Vaccination of on-farm cattle against heartwater: Safety and efficacy of *Ehrlichia ruminantium* (Welgevonden) vaccine

by

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Submitted in partial fulfilment of the requirements

for the degree Magister Scientiae

in the Faculty of Veterinary Science

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2018

DECLARATION

I, Ratselane Daniel Marumo hereby declare that the research entitled "Vaccination of on-farm cattle against heartwater: Safety and efficacy of *Ehrlichia ruminantium* (Welgevonden) vaccine" presented in this dissertation in partial fulfilment of the requirements for the degree MSc Tropical Animal Health, was executed by myself, under the guidance of my supervisors.

I further declare that this dissertation has not been submitted in the past, or is to be submitted for a degree at the University of Pretoria or any other university.

…….
Ratselane Daniel Marumo

Date…05/12/2018……..

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to the following individuals for their unique and valuable contributions made to this study:

My supervisor, **Dr. Ben Mans** for all the guidance, advice and support throughout the duration of this work. His passion about the study has inspired patience and innovation in me.

My co-supervisor, **Dr. Skhumbuzo Mbizeni** for all the support and for providing me with his deep knowledge and expert advice on tick and tick-borne diseases.

Dr. Helena Steyn from ARC-OVR (Vaccine, Diagnostic and Development department) with all her assistance with pCS20 quantitative real-time TaqMan (qPCR) work. Providing us with *Amblyomma hebraeum* nymphs from her colony, insight knowledge about tick infectivity, thesis editing and guidance.

Mr. Lefoka Molepo for all the guidance, advice, vaccine formulation, animal monitoring, providing resources to carry out this work.

All staff members at ARC-OVR Epidemiology, Parasites and Vectors for their support, especially **Mr. Christo Troskie** for all the assistance with animal monitoring, treatments and euthanasia, **Mrs. Antoinette Josemans** for guidance, advice and vaccine formulation, **Mr. Johannes Komape**, **Mr. Isaac Kale**, **Mr. David Letsoalo**, **Mr. John Bambo**, **Mr. Sello Tlatla**, **Mr. Solly Bambo**, **Mr. Daniel Chipana**, **Mr. Solly Boikanyo** and **Mr. Klaas Makinta** for daily monitoring, temperature recording, cleaning and feeding the animals.

Prof. Abdalla A Latif, University of Kwa-Zulu Natal, School of Life Sciences, for all the guidance, expert advice on tick genera and biology, statistical analysis and thesis editing.

Lastly, I would like to thank the Almighty God for His mercies, my wife **Onalenna Marumo**, my daughter **Karabo Marumo** and my sisters for all the love and support shown during my studies.

ABSTRACT

Ehrlichia ruminantium (Rickettsiales, Rickettsiaceae) is the causative agent of heartwater disease transmitted to cattle, sheep, goats and wild ruminants e.g. springbok by *Amblyomma hebraeum* in South Africa (SA). The current live blood vaccine (Ball 3) used in SA has limitations; it does not show efficacy against most field strains, it is virulent and concurrent treatment with antibiotic is necessary and it is produced in life animals which hinders its quality control. Second generation vaccines have not yet been developed to commercial stages. Previous experiments using an attenuated *E. ruminantium* (Welgevonden) tissue culture experimental vaccine in Merino sheep, Boer goats and Angora goats, administered through intramuscular (I/M) and intravenous (I/V) routes, without the use of antibiotic, showed promising results in terms of safety and efficacy. The objective of the current study was to test the safety and efficacy of this attenuated tissue culture vaccine in cattle, administered by the I/M route.

One sheep injected with 10 ml of the virulent Welgevonden heartwater strain was used to infect *Amblyomma hebraeum* nymphs. Twenty (8-24 months old) male Friesian Holstein cattle obtained from a heartwater and vector free area were used; ten were vaccinated with the attenuated Welgevonden tissue culture isolate intramuscularly and ten were untreated controls. The vaccine was prepared and inoculated with an estimated concentration of 1.11 X 10^6 *E. ruminantium* organisms in 2 ml. Tick challenge of both cattle groups was performed with 17 infected *A. hebraeum* (7 males/10 females) 35 days following vaccination. Cattle were screened serologically by the indirect fluorescent antibody test (IFAT) and by molecular tools using pCS20 quantitative real-time TaqMan (qPCR) before and after vaccination and challenge. Cattle were weighed before vaccination, during vaccination reactions and tick challenge (Days 0-77). Animals were monitored for clinical signs of heartwater disease and treated according to a score sheet when appropriate.

Samples of ticks that dropped from infected sheep on different days were highly infected (10^3) with *E. ruminantium* (Welgevonden) organisms as tested using qPCR and deemed suitable for animal challenge. The group of cattle (n=10) which were vaccinated intramuscularly, showed no clinical or local vaccine related reactions and no treatment was required. The vaccinated group was challenged 35 days post vaccination together with the untreated controls (n=10). The mean number of engorged female ticks that dropped from the vaccinated (n=7.7) and unvaccinated (n=8.3) groups showed no statistical difference. The vaccinated group did not show any clinical reactions, while 8/10 of the unvaccinated controls developed severe reactions and received treatment while one animal was euthanized following the score sheet. There was a statistical significant mean difference (p-value = 0.0003) in the final weight gain/loss between the vaccinated (mean 5.6 $\pm$ 2.84 Kg) and unvaccinated (mean -0.33 $\pm$ 2.78 Kg) groups. On Day 37 after tick challenge, 100% of the vaccinated and 80% of the unvaccinated cattle showed sero-conversion (1/180) in the IFAT.

The results of the study have demonstrated the safety and efficacy of the attenuated *E. ruminantium* (Welgevonden) experimental vaccine against homologous challenge in cattle as judged by the severe clinical reactions in the unvaccinated group. The vaccinated group also had a gain in mean body weight compared to the unvaccinated group after challenge.

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ARC-OVR	Agricultural Research Council-Onderstepoort Veterinary Research
AFGRI	Agricultural Food Growth Retail Investment
BA886	Bovine aorta endothelial cells 886
BUE	Bovine umbilical endothelial cells
bp	base pair
°C	degree Celsius
df	degree of freedom
DAFF	Department of Agriculture, Forestry and Fisheries
DoH	Department of Health
DNA	Deoxyribonucleic acid
ECF	East Coast fever
EDTA	Ethylene di-amine-tetra-acetate
ELISA	Enzyme-linked immunosorbent assay
Fig	Figure
FCS	Fetal calf serum
g	Gram
h	Hour
I/M	Intramuscular
IFAT	Indirect fluorescent antibody test
I/V	Intravenous
IU	International unit
kg	Kilogram
µl	Microlitre
µg	Microgram
LN_2	Liquid nitrogen
MAP-1	Major Antigenic Protein
MCC	Medicines Control Council
M	mean
ml	millilitre
rpm	rate per minute
S/C	subcutaneous
SADC	Southern African Development Community
n	number
PCR	Polymerase chain reaction
qPCR	pCS20 quantitative real-time TaqMan
SA	South Africa
SD	standard deviation
SPG	sucrose potassium glutamate
UF	University of Florida
UP	University of Pretoria
USAID	United States Agency for International Development
%	Percentage

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CHAPTER 1

1.1 INTRODUCTION

Ticks and tick-borne diseases pose a major constraint to livestock production in sub-Saharan Africa, including South Africa (SA). In 2003, the losses in SA due to ticks and tick-borne diseases were estimated to be $31.6 million annually (Minjauw and McLeod, 2003). Tick-borne diseases including anaplasmosis, babesiosis, theileriosis and heartwater seem to be the main limiting factor for livestock holders in Africa. Heartwater is the second major tick-borne disease of livestock in Africa after theileriosis and it occurs in most of the sub-Saharan regions, the neighbouring islands, and various small islands in the Caribbean and Atlantic Oceans (Norval, 1991; Mahan, 1992). Heartwater is an infectious, non-contagious tick-borne disease caused by the intracellular rickettsial agent *Ehrlichia ruminantium*. The disease affects mainly cattle, sheep, goats and some wild ruminants.

The vectors responsible for the transmission of the agent belong to the genus *Amblyomma*, the most important two vectors in southern Africa being *Amblyomma hebraeum* and *Amblyomma variegatum* (Walker, 1987).

According to the SA Department of Agriculture, Forestry and Fisheries (2010) there is an estimated ruminant livestock population of 13.8 million cattle, 23.9 million sheep and 5.8 million goats countrywide. A large percentage of these animals are found in the provinces where tick vectors are endemic. It has been estimated that farmers in Africa lose up to R975 million per annum due to tick-borne diseases, with SA farmers (both rural and commercial sectors) experiencing R220 million in annual losses (UF/USAID/SADC, 2005). In Southern African Development Community (SADC) countries the estimated current losses caused by heartwater alone is said to be at US$48 million (Allsopp, 2015). The economic losses due to heartwater ranges between 20 and 90% as the result of the associated high mortality rate (Carlos *et al*., 1999; Mahan *et al*., 2001; Kelly *et al*., 2011).

Currently, the control methods for heartwater in SA is through acaricide application and vaccination. Both these control mechanisms are unsatisfactory, expensive and requires excessive effort (Latif, 1993). Acaricides have undesirable residual effects on animal by-products, they are not environmentally friendly and often used inappropriately which leads to tick resistance (George *et al*., 2004; Graf *et al*., 2004). The only commercially available method of immunization is “infection and treatment” (Uilenberg, 1983). The vaccine is a cryopreserved preparation of sheep blood, which contains the virulent infective *E. ruminantium* organisms of the Ball 3 genotype (Neitz and Alexander, 1945; Haig, 1952). The vaccine is administered intravenously and daily body temperatures of all vaccinated animals must be monitored and

treated with oxytetracyclines immediately when the temperature reaction is noticed before the disease becomes irreversible (Mulinge, 1990). Problems are encountered with this procedure, namely that the blood vaccine should be kept at a temperature below -20 °C until use (Brayton *et al*., 2003). The vaccination process is a serious challenge for rural areas, as it requires intravenous administration and temperature monitoring thereof by trained staff (Allsopp, 2015). Lastly, the vaccine does not offer cross protection against many South African field strains (Oberem and Bezuidenhout, 1987b; Collins *et al*., 2003 and Zweygarth *et al*., 2005).

Zweygarth *et al*. (2005) have successfully developed an attenuated heartwater vaccine in tissue culture. When inoculated intravenously into Merino sheep or Boer goats, the attenuated culture-derived organisms did not produce disease. Some of the sheep and most of the goats reacted with a slight rise in body temperature but they remained healthy. The efficacy and safety of the vaccine as well as the duration of immunity and reversal to virulence after serial tick passages were also investigated. There are numerous advantages to an attenuated vaccine: It can induce durable immunity up to six months in tick-free conditions, it is produced *in vitro* allowing better laboratory quality assurance and it is effective. Treatment of vaccine reactions with antibiotics would not be required, which would further reduce the risks and costs. Finally, the vaccine strain has a wider spectrum of cross-immunity than that used in the current commercial blood vaccine (Ball 3), which makes it possible to use the vaccine in all endemic areas in the country (Zweygarth *et al*., 2005; 2008). Recent results generated by Haw (2013) showed that in Angora goats, which are considered the most susceptible species to heartwater, I/M vaccination with the heartwater attenuated vaccine resulted in 93% of animals that did not require antibiotic treatment after vaccination. All vaccinated Angora goats were 100% protected against lethal challenge.

Faburay *et al*. (2007) conducted a trial under controlled conditions where they had thirteen sheep (seven vaccinated and six controls). They inoculated seven sheep with 2 ml inactivated *E. ruminantium* (Gardel) subcutaneously and the same booster dose was given a month later. Three weeks after the booster vaccine, they gave both groups a heterologous challenge of 2 ml of *E. ruminantium* (Kerr Seringe) intravenously. Both groups reacted with elevated temperature and there was 43% (3/7) survival rate from vaccinated group, whereas none from the control group survived. They concluded that the inactivated Gardel Montanide ISA50 vaccine used in West Africa provided partial protection (Gardel strain has low efficacy against Kerr Keringe) since they only experienced a 57% death rate.

Previous work done by Combrink *et al*. (unpublished data) using the attenuated strain on cattle, determined the correct dose using different concentrations of 10^5, 10^6 and 10^7 organisms per 2 ml. It was found that the concentration that works well in cattle was 10^6 organisms per 2 ml, hence a similar dosage was used in the present trial. The attenuated vaccine could also be thawed and kept for up to one hour on ice before dilution and vaccination without adverse effects.

The aim of the current study was to demonstrate the safety and efficacy of the heartwater attenuated tissue culture vaccine in cattle administered by the I/M route.

CHAPTER 2

LITERATURE REVIEW

2.1 Background

Heartwater (Cowdriosis) is an infectious, non-contagious, tick-borne disease of both domestic and wild ruminants caused by a rickettsia, *E. ruminantium*. According to Allsopp (2010), ticks of the genus *Amblyomma* transmit the organism and is of serious economic importance within the distribution range of the vector ticks. This includes all of sub-Saharan Africa, and several islands in the Caribbean. There are approximately 150 million animals that are at risk in sub-Saharan Africa (Allsopp, 2010), with SA contributing 43.6 million (Meissner *et al*., 2013).

This has led to the development of the "infection and treatment" type of immunization where animals are inoculated with fully virulent *E. ruminantium* organism of the Ball 3 strain and treated with oxytetracycline when body temperature starts to rise (Bezuidenhout, 1989). While this has been the only commercially available "vaccine" for many years, the spectrum of protection of the Ball 3 blood vaccine strain against other *E. ruminantium* strains is limited (Zweygarth *et al*., 2005). Therefore, there is a need for a safe vaccine that would offer a wider cross-protection against many *E. ruminantium* field strains. Zweygarth *et al*. (2008), stated that the "infection and treatment" vaccine had many challenges which included the necessity for chemotherapeutic intervention post immunization, the need to maintain the cold chain with liquid nitrogen or dry ice during vaccine transport and the expertise required for intravenous (I/V) injection, which is a strenuous activity if a large number of animals have to be immunized.

Jongejan (1991), first reported the use of an attenuated culture-derived vaccine in which the attenuation of a Senegalese strain of *E. ruminantium* was achieved after several passages *in vitro*. It is believed that this attenuated strain provided immunity to homologous challenge, but only provided limited protection against other strains (Jongejan *et al*., 1993). In the Gambia, a field trial was conducted and the attenuated Senegalese vaccine strain protected about 75% of sheep, whereas all the unvaccinated control sheep died due to heartwater (Faburay *et al*., 2007). The virulent Welgevonden strain of *E. ruminantium* in SA was successfully attenuated and provided full protection against homologous challenge with the virulent strain when used in Merino sheep and Boer goats under controlled experimental conditions (Zweygarth *et al*., 2005).

Previous results obtained by Zweygarth *et al* (2008) demonstrated the success of the immunization using attenuated live organisms. They also demonstrated that it could be possible to use only one subcutaneous (S/C) or I/M injection for effective immunization, replacing the intravenous route of application, a factor that makes the attenuated tissue culture vaccine more attractive to the end users.

According to Allsopp (2015), Eastern Cape farmers routinely block all their goats using 3 mg/kg of short acting tetracyclines once every 14 days, showing the desperation of these farmers for an effective and safe vaccine against heartwater.

There is therefore, a need for a vaccine that will offer wider protection to heterologous challenge (Zweygarth *et al*., 2008), which is cheaper to produce and is more user friendly. The duration of immunity to heartwater may last for 4 years under field conditions (Stewart, 1987; Neitz, 1939; Du Plessis, 1981a). However, Zweygarth *et al* (2008) obtained protection in experimental animals up to 6 months under tick-free conditions, while 4 out of 5 animals challenged 12 months post immunization reacted with fever and one animal needed to be treated.

2.2 Tick vectors and distribution in Southern Africa

Heartwater is transmitted transstadially by ticks of the genus *Amblyomma* (Allsopp *et al*., 2004). Several species are proven biological vectors in Africa of which *A. variegatum* is the most widespread (Walker and Olwage, 1987; Walker *et al*., 2013), and occur in East, West, Central and parts of southern Africa (Latif, 1993; Grindatto *et al*., 2008). Other major vector species are the bont tick, *A. hebraeum* (Fig. 2.1 and 2.2), in southern Africa and SA (Fig. 2.3), *Amblyomma gemma* and *Amblyomma lepidum* in Sudan and Ethiopia. In SA, estimates based on the distribution of *A. hebraeum*, projected the numbers of cattle, sheep and goats at risk to be millions (Spickett, 2013).

Figure 2.1 *A. hebraeum* female (Heloise Heyne, ARC-OVR).

Figure 2.2 *A. hebraeum* male (Heloise Heyne, ARC-OVR).

