium*

Genetic variability exists between *E. ruminantium* organisms. There are eight different 16S ribosomal RNA genotypes currently classified as *E. ruminantium* namely Senegal, Gardel, Ball 3, Crystal Springs, Mara 87/7, Kiswani, Omatjenne and Pretoria North, and the former six of these are known to cause virulent heartwater in ruminants (Allsopp *et al.* 2004). Allsopp, Van Strijp, Faber, Josemans and Allsopp (2007) stated that it appears that there are many different *E. ruminantium* genotypes in circulation with pathogenicities varying from high to low. Cross immunity between these strains is either complete, partial or non-existent (Jongejan *et al.* 1991; Allsopp *et al.* 2004). No single effective vaccine against heartwater is available due to the limited cross protection between vaccine strains and field strains which may probably be caused by the high genetic diversity of *E. ruminantium* at different geographical locations (Cangi, Gordon, Bournez, Pinarello, Aprelon, Huber, Lefrançois, Neves, Meyer & Vachiéry 2016).

Recombination is a major driving force of genetic diversity in *E. ruminantium* (Nakao, Magona, Zhou, Jongejan & Sugimoto 2011; Cangi *et al.* 2016). Allsopp and Allsopp (2007) demonstrated that extensive inter-genome recombination occurs among *E. ruminantium* genotypes. Allsopp and Allsopp (2007) also stated that there is a larger reservoir of genetic diversity among *E. ruminantium* and related organisms that can be seen in the few well characterized heartwater causing stocks, and those genes involved in antigenicity,

infectivity, virulence and pathogenicity of the organism will likely be as susceptible to recombination as the core genes. According to Cangi *et al.* (2016) cattle movement, contact with wildlife coupled with *Amblyomma* tick dispersion have probably shaped the genetic diversity of *E. ruminantium*. These authors proposed that the origin of this genetic diversity and its ability to avoid the host's immune response, may be through the exchange of genetic material between co-infecting *E. ruminantium* strains.

Proper genotyping and characterization of field isolates of *E. ruminantium* is an important prerequisite for the development of effective vaccination strategies at regional levels (Nakao *et al.* 2011). According to these authors multi-locus sequence typing (MLST) is a powerful typing method that allows determining genetic diversity and phylogenetic diversity and the compilation of MLST data across the African continent will be particularly valuable for understanding the existing genetic diversity of field isolates in African countries. Cangi *et al.* (2016) performed MLST on *E. ruminantium* isolates from various geographic locations and identified two major distinct genetic groups of *E. ruminantium* namely a West African cluster and a worldwide cluster which includes West Africa, East Africa, Southern Africa, Indian Ocean and Caribbean.

Comprehensive information on the degree of cross protection between strains and further understanding of possible relationships between genotypes and phenotypes with respect to vaccine efficacy are expected to lead to the development of region-specific vaccination strategies (Nakao *et al.* 2011).

It is important to mention that there are virtually no strains of heartwater available with a recent isolation history (Nakao, Sugimoto & Jongejan 2016). Hence, there is an urgent need to isolate current strains of heartwater from the field. The isolation of current strains and their comparison with historical isolates will facilitate our understanding of the disease dynamics and will ultimately lead to improved disease control.

Isolation of *Ehrlichia ruminantium* from *Amblyomma hebraeum* ticks

It is possible to isolate *E. ruminantium* from *Amblyomma* ticks and various studies have been performed to demonstrate this. Theiler and Du Toit (1928) successfully isolated *E. ruminantium* from *A. hebraeum* nymphs. In a series of experiments, they demonstrated that an emulsion made from nymphs of *A. hebraeum* infected with *E. ruminantium*, and administered intravenously, produced heartwater in susceptible sheep. The concentration of the emulsion made with saline was 1 nymph/ml and dosages 0.01 ml, 0.1 ml, 1 ml and 5 ml were evaluated through intravenous administration of which the 0.1 ml, 1 ml and 5 ml dosages resulted in heartwater in susceptible sheep. The emulsions produced with saline and glycerine, at a 1:1 ratio did not induce heartwater in susceptible sheep and it was suspected that glycerine destroyed the virulence of *E. ruminantium*. Alexander (1931) performed similar experiments with emulsified *A. hebraeum* nymphs which resulted in heartwater in less than 50% of cases. Experiments conducted using emulsified adult *A. hebraeum* ticks did not induce heartwater in susceptible sheep. During these experiments, Alexander (1931) described typical anaphylaxis which occurred after intravenous administration of the emulsified *A. hebraeum*.

In order to determine the distribution of heartwater in the Caribbean islands where it has been introduced, Barré, Camus, Birnie, Burridge, Uilenberg and Provost (1984) isolated *E. ruminantium* from adult *A. variegatum* ticks by preparing tick stabilates and confirming the presence of *E. ruminantium* by inoculating susceptible sheep and goats intravenously. They confirmed heartwater on post mortem examination or brain biopsy in animals that developed a pyrexia after being inoculated.

Ngumi *et al.* (1997) successfully isolated *E. ruminantium* from three *Amblyomma* species, prepared tick homogenates and inoculated it intravenously into susceptible sheep. The isolation was confirmed by either examining brain smears to identify *E. ruminantium* or by inoculating blood stabilates, prepared from the sheep during a pyrexic reaction, intravenously into another susceptible sheep or goat.

Utilizing nested PCR to demonstrate successful detection of *Ehrlichia ruminantium*

Nested pCS20 PCR assay is used as a method to amplify a specific 279 base pair fragment from DNA of *E. ruminantium*. Martinez, Vachiéry, Stachurski, Kandasamy, Raliaina, Aprelon and Gueye (2004) validated the technique for nested pCS20 PCR and map1 gene of *E. ruminantium* and demonstrated that it is a highly sensitive method compared to standard PCR to detect the presence of *E. ruminantium* in various samples including fresh-, frozen- or ethanol preserved samples.

Nested PCR has been used in various epidemiological studies to detect *E. ruminantium*. Van Heerden, Steyn, Allsopp, Zweygarth, Josemans and Allsopp (2004), developed primers which enabled them to determine the sequences of the pCS20 region of 14 different *E. ruminantium* isolates. Steyn, McCrindle, Allsopp and Van Kleef (2006) isolated *E. ruminantium* from *A. hebraeum* ticks and blood collected from animals from heartwater endemic regions and through the use of real time PCR and nested PCR methods identified 10 new isolates. Faburay, Geysen, Munstermann, Taoufik, Postigo and Jongejan (2007) demonstrated that nested PCR is a highly sensitive diagnostic tool for the detection of *E. ruminantium* in *A. variegatum* ticks.

Preparing a vaccine from tick isolates

Various studies have been performed in order to use *A. hebraeum* ticks to produce a vaccine. Alexander (1931) discussed an attempt to produce a vaccine from emulsified *A. hebraeum* to which 40% formaldehyde had been added and saline used to dilute the mixture, with poor results being obtained. Bezuidenhout (1982) described a method to develop a heartwater vaccine from *A. hebraeum* nymphs infected with *E. ruminantium*. He concluded that although the vaccine still needs to be administered intravenously, which hold little benefit over the blood vaccine, the advantage of this vaccine is the relatively low cost of production, the simplicity of the method and the simple method of storage of *E. ruminantium* in *A. hebraeum* ticks and filtrates. Bezuidenhout and Spickett (1985)

demonstrated that the immunizing efficiency between the heartwater blood vaccine and a ground-up tick suspension vaccine is similar in calves vaccinated at 3-4 weeks of age.

