Attenuated heartwater vaccine (*Ehrlichia ruminantium* Welgevonden): Immunization of Angora goats using the intra-muscular route of administration

By

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Declaration

I, Anna Haw declare that the dissertation which I hereby submit for the degree Magister Scientiae (Veterinary Tropical Diseases) at the University of Pretoria is my own work and has not previously been submitted by me for a degree at another university.

Signature …… Date …….

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ABSTRACT

Ehrlichia ruminantium, the causative organism of heartwater infections, places severe economic constraint on the livestock industry wherever *Amblyomma* tick vectors are present. Angora goats are particularly susceptible to this disease and the current live blood vaccine cannot safely be used to protect these animals. An attenuated *E. ruminantium* (Welgevonden) experimental vaccine has previously shown promising results in Merino sheep and Boer goats. The vaccine was administered by intravenous route (i/v). The general objective of this study was to test the efficacy and safety of the attenuated heartwater vaccine *E. ruminantium* (Welgevonden) in Angora goats. The specific objectives were, firstly to assess the intra-muscular route of administration of the attenuated vaccine as compared to the standard i/v route and, secondly, to study the haematological changes in Angora goats before, during and after vaccination under controlled conditions at the Onderstepoort Veterinary Institute tick-free stables. A total of 55 Angora goats were used in this trial. They were purchased from an area in South Africa which is known to be *Amblyomma*-free and heartwater-free. Furthermore, on arrival, the goats were screened for *E. ruminantium* infection by the immunofluorescent antibody (IFA) test to confirm their disease-free status.

The Angora goats were divided into 3 groups: In Group 1, ten were vaccinated by the standard i/v route, in Group 2, 31 received the vaccine by i/m route and 10 served as untreated controls for Group 3. Five of the 10 i/v vaccinated group, 20/31 of the i/m vaccinated and 5 controls were challenged by feeding of known infected adult *A hebreaum*. The other remaining animals within the three groups were challenged using a known infected blood stabilate administered by the standard i/v route (dose $5xLD_{50}$). All animals were challenged 42 days after vaccination.

The vaccine did not produce any inflammatory reactions at the site of injection. However, 3/31 (9.7%) of i/m and 7/10 (70%) of i/v vaccinated goats developed febrile reactions starting on Day 11 post-immunisation and were treated. All vaccinated goats were fully protected against either needle i/v or tick challenge, while the control non-vaccinated goats reacted severely to the challenge materials and required oxytetracycline treatment. Despite treatment, two of the unvaccinated goats died from the challenge material.

Haematological values (packed cell volume, differential blood cells count) were obtained on blood samples taken from the treatment and control groups at different times during the course of the trial. Wide within group variations as shown by the high standard deviation values were found. As no significant changes were found between vaccinated and control animals, it is likely that the attenuated vaccine does not cause significant clinical haematological changes.

This study has demonstrated that the attenuated *E. ruminantium* (Welgevonden) vaccine is safe in 90.3% and efficacious (100% efficacy) for intramuscular administration in Angora goats. However, further laboratory and on-farms studies are needed in order to establish the lowest effective and safety dose, duration of immunity, and the vaccine's safety in young and pregnant animals.

CHAPTER 1

GENERAL INTRODUCTION

Heartwater is an infectious, non-contagious tick-borne disease, caused by the intracellular rickettsial agent *Ehrlichia ruminantium*, previously known as *Cowdria ruminantium*. It affects cattle, sheep and goats, as well as susceptible wild antelope species such as blesbok (*Damaliscus albifrons*), black wildebeest (*Connochaetes gnu*) and springbok (*Antidorcas marsupialis*) (Provost & Bezuidenhout 1987). Angora goats are particularly susceptible to this disease which places a severe constraint on mohair farmers, particularly in the valley bushveld area of the Eastern Cape Province (Du Plessis, De Waal & Stoltsz 1994).

The original distribution of the disease appears to have been sub-Saharan Africa (Norval, Andrew, Yunker & Burridge 1992) but in the past centuries it has spread to a number of islands in the Indian and Atlantic Oceans, as well as the Caribbean from where it poses a threat to the American mainland. The vectors responsible for the transmission of *E. ruminantium* belong to the genus *Amblyomma* with the most important species in southern Africa being *A. hebraeum* and *A. variegatum* (Walker 1987).

Although information on the actual economic impact of heartwater on livestock production is difficult to obtain (Mukhebi, Chamboko, O'Callaghan, Peter, Kruska, Medley, Mahan & Perry 1999), it is generally accepted that heartwater is either the most or second most important tick-borne disease in Africa (Provost & Bezuidenhout 1987). It is estimated to cause up to R975 million in financial losses in Africa, with South African farmers (commercial and emerging sector) loosing R220 million annually. There are 150 million animals that are at risk in sub-Saharan Africa, with South Africa contributing 8.6 million (Draft Country Report, UF/USAID/SADC Heartwater Research Project, 2005). The impact of the disease on the livelihood of poor African farmers is severe as it reduces their only source of income as well as further increasing food insecurity. It also presents a significant obstacle to the importation of improved breeds in heartwater endemic areas (Camus, Barré, Martínez & Uilenberg 1996).

The circumstances in which heartwater causes losses in domestic livestock has been eloquently summarised by Norval *et al.* (1992) as follows: (i) stock are moved from

heartwater-free to heartwater-endemic areas; (ii) highly susceptible stock (e.g. Angora goats) are raised in endemic areas; (iii) ecological conditions allow only low vector survival and not all stock are immunized by natural infection when young; (iv) intensive tick control is practised thus suppressing but not eradicating the disease; and (v) vectors and the disease spread to new areas. It is therefore clear that this disease places a severe constraint on livestock production in heartwater-endemic areas.

Infected animals which recover from the disease, either naturally or following treatment develop a solid immunity to homologous challenge; however, lack of cross-protection between isolates has been reported (Jongejan, Uilenberg, Franssen, Gueye & Nieuwenhuijs 1988; du Plessis, van Gas, Olivier & Bezuidenhout 1989; Jongejan, Thielemans, Briere & Uilenberg 1991; Collins, Pretorius, van Kleef, Brayton, Allsopp, Zweygarth & Allsopp 2003). The strain diversity of *E ruminantium* is particularly important when considering vaccine candidates against this disease, particularly as mixed infections with *E. ruminantium* genotypes has been found to occur in both ruminants and ticks (Faburay, Jongejan, Taoufil, Ceesay & Geysen 2008).

Currently, the only commercially available method of immunisation is "infection and treatment" (Uilenberg 1983). This "vaccine" is administered intravenously and, following inoculation, body temperature is measured and oxytetracycline treatment needs to be given when a temperature reaction is noted (Norval *et al.* 1992). This is therefore a cumbersome technique requiring veterinary skills for administration and clinical monitoring. Moreover, this type of a vaccine does not afford protection against all South African heartwater stocks (Oberem & Bezuidenhout 1987b; Collins *et al.* 2003; Zweygarth, Josemans, Van Strijp, Lopez-Robellar, van Kleef & Allsopp 2005). Other problems with live blood-derived vaccines in general include: (i) the possible spread of silent pathogens occurring in donor animals, (ii) difficulties in standardising the vaccine dose, (iii) the risk of distribution of a virulent strain to other areas, (iv) maintenance of carrier animals which might serve as reservoirs for tick transmission and (v) quality control of vaccine production, maintenance and transportation to the end user, including the necessity for a cold chain (Shkap, de Vos, Zweygarth & Jongejan 2007). Moreover the use of this vaccine is limited to South Africa and its use in Angora goats is extremely risky due to their high susceptibility to the disease. In order to overcome these problems, an ideal vaccine should be inactivated or DNA-derived (new generation vaccines). However, thus far, inactivated and DNA-derived vaccines for

heartwater have proved relatively ineffective (Allsopp 2010). Therefore, until enough knowledge and skills are gained in order to produce successful DNA vaccine candidates, an attenuated vaccine, safe enough to use in Angora goats, with wider cross-protection properties than the current "infection and treatment" technique will undoubtedly be safer and result in reduced economic losses from heartwater infection. In addition, a vaccine that can be administered intramuscularly will be cheaper and easier to use, allowing a larger number of farmers to take advantage of the benefits of vaccination.

Zweygarth, Josemans & Steyn (2008) have developed an attenuated *Ehrlichia ruminantium* (Welgevonden) experimental vaccine and obtained promising results with its use in Merino sheep and Angora goats. In their study they used relatively large numbers (62) of Merino sheep under laboratory conditions, but only few (17) Angora goats were used in optimization of a safe and effective vaccine i/v dose. The current study thus aimed to validate and refine these preliminary results obtained in Angora goats using statistically justifiable numbers of animals under laboratory conditions together with testing the intramuscular route of inoculation for safety and efficacy to replace the current intravenous route.

CHAPTER 2

LITERATURE REVIEW

2.1 Heartwater Disease

Heartwater is caused by the rickettsial organism, *Ehrlichia ruminantium*, formally known as *Cowdria ruminantium*. It occurs wherever the tick vectors are prevalent and is thought to have originated in South Africa where it was first identified in 1838 (Provost & Bezuidenhout 1987). *Ehrlichia ruminantium* is only transmitted by ticks belonging to the genus *Amblyomma* (Bezuidenhout 1987) with *A. hebraeum* being the only field vector in most parts of southern Africa (Yunker, Kocan, Norval & Burridge 1987; Walker 1987). Figure 2.1 shows the distribution of *A. hebraeum* the main vector in South Africa. Throughout the rest of sub-Saharan Africa as well as areas in the Caribbean, *A. variegatum* is the predominant vector of this disease (Walker 1987; Norval *et al.* 1992).

Ehrlichia ruminantium is transmitted transstadially by these three-host ticks (Prozesky & Du Plessis 1987; Yunker *et al.* 1987). Male *A. hebraeum* may also be responsible for intrasstadial transmission (Andrew & Norval 1989) when they move from one host to another, or transfer from a dead to a living host. Moreover, their prolonged attachment to hosts makes them particularly significant in the epidemiology of the disease (Andrew & Norval 1989). Apart from ruminants, certain animals such as the helmeted guinea fowl (*Numida meleagris*) and leopard tortoise (*Geochelone pardalis*) can become subclinical carriers of *E. ruminantium* and act as a source of organisms for the ticks (Oberem & Bezuidenhout 1987a).