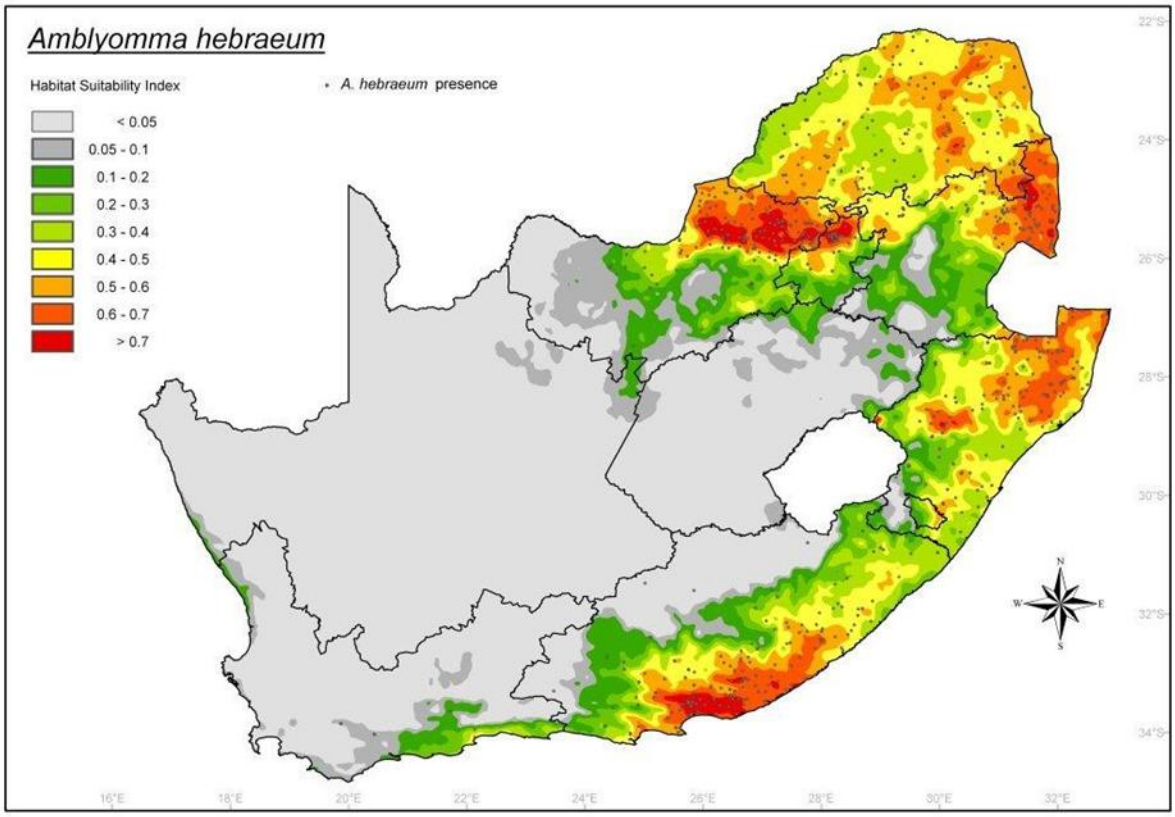

Figure 2.3. Geographic distribution of *Amblyomma hebraeum* in South Africa and its modelled habitat suitability. Black dots represent 1,147 confirmed localities (Spickett, 2013).

2.3 Epidemiology

Heartwater is an African disease and only occurs where there is a tick vector capable of transmitting the organism. There are twelve species of *Amblyomma* ticks in Africa, of which five *Amblyomma* species has been shown to transmit *E. ruminantium* (Walker and Olwage, 1987; Byaruhanga, 2017). *Amblyomma variegatum* (tropical bont tick) is the dominant tick, widely distributed across Africa, and the only tick from Africa that has established itself in the Caribbean after being exported in cattle. *Amblyomma hebraeum* (bont tick) is the prevalent tick in southern Africa. Transmission in this three-host tick occurs transstadially (Yunker *et al*., 1987), either as nymphs or as adults; larvae and nymphs acquire infection by feeding from acutely ill or subclinically infected animals.

Heartwater affects cattle, sheep, goats and some ruminant wildlife. According to Spreull (1922), sheep are more susceptible to the disease than cattle, while Angora goats are highly susceptible. Oberem and Bezuidenhout (1987a) did experiments under controlled conditions and they found out that water buffaloes (*Bubalus bubalis*), and antelopes such as eland (*Taurotragus oryx*), blesbok (*Damaliscus dorcas phillipsi*), springbok (*Antidorcas marsupialis*) and young black

wildebeest (*Connochaetes gnou*) developed clinical disease. In Africa, an increased prevalence of heartwater occurs when tick numbers reaches their peak and this is mostly during the rainy season, whereas in the Caribbean islands more especially in Guadeloupe, ticks are active throughout the year, hence clinical cases are high throughout the year with a slight decrease in autumn (Camus and Barré, 1987).

Other non-ruminant species that are important and play a role in the transmission and epidemiology of the disease are helmeted guinea fowl *(Numida meleagris*), scrub hare (*Lepus saxatilis*) and leopard tortoise (*Geochelone pardalis*). All can become subclinical carriers of *E. ruminantium* and be infective to ticks (Oberem and Bezuidenhout, 1987a).

2.4 Pathogenesis

Though heartwater has been extensively studied, the pathogenesis is still poorly understood (Prozesky and Du Plessis, 1984a,b; 1985; Du Plessis *et al*., 1987a). Most researchers believe that increased permeability of the smaller blood vessels are key lesions in the pathogenesis of the disease (Clark, 1962; Du Plessis, 1975b; Prozesky and Du Plessis, 1984).

According to Kocan *et al* (1987) and Faburay (2007), the vertebrate host is infected by an infected *Amblyomma* species. It is suggested that following infection, the infective organisms are transported to the regional lymph nodes and the initial development of the organism occurs in the restricted reticulo-endothelial cells (Du Plessis, 1970; 1975a,b). The parasitised reticulo-endothelial cells soon ruptures and the organisms eventually circulate the whole body to permeate the endothelial cells (Du Plessis, 1975a). Infection varies with the vertebrate host: while parasites have a predilection for endothelial cells in certain organs, the highest concentration of organisms is found in brain cells followed by kidneys in ruminants, whereas in mice infected with the Welgevonden strain, organisms are concentrated in the lungs (Prozesky and Du Plessis, 1985).

2.5 Clinical signs

Heartwater in clinically affected animals is characterised by sudden onset of febrile reaction, nervous signs followed by death. According to Camus *et al*. (1996) and Carlos *et al*. (1999), mortality rates differ between 5% and 100% and are higher in exotic breeds. The disease occurs in susceptible animals following natural transmission by infected *A. hebraeum* ticks. The gravity of clinical signs and mortality rate depend on several factors like breed, species, age of the ruminant host, virulence of the *E. ruminantium* isolate and route of infection (Faburay, 2007).

The duration of clinical heartwater in ruminants differ, the disease can be peracute, acute, subacute or mild. In the peracute form, there is sudden death without prior indication of the disease. In the acute form, rapid high fever is mostly followed by anorexia, dyspneoa, nervous

signs and death which can occur in 2 to 6 days. In the subacute form, signs are similar to those in the acute form but less severe and the animal may recover. In the mild form, the only sign is transient fever which may not be noticed.

2.6 Diagnosis

2.6.1 Field diagnosis

The presence of *Amblyomma* ticks, characteristic symptoms and epidemiological observation allows preliminary field diagnosis. However, the disease must still be confirmed by the demonstration of the organism, its antigens and its DNA.

2.6.1.1 Post mortem

Striking changes in most fatal cases are severe hydropericardium, hydrothorax and ascites. Hydropericardium is usually more noticeable in goats and sheep than in cattle. Large copious amounts of fluid in the thoracic cavity, which is slightly turbid to light yellow and may coagulate if exposed to air. Fluid in the thoracic cavity may be several litres in cattle and about half litre in sheep and close to 30 ml in goats. Most of the animals that suffer from the peracute and acute forms of the disease have moderate to severe lung oedema. Oedematous frothy fluid ooze from the cut surface of the lungs. Frothy serous foam often fill the trachea and bronchi. Brain oedema occurs mostly in animals suffering from the peracute and acute forms of the disease. Other distinctive symptoms in cattle include congestion and oedema in the abomasal folds.

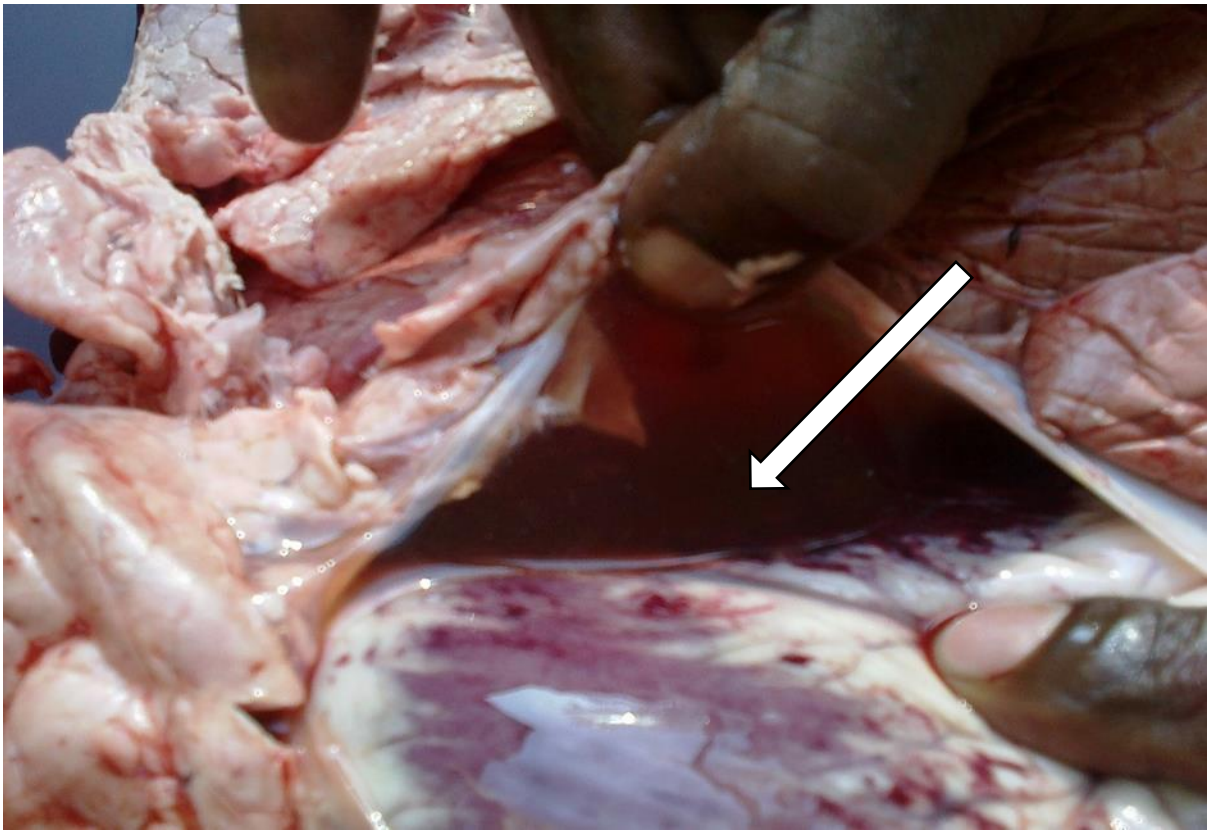

Figure 2.4 Pericardial fluid, arrow (Photo by Prof. Abdalla Latif, UKZN).

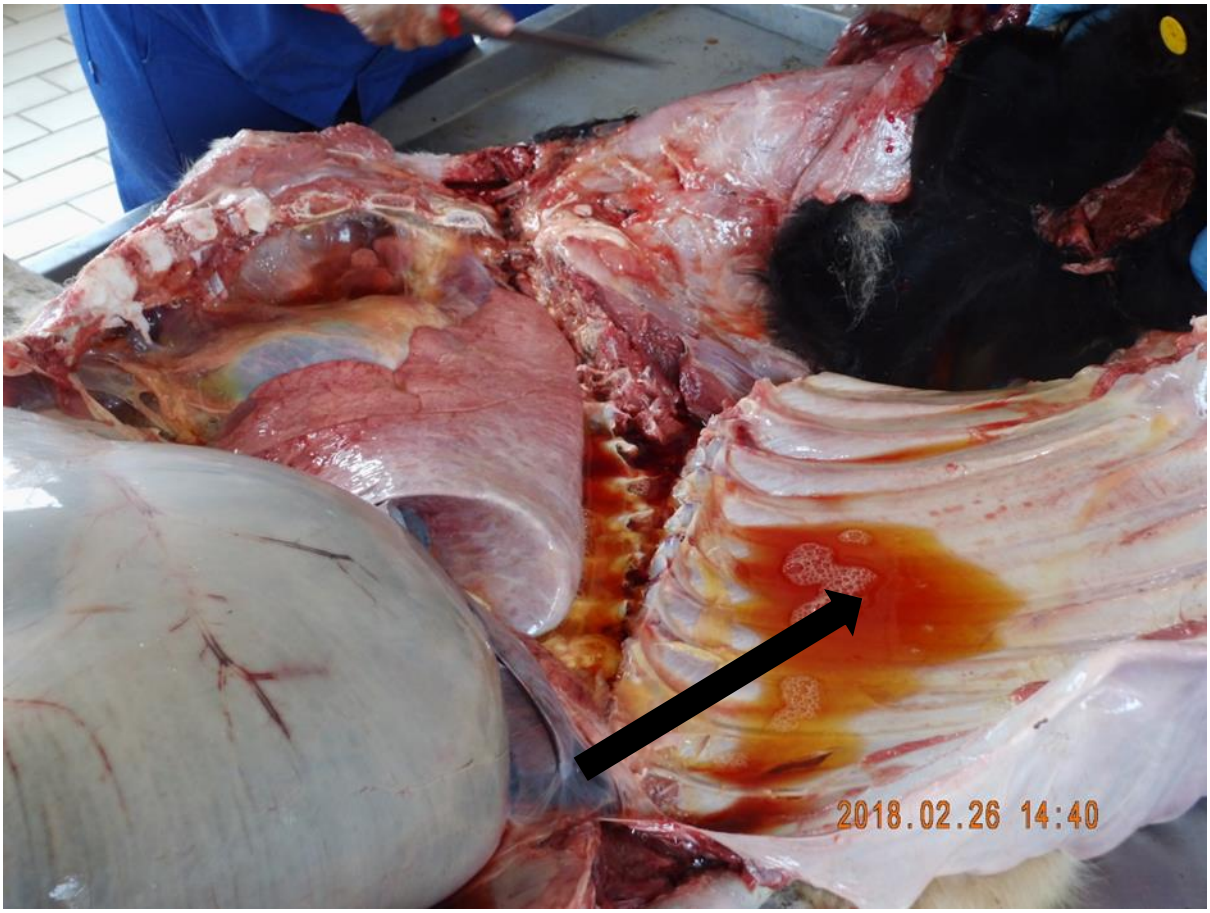

Figure 2.5 Excessive fluid (arrow) in the thoracic cavity (hydrothorax) and oedema of the lungs (Photo by Mr. Lefoka Molepo, ARC-OVR).

2.6.1.2 Experimental inoculation with tick homogenates

The detection of *E. ruminantium* infection in *Amblyomma* ticks is an integral part for understanding the dynamics of epidemiology in heartwater and for designing effective control methods (Peter *et al*., 1995). Traditional method of detecting the infection in *Amblyomma* ticks was attained by inoculating tick extracts into susceptible small ruminants (Birnie *et al*., 1985; Andrew and Norval 1989; Norval *et al*., 1990; Camus and Barre 1992), though the so called "gold standard" for infection detection was considered laborious and expensive by Peter *et al* (1995). Even so, while alternative methods have been developed, diagnosis and detection of heartwater remains complex.

2.6.1.3 Microscopic diagnosis

The traditional microscopic method of diagnosing heartwater is by the demonstration of *E. ruminantium* in the cytoplasm of endothelial cells of blood vessels of stained smears of brain

tissues through light microscopy (Allsopp, 2015). There are numerous stains used to demonstrate the heartwater organism but the most preferred is Giemsa followed by Diff-Quick.