Although these historical studies, ranging between 1931 and 1985, yielded interesting results, which even indicated that the production of a vaccine from *Amblyomma* ticks is possible, no further research on this topic has been undertaken to attempt the production of a vaccine against heartwater using *Amblyomma* ticks.

Materials and methods

Heartwater and *A. hebraeum* can be considered synonymous in the Limpopo Province of South Africa and it was therefore appropriate to follow two approaches in this study, focusing on the control of *A. hebraeum* and the detection and cryo-preservation of *E. ruminantium* from *A. hebraeum*.

The first approach consisted of a randomized controlled clinical trial to determine the efficacy and persistent efficacy of a 1.0% fipronil pour-on solution against *A. hebraeum* on boer goats, which was performed at study site A. After the initial trial using a backline application of the 1.0% fipronil pour on solution, referred to as the "backline treatment method", the trial was repeated by changing the application method to specifically target the predilection sites of *A. hebraeum*, referred to as "targeted treatment method".

The second approach consisted of the detection and cryo-preservation of *E. ruminantium* from *A. hebraeum* ticks that were collected from goats and cattle at study site B. A simplified outline of the two approaches can be seen in Figure 2.

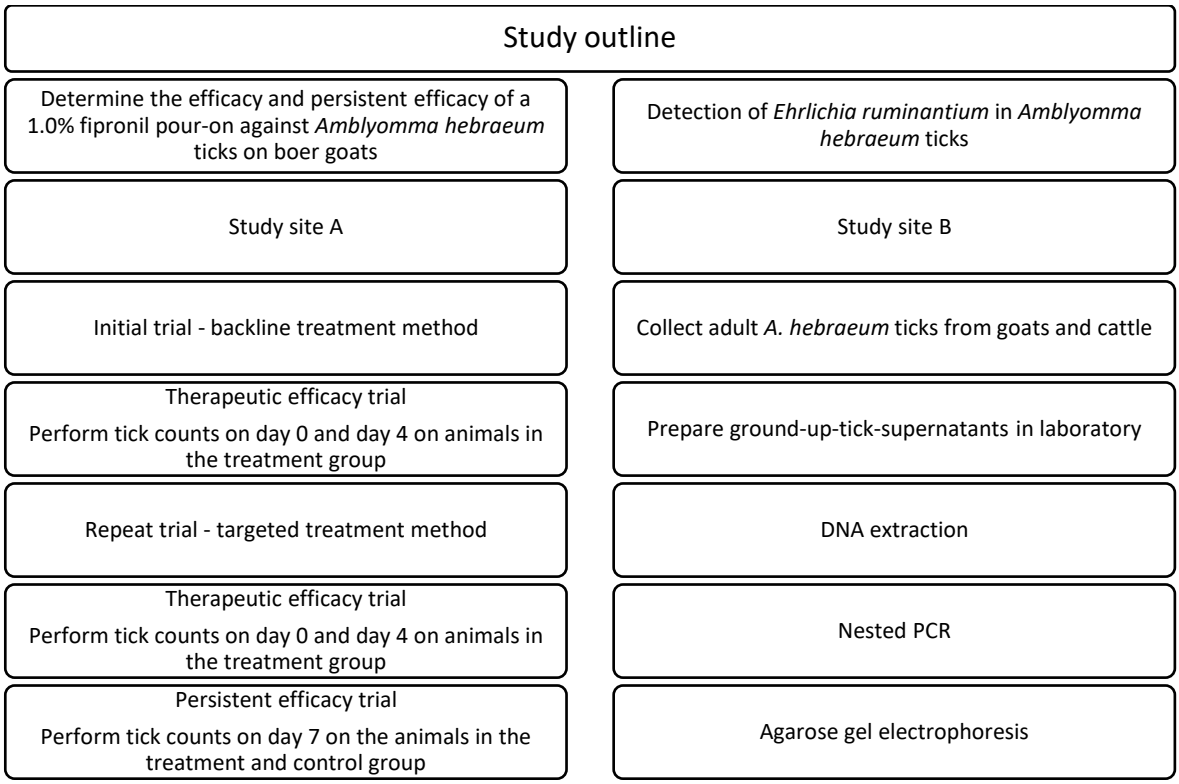

Figure 2 A simplified schematic diagram demonstrating the two approaches of the study

Determine the efficacy and persistent efficacy of a 1.0% fipronil pour-on solution against *Amblyomma hebraeum* ticks on boer goats

Selection of a commercial boergoat farm in the Limpopo Province of South Africa for study site A

The trial was conducted on a commercial boer goat farm identified in the Limpopo Province of South Africa (24° 41’ 27” S; 28° 55’ 42” E) where *A. hebraeum* ticks are prevalent or frequently encountered on goats and where heartwater is endemic.

Sample size determination, tick counts, group allocation and selection of animals

Sample size determination

The following formula, described by Sathian, Sreedharan, Baboo, Sharan, Abhilash and Rajesh (2010) to determine sample size for the difference in means, was used:

$$\mathbf{N = 2\sigma^2 (Z_\beta - Z_\alpha)^2 / difference^2}$$

Where:

N = Sample size in each group assuming equal sample size

σ = Standard deviation of the outcome variable

Z_β = Desired power

Z_α = Desired level of statistical significance

Calculation

Significance levels will be fixed at 5% (therefore there is a 5% probability of rejecting the null hypothesis when it is true). The constant of 1.96 will be used in the formula for Z_α. Power of the study will be determined at 80%. The constant of 0.84 will be used in the formula for Z_β. We assume that the mean *A. hebraeum* tick numbers in the control group will be 4 and the mean *A. hebraeum* tick numbers in the treatment group will be 0 after treatment. The expected difference in means will therefore be 4. A standard deviation of 2 will be used in the formula for σ.

N = 2 (2)2 (0.84 + 1.96)2 / (4)2

N = 3.92

Sample size should therefore include a total of at least eight animals (4 animals per group).

Tick counts

Full body tick counts were performed on every animal in the selected group of animals. Only adult *A. hebraeum* ticks were considered for the study. Tick counts were therefore only performed on adult *A. hebraeum* ticks and "tick counts" referred to the determination of the number of adult *A. hebraeum* ticks encountered on selected animals from the study group on the specified days on which tick counts were performed.

To perform the tick counts, animals were held in the standing position by an assistant. The animals' body was divided into five different regions namely the 1) head region, 2) neck, chest and axillary region, 3) perineal and peri-anal region, 4) inguinal and groin/udder region, 5) distal limb, feet and interdigital region. The number of ticks were counted and recorded for each region.

Group allocation

After initial tick counts were performed on each of the animals in the selected study group, the animals were randomly divided and allocated into a treatment group (TG) and a control group (CG). Variations between groups were reduced by forming replicates of animals with similar tick counts and then randomly allocating animals into the different groups. The animals in the CG remained untreated. Animals in the CG were kept separate from the animals in the TG to ensure that there was no contact between the animals from the respective groups and possible contact with fipronil through grooming or rubbing. Although animals from the different groups were separated to prevent contact, they remained in neighboring camps to ensure that the tick challenge was similar.

Selection of animals

Twelve boer goats were selected, to be included into the study group for the initial trial, using a backline treatment method. Eight boer goats were selected, to be included into the study group for the repeat trial, using a targeted treatment method.

A clinical examination was performed on every animal to ensure that the animal was clinically healthy before being incorporated into the study. The animals were individually identified by means of numbered and colored ear tags. The weight of every selected animal was accurately determined using a calibrated hanging scale and recorded.

Treatment

Initial trial - backline treatment method

Six animals from the TG were treated using the backline treatment method on day 0 (D0) of the study. The backline treatment method consisted of taking the total dose of the 1.0% fipronil pour-on solution, at a dosage rate of 1 ml/5 kg to administer 2.0 mg/kg fipronil, applied topically in a single line from the shoulder blades to the root of the tail. The six animals in the CG remained untreated.