Clinical signs in susceptible animals include fever, inappetence, incoordination, respiratory distress, nervous symptoms and death (Uilenberg 1983; van de Pypekamp & Prozesky 1987). These signs are mostly related to an increased capillary permeability, which leads to the excess effusion of fluid into tissues and the body cavities (Clark 1962; Prozesky & Du Plessis 1985a). The course of the disease can be quite variable, ranging from peracute to mild depending on the age, immune status, individual or breed susceptibility of the animal and virulence of the isolate (Alexander 1931; Uilenberg 1983). The incubation period may also

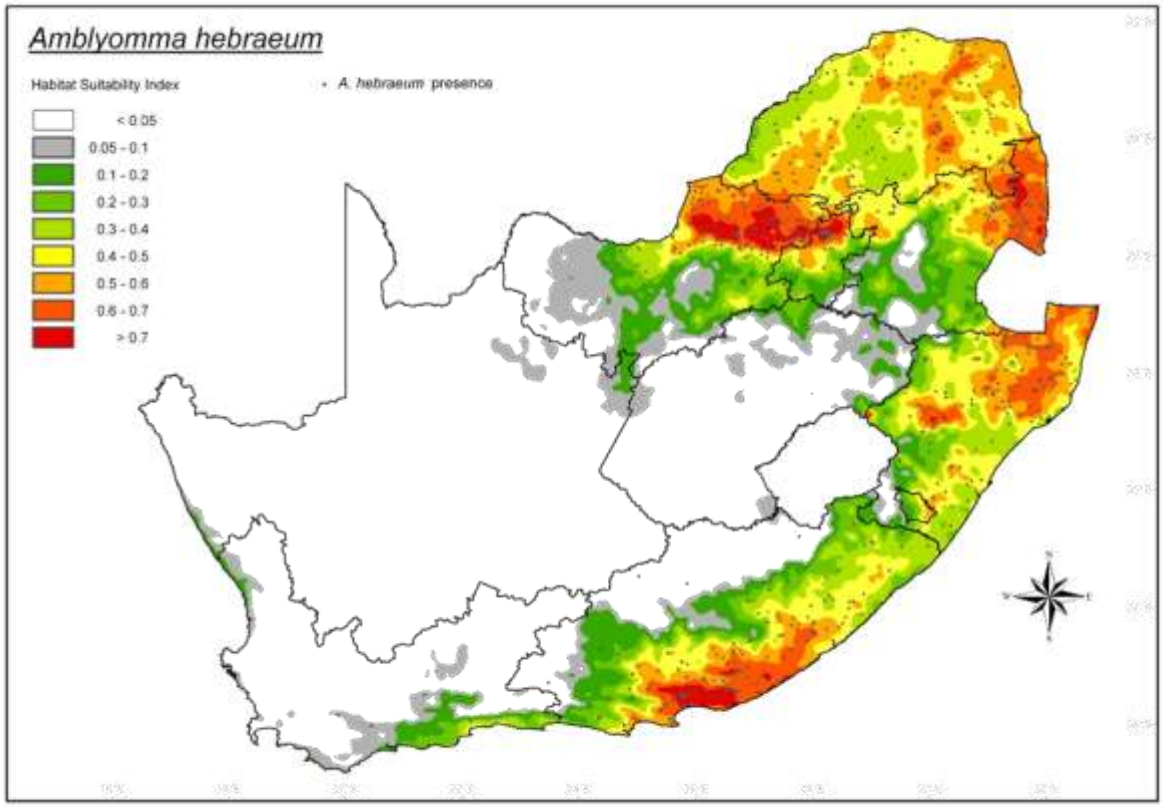

Figure 2.1 Distribution and prediction map of *A. hebraeum* in South Africa (drawn by Arthur Spickett)

be highly variable (Alexander 1931; Uilenberg 1983) but in Angora goats it has been found to be between 11 and 13 days when artificially infected with the Ball 3 strain (Prozesky & Du Plessis 1985b). In the peracute form of the disease, which is not uncommon in Angora goats (Uilenberg 1983; van de Pypekamp & Prozesky 1987), animals usually collapse suddenly and die after a few paroxysmal convulsions (Uilenberg 1983).

Acute heartwater is characterised by a fever that usually remains high with small fluctuations and drops off subnormally shortly before death (van de Pypekamp & Prozesky 1987). Initially animals appear normal before they gradually show inappetence and eventually stop feeding with reluctance to move around (Prozesky & Du Plessis 1985b; van de Pypekamp & Prozesky 1987). Respiratory rate increases as a result of lung oedema (Alexander 1931) and a progressively unsteady gait is usually noticed (Prozesky & Du Plessis 1985b). According to Alexander (1931), nervous symptoms are more pronounced in cattle than in sheep and goats.

In mild cases of heartwater, pyrexia, apathy and slight tachypnoea may be noticed and most mild cases will recover within a few days (van de Pypekamp & Prozesky 1987).

2.2 Pathogenesis of Heartwater

Although heartwater has been extensively studied, the pathogenesis is still poorly understood (Du Plessis, Malan & Kowalski 1987). It is generally accepted that the cardinal lesion in the pathogenesis of heartwater is an increased vascular permeability of smaller blood vessels (Du Plessis *et al*. 1987). However, exactly how this increased permeability is caused is not yet clearly defined.

Van Amstel, Guthrie, Reyers, Bertschinger, Oberem, Killeen & Matthee (1987) reviewed the clinical pathological changes that occur during a heartwater infection. Most of these changes coincide with the onset of the febrile reaction. A progressive anaemia develops during the course of infection and it has been suggested that this anaemia is caused by a bone marrow depression (van Amstel *et al.* 1987). Together with a drop in haematocrit, a neutropaenia, an eosinopaenia and a lymphocytosis are the most marked and consistent changes seen in the

haemogram associated with heartwater (van Amstel *et al.* 1987). However, in general, haematological findings in heartwater do not appear to be spectacular (Uilenberg 1983).

2.3 Immunity to Heartwater

Laboratory experiments as well as field observations have shown that cattle, sheep and goats are capable of developing a protective immunity against heartwater after surviving a virulent challenge (Alexander 1931). However, partial or total lack of cross protection between different isolates of *E. ruminantium* has been demonstrated (Jongejan *et al.* 1988; Du Plessis *et al.* 1989; Jongejan *et al.* 1991; Collins *et al.* 2003). Interestingly, immunity to the Welgevonden isolate of *E. ruminantium* has been shown to confer protection to a number of other virulent southern African stocks such as the Ball 3, Mara 87/7, Blaaukrans and Gardel (Du Plessis *et al.* 1989; Collins *et al*. 2003) and it is for this reason that the Welgevonden stock would be the most suitable as a vaccine candidate for South Africa.

It is also known that young animals possess an innate resistance to the disease, irrespective of the immune status of the dam. In calves this lasts up to the age of four weeks and in lambs up to at least seven days (Neitz & Alexander 1941).

The mechanism by which the immune response develops is not entirely clear, but it has been suggested that cellular immunity, rather than humoral immunity, is the predominant response (Stewart 1987; Totté, Bensaida, Mahanb, Martineza & McKeever 1999). This also became evident in a study by Mahan, Smith, Kumbula, Burridge & Barbet (2001) who demonstrated that the inactivated vaccine prepared in adjuvants that preferentially induce humoral immunity did not protect against heartwater challenge, but vaccines prepared with adjuvants which induce cellular immunity were more efficient in protecting sheep against lethal heartwater challenge. Serum antibodies are produced in response to infection but they do not appear to correlate with protection or duration of immunity (Du Plessis, Bezuidenhout & Ludemann 1984; Martinez, Maillard, Coisne, Sheikboudou & Bensaid 1994; Totté *et al.* 1999).

2.4 Vaccination against the disease

2.4.1 Live blood vaccines

Neitz and Alexander (1941) were the first to apply a practical method of protection against heartwater. It was based on the fact that calves up to the age of four weeks and lambs up to about 7 days of age possess an innate resistance to heartwater and the majority survived an injection of virulent heartwater infected blood. This method was later refined and developed for use in older animals when it was found that animals infected with live *E. ruminantium* organisms and subsequently treated with tetracyclines during the reaction developed immunity to the disease (Uilenberg 1983). This artificial method of immunization was referred to as the "infection and treatment method" and it has been practiced in South Africa for over 60 years. Currently, this expensive and cumbersome procedure is still the only commercially available method of immunisation against heartwater. This "vaccine" consists of whole blood from sheep infected with live organisms of the Ball 3 stock of *E. ruminantium* which is injected intravenously into animals. The Ball 3 isolate was chosen because it generally causes a marked febrile response a few days prior to the onset of other clinical signs (Oberem & Bezuidenhout 1987b). In this way, reacting animals can be identified and treated before debilitating clinical signs occur. While this method has aided in the control of the disease, there are a number of inherent problems preventing the widespread use of this live blood vaccine. Firstly, this method is not user-friendly as it requires a cold chain for delivery and it must be administered intravenously, which is perhaps the greatest limiting factor to its use (Oberem & Bezuidenhout 1987b). Although the Ball 3 isolate produces a good temperature reaction before the onset of clinical signs, it is not the most ideal isolate to use as a vaccine as it does not cross-protect against all of the field genotypes of *E. ruminantium* (Collins *et al*. 2003). The more virulent, Welgevonden stock, which has been shown to confer a greater level of protection against a number of stocks, would be a better isolate for a vaccine, but its virulence prohibits its use as a live blood vaccine. The vaccine is expensive to produce due to the large number (200 - 300 per year) of live sheep needed for its production (Oberem & Bezuidenhout 1987b). Moreover, the use of live animals for vaccine production means there is a risk of transmission of blood-borne pathogens and standardization of the vaccine dose is difficult. As this is a live vaccine it cannot be used in areas such as the American mainland, where the disease is not yet established for risk of introducing the disease into the area.

In a survey conducted by Du Plessis *et al.* (1994) it was shown that only 35% of cattle farmers and 15% of farmers keeping sheep and goats, vaccinate their animals against heartwater. With 9% of the farmers claiming poor protection after immunization it would seem that the current vaccine does not adequately control heartwater. The survey also showed that heartwater is seen as a severe constraint on small stock production in the endemic areas, and this is particularly true for Angora goat farmers (Du Plessis *et al.* 1994). Due to these drawbacks, improved methods of immunization against heartwater have been investigated. As a variety of immunotypes of *E. ruminantium* occur in the field, and that cross-protection between stocks varies widely from total to minimal (Jongejan *et al.* 1988; du Plessis *et al.* 1989; Jongejan *et al.* 1991; Collins *et al.* 2003) a vaccine stock that confers broad cross-protection seems to be a prerequisite for a commercial vaccine.

In summary, the production and administration of the current live blood vaccine is costly and demanding, and it confers only limited protection against some common genotypes in southern Africa. It is therefore imperative that improved methods of vaccination are investigated.

2.4.2 Inactivated vaccines

A major step in heartwater research was the successful *in vitro* cultivation of *E. ruminantium* which was first achieved in 1985 (Bezuidenhout, Paterson & Barnard 1985). For the first time, large quantities of *E. ruminantium* organisms became available which greatly facilitated research on the organism.

To overcome some of the constraints of the live vaccines, an inactivated vaccine against heartwater was developed. Inactivated heartwater vaccines consist of organisms derived from tissue culture which have been rendered non-viable by chemical treatment and combined with an adjuvant (Martinez *et al.* 1994; Mahan, Allsopp, Kocan, Palmer & Jongejan 1999; Allsopp 2010).