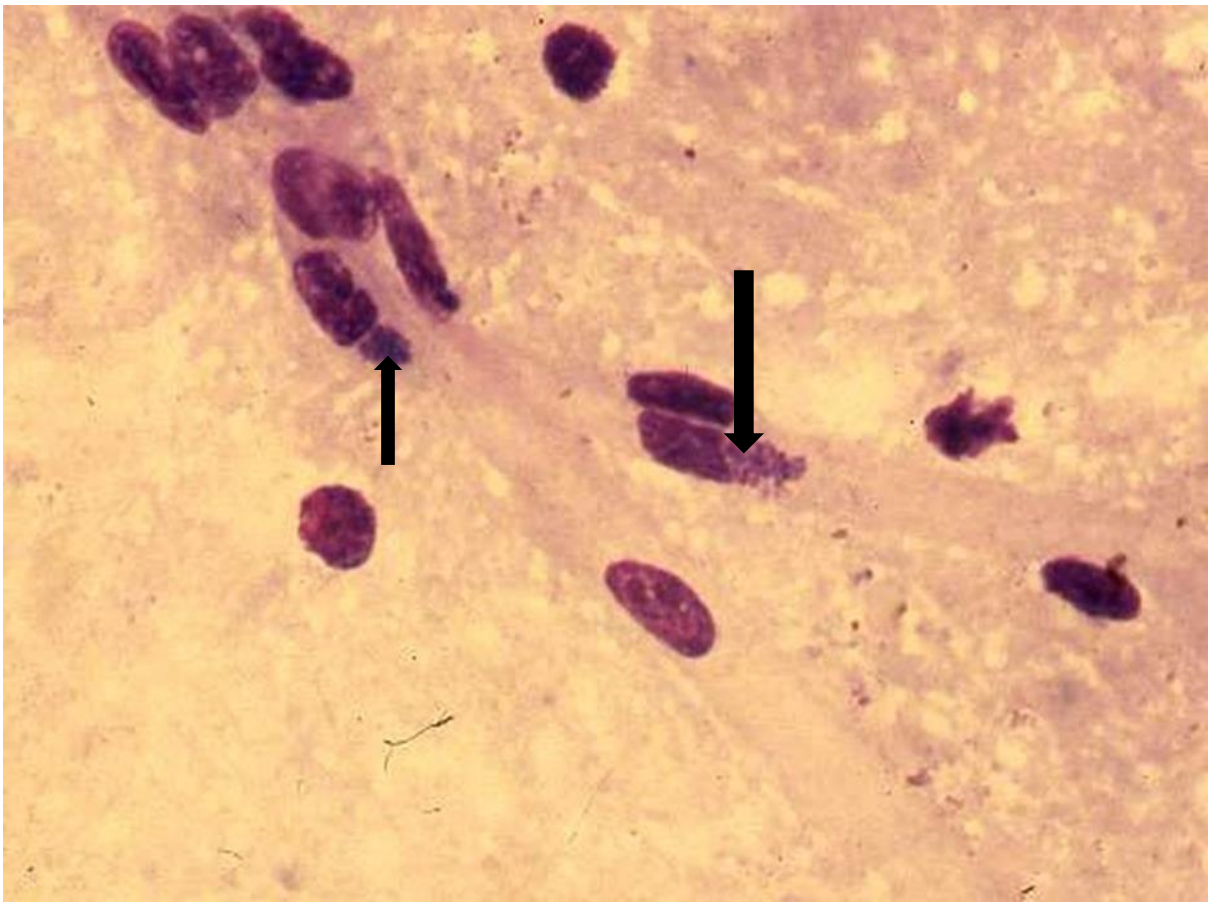

Figure 2.6 Brain smear stained with Giemsa, light microscopic morphology colonies (arrows) of *E. ruminantium* in the cytoplasm of endothelial cells of the brain. (Photo by Prof. Abdalla Latif, UKZN).

2.6.2 Serological assays (IFAT, ELISA)

Serological tests for heartwater were developed for the detection of antibodies of *E. ruminantium* by the indirect fluorescent antibody test (IFAT) (Du Plessis and Malan, 1987d) and with *in vitro* culturing of the organism for use as antigen in Enzyme-linked immunosorbent assay (ELISA) (Martinez *et al*., 1994a).

2.6.2.1 Indirect Fluorescent Antibody Test (IFAT)

Some of the uses of IFAT is to detect antibodies in the serum of animals recovered from artificial infection (Du Plessis, 1981b), to study the epidemiology of the disease (Du Plessis, 1982a,b; 1987d), and determine the prevalence of the disease (Camus and Barré, 1987). Du Plessis

(1982a) described the use of IFAT by using mouse macrophages infected with a mouse infective strain (Kümm strain) of *Ehrlichia* as antigen. However, a few years later Logan *et al* (1987) improved the specificity of the test by using primary blood neutrophil cultures as antigen. The sensitivity of the test lies in its ability to detect animals that have been infected with *E. ruminantium*, either via natural infection through a tick or artificial infection.

Basic principles of the IFAT include growing of *E. ruminantium* in mouse macrophage cultures, harvesting the antigen and fixing on antigen slides using Acetone. Diluted serum (1/40 or 1/80) are added to fixed antigen and incubated in a humid chamber at 37° C for 30 minutes before washing serum of in PBS. A secondary mAb FITC-conjugate specific for the host IgG is then added at a 1/80 dilution and incubated a further 30 minutes. The slide is washed in PBS for 10 minutes to remove unbound conjugate, slides dried and viewed under a fluorescent microscope.

2.6.2.2 Enzyme-linked immunosorbent assay (ELISA)

An ELISA assay was developed to detect *E. ruminantium* antibodies during active heartwater disease (Neitz *et al*., 1986; Viljoen *et al*., 1987). It was shown in reacting animals that the level of IgM antibody reached a peak on the 4th day after infection and started to regress until it diminished on the 7th day, whilst IgG surfaced on day 8 and continue to rise up to day 28 (Viljoen *et al*., 1987). Martinez *et al* (1994b) used organisms grown in bovine umbilical endothelial cells (BUE) cultures as antigen on infected serum of domestic ruminants; they postulated that antibodies could be detected from day 9, while the sensitivity of the test ranged between 97.3% and 98.6%.

According to Peter *et al*. (2001) the indirect MAP 1B ELISA based on the recombinant MAP 1B fragment of the immunodominant MAP 1 protein of *E. ruminantium* was a more sensitive and specific assay for serodiagnosis of heartwater. The reliability of the MAP 1B assay to detect exposure to *E. ruminantium* in field populations of domestic ruminants was tested using sera collected from cattle and goats from a heartwater endemic area. The seropravalence in cattle was significantly lower compared to the IFAT. Therefore, the indirect MAP 1B ELISA was found to be an unreliable indicator of past exposure to heartwater in the field infected cattle in Zimbabwe.

2.6.2.3 Molecular assays based on the pCS20 fragment

To address the shortcomings of microscopical and serological diagnosis, an *E. ruminantium* specific DNA probe assay that detected the pCS20 fragment was developed. This successfully detected *E. ruminantium* in *A. hebraeum* and *A. variegatum* ticks fed on clinically infected ruminants during the febrile reaction (Waghela *et al*., 1991; Yunker *et al*., 1993). The probe detected more than 92% infections from ticks that were fed on experimentally infected animals. However, it was discovered that it was unable to detect infections in ticks fed on carrier animals, therefore a PCR assay had to be developed (Peter *et al*., 1995). In an experiment designed by

Peter *et al*. (1995) that compared the detection levels of PCR and DNA probe assays on ticks fed on febrile and carrier animals, it was demonstrated that PCR was necessary for the detection of low-level infections that were considered to be below detection limit of the pCS20 DNA probe. A trial conducted by Kock *et al* (1995) demonstrated that PCR was effective in the diagnosis of heartwater during both natural and experimental infections in whole blood and bone marrow of ruminants.

Steyn *et al*. (2008) described the development of a pCS20 quantitative real-time TaqMan assay (qPCR). The qPCR assay was developed to detect *E. ruminantium* in the blood of livestock and ticks collected in the field and to overcome shortcomings of other molecular assays. The assay is based on the conserved pCS20 fragment region of *E. ruminantium* that contains two overlapping genes, *rnc* and *ctaG*. The TaqMan probe was compared to two other molecular assays, and was found to be more sensitive, specific, and had an ability to detect DNA in field samples and the blood of sheep infected under controlled conditions. However, it was discovered that all three assays also detected *E. canis* and *E. chaffeensis*. Besides its ability to detect more positive samples from the field, it was discovered that it can be performed within two hours, therefore it can be used as a useful tool for epidemiological surveillance and monitoring of infected animals.

2.7 Immunity

According to van der Merwe (1979), cattle breeds of native African origin (*Bos indicus*) are more resistant to heartwater than exotic breeds (*Bos taurus*). The situation worsens when cattle are newly introduced into endemic areas. Compared to cattle, heartwater affects sheep and goats to a greater extent (Henning, 1956). Alexandra (1931), found sufficient evidence in field and controlled experiments that farm animals develop specific immune responses post recovery from natural infection with *E. ruminantium*. Calves up to 3 weeks of age were found to possess a high degree of natural resistance (innate resistance) to the disease, and the resistance is not related to the immune status of the dam (Neitz and Alexandra, 1941; Uilenberg, 1981). According to Alexandra (1931) and Spreull (1922), Merino sheep are highly susceptible but Angora goats are the most susceptible of all domestic ruminants. Specific immune responses to heartwater were considered to be based on cellular instead of humoral immunity (Alexandra, 1931; Stewart, 1987; Totte *et al*., 1999). Animals challenged 2 months after the initial infection were found to be immune, while the level of immunity remained at a sufficient level to protect animals against fatal outcome for nearly four years (Stewart, 1987). The duration of immunity differs in animals; in cattle it could last from 6 months to 3 years (Neitz and Alexandra, 1945; Henning, 1956; Stewart, 1987), and in goats up to 205 days (Stewart, 1987). However, complete, partial and lack of cross-protection between heartwater isolates have been noticed (Jongejan *et al*., 1988; Du Plessis *et al*., 1989 and Collins *et al*., 2003). In the experiments conducted using the Welgevonden isolate of *E. ruminantium* it was shown to confer a cross-protection to a number of other virulent African isolates propagated in cell culture (Du Plessis *et al*., 1989; Collins *et al*., 2003).

2.8 Control of heartwater

2.8.1 Treatment and acaricides

Treatment of heartwater with antibiotics was found to be effective at the onset of the febrile reaction and showed to be ineffective once the nervous symptoms have been affected (Zweygarth, 2006). Antibiotics of the oxytetracyclines group are considered to be very effective chemotherapeutic agents to treat heartwater. Nevertheless, their use is severely restricted by the onset of the acute form of the disease that does not afford time to prevent fatal outcomes and is expensive too (Faburay, 2007).

Heartwater can be effectively controlled and prevented by regular dipping and spraying of livestock with acaricides. Disadvantages of using these compounds regularly are environmental degradation, pollution, animal populations that is highly susceptible to heartwater during acaricide usage breakdown or acaricide resistance, while also being expensive (Mahan *et al*., 2001; Jongejan and Uilenberg, 2004; Faburay, 2007).

2.8.2 Vaccines

2.8.2.1 Live blood vaccines

The first practical method of protection against heartwater came through Neitz and Alexander (1941). Their method was improved by Uilenberg (1983) after he discovered that animals infected with live *E. ruminantium* and treated with tetracyclines during the febrile reaction and before the onset of clinical signs developed immunity. The method was then referred to as “infection and treatment”, which required close monitoring of the vaccinated animals and treatment the moment increased temperature is noticed (Uilenberg, 1983). The immunization was standardized by establishing a procedure of producing sheep blood infected with a live strain of *E. ruminantium* and it was later renamed as the Ball 3 strain (van der Merwe, 1987). Though this “vaccine” has a number of weaknesses, it is the only commercially produced vaccine and it has been used for more than 60 years. The Ball 3 strain was selected as the most appropriate strain because of its ability to cause febrile reaction before the onset of clinical signs, hence reacting animals can be easily identified and treated accordingly. However, there are limitations to the wide use of this vaccine: it has to be injected intravenously (Oberem and Bezuidenhout, 1987b), it shows limited protection against other field isolates (Collins *et al*., 2003), it requires liquid nitrogen or dry ice to maintain the viable organism during transportation and storage and is expensive to produce (van der Merwe, 1987).

2.8.2.2 Inactivated vaccines

Successful *in vitro* tissue culture of *E. ruminantium* presented many opportunities for developing potential inactivated tissue culture based vaccines against heartwater (Faburay, 2007). An inactivated cell culture of *E. ruminantium* vaccine against heartwater was developed and administered with an adjuvant (Mahan *et al*., 2001). Inactivated elementary bodies mixed with Freund's adjuvant used in controlled trials successfully protected goats and cattle (Martinez *et al*., 1994b; 1996; Totté *et al*., 1997). After extensive clinical trials in sheep, where Freund's and Montanide ISA 50 (ISA 50) adjuvants were compared, an adjuvant that was commercial accepted was ISA 50 (Mahan *et al*., 1998). Mortalities in cattle, sheep and goats vaccinated under field conditions with inactivated vaccine was reduced remarkably (Mahan *et al*., 2001).

Although the inactivated vaccines produced promising results and proved to be safer than live vaccines, immunity is short lived and they need to be administered three times at a period of weeks or months during which animals have to be tick-free (Bell-Sakyi, 2004). A two year clinical trial (from 2002 to 2003) was conducted against heartwater in Burkina Faso, to establish the protective effect between Gardel and Welgevonden strains against local strains in sheep (Adakal *et al*., 2010). They discovered that the inactivated Gardel vaccine conferred a better protection to the local strains than Welgevonden and the authors claimed to have solved the cold chain problem. The Gardel strain can therefore be kept inside a normal freezer (-20 °C) and was chosen as a master strain and suitable for field trials in Burkina Faso.

2.8.2.3 Subunit vaccines

The use of DNA vaccines based on the *map1* gene to protect against heartwater was examined in a mouse model and it showed protection ranging from 23 % to 88 % (Nyika *et al*., 1998), while MAP1 protein boost augments protection against challenge with *E. ruminantium* (Nyika *et al*., 2002). A cocktail nucleic acid vaccine made up of four different *E. ruminantium* ORFs was used to immunize sheep and were found to be protective against subsequent lethal needle challenge of both homologous and heterologous *E. ruminantium* infective blood (Collins *et al*., 2003; Pretorius *et al*., 2007). Protection against field challenge in endemic areas was discovered to be poor (Pretorius *et al*., 2007). The difference in the outcome between the needle and field challenge demands more research to resolve this discrepancy if this vaccine is to be further developed. Recently, the study by Faburay *et al* (2017) presented an important step towards developing an efficacious heartwater subunit vaccine and defining the immunological correlates potentially associated with protection in ruminants. However, this vaccine has not been tested yet in target animals and efficacy studies need to be performed.

2.8.2.4 Attenuated vaccines

Both inactivated and nucleic acid vaccines seemed to be ideal replacements for the current commercial vaccine for protection against heartwater. However, due to their limited protection against field challenge, opened doors for further research that led to development of live attenuated vaccines. According to Zweygarth (2006), attenuation means reduced virulence in an organism by either physical or chemical interference during *in vitro* cultivation. Successful attenuation of *E. ruminantium* was first achieved on the Senegal isolate after about eleven *in vitro* passages in bovine endothelial cell cultures (Jongejan (1991). Faburay (2007) later reported that the attenuated isolate conferred protection against homologous challenge, but failed to provide cross-protection against heterologous needle challenge. The Welgevonden isolate did not attenuate after many years of effort to attenuate it (Jongejan, 1991). Subsequently attenuation was achieved using a canine monocyte-macrophage cell line (DH82) followed by re-adaptation to grow in a bovine endothelial cell line (BA 886) (Zweygarth and Josemans, 2001; Zweygarth *et al*., 2005). The re-adaptation of the attenuated Welgevonden isolate to the bovine endothelial cell line led to neither reversion to virulence nor to loss of immunogenicity (Zweygarth, 2006).

In a clinical trial, it was discovered that the attenuated Welgevonden strain protected sheep and Angora goats against virulent needle challenge with homologous and different heterologous *E. ruminantium* isolates (Zweygarth *et al*., 2005; Zweygarth *et al*., 2008; Haw, 2013). The efficacy and safety of the vaccine as well as the duration of immunity and reversal to virulence after serial tick passages were also investigated. The major advantages of an attenuated vaccine include induction of durable immunity up to six months in tick-free conditions; it is produced *in vitro* meeting the standards of laboratory quality assurance; and it is comparatively safe and effective compared to blood-based vaccine, therefore treatment of vaccine reactions with antibiotics would not be required, which would further reduce the risks and costs. Finally, the Welgevonden vaccine strain has a wider spectrum of cross-immunity than that used in the current commercial blood vaccine, which makes it possible to use the vaccine in many endemic areas in the country (Zweygarth *et al*., 2005; 2008). Recent results generated by Haw (2013) showed that when Angora goats that are considered the most susceptible species to heartwater, were vaccinated by the intra-muscular route with the heartwater attenuated vaccine, 93% did not require antibiotic treatment after vaccination. All vaccinated Angora goats were 100% protected against lethal homologous challenge.

2.9 Aim of the study

2.9.1 General aim

The aim of the study was to demonstrate the safety and efficacy of the attenuated *E. ruminantium* tissue culture vaccine (Welgevonden strain) in cattle under controlled conditions.

2.9.2 Specific objectives

a) To test the safety and efficacy of the attenuated *E. ruminantium* experimental vaccine for heartwater in cattle.