Repeat trial - targeted treatment method

Four animals from the TG were treated using the targeted treatment method on day 0 (D0) of the study. The targeted treatment method consisted of taking the total dose of the 1.0% fipronil pour-on solution, at a dosage rate of 1 ml/5 kg to administer 2.0 mg/kg fipronil and divide it into five equal parts to apply the pour-on solution to five regions on the body namely, both axillary regions, both inguinal regions and the perineal region. Since the main predilection site of *A. hebraeum* ticks where the feet and the interdigital region of the study animals, it was decided to further apply ±2 ml of the 1.0% fipronil pour-on solution to each of the four feet using a 1 liter plastic spray bottle. The four animals in the CG remained untreated.

Therapeutic efficacy testing against *Amblyomma hebraeum*

To determine the therapeutic efficacy of a 1.0% fipronil pour-on solution against *A. hebraeum,* tick counts were performed on the selected animals from the TG on the day of the treatment (D0) and then on day 4 (D4) after the first treatment in order to determine efficacy of the treatment.

Persistent efficacy testing against *Amblyomma hebraeum*

To determine the persistent efficacy of a 1.0% fipronil pour-on solution against *A. hebreaeum*, tick counts were performed on the selected animals from the TG and CG on the day of the treatment (D0) and then on day 7 (D7).

Data analysis

Tick counts and analysis was performed based on World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines for evaluating the efficacy of acaricides against ticks on ruminants (Holdsworth, Kemp, Green, Peter, De Bruin, Jonson, Letonja, Rehbein & Vercruysse 2006).

Therapeutic efficacy testing for multi-host ticks

To determine the therapeutic efficacy of a 1.0% fipronil pour-on solution against *A. hebraeum* the following formula was used:

% Control = (N_0 – N) / N_0 x 100

Where:

N_0 = number of ticks on the animal before treatment

N = number of ticks on the same animal after treatment

Persistent efficacy testing for multi-host ticks

To determine the persistent efficacy of a 1.0% fipronil pour-on solution against *A. hebraeum* the following formula was used:

% Control = (C – N) / C x 100

Where:

C = number of ticks on the CG

N = number of ticks on the TG

Data capture

Results of tick counts were recorded on raw datasheets and then processed into an Excel Spreadsheet (Microsoft®). According to Holdsworth *et al*. (2006) for a product to be effective against multi-host ticks a target therapeutic efficacy of at least 95% should be achieved within four days after treatment. And for a product to qualify for persistent efficacy against multi-host ticks a target persistent efficacy of at least 95% on the TG of animals must be achieved during weekly (7 day interval) tick counts for the period for which persistent efficacy needs to be claimed.

Detection of *Ehrlichia ruminantium in Amblyomma hebraeum* ticks

Selection of a commercial boer goat farm in the Limpopo Province of South Africa for study site B

A commercial boergoat farm was identified in the Limpopo Province of South Africa (23° 40' 02" S; 29° 28' 21" E) where *A. hebraeum* ticks are prevalent or frequently encountered on goats and cattle and where heartwater is endemic.

Amblyomma hebraeum tick collection

Adult *Amblyomma hebraeum* ticks were collected from goats and cattle. A total of eight tick collections were performed every 14-28 days between January and June 2018. To collect the ticks from goats, each goat from a flock of 30 goats, was individually restrained and evaluated for the presence of ticks, after which the ticks were removed by hand from the various predilection sites and placed in a special escape proof container. To collect ticks from cattle, six to eight Drakensberger-Holstein cross cows were restrained in a crush pen and evaluated for the presence of ticks, after which the ticks were removed by hand from the various predilection sites and placed in a special escape proof container. Afterwards, the ticks were counted, while at the same time ensuring that the ticks were alive, and placing the live ticks into smaller special escape proof containers. The ticks were then kept overnight in a polystyrene cool box with an ice pack to keep the temperature low until the laboratory procedure could be performed the following day.

Laboratory procedure to prepare ground-up-tick-supernatant samples from *Amblyomma hebraeum* ticks

In the laboratory, the ticks were rinsed under tap water for 2-3 minutes to remove excessive dirt and material. Thereafter the ticks were transferred to a mortar and triturated with a pestle with added broken glass, whilst adding 4-30 ml phosphate buffered solution. The resulting ground-up-tick-supernatant was then transferred to a 50 ml tube and allowed to settle for 15 minutes on ice in order for the coarse parts to form a sediment. The supernatant was then transferred to a 30 ml tube. Dimethylsulfoxide (DMSO) was then

slowly added until a 10% end volume was reached. The ground-up-tick-supernatant were then divided over 1 ml cryo-tubes which were clearly marked for easy identification. All procedures were performed on crushed ice in order to keep the temperature below 4°C. The 1 ml cryo-tubes were stored in a freezer at -140°C. The samples that are infected will be used for processing in further studies. One aliquot from each collection session was spotted onto FTA cards in order to bind the DNA to facilitate easy transport and subsequent PCR testing during future research studies.

Testing samples to detect *Ehrlichia ruminantium*

In order to detect *E. ruminantium*, DNA extraction and PCR testing, targeting the pCS20 region of the *E. ruminantium* genome was performed. A cryo-preserved sample from each of the eight subsequent collections was randomly selected for these procedures.

DNA extraction

DNA extraction was performed according to the guidelines of the QIAamp DNA Mini Kit (Qiagen). The samples were each marked for accurate identification and equilibrated at room temperature (15-25°C). A total of 10 samples were prepared which included eight samples from the respective collections, one positive control (*E. ruminantium* Welgevonden strain) and one negative control consisting of distilled water.

Two hundred (200) µl from each sample was transferred to a microcentrifuge tube. Each sample was subsequently homogenized using a rotor-stator homogenizer. One hundred (100) µl of Buffer ATL was added to each sample. Twenty (20) µl proteinase K was added to each sample and mixed by vortexing. The samples were then incubated at 56°C for 10 minutes in order for complete lysis to occur. Two hundred (200) µl of Buffer AL was then added to each sample, mixed by pulse vortexing for 15 minutes and incubated at 70°C for 10 minutes. Two hundred (200) µl of ethanol (96%-100%) was added to each sample and mixed by pulse vortexing for 15 seconds. Each sample mixture was then applied to a QIAamp Mini Spin column in a 2 ml collection tube and closed cap. The sample mixtures were then centrifuged at 8000 rpm for 1 minute. The QIAamp Mini spin column from each

sample was then placed in a clean 2 ml collection tube and the tubes containing the filtrate were discarded. Five hundred (500) µl Buffer AW1 was added to the QIAamp Mini spin column from each sample and centrifuged at 8000 rpm for 1 minute. The QIAamp Mini spin column from each sample was placed in a clean 2 ml collection tube and the tubes containing the filtrate were discarded. Five hundred (500) µl Buffer AW2 was added to the QIAamp Mini spin column from each sample and centrifuged at 14000 rpm for 3 minutes. The QIAamp Mini spin column from each sample was then placed in a clean 2 ml collection tube and the tubes containing the filtrate were discarded. Two hundred (200) µl Buffer AE was added to the QIAamp Mini spin column from each sample, incubated at room temperature for 1 minute and centrifuged at 8000 rpm for 1 minute.

DNA quantification

DNA quantification was performed on each of the samples according to the Qubit™ dsDNA BR Assay Kit guidelines and Qubit® 2.0 Fluorometer.