Martinez *et al.* (1994) were the first to successfully apply such a vaccine. They vaccinated goats using the Gardel isolate and found that 50-80% of the animals were protected against a homologous needle challenge which killed 100% of the negative controls (Martinez *et al.*

1994). However, in field trials, the levels of protection proved disappointing (Faburay, Geysen, Ceesay, Marcelino, Alves, Taoufik, Postigo, Bell-Sakyi & Jongejan 2007**)** especially in Angora goats where there was a 72% mortality rate in goats vaccinated with the Mbizi vaccine in South Africa **(**Mahan *et al.* 2001). Another drawback of the inactivated vaccine is that it requires several injections spread over a period of weeks or months, during which the animals have to be kept tick-free (Martinez *et al*. 1994; Adakal, Stachurski, Konkobo, Zoungrana, Meyer, Pinarello, Aprelon, Marcelino, Alves, Martinez, Lefrancois & Vachiéry 2010). Therefore, due to the limited protection against tick challenge in the field, considerable improvements will be required before inactivated heartwater vaccines could become a commercially viable option (Allsopp 2010).

2.4.3 DNA Vaccine/ Recombinant vaccines

Genetic immunisation consists of the presentation of protection-stimulating genes in a DNA vaccine vector (Collins *et al.* 2003). This type of vaccine may be ideal to protect against a number of different field isolates as genes from different isolates can be incorporated into the same vaccine. Collins *et al.* (2003) used a nucleic acid vaccine which consisted of four *E. ruminantium* genes to immunise sheep. Sheep immunised with this vaccine were completely protected against a subsequent lethal needle challenge with both homologous and heterologous *E. ruminantium*-infected blood (Collins *et al*. 2003; Pretorius, Collins, Steyn, van Strijp, van Kleef & Allsopp 2007). However, protection against a field challenge in a heartwater endemic area was relatively poor even when the vaccine was used in a prime-boost format with recombinant *E. ruminantium* proteins (Pretorius, van Kleef, Collins, Tshikudo, Louw, Faber, van Strijp & Allsopp 2008). This difference in outcome between the needle and field challenge was not thought to be due to lack of cross-protection to the field isolates, but rather due to the mode of challenge. It was thought that a needle challenge is far less acute than a tick challenge, and that immunity engendered by the DNA vaccine alone was not sufficient to protect against the natural route of infection (Collins *et al.* 2003).

2.4.4 Live attenuated vaccines

Although an inactivated or nucleic acid vaccine would provide the ideal alternative for protection against heartwater, it is clear that they currently do not afford sufficient protection against field challenge and thus focus has turned to developing live attenuated vaccines.

Attenuation is a decline in virulence imposed on a pathogen by chemical or physical interference, during the *in vitro* cultivation (Zweygarth 2006). The advantages of attenuated vaccines are that they activate all phases of the host immune system and raise immune responses to all protective antigens, thus inducing a solid immunity (Zweygarth 2006). The first successful attempt to attenuate *E. ruminantium* was achieved using the Senegal stock, which became attenuated spontaneously after several passages on bovine umbilical endothelial cell cultures (Jongejan 1991). It was shown that when these attenuated culture suspensions were used as a vaccine, they conferred strong protection against homologous challenge in sheep and goats without causing disease (Jongejan 1991). However, the attenuated Senegal stock did not provide efficient cross-protection against other virulent stocks when it was tested in field trials (Jongejan 1991; Gueye, Jongejan, Mbengue, Diouf & Uilenberg 1994) and thus it would not be suitable for widespread use. The Welgevonden isolate has been shown to provide cross-protection against a needle challenge with a range of other isolates (Collins *et al.* 2003) thus it would be more suitable for use as an attenuated vaccine.

For many years, attempts to attenuate the more virulent Welgevonden strain, which would confer wider cross-protection, had been unsuccessful. However, the stock was eventually successfully attenuated by continuous propagation in a canine-macrophage cell line (DH82) (Zweygarth & Josemans 2001) followed by re-adaption to grow in a bovine endothelial cell line (BA 886) (Zweygarth *et al.* 2005). Zweygarth (2006) demonstrated that readapting the attenuated Welgevonden stock to bovine endothelial cells (BA 886), which are derived from natural host species of *E. ruminantium*, and propagating the organisms for 64 passages, led neither to a reversion to virulence nor to a loss of immunogenicity. This attenuated *E. ruminantium* (Welgevonden) stock vaccine has shown promising results in laboratory trials in Merino sheep and Boer goats (Zweygarth *et al.* 2005). However, it has not yet been extensively tested in Angora goats and the intramuscular method of administration needs to be tested as a more user-friendly alternative to the current intravenous route.

The attenuated vaccine offers several advantages over the present infection and treatment method of immunisation. Most importantly it confers cross protection against most South African isolates (Collins *et al.* 2003; Zweygarth *et al.* 2005), a blocking treatment is not required and it is less expensive to produce than the traditional blood vaccine (Shkap *et al.* 2007).

2.5 The Angora goat (*Capra aegagrus)*

The Angora goat is considered to be the most susceptible domestic ruminant to heartwater (Spreull 1922) with mortality rates commonly around 90% in infected goats (Uilenberg 1983). Alexander (1931) indicated that the mortality amongst goats, and in particular Angora goats, is higher than amongst Merino sheep. It is particularly difficult to achieve a specific immune response to heartwater in Angora goats and immunisation with the current infection-and-treatment method is difficult, if not impossible (Du Plessis, Jansen & Prozesky 1983). Du Plessis *et al.* (1983) found that following intravenous inoculation of the Ball 3 isolate; few goats survive if treated on the second or third day of the febrile reaction while goats treated on the first day of the febrile reaction usually survived but failed to produce adequate immunity to subsequent challenge.

South Africa currently produces over 50% of the total world mohair production (Mohair South Africa 2009) making Angora goat farming an obvious economically important enterprise in the country and reducing losses due to disease is imperative. A study by Snyman (2010) in which information was obtained from 12 different Angora goat studs, revealed that the average pre-weaning mortality rate was 11.5%. It has also been shown that Angora goats are poor at coping with cold spells and other stressors as they have a reduced adrenal function in comparison to Boer goats (*Capra hirus*) and Merino sheep (*Ovis aries*) (Engelbrecht, Herselman, Louw & Swart 2000). Moreover, it has been demonstrated that the present "infection and treatment" method of immunisation cannot be safely used in Angora goats (Du Plessis *et al.* 1983). Angora goat farmers thus generally control the disease by regular fortnightly prophylactic treatment with an antibiotic, usually tetracycline. This is labour intensive, expensive and there is a risk that the organism may eventually develop resistance to the antibiotic. Therefore it is imperative to determine whether the experimental attenuated heartwater vaccine can afford a more effective and safer method of control in this species than the control methods currently available.

2.6 General and specific objectives

The general objective of the study was to test the efficacy and safety of the attenuated heatwater vaccine *E. ruminantium* (Welgevonden) in Angora goats.

Specific objectives:

1. To assess the intra-muscular route of administration of the attenuated vaccine as compared to the standard intravenous route.
2. To study the haematological changes in Angora goats before, during and after vaccination.

CHAPTER 3

MATERIALS AND METHODS

3.1 Experimental vaccine preparation

The attenuated Welgevonden stock of *E. ruminantium* was used for the immunisation of the goats. The medium used for this as well as the parental Welgevonden stock used for the virulent challenge has been described by Zweygarth *et al.* (2008). It consisted of Dulbecco's modified Eagle's medium nutrient mixture Ham F-12 (Sigma) containing 15 mM HEPES and 1.2g.l-1 sodium bicarbonate (Zweygarth *et al.* 2008). It was further supplemented with 10% (v/v) heat-inactivated foetal bovine serum, 2mM L-glutamine, 100 IU ml.-1 penicillin and 100 µg.ml-1 streptomycin (Zweygarth *et al.* 2008).

The Welgevonden stock was propagated in a continuous canine macrophage-monocyte cell line (DH82) as described elsewhere (Zweygarth & Josemans 2001). After 64 passages the infected cell cultures were harvested by resuspending the cells in fresh medium (Zweygarth *et al.* 2005). The cell suspension was centrifuged (800 x g; 10 min; room temperature) and 2.5 ml of supernatant containing elementary bodies was distributed into a culture flask containing bovine endothelial cells (BA 886) (Yunker, Byron & Semu 1988). Cells were dispersed by pipetting the suspension up and down and then transferring at various ratios (between 1:10 and 1:30) onto new endothelial cell monolayers (Zweygarth *et al.* 2008). After 24 hours all the medium was discarded and replaced with 5 ml of fresh medium containing 0.3 µg.ml-1 cycloheximide (Zweygarth *et al.* 2008). Subcultures were performed every 3 days and the attenuated Welgevonden stock was passaged 55 times in SBE 189 cells (Zweygarth *et al.* 2008). The infective, dense-cored elementary bodies of *E. ruminantium* were then harvested and cryopreserved in liquid nitrogen. Thereafter, the samples were thawed and returned to endothelial cell culture in order to determine the number of infective organisms present in the stabilate. The method for quantifying the number of elementary bodies in each immunizing dose has been described by Zweygarth *et al.* 2008. The expansion of tissue cultures and the calculations of the vaccine doses for this vaccine trial was done under phase-contrast misroscope.

For animal immunization the stabilates were diluted to a standardized number of infective organisms. The inoculation dose for the intramuscular (i/m) route was 6 x 10^5 live organisms per dose, based on previous trials (Zweygarth *et al*. 2008) and personal communication (Zweygarth 2011). Since most previous experiments with positive results were conducted using the intravenous (i/v) route (Zweygarth *et al.* 2005; Zweygarth *et al.* 2008), and this is the route of administration of the current commercially available live blood vaccine, this method was used to serve as a standard positive control. In this study, the Angora goats received a different dose for i/v route, lower than that for i/m dose, which was equivalent to 5.7 x 10^4 live organisms. The cultures were expanded to produce adequate numbers of stabilates for the vaccine trials.

3.2 Challenge of animals using infected blood

The animals were challenged with the virulent *E. ruminantium* (Welgevonden) stock ($5xLD_{50}$). Blood stabilate was prepared from an *E. ruminantium* (Welgevonden) infected sheep as described previously (Brayton, Collins, van Strijp & Allsopp 2003). Briefly, the infected sheep blood was collected on day 2 of temperature reaction, diluted in sucrose potassium glutamate buffer (SPG) (0.22 M sucrose, 0.01 M potassium phosphate pH 7.0, 0.5 mM potassium glutamate) and stored as stabilate in liquid nitrogen. The dose was titrated to determine a dose equivalent to $5xLD_{50}$ prior to challenge. Animals received the challenge in a volume of 2 ml administered via the intravenous route.