Null hypothesis (H_0): the vaccine will not control heartwater

Alternative hypothesis (H_1): the vaccine will protect against heartwater

b) To assess the intramuscular (I/M) route of administration of attenuated vaccine in cattle.

c) To confirm the infectivity of artificially infected ticks using molecular diagnostics.

d) To test the serological responses before, during and after vaccination and challenge.

e) To assess the effect of vaccination on live body weight of all the animals throughout the trial.

CHAPTER 3

MATERIAL AND METHODS

3.1 Animals in the trial

3.1.1 Sheep

One naïve sheep 18 months old of 55 kg body weight, from an area known to be heartwater free in the Free State Province (Warden) was used. The sheep was earmarked, dewormed and left for one week to acclimatise on a concrete floor and fed lucerne and commercial pellets (Agricultural Food Growth Retail Investment (AFGRI)). Blood (EDTA and serum) was collected on day 0, serum samples were tested with the Indirect Fluorescent Antibody test (IFAT) and EDTA blood samples using the pCS20 quantitative real-time TaqMan probe (qPCR). The sheep was kept in stable 100 during these procedures and later moved to the East Coast fever quarantine animal facility (ECF tick-free stables) of the Agricultural Research Council - Onderstepoort Veterinary Research (ARC-OVR).

3.1.2 Cattle

Friesian Holstein cattle, (n=20), 8-24 month old males, were purchased from the Free State Province, from areas which were designated as heartwater-free on the basis that *Amblyomma* tick vectors were absent (Dryer *et al*., 1998; Mbati *et al*., 2002; Barbet *et al*., 2009). The animals were brought and housed in concrete floor, tick-free stables at Onderstepoort Veterinary Research until the start of the trial. During the adaptation period they were castrated, dosed for gastro-intestinal parasites using Tramisol® (Afrivet Business Management), injected with Ivomec (ivermectin by Merial South Africa), ear-tagged and were fed on sterilised hay and commercial feed (pellets) from AFGRI (Fig. 3.1). EDTA-Blood and serum samples from the herd were collected for screening for the presence of an *E. ruminantium* carrier state using the pCS20 quantitative real-time assay (qPCR) (Section 3.5) and antibodies by the indirect fluorescent antibody test (Section 3.6).

3.1.3 Ticks

Ticks were obtained from a clean *A. hebraeum* colony maintained in the acaridarium of the ARC-OVR. Ticks were kept under controlled conditions of temperature (28 °C) and relative humidity (RH) of 80%.

Figure 3.1. Housing facilities at ARC-OVR during preconditioning and vaccination stages (photo by Ratselane Marumo, ARC-OVR).

3.2 Infecting sheep with the *Ehrlichia ruminantium* Welgevonden strain

The Welgevonden strain was isolated from a male tick collected on the farm Welgevonden in Bela-Bela district (Du Plessis, 1985). The virulent Welgevonden strain was used to infect *A. hebraeum* nymphs for tick challenge after immunisation with the attenuated Welgevonden experimental vaccine. The heartwater (Welgevonden) stabilate is stored in the liquid nitrogen (LN_2) and thawed in lukewarm water for 5 minutes. The stabilate was diluted at a ratio of 1:300 sucrose potassium glutamate (SPG), in a laminator cabinet to avoid contamination.

The sheep was injected with 10 ml of the diluted stabilate via the jugular vein. The rectal temperature of the sheep was recorded daily. On day 7, after inoculation, the wool at the back of the sheep was shaved, and two tick-feeding cotton cloth bags were glued using Genkem® contact adhesive glue onto the shaved area and left to dry as described before (Heyne *et al*., 1987). On Day 8, *A. hebraeum* nymphal ticks (n=300) were applied to the tick feeding bags. The nymphs

were placed inside the bags and the bags were secured using rubber rings. The nymphal ticks were allowed to feed over the febrile period of animal.

Only those nymphs that had fully engorged and dropped off the host during the febrile reaction were used for tick challenge. The engorged nymphs were stored in the acaridarium as described above. All the infected engorged nymphs moulted to adults and the infected adults were used for challenge.

3.3 Extracting genomic DNA from infected ticks

The infected adults were pooled from three batches that dropped after engorgement from the sheep. Infected adult ticks (5 males and 5 females) were randomly selected from the ventilated plastic containers. All the selected ticks were tested to detect *E. ruminantium* (Welgevonden) genomic DNA and infection rates using the pCS20 quantitative real-time TaqMan assay (qPCR). All ticks were washed in 70% ethanol, rinsed in distilled water and dried with a paper towel. The protocol for genomic DNA isolation kit (QIAamp® DNA Mini and Blood Mini Handbook) were followed according to the manufacturer's instructions. For genomic DNA extraction each tick was cut in half and placed into a 1.5 ml Eppendorf tube and 180 µl of Buffer ATL was added to assist with tissue lysis. Twenty µl Proteinase K was added and the sample incubated overnight at 56 °C for tissue lysis. The lysed tick mixture was transferred into a new tube and 200 µl of Buffer AL was added and mixed by pulse-vortexing for 15 seconds to yield a homogeneous solution and incubated at 70 °C for 10 minutes. The mixture was transferred into another tube and 200 µl of ethanol (96-100%) was added and mixed by pulse-vortexing for 15 seconds. The mixture was applied to the QIAamp Mini spin column in a 2 ml collection tube and centrifuged at 6000 x g (8000 rmp) for 60 seconds. The QIAamp Mini spin column was placed in a clean 2 ml column, the filtrate was discarded and 500 µl Buffer AW1 was added and centrifuged at 6000 x g (8000 rmp) for 60 seconds. The QIAamp Mini spin column was placed in a clean 2 ml column, filtrate discarded and 500 µl Buffer AW2 was added and centrifuged at full speed for three minutes. QIAamp Mini spin column was placed in a new 2 ml collection tube, filtrate in the old collection tubes discarded and centrifuged at full speed for 60 seconds. QIAamp Mini spin column placed in a clean 1.5 ml microcentrifuge tube, filtrate discarded and 200 µl Buffer AE was added and incubated at room temperature for 1 minute before centrifugation at 6000 x g (8000 rpm) for 1 minute. The eluted DNA from *A. hebraeum* adult ticks were then tested with the qPCR pCS20 assay.

3.4 Extracting genomic DNA from blood

Blood samples were collected from experimental cattle (n=20) before vaccination. The blood (5 ml) was collected into sterile Vacuette® tubes K3E crossmatch containing ethylene diamine tetra-acetic acid (EDTA) as anticoagulant. All the blood samples were extracted using the Generation® capture column kit (QIAgen) and procedures and instructions from the manufacturer were

followed. Fresh blood in EDTA was used to reduce DNA degradation and the sample was stored at 4 °C. Two-hundred µl EDTA blood sample was added to GENERATION® Capture Column using a pipette and allowed to mix for 1 minute and 400 µl DNA Purification Solution (Solution 1) was added and thoroughly mixed. Samples were incubated at room temperature for one minute, the solution allowed to drain into the Waste Collection Tube and centrifuged for 10 seconds at 6000 x g. The GENERATION® Capture Column was transferred to the second Waste Collection Tube. Another 400 µl DNA Purification Solution (Solution 1) was added, incubated for 1 minute and centrifuged at 6000 x g for 10 seconds. Two-hundred µl DNA Elution Solution (Solution 2) was added after removing the Waste Collection Tube, centrifuged for 10 seconds at 6000 x g and DNA was eluted in 200 µl elution buffer. Eluted sample was transferred to a DNA Collection Tube. Another 200 µl DNA Elution Solution (Solution 2) was added to the binding column, incubated for 10 minutes in a dry block heater pre-heated to 99 °C, centrifuged for 20 seconds at 6000 x g following heating to release purified DNA from GENERATION® Capture Column. The two eluates were combined to give 400 µl (200 µl X 2) eluate.

3.5 pCS20 quantitative real-time PCR assay (qPCR) of *Ehrlichia ruminantium*

The *E. ruminantium* DNA was successfully amplified using the real-time PCR method for amplification of the pCS20 fragment as described previously (Steyn *et al*., 2008). Briefly, the CowF forward (CAA AAC TAG TAG AAA TTG CAC A) and CowR reverse (TGC ATC TTG TGG TGG TAC) primers and Cow probe (6FAM TCC TCC ATC AAG ATA TAT AGC ACC TAT TA XT-PH) was used for amplification and detection of a 226 bp fragment of the conserved region of the pCS20 fragment. The PCR mixture consisted of 2 µl DNA template, 0.5 µM of each primer, 04. µM probe, 5 U Taq polymerase (Roche), 4 mM $MgCl_2$, 1U Uracil DNA N-Glycosylase (UNG) and DNAse free water up to a total volume of 20 µl. The PCR conditions consisted of incubation for 10 min at 40 °C, followed by denaturing for 10 min at 95 °C. Thirty-eight cycles of denaturing at 95°C, annealing at 48°C for 10 seconds, elongation at 58 °C for 30 seconds followed. Samples were then cooled to 4 °C. Data was acquired at the end of the elongation step and analysed using the Roche LightCycler software version 4.0. The cutoff threshold cycle (Ct) value was set at 38 cycles.

3.6 Indirect fluorescent antibody test (IFAT)

Serum samples were collected from all animals on Day 0, before tick challenge, Day 11 post challenge, Day 15 post challenge and Day 37 post tick challenge. All the serum samples were tested using the indirect fluorescent antibody test (IFAT) which detects anti-*Ehrlichia* antibodies (Zweygarth *et al*., 2005). Du Plessis (1981b), Du Plessis and Malan (1987d) and Zweygarth *et al*., (2005), described the application of IFA test. Briefly, two-fold dilutions (1:40 and 1:80) of the test samples were applied to the wells of antigen slides with Welgevonden as an antigen and incubated at 37°C for 30 min. Following incubation, serum was washed off the slides and slides washed on magnetic stirrer at very low revolutions (50-70 rpm) for 10 minutes in 200-300 ml PBS. Slides were washed further with distilled water for 5 minutes on a magnetic stirrer. A drop

(20-25 µl) of diluted conjugate (0,02% Evans blue in PBS plus Sigma Aldrich FITC conjugated anti-bovine/anti-goat/anti-sheep diluted to 1/80 dilution) were placed on each well of the slide. Slides were incubated in a humid chamber at 37 °C for 30 minutes. After incubation, conjugate was washed off from the slide, washed in PBS for 10 minutes on magnetic stirrer and slides left to dry. The slides were evaluated under a Leitz Orthoplan fluorescence microscope. Positive samples showed fluorescence at dilutions higher than 1:40.

3.7 Vaccine preparation

The attenuated Welgevonden vaccine was prepared according to the procedure outlined by Zweygarth (2007). Briefly, vaccine preparation and dilutions were as follows; the vaccine was prepared and the concentration was 4, 44 X 10^6 *E. ruminantium* organisms in 1 ml as indicated in Table 4.1. The vaccine was further diluted in SPG to 1, 11 X10^6 so that 2 ml could be used. All the materials and equipment used were handled aseptically to maintain the sterility of the vaccine.

The virulent *E. ruminantium* Welgevonden isolate was used for lethal challenge of the Friesian Holstein cattle, whilst the attenuated *E. ruminantium* Welgevonden isolate was used for vaccination. Both antigen isolates were grown in Dulbecco's modified Eagle's medium nutrient mixture Ham F-12 (DME/F-12) (Sigma, St. Louis, MO, USA; catalogue no. D 0547) containing 15 mM HEPES and 1, 2 g/L sodium bicarbonate and supplemented with 10% (v/v) heat-inactivated foetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin, 100 g/ml streptomycin, and 2,5 µg/ml Amphotericin B.

The attenuated *E. ruminantium* Welgevonden vaccine isolate was propagated in bovine aorta endothelial cells (BA 886), using 176 cm tissue culture flasks incubated at 37°C without shaking. The medium was discarded 24 h post inoculation and replaced with fresh medium containing 0.3 µg/ml cycloheximide.

The vaccine antigen was harvested 2 days later at passage #92 by scrapping off the inoculated cells with a cell scraper and lysing the cells using a Perifill Jencons Machine set on slow automatic volume 10 for 2 minutes. The cell suspension was centrifuged for 10 minutes at 800 x g at room temperature. The resulting supernatant containing elementary bodies was pipetted into oak ridge polycarbonate tubes and spun for 20 minutes at 20 000 x g in a refrigerated centrifuge (4° C). Following this, the supernatant was discarded and resulting pellet reconstituted in 1ml sucrose potassium glutamate/ fetal calf serum (SPG/FCS). This was made to 100 ml with SPG/FCS, stirred for 30 minutes, and sampled for flow cytometer analysis.

3.8 Vaccination and challenge

3.8.1 Vaccination of cattle

Ten Friesian Holstein cattle were vaccinated with attenuated heartwater (Welgevonden) tissue culture experimental vaccine intramuscularly in the rump area (Table 3.1). Daily body temperatures were recorded (Fig. 3.2) using rectal thermometers for the whole month for vaccine related adverse reactions. Ten cattle per group was considered the minimum to achieve the scientific objectives and the minimum number of animals required to obtain valid data.

Twenty animals were randomly allocated to two treatments (control and *E. ruminantium*), in other words ten animals per treatment. T-test was performed to test the significance difference between means of the two treatments. Significant effects in all tests were declared at the probability level of $P< 0,05$. All tests was done using InStat3 software.

Data were analysed for the effects of treatments with the model:

$$Y_ij = \mu + t_i + \varepsilon_{ij}$$

where: Y_{ij} is the individual observations of the i-th treatment and the j-th replicate, μ is the overall mean, t_i is the effect of the i-th treatment and ε_{ij} is the residual error.

Table 3.1. Number of cattle per group.

Groups	Category	Immunisation route	Challenge route & type	Total animals
1	Experimental	Intramuscular	Ticks	10
2	Control	-	Ticks	10
Total				**20**

Figure 3.2. Technician recording daily morning body temperatures from both groups (photo by Ratselane Marumo, ARC-OVR).

3.8.2 Challenge of cattle

Tick challenge was performed with infected adult *A. hebraeum* ticks one month following vaccination. Twenty cattle (vaccinated and control animals) were shaved on the back between the shoulders and one tick feeding bag was glued as explained above. All the animals were moved to the East Coast Fever (ECF) stables for tick challenge. Infected adult *A. hebraeum* male ticks (n=7) were applied to each tick feeding bag on each cattle. Seven infected males were allowed to feed for 2 days before applying ten infected females to each bag. During this two day period, the males were producing attraction pheromones for the females to attach (Yunker *et al*., 1990).

3.8.3 Animal monitoring during and after challenge

Daily rectal temperatures were recorded from the two groups of vaccinated cattle and controls using rectal thermometers for the whole period of vaccination and challenge (total 77 days). Animals were monitored for clinical signs of heartwater disease and treated according to the score and monitoring sheets for heartwater research (Table 3.2 and Appendix 1).

Cattle were weighed before vaccination, during vaccination reactions and during tick challenge (Days 0-77). Weights were recorded using the "scale tape" weighing band procedure. The tape is mostly used in cattle, horses, sheep, goats and pigs, by commercial and communal farmers when determining proper feed rations, treatment dosages or monitoring weight loss or gain in individual animal (Wangchuk *et al*., 2018).

Table 3.2 Score sheet for heartwater research (Combrink *et al*., 1997; Pretorius *et al*., 2007).

1) Before vaccination: Average temperature of animals before treatment over 10 days calculated for each cattle		
2) After vaccination or challenge: During observation period of reaction		
Criteria	Parameter	Points scored
Temperature	For every 0.1 above the average calculated temperature	0.1
Symptoms	Loss of appetite, heavy breathing, hanging head, stiff gait, depression	5
Treatment/death/euthanasia	Treatment (oxytetracycline)	20

3.9 Animal Ethics Approval

The Animal Use and Care Committees of the ARC-OVR (AEC 21.17), University of Pretoria (V071-16), Department of Agriculture Forestry and Fisheries (DAFF, 12/11/1/1) and Department of Health (Medicines Control Council, VCT/04/2017) approved all the experimental procedures and protocol.

3.10 Statistical analysis

The number of heartwater infected engorged *A. hebraeum* females that dropped from the vaccinated group and control group were statistically compared. A two-sample t-test was used to determine whether there was a difference between the two groups and 95% confidence intervals were used. The p-value was tested against a significance level of 0, 05, and standard errors were determined.

A two-sample *t*-test analysis of variance was used to compare mean variances of original body weight, mean and average mean weight gain/loss to determine whether there was a difference between the two groups.