PCR analysis

Samples were prepared for PCR according to the Thermo Scientific™ Phusion™ Flash High-Fidelity PCR Master Mix guidelines. For a nested PCR targeting the pCS20 gene of *E. ruminantium*, two PCR reactions were performed. For the first reaction, to achieve a total volume of 20 µl for each sample, each sample was prepared to consist of seven (7) µl H_2O, 10 µl 2X Phusion Flash Master Mix, 1 µl forward primer (AB128), 1 µl reverse primer (AB130) and 1 µl of DNA each prepared during the DNA extraction process. Ten samples were prepared which included the eight field samples, one positive control and one negative control.

The first PCR reaction was performed according to specified guidelines. Initial denaturation at 98°C for 10 seconds, denaturation at 98°C for 1 second, annealing at 50°C for 5 seconds, extension at 72°C for 15 seconds and final extension at 70°C for 1 minute for a total of 30 cycles and held at 4°C until collection.

For the second PCR reaction, to achieve a total volume of 20 µl for each sample, each sample was prepared to consist of 7.5 µl H_2O, 10 µl Phusion Flash Master Mix, 1 µl forward primer (AB128), 1 µl reverse primer (AB129) and 0.5 µl of the pure DNA amplicons generated during the first PCR reaction.

The second PCR reaction was performed according to specified guidelines. Initial denaturation at 98°C for 10 seconds, denaturation at 98°C for 1 second, annealing at 50°C for 5 seconds, extension at 72°C for 15 seconds and final extension at 70°C for 1 minute for a total of 30 cycles and held at 4°C until collection.

Samples were analyzed by agarose gel electrophoresis and ethidium bromide staining to determine whether they contained *E. ruminantium* by demonstrating the amplified DNA from the organism.

Results

Determine the efficacy and persistent efficacy of a 1.0% fipronil pour-on solution against *Amblyomma hebraeum* ticks on boer goats

Initial trial – backline treatment method

A total of 28 and 25 *A. hebraeum* ticks were counted on D0 of the initial trial on the animals in the TG and CG respectively. A total of 18 and 25 *A. hebraeum* ticks were counted on D4 of the initial trial on the animals in the TG and CG respectively. A summary of animals in the TG and their respective tick counts on D0 and D4 can be seen in Table 1 and Table 2. Using the calculations from Holdsworth *et al.* (2006) to determine the therapeutic- and persistent efficacy of an acaricide against multi-host ticks, the efficacy of a 1.0% fipronil pour-on solution against *A. hebraeum*, four days after treatment, when applied topically in a single line from the shoulder blades to the root of the tail was 35.71% and 28% respectively.

Table 1 Summary of study animals in TG and the number of *A. hebraeum* ticks counted per region on day 0 of the initial trial

Treated Group (TG)						
	Number of *A. hebraeum* ticks counted per region					
Animal ID	**Head, Neck & chest**	**Axilla & ventral abdomen**	**Perineal & Peri-anal**	**Inguinal & Groin**	**Distal limb, feet & interdigital**	**Total D0 (N_0)**
TG1				3	3	6
TG2				4	6	10
TG3					1	1
TG4		1			2	3
TG5					3	3
TG6					5	5
Total count	**0**	**1**	**0**	**7**	**20**	**28**
Mean count	**0**	**0.17**	**0**	**1.17**	**3.3**	**4.7**

Table 2 Summary of study animals in TG and the number of *A. hebraeum* ticks counted per region on day 4 of the initial trial

Treated Group (TG)						
	Number of *A. hebraeum* ticks counted per region					
Animal ID	**Head, Neck & chest**	**Axilla & ventral abdomen**	**Perineal & Peri-anal**	**Inguinal & Groin**	**Distal limb, feet & interdigital**	**Total D4 (N_0)**
TG1				2	2	4
TG2				3	6	9
TG3				1		1
TG4		1		1		2
TG5						0
TG6					2	2
Total count	**0**	**1**	**0**	**7**	**10**	**18**
Mean count	**0**	**0.17**	**0**	**1.17**	**1.7**	**3**

Repeat trial – targeted treatment method

A total of 14 *A. hebraeum* ticks were counted on D0 of the repeat trial on animals selected in the TG. A total of 0 *A. hebraeum* ticks were counted on D4 of the repeat trial. A summary of animals and their respective tick counts on D0 and D4 of the repeat trial can be seen in Table 3 and Table 4.

Table 3 Summary of study animals in TG and the number of *A. hebraeum* ticks counted per region on day 0 of the repeat trial

Treated Group (TG)						
	Number of *A. hebraeum* ticks counted per region					
Animal ID	**Head, Neck & chest**	**Axilla & ventral abdomen**	**Perineal & Peri-anal**	**Inguinal & Groin**	**Distal limb, feet & interdigital**	**Total D0 (N_0)**
TG1					2	2
TG2	1			1		2
TG3				1		1
TG4				3	6	9
Total count	**1**	**0**	**0**	**5**	**8**	**14**
Mean count	**0.25**	**0**	**0**	**1.25**	**2**	**3.5**

Table 4 Summary of study animals in TG and the number of *A. hebraeum* ticks counted per region on day 4 of the repeat trial

Treated Group (TG)						
	Number of *A. hebraeum* ticks counted per region					
Animal ID	**Head, Neck & chest**	**Axilla & ventral abdomen**	**Perineal & Peri-anal**	**Inguinal & Groin**	**Distal limb, feet & interdigital**	**Total D4 (N_0)**
TG1	0	0	0	0	0	0
TG2	0	0	0	0	0	0
TG3	0	0	0	0	0	0
TG4	0	0	0	0	0	0
Total count	**0**	**0**	**0**	**0**	**0**	**0**
Mean count	**0**	**0**	**0**	**0**	**0**	**0**

Using the calculation from Holdsworth *et al.* (2006) to determine the therapeutic efficacy of an acaricide against multi-host ticks, the efficacy of a 1.0% fipronil pour-on solution against *A. hebraeum*, when applied in both axillary regions, both inguinal regions, perineal region and on all four feet of an animal was 100%. A total of 0 *A. hebraeum* ticks were counted on D7 of the repeat trial on the animals selected in the TG and a total of 13 *A. hebraeum* ticks were counted on D0 and D7 on animals selected in the CG. A summary of animals included into the TG and CG and their respective tick counts on D7 of the repeat trial can be seen in Table 5 and Table 6.

Table 5 Summary of study animals in TG and the number of *A. hebraeum* ticks counted per region on day 7 of the repeat trial

Treated Group (TG)						
	Number of *A. hebraeum* ticks counted per region					
Animal ID	**Head, Neck & chest**	**Axilla & ventral abdomen**	**Perineal & Peri-anal**	**Inguinal & Groin**	**Distal limb, feet & interdigital**	**Total D7 (N_0)**
TG1	0	0	0	0	0	0
TG2	0	0	0	0	0	0
TG3	0	0	0	0	0	0
TG4	0	0	0	0	0	0
Total count	**0**	**0**	**0**	**0**	**0**	**0**
Mean count	**0**	**0**	**0**	**0**	**0**	**0**

Table 6 Summary of study animals in CG and the number of *A. hebraeum* ticks counted per region on day 7 of the repeat trial

Control Group (CG)						
	Number of *A. hebraeum* ticks counted per region					
Animal ID	**Head, Neck & chest**	**Axilla & ventral abdomen**	**Perineal & Peri-anal**	**Inguinal & Groin**	**Distal limb, feet & interdigital**	**Total D7 (N_0)**
CG1		1			1	2
CG2					4	4
CG3			1		5	6
CG4					1	1
Total count	**0**	**1**	**1**	**0**	**11**	**13**
Mean count	**0**	**0.25**	**0.25**	**0**	**2.75**	**3.25**

Using the calculation from Holdsworth *et al.* (2006) to determine the persistent efficacy of an acaricide against multi-host ticks, the persistent efficacy of a 1.0% fipronil pour-on solution against *A. hebraeum*, when applied to both axillary regions, both inguinal regions, perineal region and all four feet and interdigital region of an animal seven days after treatment was 100%.