3.3 Challenge of animals by feeding of infected ticks

A total of 5 infected *A. hebraeum* adult ticks which dropped as nymphs from an infected sheep with the virulent Welgevonden stock coinciding with the duration of high temperature were used to infect each goat. Three males were placed in linen bags fitted on the backs of goats and allowed to feed for three days before two females were placed in the sack. This allows faster attachment of females in response to the released attachment-aggregation-pheromone secreted by feeding males. Ticks were allowed to feed to engorgement.

Figure 3.1 Ticks for feeding contained and secured in a bag fixed on the back of a goat

3.4 Origin, maintenance and monitoring of experimental animals

A total of 55 Angora goats were used in this experiment. The Angora goats were purchased from an area in South Africa which is known to be *Amblyomma*-free and heartwater-free. On arrival, the goats were screened for *E. ruminantium* infection by the immunofluorescent antibody (IFA) test (Semu, Mahan, Yunker & Burridge 1992) to verify that all animals were negative before the start of the experiment. Ten ml of blood were drawn from all animals on arrival for the above mentioned serological diagnostic test. Blood for IFA tests were also drawn from experimental animals on day 0, before inoculation, Day 14, 42 and 64 as a monitoring procedure.

The animals were kept in stables with provision of shade and shelter at the Onderstepoort Veterinary Institute. They were housed for a period of two months before the experiment commenced. This was to ensure that they became accustomed to the new surroundings and daily handling. In addition, faecal worm egg counts showed evidence of high roundworm and coccidia loads. Therefore the goats were all treated with Tramisol® Plus (rafoxanide + levamisole hydrochloride [Afrivet]) and Sulfazine (sulphadimidine sodium) to ensure they were free from internal parasites before proceeding with inoculations. In addition, all animals were dipped with a pyrethroid-based acaricide against ticks and lice. Faecal worm egg counts followed by deworming treatments when needed were continued throughout the experimental period as Angora goats are particularly susceptible to internal parasites (Hoste, Leveque & Dorchies 2001).

Blood samples in EDTA vacutainers were taken to measure haematocrit/packed cell volume (PCV) every two weeks starting Day 0, before inoculation. PCV was measured to determine whether or not the attenuated vaccine or challenge material had a significant influence on the animals' haematocrit as heartwater infection is said to cause a drop in haematocrit values (van Amstel *et al.* 1987). Differential blood cell counts were also conducted during the vaccine-related temperature reaction and a month after the end of the challenge period.

Morning rectal temperatures and clinical signs of all goats were monitored daily for the duration of the experiment. Goats were confined to a crush while the rectal temperatures were being taken. Once all readings were recorded, the goats were released into the large pen where a strip of pellets was spread along the length of the pen. In this way, the goats' general demeanour could easily be assessed by watching the way in which each goat ran towards the

pellets and started eating (Figure 3.2). After immunisation and challenge, animals were scored daily according to a reaction index (RI) based on their temperature reaction, symptoms displayed and treatment received (Table 1). In compliance with the institute's animal ethics regulations, oxytetracycline was administered to any animal showing severe clinical signs, such as depression, anorexia, laboured respiration, recumbence or in-coordination. The need to administer treatment was assessed by a veterinarian (Dr Haw) on an individual goat basis taking into account the temperature reaction together with clinical signs. Treatment was also given to those animals that had rectal temperatures of two or more degrees Celsius above their pre-immunisation averages, regardless of whether or not they were showing any other clinical signs. Scores were totalled at the end of each reaction period following either immunisation or challenge.

Table 3.1: Reactions Index

1) Before vaccination: Average temperature over 10 days calculated for each goat		
2) **After vaccination or challenge:** During observed period of reaction (16-19 days)		
Criteria	Parameter	Points scored
Temperature	For every 0.1˚C above the average calculated temperature	0.1
Symptoms	Loss of appetite, heavy breathing, hanging head, stiff gait, depression	5
Treatment/death/ euthanasia	Intravenous treatment (short-acting oxytetracycline)	20

Figure 3.2 Food pellets distributed in a straight line to allow clinical observation of goats while eating

3.5 Assessment of intramuscular injection site

Hair was clipped from a 5 cm by 5 cm square area over the right semitendinosus muscle on those goats (31) that were to receive intramuscular inoculations. These goats were turned on their backs and the area was palpated and visually assessed for any swelling or inflammatory reactions daily for a period of 10 days post immunisation.

3.6 Haematocrit and differential blood cell analyses

Blood samples in EDTA vacutainers were taken to measure haematocrit/packed cell volume (PCV) every two weeks starting on Day 0, before inoculation. Samples were centrifuged in capillary tubes after which the PCV result was manually read off the haematocrit reader and recorded by the veterinarian in charge to ensure consistency.

Differential blood cell counts were also conducted during the vaccine-related temperature reaction as well as one month after all reactions had resolved. These were analysed using an ADVIA 2120 Haematology System (Siemens) kept by the Onderstepoort Veterinary Pathology Department. Blood samples from nine goats in Groups 1 and 2 were taken during the temperature reaction on day 12 post immunisation. A further 17 goats in groups 3 and 4 were sampled. These samples were taken between 12 and 16 days post immunisation depending on the temperature reaction of individual animals. Five females and five males of the untreated negative control animals were sampled during this same period. One month after all challenge related reactions had subsided, these same animals were re-sampled.

3.7 Antibody analysis by an indirect fluorescent antibody test (IFAT)

Serum samples of all goats were taken on Day 0, 14 days post immunisation and 42 days post immunisation (pre-challenge). These serum samples were tested using the immunofluorescent antibody test (IFAT) which detects anti-*Ehrlichia* antibodies (Semu *et al.* 1992; Zweygarth *et al.* 2005). The application of the IFA test was described by Zweygarth *et al.* (2005). Two-fold dilutions (1:40; 1:80; 1:160 and 1:320) of the test sera were applied to the wells of antigen slides and were incubated at 37˚C for 30min. The second antibody was rabbit anti goat IgG, labelled with fluorescein isothiocyanate (Sigma), and diluted 1:80 in 0.1% Evans blue solution (Zweygarth *et al.* 2005). The slides were evaluated under a Leitz

Orthoplan fluorescence microscope. Positive and negative control sera were included in each test. Titres higher than 1:40 were considered *Ehrlichia*-positive. A further serum sample was taken from the negative control animals (Groups 5 and 6) 64 days after the needle challenge in order to detect a rise in antibodies as a result of the challenge material.

3.8 Intramuscular and intravenous vaccine applications

Four groups of Angora goats were immunized with the attenuated heartwater vaccine using two routes of administration i.e. intravenous (i/v) (Groups 1 & 2) and intramuscular (i/m) (Groups 3 & 4). The intravenous route has been demonstrated in several experiments to be safe and efficacious (Zweygarth *et al.* 2005; Zweygarth *et al.* 2008) and thus was included as a standard control procedure. Groups 5 and 6 served as negative controls for animals challenged while animals in Group 7 received neither vaccine nor challenge and served as untreated negative controls (Table 2).

Table 3.2 Summary of different treatment groups

Groups		**Immunisation Route**	**Challenge Route**	**Total animals**
1	Positive controls	i/v	i/v	5
2	Positive controls	i/v	Ticks	5
3	Experiment	i/m	i/v	11
4	Experiment	i/m	Ticks	20
5	Negative control	Untreated control	i/v	5
6	Negative control	Untreated control	Ticks	5
7	Negative control	Untreated	Untreated	4
Total				**55**

The goats were immunised intravenously with a dose of 2ml per animal equivalent to $5.7x10^4$ culture-derived elementary bodies. The dose for the goats that received the intramuscular suspension (2ml per animal) was equivalent to $6x10^5$ culture derived elementary bodies. This injection was given into the right semitendinosus muscle.

All vaccinated animals and negative controls received a homologous *E. ruminantium* (Welgevonden) challenge six weeks after immunization. Groups 1, 3 and 5 received the challenge material via an intravenous injection of $5xLD_{50}$ of the virulent *E. ruminantium* Welgevonden stock as a blood stabilate. Groups 2, 4 and 6 were challenged by feeding of infected ticks.

3.9 Statistical analysis

A one-way analysis of variance (ANOVA) was performed on the vaccine reaction indices of the goats to test for significant differences between the seven (IV/IV, IV/Tick, IM/IV, IM/Tick, Neg/IV, Neg/Tick, Neg/Neg) experimental groups. Treatment Group means were separated using Fishers' protected t-LSD (least significant difference) at the 5 % level of significance (Snedecor & Cochran 1980). The data was analysed with SAS statistical software version 9.2 (SAS 1999).

CHAPTER 4

RESULTS

4.1 Local reactions at intramuscular site

Thirty-one Angora goats (Groups 3 and 4) were vaccinated via deep intramuscular injection in the right semitendinosus muscle. Reactions such as erythema, swelling, skin sloughing or pain were not noted to any degree over the injection site which was assessed daily for a period of 10 days following inoculation.

4.2 Reaction Index Results

4.2.1 Post immunisation reactions (Appendix 1)

A rise in rectal temperatures started occurring in vaccinated goats seven days post immunisation (PI) with the longest incubation period being 17 days (see Appendix 1). This incubation period was similar for both intravenous (i/v) and intramuscular (i/m) immunisation. The highest temperature in the two vaccinated positive control groups which received the vaccine via the i/v route (Groups 1 and 2) was 42˚C. On day 11 PI, six of the ten goats immunised via the intravenous route needed to be treated due to severe clinical signs of heartwater including high rectal temperatures, anorexia, separation from the group and increased respiratory rates. Reaction index scores of these goats on the day of treatment, but excluding the treatment scores, ranged from 2.1 to 8.9. Three goats were treated due to severe pyrexia (>2˚C above their pre-immunisation average), while the other three goats had pyrexia combined with anorexia and increased respiratory rate. Treatment consisted of a single intravenous injection of short-acting oxytetracycline (Terramycin® 100, Pfizer) at a dose rate of 10mg per kg body mass. On day 14, a 7th goat in the i/v group needed to be treated with oxytetracycline, as the reaction index score, prior to treatment, was 7.1 on that day, as a result of a high rectal temperature and clinical signs including anorexia and separation from the group. Thus a total of seven out of ten goats immunised via the intravenous route received one dose of short-acting oxytetracycline as a result of severe vaccine related reactions. All these goats recovered fully after treatment.

In the goats vaccinated via the intramuscular route (Groups 3 and 4), the highest temperature recorded was 41.5˚C. This occurred on day 11 in goat #2618 and Day 14 in goat #2612 post immunisation. Goat #2618 was treated on day 11 PI together with two other goats (#2626, #2631) that had temperature reactions of more than 2˚C above their pre-inoculation averages. Treatment consisted of a single intravenous dose of oxytetracycline at 10mg/kg.

Groups 5, 6 and 7 were the negative control animals and did not receive any vaccine and therefore did not display any temperature reactions during the post immunisation reaction period.