CHAPTER 4

RESULTS

4.1 Tick infectivity

Adult ticks (5 males and 5 females) were randomly chosen from ticks that dropped off the infected sheep, 45 days post detachment. Detection of the genomic DNA of *E. ruminantium* (Welgevonden) was conducted using the pCS20 qPCR to determine the infection rates of both males and females (Fig. 4.1 and 4.2). The fluorescence profiles of the positive tick samples are shown in figures 4.1 and 4.2, the horizontal red line is used as a threshold to determine whether true amplification occurs. The signal above the red line is considered amplification, while no amplification occurs below this line. The cutoff for the cycle threshold value was set at 38 cycles. Samples with crossing point values below this LOD were considered true positive, while crossing points above the LOD were considered false positive. The fluorescence was detected at an average crossing point (cycle threshold) between 15.53 and 20.96. The positive control (A) shows that the qPCR is sensitive to genomic DNA detection of the Welgevonden strain and can be used as a tool to show infection rates of the ticks. The negative control (B) signifies that the mix was not contaminated. All the ticks were positive and sampled ticks had high level of *E. ruminantium* (Welgevonden strain) as indicated by qPCR, and the infection rates were 100% (Fig. 4.3)

Table 4.1 below indicates the results of culture counts. The flow cytometry live elementary bodies events count was at 4, 44 X 10^6 organisms per ml. This was diluted to 0, 55 X 10^6 organisms per ml in a dosage of 2ml using SPG/FCS. This brings the number of organisms inoculated per animal to 1, 1 X 10^6 organisms in total.

Table 4.1. Flow cytometric events count.

		Live					
		P9	Dilution factor	Total (Ave X dilution)	Convert to		
		Events/µl		Events/µl	Events/ml		Events/ml
	E1 water	1	10	10	$1x10^4$		
Sample: 1frozen	A02	397	10	3970	$3.97x10^6$	$3.96x10^6$	
	A03	491	10	4910	$4.91x10^6$	$4.90x10^6$	
	A03	444	10	4440	$4.44x10^6$	$4.43x10^6$	**$4.43x10^6$**

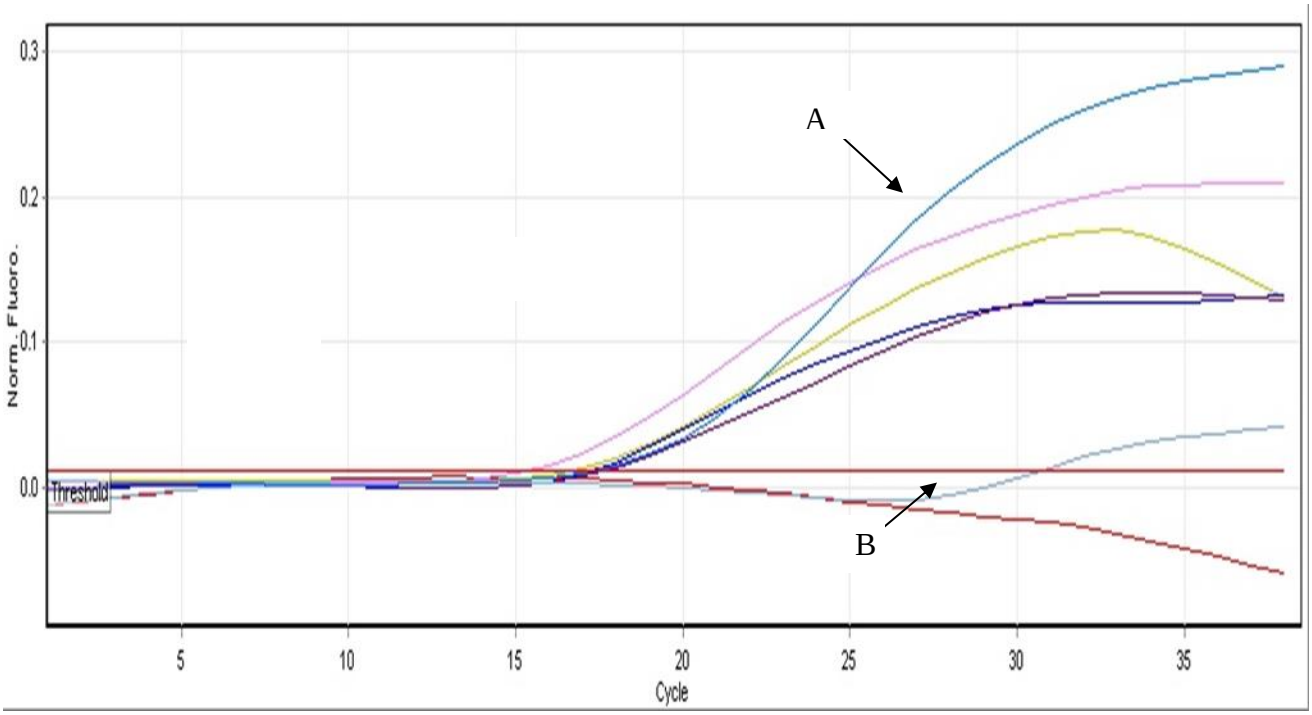

Figure 4.1. Detection of *E. ruminantium* (Welgevonden) genomic DNA by qPCR in *A. hebraeum* males to determine their infection rate. A indicates the positive control and B the negative control.

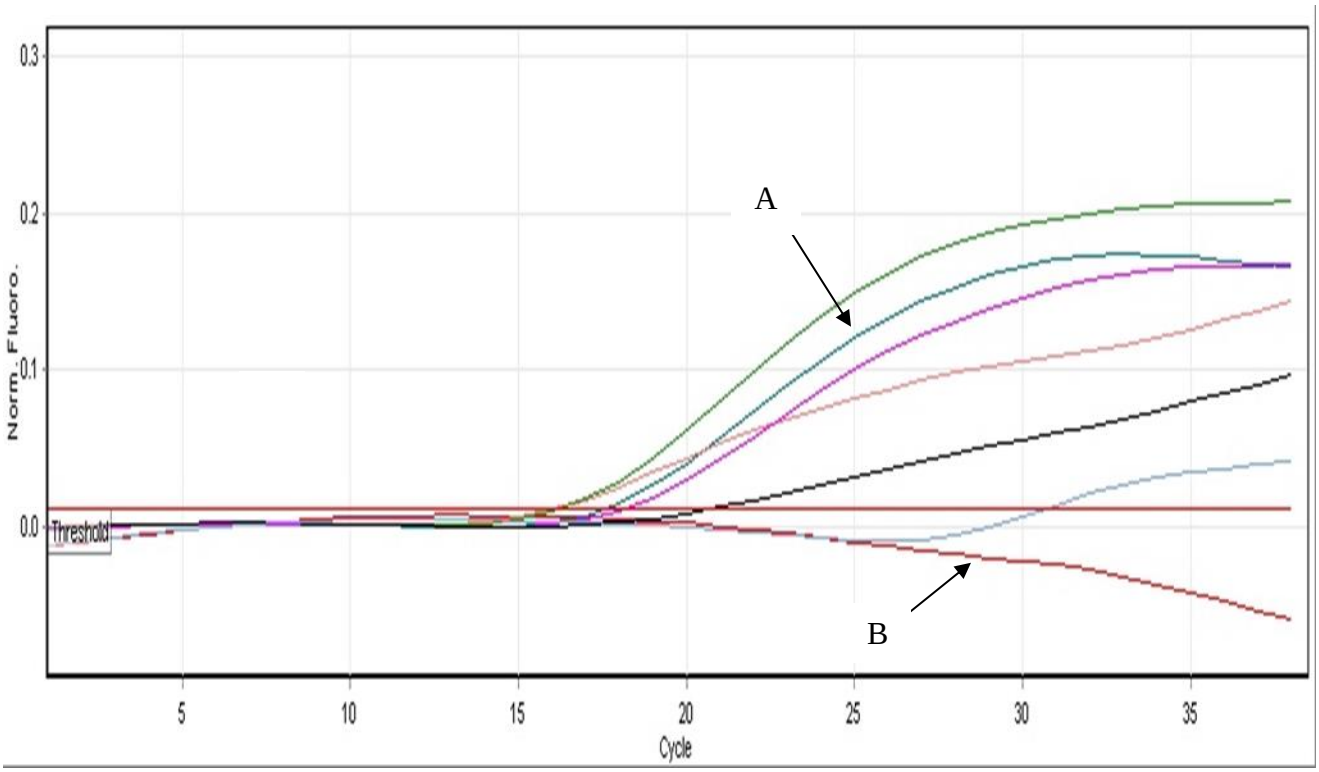

Figure 4.2. Detection of *E. ruminantium* (Welgevonden) genomic DNA by qPCR in *A. hebraeum* females to determine their infection rate. A indicates the positive control and B the negative control.

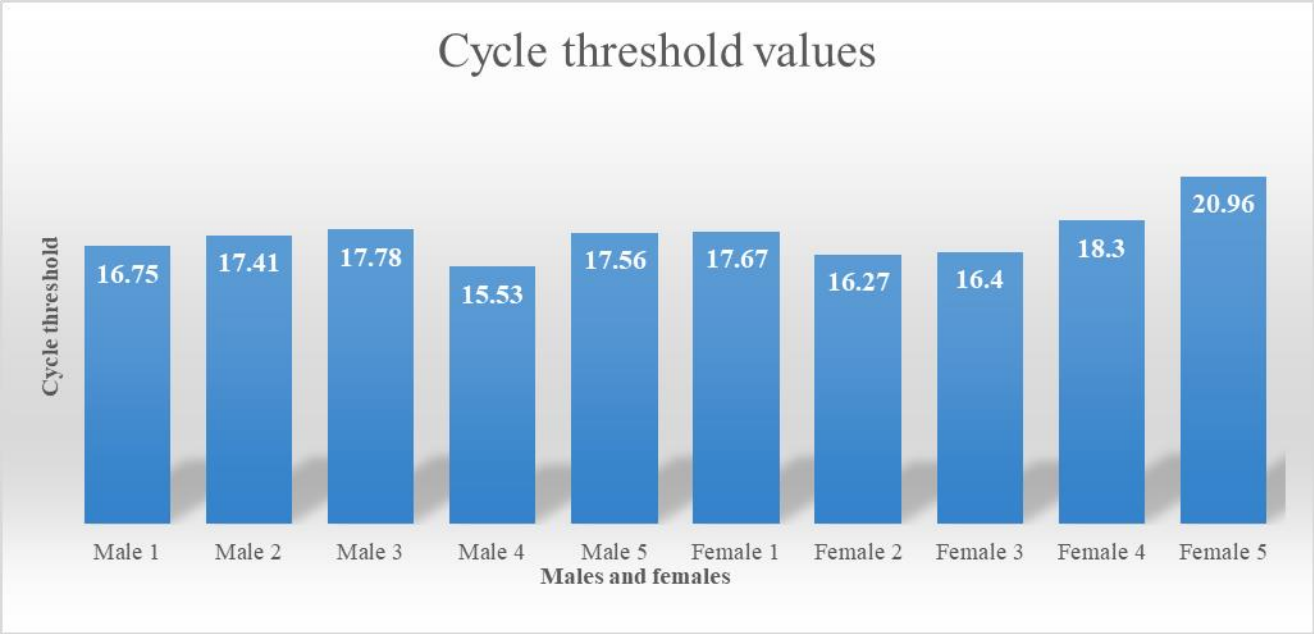

Figure 4.3. pCS20 quantitative real-time TaqMan assay (qPCR) results for *E. ruminantium* (Welgevonden) genomic DNA (organism) in *A. hebraeum* ticks for both males and females. Values indicate crossing-point values (Cp).

According to Table 4.2, there was no statistical significant mean difference (p-value = 0.3609) in the genomic DNA concentration of *E. ruminantium* genomic DNA detected in females and males at 5% level of significance. The results shows that the females had a slightly higher concentration (mean = 17.920) than the males (mean = 17.006).

Table 4.2: t-test results comparing the mean crossing point values in *A. hebraeum* in males and females.

Groups						95% CI for Mean Difference			
Males			Females						
M	SD	n	M	SD	n		t	P-value	df
17.006	0.9101	5	17.920	1.903	5	-1.261, 3.089	0.9690	0.3609	8

P-value at 95% = 0.3609 (There was not a statistically significant mean difference (p-value = 0.3609).

The experimental cattle (n=10) which were vaccinated intramuscularly with attenuated *E. ruminantium* (Welgevonden) experimental vaccine, showed no post vaccine related reactions when monitored daily after vaccination. There was no pain on palpation of the vaccine injected sites, no redness, no edematous fluid oozing out or sloughing. There was no treatment required for any vaccinated cattle as there was no febrile reaction (Table 4.3).

Table 4.3. Clinical reaction in cattle after vaccination with attenuated *E. ruminantium* (Welgevonden) vaccine and tick challenge with virulent *E. ruminantium*. A temperature ≥ 39.5 °C were defined as high.

Cattle No.	Group	Vaccination			Tick challenge			Outcome
		Days to high temp	t_{max} (°C)	Treatment	Days to high temp	t_{max} (°C)	Duration of fever (days)	Reaction
23	V	0	0	0	0	0	0	N/R
36	V	0	0	0	0	0	0	N/R
37	V	0	0	0	0	0	0	N/R
38	V	0	0	0	0	0	0	N/R
47	V	0	0	0	0	0	0	N/R
65	V	0	0	0	0	0	0	N/R
68	V	0	0	0	0	0	0	N/R
80	V	0	0	0	0	0	0	N/R
89	V	0	0	0	0	0	0	N/R
99	V	0	0	0	0	0	0	N/R
3	C	0	0	0	18	41,2 (T)	4	S/T
4	C	0	0	0	15	40,2 (T)	5	S/T
15	C	0	0	0	17	40,5 (T)	4	S/T
20	C	0	0	0	17	40,7 (T)	4	S/T
32	C	0	0	0	0	0	0	N/R
51	C	0	0	0	14	41 (T)	7	S/T
64	C	0	0	0	13	41 (T)	9	S/T
75	C	0	0	0	0	0	0	N/R
93	C	0	0	0	15	41 (T)	5	S/D
104	C	0	0	0	15	40,3 (T)	5	S/T

C (controls); V (vaccinated); (T) (treatment); S/T (Severe signs, Treated); S/D (Severe signs, Died); N/R (No reaction, recovered); High Temp (39.5 °C)

4.2 Tick challenge

4.2.1 Non-vaccinated animals

One month following immunization, both experimental and control cattle were exposed to tick challenge (Table 4.3). All the males seemed to have attached on the same day (Day 0) and were allowed to feed. On Day 2, when the females were applied to feed, two male ticks were found dead and were removed from cattle #3 and #37. On Day 7, four males were found dead and were removed from cattle #23, #47, #65 and #93, respectively. On Day 7, ten unfed females were found dead and were removed from cattle #20, #23, #32, #68 and #80 and four more females were dead two days later and were removed from cattle #51 and #75.

The first batch of engorged females dropped by Day 9 and the last batch dropped on Day 16. The number of female ticks that fed only partially and detached from all animals was 26 and they detached between Day 13 and Day 24 of feeding (Table 4.3). All the remaining males fed from Day 0 to Day 31 and cattle were dipped on Day 32.

The incubation periods varied between Day 13-18 as expressed by a rise in rectal temperature of up to 41,2°C, which lasted from 4 to 8 days (Fig 4.4).

On Day 19, following challenge, bovine #64 reacted with elevated body temperature and for the first few days this was the only symptom noticed (Fig 4.4). Fever lasted for nine days with the highest temperature being 41 °C. The animal was treated on Day 4 of febrile reaction using short acting oxytetracycline (Hi-Tet 120®, Bayer Animal Health) at a dose of 1 ml/10kg body mass. On Day 4 of febrile reaction, the animal started showing mild heartwater symptoms that included anorexia. It was thus treated for three days according to score and monitoring sheets for heartwater research and as per protocol. The animal continued to eat and act normally again.

On Day 20, bovine #51 reacted with hyperthermia (Fig 4.4), it persisted for seven days; on the fourth day of febrile reaction it had the highest temperature (41 °C), stopped eating, hung its head, had moderate dyspnea, and the animal was treated according to score sheet as well and recovered within three days.

On Day 21, three animals (#4, #93 and #104) reacted with hyperthermia and all of them had fever as the only symptom but they continued to eat and drink. On Day 4 of febrile reaction, cattle #93 started showing severe symptoms like anorexia, labored breathing, nervous signs and sternal recumbency. The three animals were treated as per score sheet and protocol. Despite treatment, on day 5 of persistent febrile reaction (41° C) bovine #93 was suffering greatly from the virulent *E. ruminantium* challenge and since it was not responding to treatment, the animal was euthanized following the score sheet recommendation of human intervention to prevent further suffering. It was euthanized by using 200 mg sodium pentobarbitone (Eutha-Nase®, Bayer Animal Health) per kg body mass.

A post mortem was conducted on cattle #93 that was euthanized and the animal exhibited post mortem lesions typical of heartwater: hydrothorax (400 ml), hydropericadium (250 ml) and ascites (800 ml). It was confirmed positive for *E. ruminantium* infection in brain crushed smears and by pCS20 qPCR on blood for genomic DNA extracts.

On Day 22, bovines #15 and #20 showed elevated body temperatures. On day three of elevated temperature, animal #15 stopped eating, started breathing with difficulty and got up slowly and it was treated like the previous cattle. On day four of the fever, cattle #20 showed reduced food and water intake and it had to be treated.