Detection of *Ehrlichia ruminantium* in *Amblyomma hebraeum* ticks

A summary of the total number of *A. hebraeum* ticks collected from goats and cattle at study site B during each of the eight collection sessions is shown in Table 7. Between 85 and 183 *A. hebraeum* ticks that were collected during each collection session, and which were confirmed to be alive, was used to facilitate the detection and cryo-preservation of *E. ruminantium*.

Table 7 Summary of the total number of *A. hebraeum* ticks collected from goats and cattle at study site B during each of the eight collection sessions (B-I)

Collection session	B	C	D	E	F	G	H	I
Number of ticks collected from goats	15	35	20	17	3	10	6	0
Number of ticks collected from cattle	70	70	90	97	180	86	86	117
Total number of ticks collected	**85**	**105**	**110**	**114**	**183**	**96**	**92**	**117**

The results of the agarose gel electrophoresis is shown in Figure 3. Based on these findings of the samples prepared from the collected field samples, it was concluded that *A. hebraeum* collected from goats and cattle at this particular study site were infected with *E. ruminantium*. The *E. ruminantium*-positive samples prepared from the collected *A. hebraeum* ticks during each of the eight subsequent collections were cryo-preserved.

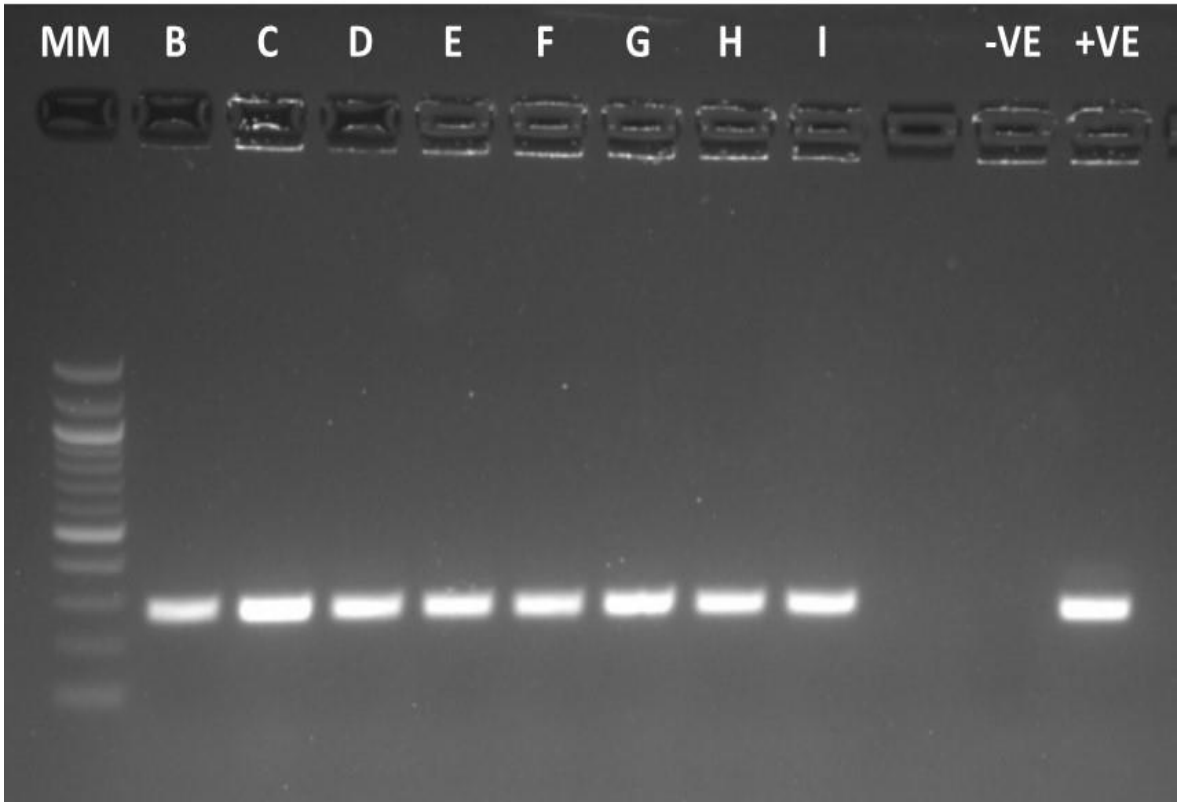

Figure 3 PCR products obtained and visualized by agarose gel electrophoresis. Lane M: 100 Bp (Base pair) ladder. Lane B-I: is the DNA amplification products from the isolates prepared from the respective tick collection sessions. Lane -VE: is the negative control sample. Lane +VE: is the DNA amplification products of the positive control sample.

Discussion

Determine the efficacy and persistent efficacy of a 1.0% fipronil pour-on solution against *Amblyomma hebraeum* ticks on boer goats

According to Holdsworth *et al*. (2006) for a product to be effective against multi-host ticks a target therapeutic efficacy of at least 95% should be achieved within four days after treatment. And for a product to qualify for persistent efficacy against multi-host ticks a target persistent efficacy of at least 95% on the TG of animals must be achieved during weekly (7 day interval) tick counts for the period for which persistent efficacy needs to be claimed. As demonstrated in this study, the efficacy of a 1.0% fipronil pour-on solution applied topically in a single line from the shoulder blades to the root of the tail ensuring that maximum contact with the skin is obtained was 35.71% and can therefore not be considered effective for the treatment of *A. hebraeum* on goats. This result was to some extent expected, since the main attachment site for *A. hebraeum* ticks on the study animals were around the feet and interdigital region. Even though fipronil has been shown to distribute across the skin in the beagle dog (Cochet, Birckel, Bromet-Petit, Bromet & Weil 1997), no studies have been conducted to demonstrate the distribution of fipronil across the skin of goats. Since the attachment sites of *A. hebraeum* is a relative distance from the site of application, the fipronil was not able to reach these distant attachment sites and was therefore not effective.

In order to further determine the efficacy of a 1.0% fipronil pour-on solution against *A. hebraeum* a different treatment method, which targeted the attachments sites of *A. hebraeum* was followed. As can be seen from the results, by following this targeted treatment method, a 1.0% fipronil pour-on solution is 100% effective against *A. hebraeum* and also have a persistent efficacy of seven days against *A. hebraeum* after treatment. The trial was not extended further to evaluate whether a 1.0% fipronil pour-on solution will have a persistent efficacy of more than seven days, since by the time this trial was performed, the tick season was already at an end and further evaluation may have resulted in a false positive result.

This study demonstrated that for boer goat farmers to control *A. hebraeum* with a 1.0% fipronil pour-on solution, a more targeted treatment method should be considered. Since the predilection sites of *A. hebraeum* is in the interdigital region and around the feet of goats, as well as the axillary region, the inguinal region and the perineal region, treatment should be applied directly to these attachment sites. Not only will this ensure that the success rate of treatment will be higher, but from an economic perspective the acaricide can be used more efficiently with minimal wastage. From a practical point of view however, this treatment method is more labor intensive since it requires animals to be individually handled and restrained in order to apply the acaricide directly to the targeted areas on the body.