The reaction period post immunisation spanned a period of 16 days with the maximum duration of any single goat being a period of 7 days. Reaction indices were thus calculated and totalled during this 16 day period. The average total reaction scores are presented in Table 4.1. In general, Groups 1 and 2 that were vaccinated intravenously had almost twice the average reaction score of Groups 3 and 4, which received the vaccine via deep intramuscular injection. However the i/m groups still had a significantly higher average reaction index compared to the negative control goats (Groups 5, 6 and 7). The mean reaction index for the intravenous group was 20.1 (range 7.6-26.8), while for the intramuscular group it was 10.3 (range 0.5-27.2). The goats that did not receive any vaccine had an average reaction score of 2.1 (range 0.2-5.5).

Table 4.1 Average total reaction scores during the reaction periods post immunisation and post challenge.

Post-Immunisation			Post-Challenge		
Group	**Ave total reaction index**	**Number treated**	**Ave total reaction index**	**Number treated**	**Number protected**
1	17.5	2/5	3.8	0/5	5/5
2	22.7	5/5	1.8	0/5	5/5
3	10.9	1/11	1.9	0/11	11/11
4	9.9	2/20	3.5	0/20	20/20
5	2.3	0/5	55.6	5/5	0/5
6	1.7	0/5	52.1	4/4*	0/4
7	2.3	0/4	1.7	0/4	-

* One goat did not receive a challenge as ticks died *in situ* thus this goat was excluded, reducing the number of goats in this group to four.

4.2.2 Post challenge reactions (Appendix 2)

Group 1: The 5 Angora goats that were immunised via the intravenous route and received the virulent Welgevonden challenge material via needle challenge (i/v) were all protected with no temperature reactions or any other apparent heartwater symptoms. The average total reaction index over the 19 day reaction period was 3.8.

Group 2: The 5 Angora goats that were immunised via the intravenous route and received a tick challenge were also fully protected and did not display any temperature reactions or other signs of heartwater infection. The average total reaction index was 1.8.

Group 3: Goats in this group were immunised via the intramuscular (i/m) route and challenged with a needle challenge. All the animals in this group were fully protected and no clinical signs were observed. The average total reaction index was 1.9.

Group 4: These 20 Angora goats were immunised via i/m route and received challenge with 5 infected *A. hebraeum* adults. All animals in this group were fully protected and no clinical signs were observed. The average total reaction index was 3.5.

Group 5: In this group, the 5 Angora goats did not receive an immunisation but were given a needle challenge of virulent *E. ruminantium* (Welgevonden stock). All the goats developed severe temperature reactions and displayed clinical signs; increased respiratory rate, inappetance and incoordination. These goats had to be treated with a course of three days of oxytetracycline starting on day 13 post infection. Despite treatment, one goat in this group died from the heartwater infection. The average total reaction index was 55.6.

Group 6: The 5 Angora goats in this group were not immunised but were challenged with 5 infected adult *A. hebraeum* ticks. One goat did not receive the expected tick challenge as the ticks died *in situ* before attachment. This goat was therefore excluded from the group, bringing the total number of goats in this group to four. All four of the challenged goats developed severe temperature reactions and displayed clinical signs associated with acute heartwater infection. They were thus treated for 3 days with oxytetracycline. Despite this treatment, one goat did not recover and needed to be euthanized on humane grounds to prevent unnecessary suffering. The average total reaction index was 52.1.

Group 7: 4 negative control Angora goats were neither immunised nor challenged and remained healthy. The average total reaction index was 1.7.

4.2.3 Reaction Index Statistical Analyses

There was a significant difference ($p < 0.001$) between the mean reaction indices following both immunisation and challenge. Fishers' protected t-LSD (least significant difference) at the 5% level of significance of the mean of the reaction induces following immunisation was 7.211 (Table 4.2). Therefore a difference greater than 7.211 was significant. Following immunisation, Groups 1 and 2 were significantly different from Group 4, and Groups 3 and 4 were significantly different from Group 2. However, although the difference between some of these groups was greater than 7.211, the results also indicate that there was no significant difference between Groups 1 and 2, 1 and 3, and 3 and 4, suggesting that the difference between all of the first four groups is small (Table 402). Moreover, there was no significant variation between Groups 5, 6 and 7; however, groups 5, 6 and 7 did differ significantly from the first 4 groups. Thus, goats that were vaccinated either via the intramuscular or intravenous route reacted significantly more than those that were not vaccinated.

The $LSD_{(p = 0.05)}$ between the means of the reaction indices following challenge was 6.5099. Therefore, a difference greater than 6.5099 between group means was significant. Following challenge, the mean reaction indices for groups 5 and 6 were significantly higher than the mean reaction indices for all the other groups (1, 2, 3, 4 and 7) which were not significantly different from each other (see Table 4.3). Therefore, following challenge, those goats that were vaccinated (Groups 1,2,3 and 4) were not significantly different from the negative controls (Group 7) but they were significantly different from the goats that were not vaccinated (Groups 5 and 6) (Figure 4.2).

Table 4.2 Reaction indices post immunisation: statistical analysis

Group	Immunisation	Mean RI	Std Dev[1]
1	IV/IV	17.5 [ab]	11.9
2	IV/Tick	22.7 [a]	3.6
3	IM/IV	10.9 [bc]	7.5
4	IM/Tick	9.9 [c]	5.1
5	Neg/IV	2.3 [d]	1.9
6	Neg/Tick	1.7 [d]	1.2
7	Neg/Neg	2.3 [d]	2.1
[2]F-Prob		<0.001	
[3]$LSD_{p=0.05}$	7.211	7.211	

[1]Std Dev = standard deviation
[2] An F-Prob≤ 0.05 is considered as significant
[3]$LSD_{p=0.05}$ = Fisher's Least significant difference at a 5% significance level. Means within columns with the same letter or letters do not differ significantly at the 5% level

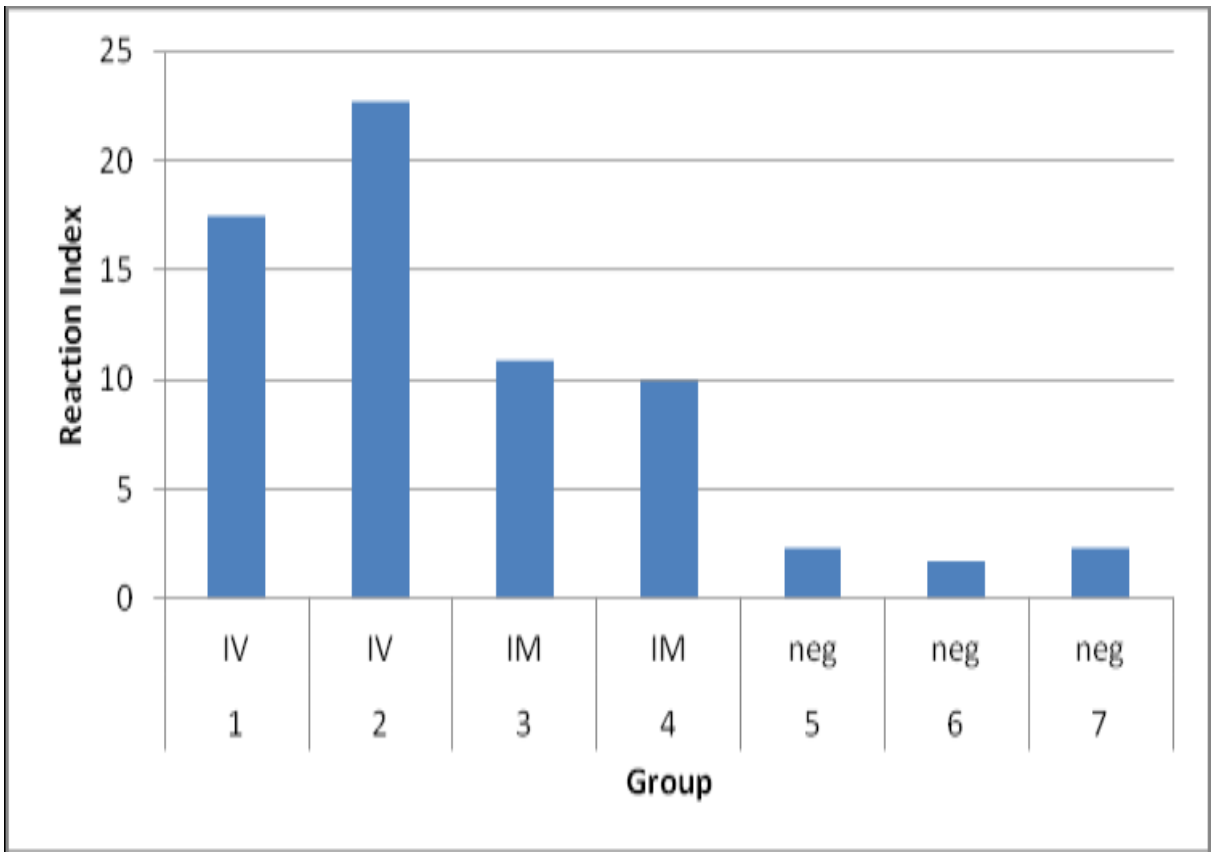

Figure 4.1 The mean reaction scores of the different groups post immunisation

Table 4.3 Mean reaction indices post challenge

Group	Challenge	Mean RI	Std Dev[1]
1	IV/IV	3.8 [b]	3.5
2	IV/Tick	1.8 [b]	1.7
3	IM/IV	1.8 [b]	1.4
4	IM/Tick	3.5 [b]	2.6
5	Neg/IV	55.6 [a]	9.6
6	Neg/Tick	52.1 [a]	16.4
7	Neg/Neg	1.7 [b]	0.7
[2]F-Prob		<0.001	
[3]$LSD_{p=0.05}$		6.5099	

[1]Std Dev = standard deviation
[2] An F-Prob≤ 0.05 is considered as significant
[3]$LSD_{p=0.05}$ = Fisher's Least significant difference at a 5% significance level. Means within columns with the same letter or letters do not differ significantly at the 5% level