On Day 23, bovine #3 developed a febrile reaction that lasted for four days and was the highest recorded febrile reaction (41,2 °C). It had early symptoms of heartwater and required treatment. Cattle #32 and #75 from the control group did not react to tick challenge, despite being fed with infected ticks. All the treated animals recovered fully after treatment except #93.

4.2.2 Vaccinated cattle

All the vaccinated cattle were monitored for febrile reaction post vaccination (21 days) and non reacted with elevated temperature (Fig 4.5) and they were all challenged with infected ticks one month post vaccination. They were challenged with seven infected males and ten infected females as for control animals. They were monitored for daily rectal temperatures and the signs of heartwater for the whole month. None of the vaccinated animals developed high fever post tick challenge and all animals recorded normal body temperature for the duration of the tick challenge (Fig. 4.6). All the animals in this group were fully protected as no clinical symptoms were noticed.

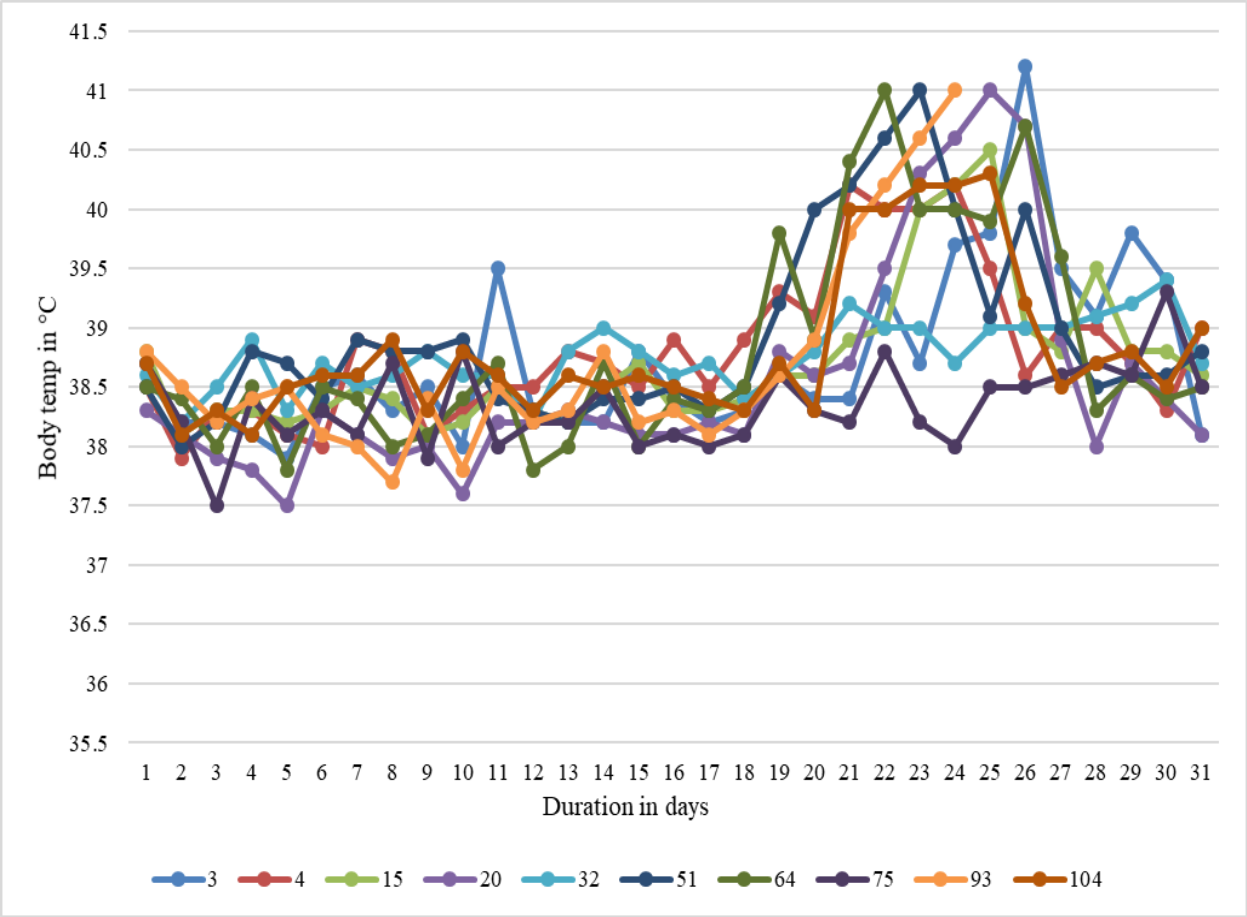

Figure 4.4. Body temperatures of the unvaccinated cattle during tick challenge from Day 0 to Day 30. Temperatures above 39.5° C was recorded as a temperature reaction

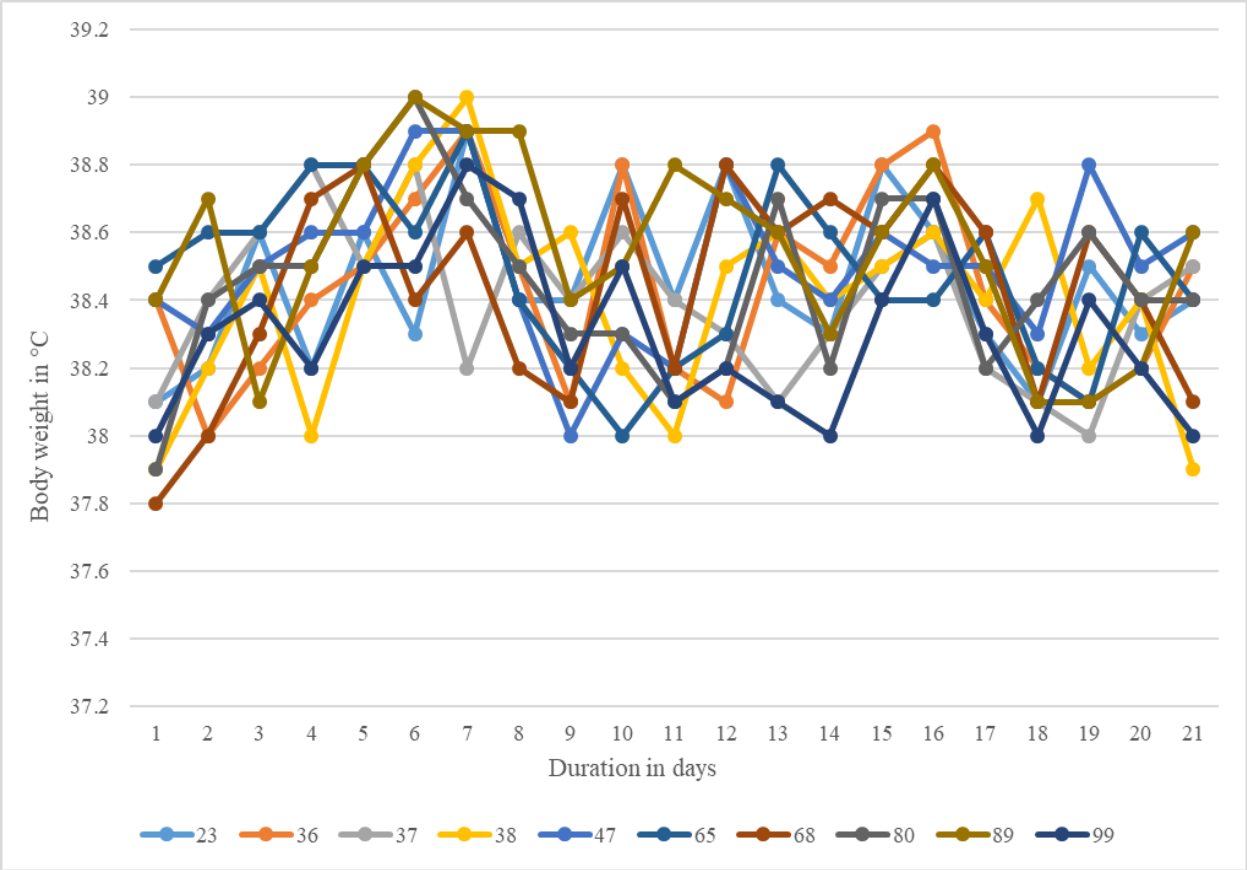

Figure 4.5. Body temperatures of the vaccinated cattle from Day 0 to Day 21 post vaccination with attenuated (Welgevonden) *E. ruminantium* experimental vaccine. Temperatures above 39.5° C was recorded as a temperature reaction.

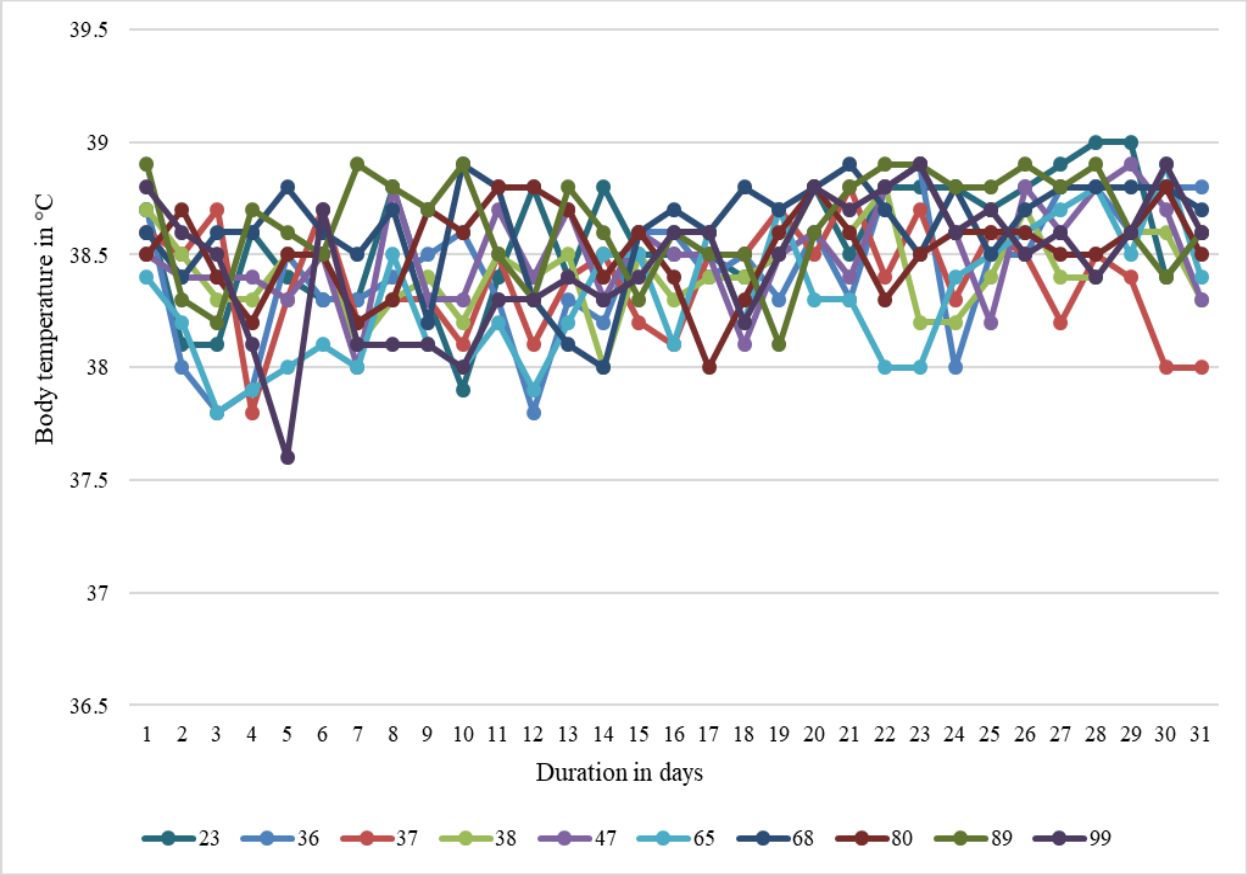

Figure 4.6. Body temperatures of the vaccinated cattle during tick challenge from Day 0 to Day 30. Temperatures above 39.5° C was recorded as a temperature reaction.

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Table 4.4. Infected tick challenge: tick feeding performance on vaccinated and unvaccinated groups.

Animal No	Group	No of ticks applied		No of ticks didn't attach		No of female partial engorged detached	No of female engorged and dropped
		Males	Females	Males	Females		
23	Vaccinated	7	10	1	2	1	7
36	Vaccinated	7	10	0	0	0	10
37	Vaccinated	7	10	1	0	3	7
38	Vaccinated	7	10	0	0	0	10
47	Vaccinated	7	10	1	0	2	8
65	Vaccinated	7	10	1	0	0	10
68	Vaccinated	7	10	0	2	1	7
80	Vaccinated	7	10	0	1	3	6
89	Vaccinated	7	10	0	0	0	10
99	Vaccinated	7	10	0	0	2	8
Total		70	100	4	5	12	83
Mean							**8.3**
3	Control	7	10	1	0	2	8
4	Control	7	10	0	0	3	7
15	Control	7	10	0	0	3	7
20	Control	7	10	0	2	1	7
32	Control	7	10	0	3	1	6
51	Control	7	10	0	1	0	9
64	Control	7	10	0	0	0	10
75	Control	7	10	0	3	0	7
93	Control	7	10	1	0	2	8
104	Control	7	10	0	0	2	8
Total		70	100	2	9	14	77
Mean							**7.7**

Table 4.5 shows the tick feeding challenge on the vaccinated group (V) and control group (C). The male ticks had high attachment rates on all animals that varied between 86-100% (only one tick per animal of 6 animals failed to attach). The percentage of females engorged and dropped from vaccinated cattle was 83% and the average was 8.3 per animal. The percentage of females engorged and dropped from the control cattle was 77%, average 7.7 per animal.
There was no statistical significant mean difference (p-value = 0.3433) in the number of females engorged and dropped between the control group and vaccinated group at 5% level of significance (Table 4.5). Results show that the vaccinated group had a slightly larger number of females engorged and dropped (mean = 8.3) than the control group (mean = 7.7).

Table 4.5: t-test results comparing number of *A. hebraeum* females engorged and dropped between vaccinated group (V) and the control unvaccinated group (C).

	Groups						95% CI for Mean Difference			
	Control			Vaccinated						
	M	SD	n	M	SD	n		t	P-value	df
No. of females engorged and dropped	7.7	1.160	10	8.3	1.567	10	-1.895, 0.695	0.9733*	0.3433	18

P-value at 95% = 0.34 (There was no statistical significant difference between means).

Table 4.6 shows hyperthermia reactions between the two groups post tick challenge. There was statistical significant difference ($p < 0.0001$) in the mean temperatures of animals at 5% level of significance. Results shows that the vaccinated group had no temperature reactions (mean = 0) and the control group reacted with elevated temperatures (mean = 40.738).

Table 4.6: Statistical analysis to show hyperthermia between vaccinated and control groups. Only temperatures above 39.5 °C was taken into account to determine mean temperature.

	Groups						95% CI for Mean Difference				
	Control			Vaccinated							
	M	SD	n	M	SD	n			t	P-value	df
Mean temperature of animals	40.738	0.3701	8	0	0	0	40.428,	41.047	311.34	< 0.0001	7

P-value at 95% < 0.0001 shows statistical significant difference between the two groups.

4.3 Serology

Whole blood samples were collected from twenty cattle and analyzed using IFAT assay. Sera was collected two weeks (Day 15) following vaccination from all the cattle to detect development of antibodies using IFAT. Results of the serology is shown in Table 4.7.

Table 4.7. Results of the serological test (IFAT) performed on Day 0 (before tick challenge) and on Day 37 (after tick challenge).

Animal number	Group	Day 0	Day 15 post vaccination	Day 37 post challenge (IFAT Titres)
23	Vaccinated	Neg	Pos (1/80)	Pos (1/80)
36	Vaccinated	Neg	Neg	Pos (1/80)
37	Vaccinated	Pos (1/40)	Pos (1/80)	Pos (1/80)
38	Vaccinated	Pos (1/40)	Pos (1/80)	Pos (1/80)
47	Vaccinated	Neg	Neg	Pos (1/80)
65	Vaccinated	Neg	Pos (1/80)	Pos (1/80)
68	Vaccinated	Neg	Pos (1/80)	Pos (1/80)
80	Vaccinated	Neg	Pos (1/40)	Pos (1/80)
89	Vaccinated	Neg	Pos (1/80)	Pos (1/80)
99	Vaccinated	Neg	Pos (1/40)	Pos (1/80)
3	Control	Neg	Neg	Pos (1/80)
4	Control	Neg	Neg	Pos (1/80)
15	Control	Neg	Neg	Pos (1/80)
20	Control	Neg	Neg	Pos (1/80)
32	Control	Neg	Neg	Neg
51	Control	Neg	Neg	Pos (1/80)
64	Control	Neg	Neg	Pos (1/80)
75	Control	Pos (1/40)	Neg	Pos (1/80)
93	Control	Neg	Neg	Died
104	Control	Neg	Neg	Neg

On Day 0, before vaccination, 3/20 (15%) of the animals were serologically positive with low titres (1/40). Therefore, IFAT specificity is 17/20 (85%). Two weeks post vaccination, cattle #36 and #47 were still serologically negative and the rest of the vaccinated animals had a positive heartwater antibody titre. Cattle #32 and #104, both from the control group were the only bovines which were serologically negative after tick challenge. In summary, 2/20 (10%) of challenged animals from the two groups did not develop significant antibodies; 0/10 (0%) of the vaccinated group and 2/10 (20%) of the control/challenged. All the positive cattle had high titres (1/80) including the two animals from the vaccinated group that previously gave negative results.