This study also demonstrated that, compared to cattle, the number of *A. hebraeum* ticks on goats may not be very high. This is in accordance with the findings of various other reviews and studies (Nyangiwe & Horak 2007; Ngumi *et al.* 1997 Baker & DuCasse 1968; Hove *et al.* 2008; Rechav & De Jager 1991). However even a small number of *A. hebraeum* ticks may result in the development of wounds, secondary abscess formation and severe lameness. The transmission of *E. ruminantium* may also be facilitated, even with low *A. hebraeum* tick infestations (Howard & Norval 1989). *Amblyomma hebraeum* should therefore be controlled in goats in order to reduce the negative effects associated with these ticks on goats. During this trial *Rhipicephalus eversti,* the red legged tick, were numerous and mainly found in the perineal region of the study animals.

A 1.0% fipronil pour-on solution can be used strategically, by using an approach to target the main predilection sites of *A. hebraeum* on goats. Since fipronil has a relatively long meat withdrawal period of 100 days in cattle, the use of fipronil in especially meat producing goats like the boer goat may be a concern. However, a 1.0% fipronil pour-on solution may be advocated to be used in breeding animals that is not destined for slaughter.

MacIvor and Horak (1987) advocated the use of regular acaricide application to the feet of goats in the Eastern Cape Province of South Africa, during the spring and summer periods of August to December in order to reduce the incidence of abscess formation. During the current study, conducted in the Limpopo Province of South Africa, *A. hebraeum* was still

present on goats during the late autumn and early winter period. Therefore goats in the Limpopo Province, should be evaluated for the presence of ticks and acaricide should be applied, not only during the spring and summer periods but also during the autumn and even early winter periods to reduce the incidence of abscess formation.

MacIvor and Horak (1987) also stated that the efficacy of the acaricide applied to the feet may be reduced since it is constantly exposed to soil, vegetation and moisture which may remove or dilute the acaricide at this site. This is an important practical consideration to take into account when a pour-on solution is applied to the feet of goats, since this constant exposure to soil, vegetation and moisture, may have a negative effect on the therapeutic- and persistent efficacy of the acaricide.

To control *A. hebraeum* on goats in the Limpopo Province, a more targeted treatment approach is recommended. The known predilection sites where *A. hebraeum* attach on goats should be targeted namely the chest and axillary region, the inguinal region including the udder and teats in does and testes in rams, the perineal region and most importantly the feet and interdigital space. Targeted hand spraying, foot and belly baths or pour-on formulations can be used. Another approach that warrants further investigation is the use of aerosolized acaricides. It is possible to formulate acaricides in an aerosolized container to spray or expel the acaricide with higher pressure. This is especially beneficial for the application to the feet and interdigital space of goats since the expulsion of the acaricide with pressure ensures that the acaricide reaches these attachment sites.

Detection of *Ehrlichia ruminantium* in *Amblyomma hebraeum* ticks

This part of the study was conducted to detect and cryo-preserve *E. ruminantium* from *A. hebraeum* ticks from a heartwater endemic region. Adult *A. hebraeum* ticks were collected from goats and cattle on a commercial farm in the Limpopo province of South Africa where these ticks occur and where heartwater is endemic. Cattle were included in the study to ensure that a minimum of 50 *A. hebraeum* ticks could be collected during each tick collection session.

Samples were prepared in the laboratory as described above. To detect *E. ruminantium* in the samples, DNA extraction, nested PCR analysis which targeted the pCS20 gene of *E. ruminantium* was performed. Agarose gel electrophoresis was performed to visualize the amplified DNA. As demonstrated in the results *E. ruminantium* was present in eight out of eight ground-up-tick-supernatant samples. *Ehrlichia ruminantium* was therefore successfully detected and cryo-preserved from *A. hebraeum* ticks.

Nested pCS20 PCR assay is used as a method to amplify a specific 279 base pair fragment from DNA of *E. ruminantium*. Nested PCR is a highly sensitive method to determine the presence of *E. ruminantium* in various samples, including *Amblyomma* ticks (Martinez *et al.* 2004; Faburay *et al.* 2007). For the purpose of this study it was therefore an appropriate method to determine whether *A. hebraeum* ticks are infected with *E. ruminantium*. The pCS20 region is especially useful for epidemiological studies to identify different isolates of *E. ruminantium* since this region contains polymorphism (Allsopp, Hatting, Vogel & Allsopp 1999) and by performing DNA sequencing on this region, single nucleotide polymorphisms (SNPs) can be identified to ultimately identify different isolates of *E. ruminantium* (Steyn *et al.* 2006).

Various studies have been performed whereby *E. ruminantium* was isolated from *Amblyomma* ticks (Theiler & Du Toit 1928; Alexander 1931; Barré *et al.* 1984; Ngumi *et al.* 1997). These eight novel cryo-preserved samples, which originate from a specific geographic location in the Limpopo Province of South Africa, may now be used in further research studies to attempt to isolate *E. ruminantium* by experimentally infecting a susceptible host, by administering the cryopreserved sample intravenously, and isolating the *E. ruminantium* in the blood of the susceptible host during the pyrexic stage (Theiler & Du Toit 1928; Alexander 1931; Ngumi *et al.* 1997). If the isolation of *E. ruminantium* is successful an attempt may also be made to cultivate *E. ruminantium* on appropriate cell culture like mammalian endothelial cell lines (Bezuidenhout 1987). It has been demonstrated that in vitro cultivation of *E. ruminantium* from *A. hebraeum* tick stabilates is possible (Bezuidenhout, Paterson & Barnard 1985) and in vitro cultivation of *E. ruminantium* from the cryopreserved samples may also be attempted.

An additional advantage of the study was that it provided an opportunity to study the dynamics of *A. hebraeum* on boer goats and cattle during the summer, autumn and early winter period in the heartwater endemic region of the Limpopo Province of South Africa. As demonstrated, *A. hebraeum* was present on goats and cattle during this whole period. Another interesting observation was the attachment of *Hyalomma truncatum*, the bont-legged tick, along with *A. hebraeum* in the interdigital region and feet, the axillary and chest region and on the tail end of the goats (Figure 4). *Hyalomma truncatum* also have long mouthparts and contributed towards the formation of wounds, abscess formation and severe lameness.

Figure 4 *Amblyomma hebraeum* and *Hyalomma truncatum* ticks attached in the interdigital region of a boer goat

Another interesting observation, demonstrated in Figure 5, was the attachment of *A. hebraeum* ticks to the tails of goats. This demonstrates that animals should be thoroughly evaluated to determine the presence of *A. hebraeum* ticks and that acaricide treatment should also be applied to this region to control this tick.