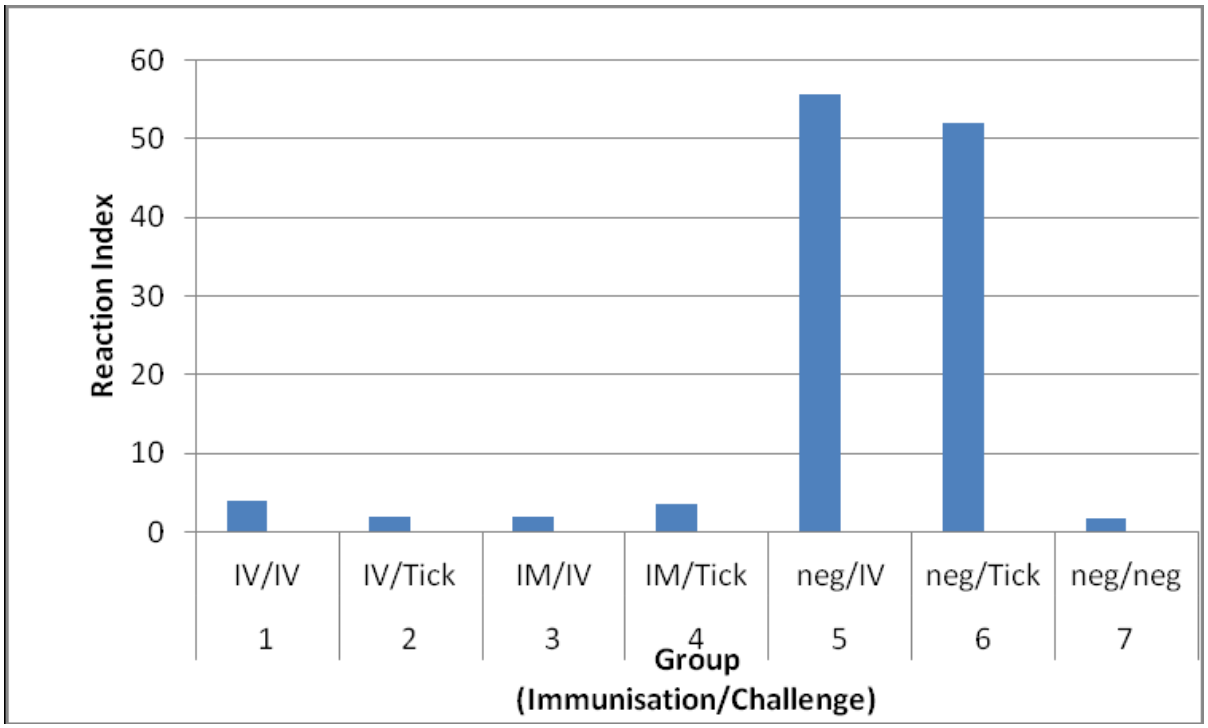

Figure 4.2 The mean reaction index scores of the different groups following challenge.

4.3 Differential blood cell counts (Appendices 3, 4)

Haematological evaluation was performed on blood samples taken from animals suffering a vaccine-related febrile reaction. The untreated control animals were also bled at the same time (see Appendix 3). These same animals were sampled once again one month after the end of the trial period (Appendix 4). Wide within group variation was found, thus hindering interpretation of these blood results. For example, during the vaccine-related febrile reaction of the goats that were immunised via the intravenous route, the total white cell count (WCC) ranged from 9.69 – 18.19 x 10^9/l. In this same group, the percentage mature neutrophils ranged from 14 – 56% with a standard deviation of ± 12.54 around the mean of 31.11%. Similarly, the lymphocyte percentage ranged from 38 – 78% with a standard deviation of ± 12.04 around the mean of 62.89%. The range of WCC for the intramuscular immunised group and control group was 9.46-18.33 x 10^9/l and 13.23-20.28 x 10^9/l, respectively.

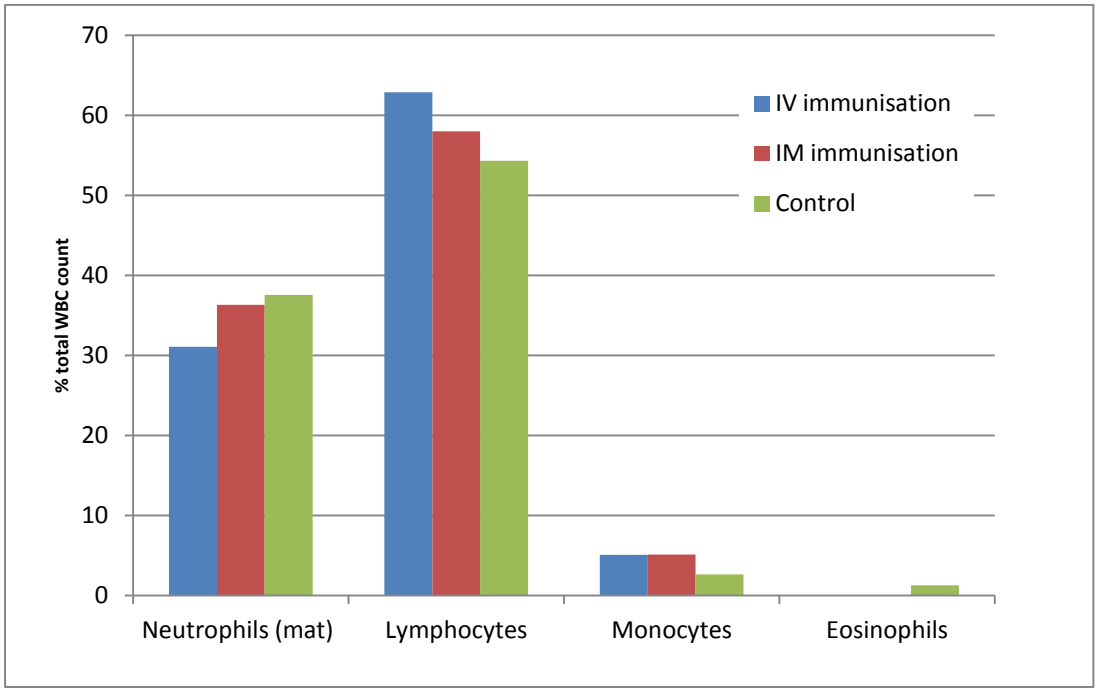

Figure 4.3 Mean proportional values of mature neutrophils, lymphocytes, monocytes and eosinophils during vaccine-related temperature reactions

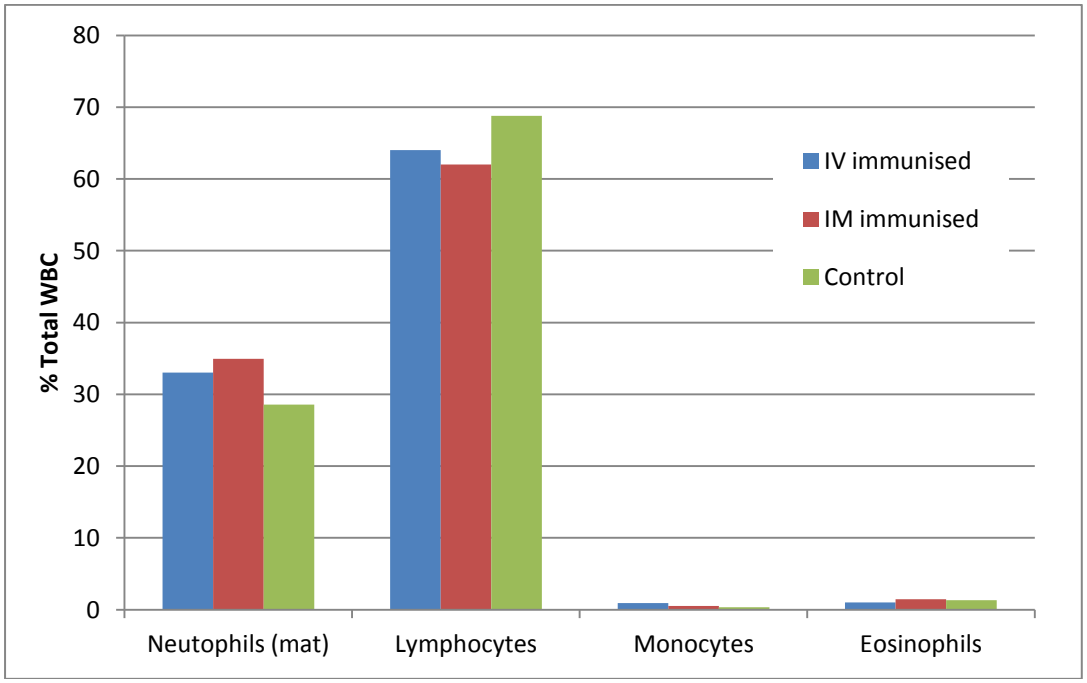

Figure 4.4 Mean proportional values of mature neutrophils, lymphocytes, monocytes and eosinophils one month after all experimental-related febrile reactions had resolved.

4.4 Packed Cell Volume (PCV) (Appendix 5)

A one-way analysis of variance (ANOVA) on the PCV results that were taken on the 20th July (following immunisation) indicated that there was a significant difference (p = 0.0192) between the group means. Treatment Group means were separated using Fishers' protected t-LSD (least significant difference) at the 5 % level of significance. The least significant difference was 4.1152 (see Table 4.4). However, as seen in the T groupings, the differences observed are most likely due to normal variation as the differences do not correlate with whether or not the goats were immunised. This is illustrated by Group 1 (i/v immunisation) having no significant difference from Group 5 (not immunised).

There was no significant difference between the group means of the PCV prior to vaccination (5th July) (p = 0.3368) and after vaccination i.e. on 3rd August (p = 0.8439) and 31st August (p = 0.2837). From the PCV values obtained on 17th August (post-challenge), the p-value from the ANOVA of the mean of the PCV values was 0.049, indicating that there was significant differences between the group means and that the null hypothesis should be rejected ($p<0.05$). However, as with the PCV results on 20th July, these differences are not clinically significant. On 14th September, the p-value from the ANOVA of the mean on the PCV values was 0.0002, indicating that there was significant differences between the group means and therefore the null hypothesis should be rejected ($p<0.05$). However, this difference is again not clinically significant when looking at the groupings (Table 4.5).

Table 4.4 Mean PCV comparisons (20th July 2011, 2 weeks post immunisation)

Group	Immunisation	Mean PCV	Std Dev[1]
1	IV/IV	22.0 [c]	4.8
2	IV/Tick	25.0 [bc]	6.0
3	IM/IV	24.8 [c]	3.1
4	IM/Tick	26.5 [ab]	3.1
5	Neg/IV	27.8 [bc]	2.9
6	Neg/Tick	30.0 [a]	2.8
7	Neg/Neg	28.8 [ab]	2.2
[2]F-prob		0.0192	
[3]$LSD_{p=0.05}$		4.115	

- [1]Std Dev = standard deviation
- [2] An F-Prob≤ 0.05 is considered as significant
- [3]$LSD_{p=0.05}$ = Fisher's Least significant difference at a 5% significance level. Means within columns with the same letter or letters do not differ significantly at the 5% level.

Table 4.5 Mean PCV comparisons (14th September 2011, >1month post-challenge)

Group	Challenge	Mean PCV	Std Dev[1]
1	IV/IV	23.0 [bc]	1.9
2	IV/Tick	27.8 [a]	2.2
3	IM/IV	25.1 [ab]	2.3
4	IM/Tick	27.0 [a]	2.8
5	Neg/IV	21.5 [c]	2.6
6	Neg/Tick	21.3 [cb]	4.0
7	Neg/Neg	27.0 [a]	2.4
[2]F- prob		<0.001	
[3]LSD	3.282	3.282	

- [1]Std Dev = standard deviation
- [2] An F-Prob≤ 0.05 is considered as significant
- [3]$LSD_{p=0.05}$ = Fisher's Least significant difference at a 5% significance level. Means within column with the same letter or letters do not differ significantly at the 5% level.