Cattle #104 (control group) showed severe reactions after challenge and required treatment. However, the test were negative on Day 37. Therefore, IFAT sensitivity is 80%.

4.4 Average weight gain/loss for vaccinated and control groups

The weekly body weights and weight gain/loss of all the animals were recorded during the entire duration of the trial using the tape technique. Weight changes were seen during the cattle adaptation period, before and after vaccination, after infected tick challenge and after treatment of reacting cattle. Records of actual weights and averages of body mass gain/loss and data analysis are shown in Tables 4.8, 4.9, 4.10 and Figures 4.7. 4.8.

During the adaptation period, i.e., the period before animals were used in the trial, 16/20 bovines recorded a loss in body weight, ranging at 1-4 kg, between week 1 and week 2. Thereafter, between week 2 and week 3, there was an improvement of average weight gain and only 7/20 cattle showed slight loss of weight (1-3 Kg) (Table 4.8). The initial weights between the control group (mean 333.9 kg) and the vaccinated group (mean 349.6 kg) did not differ significantly (p-value = 0.3785) (Table 4.9). The final weight gain/loss between the vaccinated and unvaccinated groups showed a statistically significant difference (p-value = 0.0003) (Table 4.10). The vaccinated group had a mean weight gain of 5.6 $\pm$ 2.84 kg whereas the control unvaccinated group had a mean weight loss of -0.33 $\pm$ 2.78 kg. There were only three bovines in the vaccinated group which lost weight of 2-4 kg after vaccination, and two of these consistently showed loss of weight of 1-2 kg during the six weeks after challenge (Fig 4.7). These two bovines started to gain weight by the 7^{th} week. In contrast, only three bovines in the unvaccinated group started to gain weight at the termination of the trial (Table 4.8; Fig 4.9)

Table 4.8. Weekly body weight (kg) recorded for the duration of the experiment.

No of days	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49	Day 56	Day 63	Day 70	Day 77	
Vaccinated animals													**Gain/loss**
23	336	334	336	336	338	339	340	339	341	342	342	344	+8
36	357	355	358	358	357	359	360	360	362	363	364	364	+7
37	338	335	338	337	338	338	339	338	339	340	341	341	+3
38	379	375	380	379	380	380	381	380	382	382	383	384	+5
47	368	364	365	364	366	367	368	367	368	368	370	370	+2
65	390	391	393	393	394	394	395	394	394	395	396	396	+6
68	346	342	345	344	345	344	345	344	344	345	345	347	+1
80	277	278	280	281	283	283	284	283	284	284	286	286	+9
89	315	315	317	316	318	317	318	317	317	318	320	321	+6
99	390	392	394	395	394	395	396	395	397	397	398	399	+9
Mean	**349.6**											**355.2**	**5.6**
Non-vaccinated animals													
3	268	269	271	274	273	275	275	272	270	268	269	272	+4
4	296	294	294	296	295	296	296	294	292	290	291	293	-3
15	401	399	402	404	405	407	408	405	403	402	403	404	+3
20	390	387	388	391	392	392	393	392	390	388	387	388	-2
32	315	312	310	313	314	316	317	315	314	313	311	313	-2
51	315	313	314	315	318	319	318	316	315	314	314	314	-1
64	368	365	367	368	369	370	369	368	366	365	364	364	-4
75	346	346	348	345	346	347	346	345	345	346	347	348	+2
93	315	314	315	316	316	318	317	315					-
104	325	324	326	328	327	328	326	325	323	324	324	325	0
Mean	**333.9**											**335.7**	**-0.33**

Table 4.9: t-test results comparing initial weight at the start of the trial between vaccinated group (V) and the control unvaccinated group (C).

Groups						95% CI for Mean Difference			
Control			Vaccinated						
M	SD	n	M	SD	n		t	P-value	df
333.90	42.017	10	349.60	35.47	10	-52.321, 20.831	0.9029	0.3785	18

P-value at 95% = 0.3785 (There was not a statistically significant mean difference (p-value = 0.3785).

Table 4.10: t-test results comparing average mean weight gain/loss between vaccinated group (V) and the control unvaccinated group (C).

Groups						95% CI for Mean Difference			
Control			Vaccinated						
M	SD	n	M	SD	n		t	P-value	df
-0.33	2.784	9	5.6	2.836	10	-8.659, -3.208	4.593	0.0003	17

P-value at 95% = 0.0003 (There was a highly statistically significant mean difference (p-value = 0.0003).

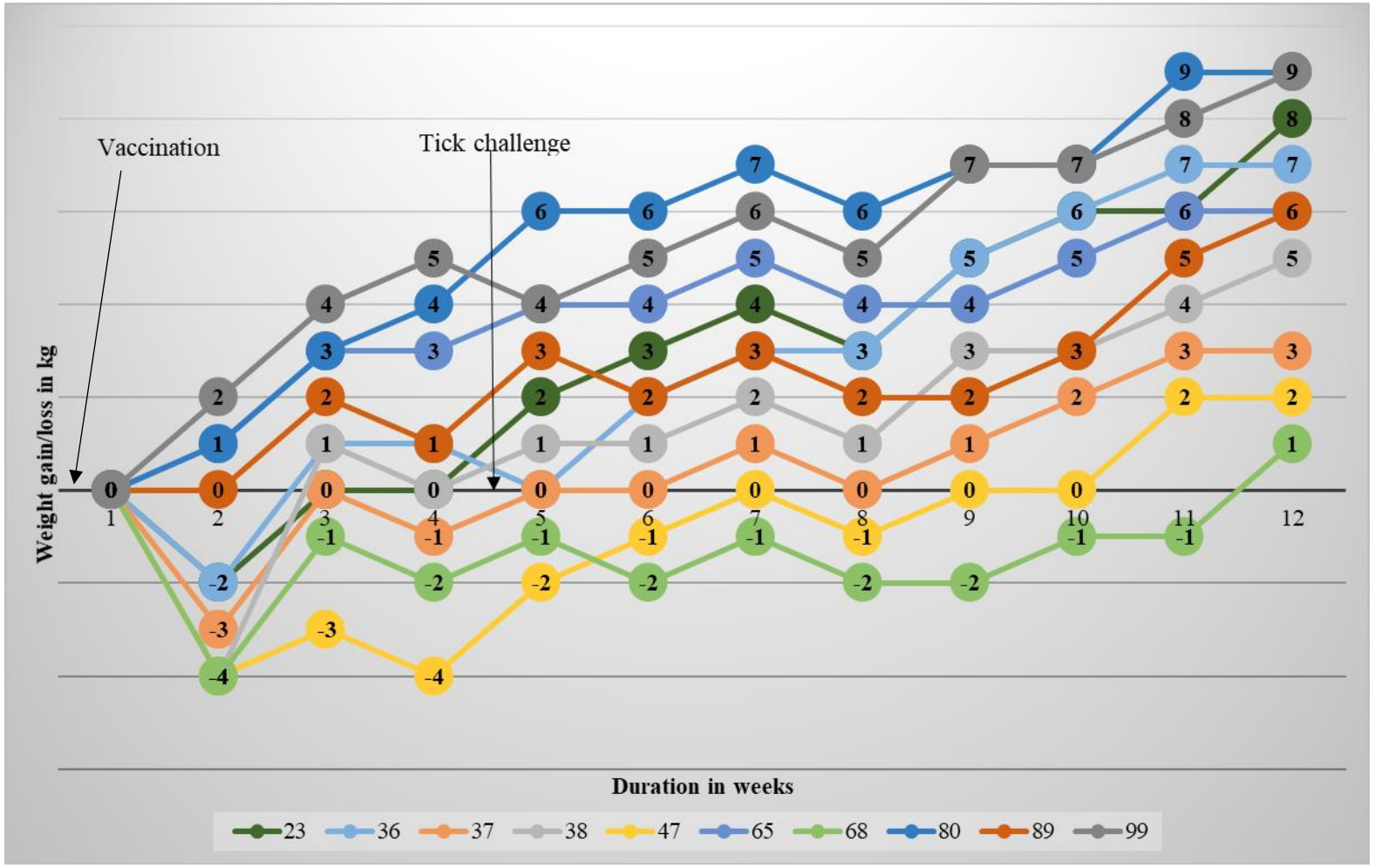

Figure 4.7. Average weight gain/loss (kg) of vaccinated cattle during the entire period of observation.

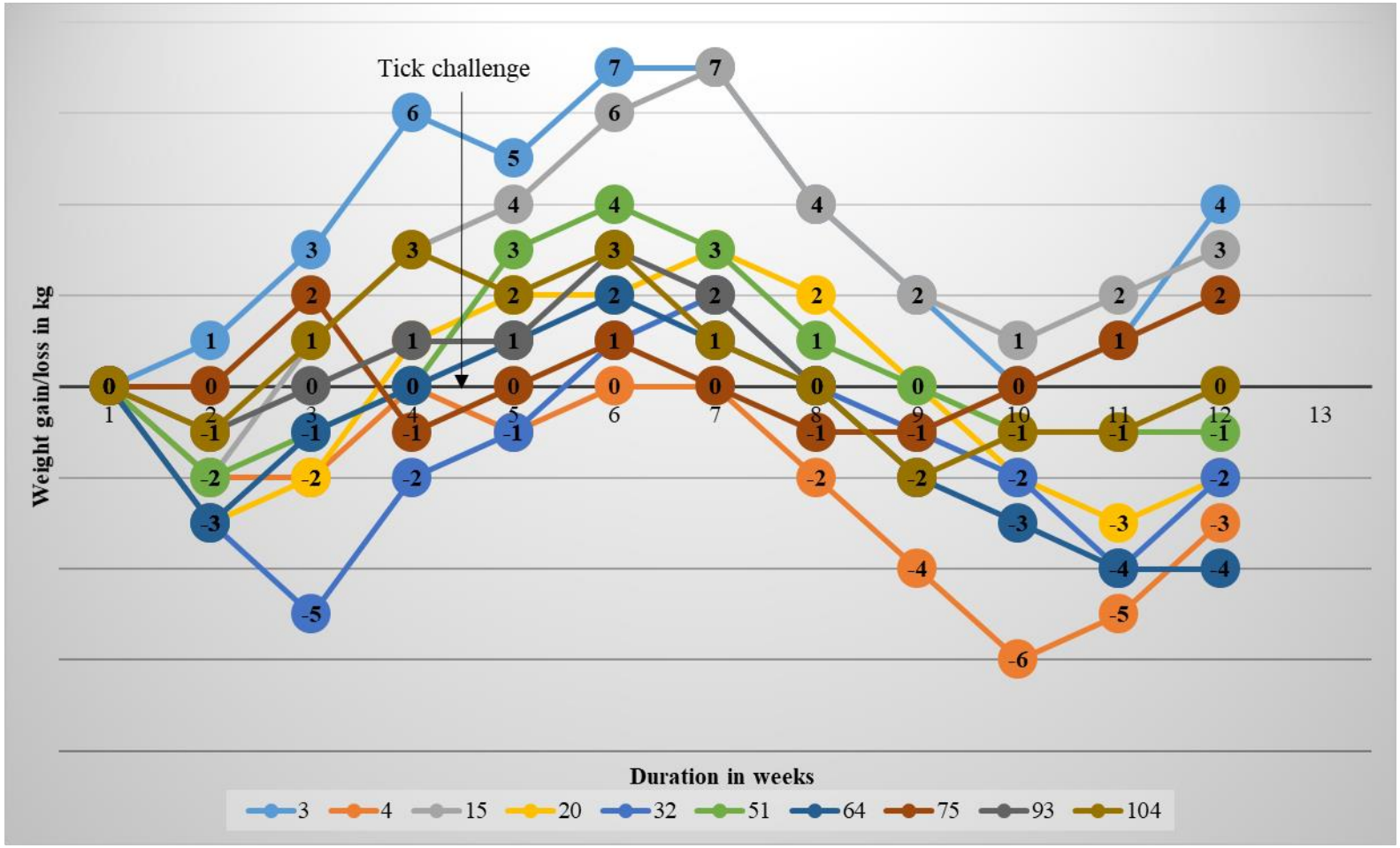

Figure 4.8. Average weight gain/loss (kg) of unvaccinated control cattle during the entire period of observation.

4.5 Assessment of the intramuscular immunization site

Before cattle (n=10) were vaccinated, the area over the left rump region was shaved where the deep I/M inoculations were to be made. Daily palpation and visual assessment of the injection sites were carried out for a period of ten days post immunization for any swelling, inflammation response, pain, skin sloughing or erythema. None of these parameters were noted over the injection sites for the entire observation period.

4.6 Safety testing of the attenuated experimental vaccine

The experimental vaccine was produced and tested for safety and efficacy in cattle. Normal body temperatures were recorded in all the vaccinated cattle post immunization. No abnormal behaviour, clinical signs or local reactions at the injection sites were observed in any of the vaccinated cattle. Therefore, the vaccine was found to be safe for use in cattle given by the intra-muscular route.

4.7 Efficacy of the vaccine

After infected tick challenge, daily rectal temperatures were recorded and animals monitored daily for clinical signs including anorexia, respiratory distress, inappetence, depression, incoordination or recumbence and nervous signs. No treatment intervention was required for the vaccinated group post tick challenge. Therefore, the vaccine was efficacious since all the cattle were protected.

CHAPTER 5

5.1 DISCUSSIONS

The only commercially available vaccine against heartwater in SA is the Onderstepoort live blood vaccine, Ball 3 isolate. Only the intravenous route is recommended to vaccinate animals with cryopreserved blood from sheep infected with the virulent Ball 3 isolate of *E. ruminantuim* (Bezuidenhout, 1989), and monitoring of febrile reaction begin immediately post immunization to block with oxyteracycline treatment at the time when animals show a rise in temperature. Other shortcomings of this infection and treatment regimen (Uilenberg, 1983), which is not necessarily a vaccine, includes cold storage in liquid nitrogen and transportation of frozen vaccine on dry ice which limits its usage in the rural communities (communal farmers). The infection and treatment method is also labour intensive, as the animals need to be monitored daily for febrile reaction, while the vaccination procedure requires administration by the intravenous route which is a specialized skill. The Ball 3 isolate also does not offer wide protection to other field isolates (Jongejan *et al*., 1988; Du Plessis *et al*., 1989). Zweygarth (2007) recognized the administration of Ball 3 vaccine as being costly as it adds to the cost of purchasing oxytetracyclines to block the vaccine reaction.

In West Africa, the current methods available used to protect sheep and goats against heartwater is based on inactivated (Martinez *et al*., 1994; Martinez *et al*., 1996; Mahan *et al*., 1998) and live attenuated vaccines (Jongejan, 1991; Zweygarth *et al*., 2005).

Faburay (2007), immunized twelve sheep with attenuated *E. ruminantium* (Senegal) vaccine under field conditions and a month later exposed them to natural *E. ruminantium* tick challenge including the control group (n=12). According to his findings, all the animals in the latter group died between 14 and 29 days, seven of the vaccinated group reacted to natural tick challenge and three of the twelve vaccinated sheep died, showing (9/12) 75% survival rate at the end of the study. He concluded that the death of three immunized sheep was due to different field genotypes. Under controlled conditions, he tested similar vaccine in 12 sheep (6 vaccinated and 6 controls) and challenged them with the Kerr Seringe isolate, all the sheep in the control group died whereas none of the vaccinated developed fever or died. He concluded that the attenuated Senegal isolate conferred protection against the local Kerr Seringe isolate.

The safety and efficacy of the attenuated *E. ruminantium* Welgevonden isolate using the I/M route in cattle was evaluated in the current study. Attenuation means reduced virulence in a pathogen by either physical or chemical interference during *in vitro* cultivation (Zweygarth, 2007). Initially, the attenuated organisms were derived from DH82 cells, which produced no

disease when inoculated into sheep or mice, and the animals were fully protected against lethal homologous needle challenge. Since the DH82 cells are carcinogenic cells, they are inappropriate to be used for commercial mass production, therefore attenuated Welgevonden isolate had to be re-adapted to the bovine endothelial cell culture line BA 886 (Zweygarth, 2007). After several passages of the attenuated organism in BA 886 cells, sheep were injected with 2 ml of BA 886 culture derived material and showed increased body temperature with no other clinical symptoms.