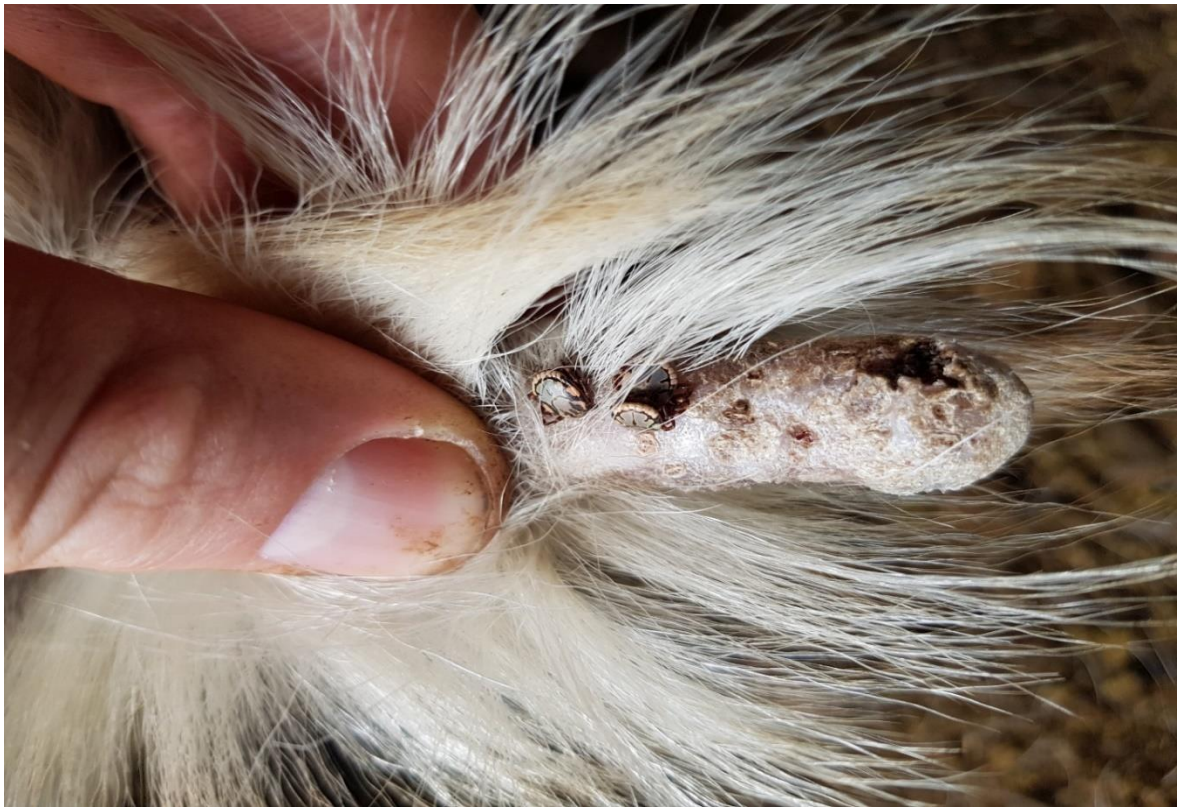

Figure 5 Adult male *Amblyomma hebraeum* ticks attached to the tail of a goat

Various studies have been performed to demonstrate the use *A. hebraeum* ticks for the production of a vaccine against heartwater (Alexander 1931; Bezuidenhout 1982; Bezuidenhout 1985). Very few, if any further research has been performed since then using *A. hebraeum* ticks to prepare a heartwater vaccine.

Since extensive inter-genome recombination occurs, which is the driving force behind genetic diversity *of E. ruminantium* and its ability to evade the host's immune system, there are various strains of *E. ruminantium* (Allsopp & Allsopp 2007; Nakao *et al*. 2011; Cangi *et al.* 2016). Any given sample may contain more than one genus and/or genotype (Allsopp,

Visser, Du Plessis, Vogel, Allsopp 1997 cited by Allsopp *et al.* 1999). Since multiple *A. hebraeum* ticks were included to prepare these cryopreserved samples, they may contain multiple strains of *E. ruminantium*, and these cryopreserved samples may therefore consist of a "cocktail of strains" with varying genotypes. Molecular methods can be used to attempt to determine and classify the genotype of *E. ruminantium*. DNA extraction and PCR first have to be performed to produce amplicons of specific target DNA. Target DNA regions, specific for *E. ruminantium*, that can be targeted for amplification and molecular probing to distinguish between strains include the 16S rRNA gene (Allsopp *et al.* 1999), the map1 gene (Martinez *et al.* 2004; Raliniaina, Meyer, Pinarello, Sheikboudou, Emboulé, Kandassamy, Adakal, Stachurski, Martinez, Lefrançois, Vachiéry 2010) and the pCS20 genomic region (Steyn *et al.* 2006). Sequencing of the amplified DNA may also be used to distinguish and identify new strains or isolates (Steyn *et al.* 2006). More advanced methods like MLST can also be used to determine genetic and phylogenetic diversity and to generate data across the African continent (Nakao *et al.* 2011; Cangi *et al.* 2016).

Should the isolation of *E. ruminantium* from these cryo-preserved samples be successful and possible strain identification or genotyping can be accomplished, it may pave the way for future virulence studies, cross-protection studies and region specific vaccination strategies.

Conclusion

The results in this study indicated that a 1.0% fipronil pour-on solution is not effective against adult *A. hebreum* ticks when applied only on the backline of boer goats. The reason for this is most likely that the active ingredient fipronil does not reach the predilection sites of these ticks which is predominantly the interdigital region and the feet, as well as the axillary region, the inguinal region and perineal region. The trial was repeated and a more targeted treatment approach was followed. It was demonstrated that a 1.0% fipronil pour-on solution was 100% effective against adult *A. hebreum* ticks and has a persistent efficacy of seven days through strategic application, targeted at the predilection sites of *A. hebraeum*. It is possible for this persistent effect to be longer and further research needs to be conducted by repeating this trial during the peak seasonal occurrence of *A. hebraeum*. A 1.0% fipronil pour-on formulation can therefore be used by boer goat farmers as an effective acaricide to control *A. hebraeum*. The study also demonstrated that *E. ruminantium* can be successfully detected and cryo-preserved from *A. hebraeum* ticks collected from cattle and goats in a region where heartwater is endemic. DNA extraction, nested PCR analysis targeting the pCS20 region of the genome of *E. ruminantium* and visualizing the amplified DNA by agarose gel electrophoresis is an appropriate method to demonstrate that the detection and cryo-preservation *E. ruminantium* from adult *A. hebraeum* ticks was successful. The eight novel cryo-preserved samples can be used for further research including the isolation and cultivation of *E. ruminantium*, virulence studies and cross protection studies as well as the identification of genetic diversity and comparing current strains with historical isolates in order to ultimately improve our understanding of the dynamics of heartwater. It may also facilitate the implementation of novel disease control methods like region specific vaccination strategies.

Limitations

Although the sample size calculated and used for the acaricide efficacy trial was adequate, the results would have been much more powerful and significant if there were more ticks present on the study animals. Some individual animals only had one or two ticks attached whilst other animals had more ticks attached at specific attachment sites. This was however a direct reflection of the dynamics of the occurrence of *A. hebraeum* and the current situation at farm level. Factors that may have influenced this variable include the seasonal occurrence of *A. hebraeum* ticks, with lower numbers of *A. hebraeum* present during the late autumn period when this trial was performed as well as the fact that there are relatively fewer *A. hebraeum* ticks found on goats compared to cattle. A definite challenge was to locate a suitable study site at which the study animals harbored enough *A. hebraeum* ticks and where the study animals can be divided into a TG and CG, where they can be kept in separate camps in close proximity to each other to ensure that the tick challenge is similar between both groups. The lower tick challenge however had the advantage that the tick load on the study animals in the CG was low enough to prevent severe damage, pain and subsequent welfare concerns.