Two weeks following immunisation, all goats, including the negative controls, showed an average drop in haematocrit from their pre-injection values (see Appendix 5). Moreover, some goats that were vaccinated intravenously (Groups 1 and 2) had severely low values in comparison to those vaccinated intramuscularly (Groups 3 and 4) and the negative controls had only moderately low values. The mean PCV value of Group 1 dropped from 28.8% to 22%; that of Group 2 dropped from 28.8% to 25%; Group 3 dropped from 32.5% to 24.8%; Group 4 dropped from 30.3% to 26.5%; Group 5 dropped from 31.7% to 27.8%; Group 6 dropped from 31.3% to 30.2% and the mean of Group 7 dropped from 32.3% to 28.8%. Two weeks after immunisation, the lowest value in the intravenous group was 16%, intramuscular group was 20% and the negative controls 24%. However, this difference is not statistically significant.

Groups 1, 3, and 5 were challenged with a virulent needle challenge. The changes in PCV values from pre-challenge to two weeks post challenge are not clinically significant. In Group 1, in which the goats were given an i/v vaccination, 2/5 goats showed a mild drop in PCV values with the lowest value being 20%, and 3/5 goats had raised PCV's two weeks following challenge. The goats in Group 3 received i/m immunisation. 5/11 goats in this group showed a drop in PCV two weeks after challenge with the lowest value being 21%. Five out of 11 goats had raised PCV values while the PCV of one goat in this group did not change between pre-challenge and two weeks post-challenge. Group 5 was not immunised but did receive i/v challenge. All 5 goats in this group showed a drop in PCV two weeks following challenge with the lowest value being 20%.

Groups 2, 4 and 6 received challenge via infected ticks. The changes in PCV values between pre-challenge and 4 weeks after the ticks were placed on the goats are as follows. In Group 2 (i/v immunised) three out of five goats showed a drop in PCV while 2/5 showed increased PCV values. In Group 4 (i/m immunised) 6/11 had a drop in PCV and 5/11 had increased PCV values. In Group 6 (not immunised) 4/5 goats had a drop in PCV with the lowest value of 19% and the 5th goat died from the challenge.

4.5 Antibody analysis by the indirect fluorescent antibody test (IFAT) (Appendix 6)

Fourteen days after immunisation, 38 of the 41 vaccinated goats had positive heartwater antibody titres. Two goats missed sampling while the third one gave negative titres.

However, on Day 42 post immunisation, all immunised goats, including the one that was negative on Day 14 post-immunisation, had sero-converted at the highest dilution tested (1:320).

Eight out of the 10 goats that were not immunised, but were either needle or tick challenged (groups 5 and 6) were still alive 64 days post challenge. One of these eight goats (#2677) did not receive a challenge as the ticks died *in situ* and therefore this goat was excluded from the group. The remaining seven goats all had positive antibody titres on the IFAT 64 days post challenge.

CHAPTER 5

DISCUSSION and CONCLUSIONS

The Onderstepoort live blood vaccine (Ball 3 isolate) is the only commercially available vaccine against heartwater. Animals are inoculated intravenously with cryopreserved blood from sheep infected with the virulent Ball 3 stock of *E. ruminantium* (Bezuidenhout 1989), their body temperature is monitored and tetracycline antibiotic treatment has to be administered at the appropriate time. The vaccine must be administered intravenously which requires specialised skills and the procedure is labour intensive, as the animals have to be closely monitored for a temperature reaction. A further cost is incurred on the farmers as tetracyclines are needed to block the infection. Moreover the Ball 3 stock does not protect against all isolates present in the field (Collins *et al.* 2003). The Ball 3 stock is mainly used because it produces a temperature rise several days before serious clinical disease occurs, making it relatively easy to decide when to treat. All of these factors make the infection-and-treatment procedure far from what would be regarded as an ideal vaccine. The virulent Welgevonden isolate, which provides wider cross-protection against other stocks, can cause death very shortly after a rapid temperature rise, and therefore cannot be used safely, in its present state, for the infection-and-treatment method of vaccination (Du Plessis *et. al.*1983).

The current infection-and-treatment method of immunisation is particularly difficult and hazardous to apply to Angora goats. Du Plessis *et al.* (1983) found that few Angora goats survived when they were treated on the 2nd or 3rd day of the febrile reaction following inoculation of the live blood vaccine. When treatment was given on the first day of the reaction, the survival rate was high but the immunity of the goats to subsequent challenge was poor. For this reason, Angora goats in South Africa are not generally vaccinated with the current Onderstepoort vaccine (Zweygarth 2006).

5.1 Intramuscular immunisation and vaccine-related reactions

The results clearly demonstrated that the intramuscular route of administration of the attenuated heartwater (Welgevonden strain) vaccine in Angora goats is safe and efficacious. The total lack of inflammatory reactions at the site of injection confirms that this vaccine is non-irritant. Moreover, goats vaccinated via the intramuscular route had less severe post-

immunisation reactions than those vaccinated intravenously, yet they resisted a virulent challenge just as well as the animals that were vaccinated intravenously. Therefore the intramuscular route of administration would prove to be safer than the intravenous route for administration of this attenuated vaccine. This is an important finding as specialised skills are therefore not needed for the application of this vaccine.

Ideally, no severe reactions requiring treatment should occur following immunisation as the aim is to establish a vaccine that stimulates an immune response without causing a severe clinical response. Unfortunately, 70% of the goats immunised intravenously and 9.7% of the goats immunised intramuscularly needed treatment to control the vaccine-related reactions. This firstly illustrates that the intravenous route is a more virulent method of immunisation and that the intramuscular route of administration is therefore safer. Moreover, the vaccine doses used in this experiment (10^5) were not optimal and it is thus suggested that a titration study be undertaken using the intramuscular route of administration in Angora goats in order to determine the lowest effective dose resulting in 100% safety rather than the 90.3% obtained in this study.

5.2 Attenuated vaccine efficacy in Angora goats

All the Angora goats that were immunised, both intravenously and intramuscularly, with the attenuated *E. ruminantium* (Welgevonden) vaccine were fully protected against a virulent homologous needle challenge as well as by feeding of Welgevonden-infected ticks. This confirms the results of a previous preliminary experiment (Zweygarth *et al*. 2008) but we added that the attenuated *E. ruminantium* (Welgevonden) vaccine stimulates a productive immune response that can afford protection to natural tick challenge. This is a significant finding as the Welgevonden strain has broader cross-protection than the Ball 3 strain (du Plessis *et al.* 1989; Collins *et al.* 2003; Zweygarth *et al*. 2005), thus it is likely that this attenuated vaccine will also protect Angora goats against a number of other field isolates with expected wider use in Southern Africa.

It must be remembered that this study was carried out under controlled laboratory conditions and that the goats were closely observed and kept free from any other diseases. In a field situation, goats may be suffering from high burdens of internal parasites as well as other subclinical conditions which could result in more severe post-immunisation reactions. It is

therefore necessary to conduct a thorough field trial with the suggested vaccine dose in order to determine if the vaccine is safe enough to use without the need for post-immunisation treatment.

5.3 Haematology

Van Amstel *et al*. (1987) reviewed the clinical pathological changes that occurred during a heartwater infection and concluded that a progressive anaemia develops together with a neutropaenia, an eosinopaenia and a lymphocytosis. These changes have been found to coincide with the onset of the febrile reaction. Therefore, differential white blood cell counts were done in selected goats during the vaccine-related febrile reaction in order to assess whether or not the vaccine caused any unwanted haematological changes. However, the results of the differential blood cell counts and the haematocrit readings were inconclusive in this experiment, partly as a result of wide within-group ranges. Perhaps a clearer haematocrit pattern would have been observed if PCV readings were taken at more regular and shorter intervals. It is also important to note that there are a number of factors that could influence PCV results including infection with the helminth, *Haemonchus contortus*. When the goats arrived at Onderstepoort Veterinary Institute, some of the animals had severe internal parasite loads with faecal worm egg counts (FWEC) as high as 47900 eggs per gram (epg), combined with severe coccidial infestations. The goats were treated every two to three weeks until faecal worm egg counts of 200 epg or less were achieved, with the majority of the goats having FWEC of zero before commencement of the experiment. In the field situation, Angora goats may have much higher parasite loads at the time of vaccination which could make them more susceptible to vaccine related adverse reactions.

However, comparing the PCV results in the different groups, it can be concluded that neither the vaccine material nor the challenge material has a significant influence on the haematocrit values. Therefore, as no significant changes were found between vaccinated and control animals, it is likely that the attenuated vaccine does not cause unwanted haematological changes. Indeed, Uilenberg (1983) states that in general, the haematological findings in heartwater do not appear to be spectacular, and our results would tend to agree with this statement.

5.4 Antibody analysis by IFAT

The 55 goats were tested by the IFAT on arrival at the Onderstepoort Veterinary Institute. Three of these goats were found serologically positive (with titres of 1:80), which presumably indicated some form of cross-reaction with other unidentified *Ehrlicha* organisms as the goats were purchased from a known *Amblyomma*- and heartwater-free area. These goats presented clinical heartwater reactions during the experiments which confirmed their susceptibility to the disease.

On Day 14 post immunisation, six out of the 41 immunised goats had not shown a vaccine-related temperature reaction. However, all goats apart from one (number 2606) had positive antibody titres. This demonstrates that the IFAT may be able to detect vaccine-induced antibodies before a temperature reaction occurs. Goat #2606 had a delayed temperature reaction as well as a delayed rise in antibody titre. By Day 42 post immunisation, all vaccinated goats had positive antibody titres at the highest dilution tested (1:320).

The eight negative control goats that were serologically tested 64 days post-challenge all had positive titres. The positive results of these goats illustrates that the challenge material via both infected ticks and intravenous injection stimulated an immune response.

CONCLUSIONS

This experimental attenuated vaccine has overcome some of the problems associated with the live blood vaccine such as lack of cross-protection and severe vaccine-related reactions. It is also cheaper to produce as live animals are not needed. However, it still possesses problems associated with live vaccines such as the need for a continuous cold chain. Zweygarth *et al.* (2005) showed in one case using one sheep that *A. hebraeum* nymphs were able to pick up the attenuated heartwater vaccine organisms from an immunized sheep. The subsequent adult stages of the tick successfully transmitted heartwater to a recipient sheep with no indication of reversal to virulence. Interestingly, this recipient sheep resisted a virulent challenge and confirmed to be immune. Unfortunately, inactivated and DNA-derived vaccines against heartwater have thus far been unsuccessful and therefore the attenuated vaccine, which shows promising results, would be the next best option for widespread use until more improved vaccines are produced. The fact that the “infection-and-treatment” method of immunisation

has been used for over 60 years illustrates that developing an ideal vaccine against heartwater disease is an extremely challenging task.