In the current study, ten cattle were vaccinated with attenuated *E. ruminantium* (Welgevonden) tissue culture experimental vaccine. All the cattle were immunized via the deep I/M route. Close monitoring post vaccination indicated no vaccine related reactions. Zweygarth *et al*. (2008) conducted a clinical trial and they determined the effectiveness and safety of both subcutaneous and I/M routes in small ruminants using the attenuated inoculum. In this latter study, two out of five sheep vaccinated intramuscularly reacted with fever for one day, 16 and 18 days post vaccination. Faburay (2007) recorded similar findings, where six sheep were immunized intravenously using the attenuated *E. ruminantium* Senegal isolate and half showed hyperthermia up to 5 days with a maximum temperature of 41.4 °C. Haw (2013) immunized 31 Angora goats using attenuated *E. ruminantium* Welgevonden vaccine through the deep I/M route of which 27 developed febrile reactions following immunization and three had to be treated. Therefore, according to these previous findings it was expected that vaccinated cattle will react with febrile reaction, but none were observed.

No post vaccination adverse reactions should occur with the use of this vaccine in cattle that might require treatment, while immune response stimulation occurred without causing severe clinical response. Fortunately, 100% of animals immunized intramuscularly never required treatment to manage the vaccine-related reactions; which means this route is safe. The vaccine dose ($1.1x10^6$) that was used in this study was the optimum effective dose according to Combrink *et al*. (unpublished data) and it resulted in 100% safety.

Eight of the ten unvaccinated control animals developed pyrexia post tick challenge; all had to be treated as they had elevated temperatures and severe heartwater symptoms. One animal from the control group died and post mortem was conducted on that animal, which exhibited post mortem lesions typical of heartwater (severe hydrothorax, hydropericadium and ascites). This animal was confirmed positive for *E. ruminantium* infection in brain crushed smears examination and by pCS20 quantitative real-time TaqMan assay (qPCR) on brain tissue DNA extracts (Faburay, 2007).

Zweygarth *et al*. (2008) and Haw (2013) found that some of the small stock required treatment for vaccine related reactions and protection to needle and infected tick challenge in their preliminary experiments. All the cattle that were immunized by I/M route with the

attenuated *E. ruminantium* (Welgevonden) vaccine were fully protected against feeding of Welgevonden-infected *A. hebraeum* ticks. This is the greatest benchmark reached for the attenuated vaccine, where vaccinated animals were 100% immune to homologous tick challenge, thus simulating natural challenge.

Since the food intake was the primary monitoring parameter in the study, body weight of all the animals from both groups were recorded throughout the trial. The critical stages of the trial included vaccination, tick challenge and post tick challenge. These stages were closely monitored for any increase or decrease of individual cattle body weight. Only the average body weight of the control cattle were severely affected during tick challenge and post tick challenge. All the animals that had febrile reaction showed a decreased appetite, while some stopped eating and drinking for a few days. The tick challenge took place between week 5 and 6 from the start of the trial and the reaction period was at the end of week 7 to 10. Cattle #32 and #75 did not react to tick challenge with elevated temperature and did not require treatment. However, they also had reduced appetites and only #32 lost weight while #75 maintained its weight during the tick challenge period but recovered more quickly than those requiring treatment. The body weight of all animals that were subjected to treatment started improving on the third week after the onset of the febrile reaction.

Du Plessis and Malan. (1987c,d) and Semu *et al*. (1992) developed the *E. ruminantium* infected cultures as antigen in IFATs to detect similar *E. ruminantium* specific antibody responses in the field in clinically sick, recovered and carrier animals. Du Plessis *et al*. (1987b) questioned the specificity of the IFA test when sera of cattle and small stock from the Caribbean islands where the vector tick occurs but clinical heartwater had never been diagnosed before, and cattle sera from Namibia where tick vector of heartwater does not occur, reacted positively. An infected animal with *E. ruminantium* Ball 3 which did not show clinical febrile reactions were found to demonstrate specific positive sero-conversion in the IFAT test. Infected cattle had antibody titres peaked between 3-6 weeks post infection. Non-specific cross-reactivity in the IFAT was related to cross-reactions with *Rickettisa conorii* demonstrated experimentally in 8/24 cattle (Du Plessis *et al*., 1987c; Sumption *et al*., 2003). Du Plessis *et al.* (1994), tested 100 sera collected from cattle in the Free State Province (SA) where *Amblyomma* does not occur and all their results were negative.

In the current study, all the Friesian Holstein cattle (n=20) were tested using the IFAT on arrival at the Onderstepoort Veterinary Research for screening purposes. Three of these cattle were found to be serologically positive namely cattle #37, #38 and #75 had 1/40 titres, respectively. This could presumably be some form of cross-reaction with other unidentified species of *Ehrlichia* organisms as these animals were purchased in an area known to be heartwater and *Amblyomma* free. Eight out of ten control animals presented typical early to severe clinical heartwater reactions during the course of the experiment and this confirms

their susceptibility to the disease. Cattle #75 could have been an outlier, as the origin supports that it could have had cross-reaction (Du Plessis *et al*., 1987b). On the other hand, the second bovine in the same unvaccinated control group did not react to the challenge with the infected ticks. As such, the reactions in the supposedly positive bovine (#75) could not be related to previous immunity of this animal as they originated from the same locality but most probably to certain immune factors in the animal or less probable to non-tick transmission. The two non-reacted bovines did not show any sero-positivity in the IFAT following the challenge. Several field studies had demonstrated that not all of the controlled cattle introduced together with vaccinated animals succumb to the heartwater field challenge. Under field challenge in a heartwater-endemic area in Zimbabwe, the Mbizi inactivated vaccine significantly protected 17 of 21 cattle against heartwater challenge while 7 of 21 (33.3%) unvaccinated control cattle survived (Mahan *et al*., 2001).

5.2 CONCLUSIONS

In conclusion, the attenuated Welgevonden tissue culture vaccine against heartwater proved to be safe and efficacious in cattle. No post vaccination related reactions were observed in this species. The absence of clinical reaction to the vaccine suggests cattle immunized with the vaccine would not require monitoring of temperature reactions and subsequent treatment. The results above denote that administration of the attenuated heartwater (Welgevonden) vaccine in cattle through the I/M route is safe and efficacious, with no inflammatory reactions observed at the injection site. Moreover, the I/M route is more convenient to apply than intravenous, as it has been one of the limiting factors for Ball 3 usage countrywide. Therefore, the use of attenuated heartwater (Welgevonden) vaccine to control heartwater in cattle will be a great advantage especially to rural farmers because of the ease of administration. All the vaccinated animals were fully protected (100%) to homologous virulent tick challenge, suggesting the vaccine would overcome the shortcoming of Ball 3 of limited protection and can be considered a candidate vaccine for protection against heterologous field.

5.3 Recommendations

Field experiments should be conducted to assess the efficacy of the attenuated vaccine to protect cattle against heterologous field challenge. Safety of the vaccine needs to be assessed in different stages of reproduction under controlled conditions. Although the vaccine was 100% efficacious against homologous challenge, the majority of farmers are reluctant to adopt use due to its storage requirement. Therefore, possible innovative ways of keeping the *E. ruminantium* organism viable at 4° C for lengthy period needs to be explored. With the current data from the qPCR analysis, it was evident that the ticks used for challenge were infected. Even so, two control animals survived. In future studies and additional quality control measure that may be utilized may include detection of the presence of *E. ruminantium* in the blood during the challenge phase using the pCS20 qPCR.

CHAPTER 6

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APPENDICES

Appendix 1: Monitoring Sheet

Group:	Cattle #	Temperature increase	Early heartwater symptoms	Severe heartwater symptoms	Treated	DAY 1	DAY2	...DAY45	Total	Average	STDEV
									RI Score	RI	
GROUP 1	1	0	0	0	0	0	0	0	0		
IM inoc; Tick chall	2	0	0	0	0	0	0	0	0		
	3	0	0	0	0	0	0	0	0		
Experiment	4	0	0	0	0	0	0	0	0		
Route Im 10^6	5	0	0	0	0	0	0	0	0		
Tick challenge	6	0	0	0	0	0	0	0	0		
	7	0	0	0	0	0	0	0	0		
	8	0	0	0	0	0	0	0	0		
	9	0	0	0	0	0	0	0	0		
	10	0	0	0	0	0	0	0	0		
GROUP 2	11	0	0	0	0	0	0	0	0		
Negative Control; Tick chall	12	0	0	0	0	0	0	0	0		
	13	0	0	0	0	0	0	0	0		
Negative Control	14	0	0	0	0	0	0	0	0		
Untreated	15	0	0	0	0	0	0	0	0	0	0
Tick Challenge	16	0	0	0	0	0	0	0	0		
	17	0	0	0	0	0	0	0	0		
	18	0	0	0	0	0	0	0	0		
	19	0	0	0	0	0	0	0	0		
	20	0	0	0	0	0	0	0	0		

Appendix 2: Animal Ethics Approval – University of Pretoria (UP)

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

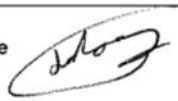
PROJECT TITLE	**Vaccination of on-farm cattle against heartwater using an attenuated tissue culture vaccine**
PROJECT NUMBER	**V071-16**
RESEARCHER/PRINCIPAL INVESTIGATOR	**RD Marumo**

STUDENT NUMBER (where applicable)	**UP_28069944**
DISSERTATION/THESIS SUBMITTED FOR	**MSc**

ANIMAL SPECIES	**Cattle**	
NUMBER OF ANIMALS	**20**	
Approval period to use animals for research/testing purposes		**21 October 2016 – 31 October 2017**
SUPERVISOR	**Dr. B Mans**	

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 21 October 2016
CHAIRMAN: UP Animal Ethics Committee	Signature 

S4260/16

Appendix 2.1 Animal Ethics Approval (UP) extension

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

Extension No. 1

PROJECT TITLE	**Vaccination of on-farm cattle against heartwater using an attenuated tissue culture vaccine**
PROJECT NUMBER	**V071-16**
RESEARCHER/PRINCIPAL INVESTIGATOR	**RD Marumo**

STUDENT NUMBER (where applicable)	**UP_28069944**
DISSERTATION/THESIS SUBMITTED FOR	**MSc**

ANIMAL SPECIES	**Cattle**	
NUMBER OF ANIMALS	**20**	
Approval period to use animals for research/testing purposes		**January 2017-Janury 2018**
SUPERVISOR	**Dr. B Mans**	

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 22 May 2017
CHAIRMAN: UP Animal Ethics Committee	Signature

Appendix 3: Animal Ethics Approval (ARC-OVR)

AEC 21.17

ARC • LNR

APPROVED

Onderstepoort Veterinary Institute

Animal Ethics

Decision of the Animal Ethics Committee for the use of living vertebrates for research, diagnostic procedures and product development

TRAIL PERIOD
STARTING DATE: August 2017 ENDING DATE: March 2018

PROJECT NUMBER:	P10000017			
PROJECT TITLE:	Vaccination of cattle housed on ARC-OVR research premises against heartwater using attenuated tissue culture vaccine			
PROJECT LEADER:	Mr R.D. Marumo			
DIVISION:	EPV			
CATEGORY:	D			
SPECIES OF ANIMAL:	Bovine			
NUMBER OF ANIMALS:	20			

RECOMMENDATIONS BY ANIMAL ETHICS COMMITTEE

Date of AEC meeting for consideration:	Action Taken:	SIGNATURE: AEC-Chairperson Dr P. Mutowembwa
19-10-2017	APPROVED	

PLEASE NOTE: Should the number or species of animal(s) required, or the experimental procedure(s) change, please submit a revised animal ethics clearance form to the animal ethics committee for approval before commencing with the experiment

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Appendix 4: Section 20 approval (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: 12/11/1/1

Mr. Ratselane Daniel Marumo
ARC-OVI
Tel: (012) 529 9211
Fax: (012) 529 9434
E-mail: MarumoR@arc.agric.za

RE: PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984)

Dear Mr. Marumo,

Your application sent with the email on 7 April 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 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. Written permission from the Director: Animal Health must be obtained prior to any deviation from the conditions approved for this study under this Section 20 permit. Please apply in writing to HerryG@daff.gov.za;
3. All potentially infectious material utilised, collected or generated during the study is to be destroyed at the completion of the study. Records must be kept for five years for auditing purposes. A dispensation application may be considered by the Director Animal Health in the event that any of the above is to be stored or distributed;
4. Only aliquots of cryopreserved *Ehrlichia ruminantium* from bio-banked at the ARC-OVI laboratory may be used in this study. No samples may be obtained from another biobank or another species without written permission from the Director: Animal Health;

– 1 –

5. The study must be conducted in compliance with the Veterinary and Para-Veterinary Professions Act 1982 (Act No. 19 of 82);
6. Only animals currently on ARC-OVI property may be used for the study. No outside animals may be sourced without written permission from the Director: Animal Health;
7. Only laboratory bred, disease free *Amblyomma hebraeum* ticks may be used in this study and must be obtained from the tick colony bank, Parasitology section, ARC-OVI;
8. Animals vaccinated with the attenuated tissue culture heartwater vaccine may not leave the property of the ARC-OVI or enter the human or animal food chain in any way within 30 days after vaccination. Any mortalities within 30 days should be incinerated;
9. In the case of any mortalities, carcasses must be sprayed with an acaracide and the carcasses double bagged in leak proof bags prior to removal to the post mortem facility. Carcasses are also to be double bagged in leak proof bags prior to being moved from the post mortem facility to the incinerator;
10. The experimental status of the animals should be declared to the Veterinary Public Health veterinarian responsible for the abattoir at which the animals are to be slaughtered;
11. No part of the study may begin until the valid ethical approval has been obtained from the relevant authority;
12. Cattle may only be challenged with infected *Amblyomma hebraeum* ticks while being quarantined in the tick-free East Coast fever complex at the ARC-OVI;
13. Appropriate personal protective equipment must be worn when collecting samples or handling animals to ensure that no ticks are removed from the tick-free East Coast fever complex;
14. This Section 20 approval is valid up to and including 31 January 2018. An application for an extension must be made by the responsible researcher at least one month prior to the expiry of this Section 20 approval.

Title of research/study: Vaccination of cattle housed on ARC-OVI research premises against heartwater using attenuated tissue culture vaccine
Researcher: Mr. RD Marumo
Institution: ARC-OVI
Our ref Number: 12/11/1/1
Your ref:
Expiry date: 31 January 2018

Kind regards,

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date: 2017 -06- 0 6

SUBJECT: *PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO. 35 OF 1984)*

Appendix 5: Section 21 approval (MCC/DoH)

health
Department:
Health
REPUBLIC OF SOUTH AFRICA

MEDICINES CONTROL COUNCIL
The Registrar of Medicines, Private Bag X828, PRETORIA, 0001
Tel 012 395 8000 Fax 012 395 9201
Tel: (012) 395 8353 *Enquiries:*
Fax: 0866 329 637 *Reference:*

Enquiries: Dr A T Sigobodhla
References: 26/2/2 VCT/04/2017)
01 August 2017

UNIVERSITY OF PRETORIA (ARC-OVR)
100 Old South Road
ONDERSTEPOORT
0110

Att: Mr R.D. Marumo
Tel: 012 529 9211
Fax: 012 529 9434
Email: MarumoR@arc.agric.za

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

Your application refers:

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

Name of product:	Attenuated tissue culture vaccine 10 ml x 2
Species:	Bovine and Ovine
Description of patients:	Cattle and Sheep
Diagnosis/purpose:	Vaccination of on-farm cattle against heartwater
Authorisation number:	VCT/04/2017

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
for and on behalf of the REGISTRAR

MEDISYNEBEHEERRAAD

Republiek van Suid-Afrika

MEDICINES CONTROL COUNCIL

Republic of South Africa

DIE REGISTRATEUR VAN MEDISYNE
DEPARTEMENT VAN GESONDHEID
PRIVAATSAK X828
PRETORIA
0001

Telefoon: **(012) 395 8351**
Telephone:

Fax: **0866 330 298**

THE REGISTRAR OF MEDICINES
DEPARTMENT OF HEALTH
PRIVATE BAG X828
PRETORIA
0001

- Inquiries: Dr A.T. Sigobodhla
- Reference: V071-16

Date: 01st August 2017

UNIVERSITY OF PRETORIA (ARC-OVR)
100 Old South Road
ONDERSTEPOORT
0110

Tel: 012 529 9211
Fax: 012 529 9434
Email: MarumoR@arc.agric.za

APPLICATION TO CONDUCT A CLINICAL STUDY: V071-16

Your application to conduct the clinical study refers:

Please find the attached recommendations from the Veterinary Clinical Committee for your attention.

YOURS FAITHFULLY

REGISTRAR OF MEDICINES

PRODUCT NAME: Attenuated tissue culture vaccine

SPECIES: Bovine and Ovine

APPLICANT: University of Pretoria

MCC Approval Number: VCT 04/2017

STUDY TITLE: Vaccination of on-farm cattle against heartwater using an attenuated tissue culture vaccine

VCC RECOMMENDATIONS

1. The study protocol is recommended.
2. Applicant may commence the study.