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Appendix 1

Approval from the Animal Ethics Committee (AEC) of the University of Pretoria (Project number V109-17)

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Determining the efficacy of a 1,0% Fipronil pour-on solution against *Ambylomma hebraeum* on boergoats and isolation of *Ehrlichia ruminantium* strains from different commercial farms in the Limpopo Province of South Africa to facilitate the implementation of novel disease control methods**
PROJECT NUMBER	**V109-17**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr. JG Nel**

STUDENT NUMBER (where applicable)	**U_28100141**
DISSERTATION/THESIS SUBMITTED FOR	**MSc**

ANIMAL SPECIES	**Caprine**	
NUMBER OF ANIMALS	**12**	
Approval period to use animals for research/testing purposes		**October 2017 – October 2018**
SUPERVISOR	**Prof. F Jongejan**	

KINDLY NOTE:

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

APPROVED	Date 23 November 2017
CHAIRMAN: UP Animal Ethics Committee	Signature

S4285-15

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

Extension No. 1

PROJECT TITLE	**Determining the efficacy of a 1,0% Fipronil pour-on solution against *Ambylomma hebraeum* on boergoats and isolation of *Ehrlichia ruminantium* strains from different commercial farms in the Limpopo Province of South Africa to facilitate the implementation of novel disease control methods**
PROJECT NUMBER	**V109-17**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr. JG Nel**

STUDENT NUMBER (where applicable)	**U_28100141**
DISSERTATION/THESIS SUBMITTED FOR	**MSc**

ANIMAL SPECIES	**Caprine**	
NUMBER OF ANIMALS	**12 used**	
Approval period to use animals for research/testing purposes		**March 2018 – March 2019**
SUPERVISOR	**Prof. F Jongejan**	

KINDLY NOTE:

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

APPROVED	Date 28 March 2018
CHAIRMAN: UP Animal Ethics Committee	Signature

S4285-15

Appendix 2

Permission to conduct research in terms of section 20 of the Animal Diseases Act (Act 35 of 1984) from the Department of Agriculture Forestry and Fisheries (DAFF)

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

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

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
Reference: 12/11/1/6

Dr Johan Gerhard Nel
Faculty of Veterinary Science
University of Pretoria

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

Dear Dr Nel

Your fax / memo / letter/ Email dated 1 November 2017, requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers.

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

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. All potentially infectious material utilised or collected during the study is to be destroyed at the completion of the study. Records must be kept for five years for audit purposes. A dispensation application may be made to the Director Animal Health in the event that any of the above is to be stored or distributed;
3. Approval is needed from the Medicines and Related Substances Control Act, 1965 (Act No 101 of 1965) for the importation of the unregistered Fipronil 1.0% pour-on;
4. Permission in terms of the Fertilizers, Farm Feeds, Agricultural Remedies and Stock Remedies Act, 1947 (Act No 36 of 1947) and/or the Medicines and

– 1 –

Related Substances Control Act, 1965 (Act No 101 of 1965) may be needed prior to the start of the study;

5. The study may only be performed in the Capricorn District of Limpopo Province as per the letter from Dr Mampane, Director of Veterinary Services, Limpopo Province;
6. Ticks must be transported in escape proof containers and packaged in compliance with the Regulations of the National Road Traffic Act, 1996 (Act No 93 of 1996);
7. All potentially infectious waste must be autoclaved prior to disposal by The Waste Group as accredited waste management company;
8. A meat withdrawal of 200 days must be adhered to;
9. No milk from these goats may be utilised for human consumption;
10. This Section 20 approval is valid until 30 October 2018.

Title of research/study: Determining the efficacy of a 1.0% Fipronil pour-on solution against *Ambylomma hebreaeum* on Boergoats and isolation of *Ehrlichia ruminantium* strains from different commercial farms in the Limpopo Province of South Africa to facilitate the implementeation of novel disease control methods
Researcher (s): Dr Johan Gerhard Nel
Institution: Faculty of Veterinary Science, UP
Your Ref./ Project Number: V109-17
Our ref Number: 12/11/16

Kind regards,

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH

Date: 2018 -01- 17

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

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

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

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
Reference: 12/11/1/6

Dr Johan Gerhard Nel
Faculty of Veterinary Science
University of Pretoria

Dear Dr Nel,

RE: Dispensation on Section 20 Approval in Terms of the Animal Diseases Act, 1984 (Act No 35 of 1984) for: Determining the Efficacy of a 1.0% Fipronil Pour-on Solution Against *Amblyomma hebreaeum* on Boergoats and Isolation of *Ehrlichia ruminantium* Strains from Different Commercial Farms in the Limpopo Province of South Africa to Facilitate the Implementation of Novel Disease Control Methods

Your email dated 1 November 2017 refers.

A dispensation is hereby granted on point 2 of the Section 20 approval that was issued for the above mentioned study (attached):

i) The ground-up tick-supernatant may be stored at the DVTD Biobank, Faculty of Veterinary Science, University of Pretoria;
ii) The stored supernatant may only be used for further research after having obtained new Section 20 approval;
iii) The stored supernatant may not be outsourced without prior written approval from DAFF.

Kind regards,

DR. MPHO MAJA
DIRECTOR: ANIMAL HEALTH
Date: 2018 -01- 17

Appendix 3

Approval from the Veterinary Clinical Committee (VCC) of the Department of Health (DoH) to conduct a research trial and to use an unregistered veterinary pharmaceutical product

Republiek van Suid-Afrika

Republic of South Africa

DEPARTEMENT VAN GESONDHEID
PRIVAATSAK X828
PRETORIA
0001

Telefoon:
Telephone: 012 395 8351

Faks:
Fax: 0866 330 298

DEPARTMENT OF HEALTH
PRIVATE BAG X828
PRETORIA
0001

- Inquiries Dr A T Sigobodhla
- Reference: Fiprocules

Date: 02/03/2018

67 Nicholson Street
BROOKLYN
1776

Att: **Dr JG Nel**
Tel: 082 785 1776
Fax: 012 346 2095
Email: **neljg7@gmail.com**

RE: FIPROCULES

Your response on the above matter refers.

Your product has been discussed by the Veterinary Clinical Committee and the following is the outcome:

Yours faithfully

For and on behalf of the CEO of SAHPRA

Product Name: Fiprocules

Species:	**Caprine**
Applicant:	**Dr J G Nel**
Study Title:	Determining the efficacy of a 1.0 % fipronil pour on solution against Amblyoma hebreaeum on boergoats and isolation of Ehrlichia ruminantium strains from different commercial farms in the Limpopo province of South Africa to facilitate the implementation of novel disease control methods

VCC recommendations 23/02/2018

1. The study protocol is approved.
2. Applicant may commence study once approval has been granted also by DAFF (Section 20).

Appendix 4

Permission to import and use an unregistered veterinary pharmaceutical product in terms of the provisions under section 21 of Act 101 of 1965 from the Medicines Control Council (Permit reference number 26/2/2 VCT/01/2018)

health
Department:
Health
REPUBLIC OF SOUTH AFRICA

Private Bag X828, PRETORIA, 0001. 27th Floor, Room 2710, Civitas, Cnr Thabo Sehume & Struben Street, PRETORIA, 0001
Tel: +27 (0) 12 395 8000, Fax: +27 (0) 12 395 8422

Tel: (012) 395 8353
Fax: 0866 329 637

Enquiries: Dr A T Sigobodhla
References: 26/2/2 VCT/01/2018)
05 March 2018

67 Nicholson Street
BROOKLYN PRETORIA
0181

Att: Dr J.G. Nel
Tel: 082 785 1776
Fax: 012 346 2095
Email: neljg7@gmail.com

PERMISSION FOR USE OF UNREGISTERED MEDICINES IN TERMS OF THE PROVISIONS SECTION 21 OF ACT 101 OF 1965.

Your application refers:

Authorisation is hereby granted for the import / purchase of the following unregistered product on condition that:

Name of product:	Fiprocules x 5 Bottles
Species:	Caprine
Description of patients:	Boergoat, Male & female, 6-12 mnts
Diagnosis/purpose:	Tick Control – Efficacy & persistent efficacy trail
Authorisation number:	VCT/01/2018

Kindly note that this permit **allows you a once-off supply** of the imported product for use in the clinical trial. You are reminded to furnish this office with an interim report on the clinical trial.

Yours faithfully

Dr A T Sigobodhla