We have successfully demonstrated that the Angora goats can be safely vaccinated with the experimental attenuated heartwater (Welgevonden) vaccine via the intramuscular route of administration. Not only is the administration of this vaccine far safer than the present "infection and treatment" method of immunisation, but it also conferred 100% protection against a subsequent homologous virulent needle and tick challenge.

RECOMMENDATIONS

The results of this trial aid in bringing this attenuated vaccine a step closer to becoming a suitable commercial product. However, while the results are promising, it is necessary that a number of other studies are undertaken before this product can become suitable for the commercial market. Firstly, duration of immunity trial under controlled tick-free conditions needs to be conducted and the vaccine needs to be tested in young kids as well as pregnant does. Then, as already mentioned, the vaccine needs to be tested on animals in the field as the field situation may present additional problems that are not encountered in laboratory circumstances. Finally, it is also imperative to confirm that the vaccine is transmissible and will not revert to virulence after it is administered to the host animal, though promising results have been obtained from a single case (Zweygarth et al. 2005).

CHAPTER 6

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APPENDICES

Appendix 1

Febrile reactions recorded in the goats following immunisation with the attenuated heartwater (Welgevonden strain) vaccine

Group	Goat number	Immunisation route	Incubation period (days)	T_{max} (˚C)	Duration of fever (days) *[]	Treatment required
1	2584	IV	10	40.8	4	No
1	2585	IV	10	41.6	3[1]1[2]1	No
1	2587	IV	8	42	5[1]2[1]	Yes
1	2589	IV	8	41.8	4[2]1	Yes
1	2592	IV	13	41.2	4	No
2	2593	IV	10	40.7	2	Yes
2	2596	IV	10	41.5	5	Yes
2	2598	IV	8	41.8	5	Yes
2	2599	IV	8	41.4	4	Yes
2	2603	IV	9	41.1	4	Yes
3	2605	IM	8	41.5	1[2]6	No
3	2606	IM	No febrile reaction observed			
3	2609	IM	11	41.3	6	No
3	2610	IM	13	40.7	4	No
3	2611	IM	No febrile reaction observed			
3	2612	IM	11	41.5	7	No
3	2613	IM	11	40.3	1[2]1	No
3	2618	IM	8	41.5	5[1]1	Yes
3	2619	IM	10	41.3	6	No
3	2620	IM	14	40.6	1	No
3	2622	IM	10	41.3	6	No
4	2624	IM	11	40	1	No
4	2626	IM	8	41	4[2]1	Yes
4	2628	IM	14	40.1	1	No
4	2629	IM	11	41	1	No
4	2631	IM	10	41.3	2[2]1	Yes
4	2633	IM	14	40.1	1	No
4	2636	IM	17	41.1	1[1]1	No
4	2637	IM	11	41.1	1[1]5	No
4	2639	IM	11	41.6	5	No
4	2640	IM	No febrile reaction observed			
4	2641	IM	13	40.2	3	No
4	2644	IM	10	41.2	6	No
4	2645	IM	10	41.1	7	No
4	2649	IM	7	40.3	2[2]2[1]1	No
4	2650	IM	11	40.2	1[4]1	No
4	2652	IM	17	40.2	1	No
4	2655	IM	11	40.5	4	No
4	2656	IM	No febrile reaction observed			

4	2661	IM	8	40.8	1[4]4	No
4	2663	IM	14	40.9	5	No
5	2665	None				
5	2700	None				
5	2669	None				
5	2671	None				
5	2672	None				
6	2673	None				
6	2676	None				
6	2677	None				
6	2680	None				
6	2683	None				
7	2685	None				
7	2692	None				
7	2693	None				
7	2666	None				

*[number of days when rectal temperature was within normal limits]

Appendix 2

Febrile reactions recorded in the goats following challenge with *Ehrlichia ruminantium*, Welgevonden strain.

Group	Goat number	Immunisation route	Challenge route	Incubation period (days)	T_{max} (˚C) *[]	Duration of fever (days)	Treatment (#)	Animals protected
1	2584	IV	IV	No reaction	-	-	No	Yes
1	2585	IV	IV	No reaction	-	-	No	Yes
1	2587	IV	IV	No reaction	-	-	No	Yes
1	2589	IV	IV	No reaction	-	-	No	Yes
1	2592	IV	IV	No reaction	-	-	No	Yes
2	2593	IV	Ticks	No reaction	-	-	No	Yes
2	2596	IV	Ticks	No reaction	-	-	No	Yes
2	2598	IV	Ticks	No reaction	-	-	No	Yes
2	2599	IV	Ticks	No reaction	-	-	No	Yes
2	2603	IV	Ticks	No reaction	-	-	No	Yes
3	2605	IM	IV	No reaction	-	-	No	Yes
3	2606	IM	IV	No reaction	-	-	No	Yes
3	2609	IM	IV	No reaction	-	-	No	Yes
3	2610	IM	IV	No reaction	-	-	No	Yes
3	2611	IM	IV	No reaction	-	-	No	Yes
3	2612	IM	IV	No reaction	-	-	No	Yes
3	2613	IM	IV	No reaction	-	-	No	Yes
3	2618	IM	IV	No reaction	-	-	No	Yes
3	2619	IM	IV	No reaction	-	-	No	Yes
3	2620	IM	IV	No reaction	-	-	No	Yes
3	2622	IM	IV	No reaction	-	-	No	Yes

4	2624	IM	Ticks	No reaction	-	-	No	Yes
4	2626	IM	Ticks	No reaction	-	-	No	Yes
4	2628	IM	Ticks	No reaction	-	-	No	Yes
4	2629	IM	Ticks	No reaction	-	-	No	Yes
4	2631	IM	Ticks	No reaction	-	-	No	Yes
4	2633	IM	Ticks	No reaction	-	-	No	Yes
4	2636	IM	Ticks	No reaction	-	-	No	Yes
4	2637	IM	Ticks	No reaction	-	-	No	Yes
4	2639	IM	Ticks	No reaction	-	-	No	Yes
4	2640	IM	Ticks	No reaction	-	-	No	Yes
4	2641	IM	Ticks	No reaction	-	-	No	Yes
4	2644	IM	Ticks	No reaction	-	-	No	Yes
4	2645	IM	Ticks	No reaction	-	-	No	Yes
4	2649	IM	Ticks	No reaction	-	-	No	Yes
4	2650	IM	Ticks	No reaction	-	-	No	Yes
4	2652	IM	Ticks	No reaction	-	-	No	Yes
4	2655	IM	Ticks	No reaction	-	-	No	Yes
4	2656	IM	Ticks	No reaction	-	-	No	Yes
4	2661	IM	Ticks	No reaction	-	-	No	Yes
4	2663	IM	Ticks	No reaction	-	-	No	Yes
5	2665	None	IV	13	41.5	4[2]1	Yes (3)	No
5	2700	None	IV	12	42	6	Yes (3)	No
5	2669	None	IV	14	41.1	2 then died	Yes (3)	No
5	2671	None	IV	12	42	8[1]1	Yes (3)	No
5	2672	None	IV	13	42	3	Yes (3)	No
6	2673	None	Ticks	18	42	6	Yes (3)	No
6	2676	None	Ticks	18	41.9	6 then euthanized	Yes (3)	No
6	2677	None	Ticks	*				

6	2680	None	Ticks	21	42	8	Yes (3)	No
6	2683	None	Ticks	26	42	4	Yes	No
7	2685	None	None					
7	2692	None	None					
7	2693	None	None					
7	2666	None	None					

*[] number of days when rectal temperature was within normal limits
(#) Number of oxytetracycline injections given at 24hr intervals
Goat 2677: Not included in statistical analyses as ticks died *in situ* before attachment

Appendix 3

Haematological results from goats undergoing vaccine-related temperature reactions

Group	Goat ID	Date	Rectal Temp	Hb	RCC	Ht	WCC	Neuts(mat)	Neut (immat)	Lymph	Mono	Eosinophil	Basophil
			˚C	*g/l*	*x 1012/l*	*l/l*	*x 109/l*	*%*	*%*	*%*	*%*	*%*	*%*
IV immunisation	2584	18-Jul	40.8	64	12.69	0.2	11.7	26	0	66	8	0	0
	2585	18-Jul	41.1	81	13.9	0.24	13.82	34	0	62	4	0	0
	2587	18-Jul	39.9	83	18.55	0.26	11.13	22	0	72	6	0	0
	2589	18-Jul	39.7	82	14.86	0.24	17.73	14	4	78	4	0	0
	2592	20-Jul	40.8	83	15.4	0.27	18.19	56	0	38	6	0	0
	2593	18-Jul	39.4	87	16.08	0.28	9.69	34	0	62	4	0	0
	2596	18-Jul	41.2	69	12.77	0.21	11.19	48	0	46	6	0	0
	2598	18-Jul	40.2	94	18.18	0.29	11.69	28	0	68	4	0	0
	2603	18-Jul	40.1	90	18.95	0.29	12.64	18	4	74	4	0	0
	Mean		40.4	81.44	15.71	0.25	13.09	31.11	0.89	62.89	5.11	0	0
	Standard deviation		± 0.64	± 9.53	±2.76	± 0.04	± 1.23	± 12.54	± 1.76	± 12.04	± 1.00	0	0
	Range		39.4-41.2	64-94	12.69-18.95	0.2-0.29	9.69 - 18.19	14-56	0-4	38-78	4-8	0	0
IM immunisation	2605	18-Jul	41.1	96	20.94	0.3	9.46	36	0	50	14	0	0
	2606	22-Jul	40	88	19.42	0.27	9.95	22	1	66	11	0	0
	2609	18-Jul	40	83	17.39	0.26	9.79	40	0	51.5	5.4	1	1.3
	2610	22-Jul	40.6	82	14.43	0.23	16.48	30	2	60	8	0	0
	2612	18-Jul	40	103	19.92	0.34	10.16	54	0	42	4	0	0
	2618	18-Jul	39.9	66	12.97	0.21	16.35	24	0	68	8	0	0
	2619	18-Jul	40.1	112	22.33	0.36	15.74	44	0	54	2	0	0

	2622	18-Jul	41.3	107	19.57	0.34	13.47	36	0	60	4	0	0
	2624	18-Jul	39.6	85	16.87	0.27	15.49	28	0	70	2	0	0
	2637	20-Jul	40.6	100	19.01	0.32	13.35	40	0	58	2	0	0
	2639	18-Jul	41	96	18.73	0.31	16.69	56	0	42	2	0	0
	2641	20-Jul	40.2	82	15.62	0.26	11.79	28	0	60	12	0	0
	2644	18-Jul	40.6	90	18.76	0.28	13.59	44	0	54	2	0	0
	2645	18-Jul	40.5	95	17.45	0.3	18.33	26	0	72	2	0	0
	2649	18-Jul	39.6	110	19.5	0.35	13.14	32	1	61	5	0	0
	2655	18-Jul	40.3	90	15.68	0.27	11.68	42	0	56	2	0	0
	2661	20-Jul	40.2	87	16.5	0.27	16.42	36	0	62	2	0	0
	Mean		40.3	92.47	17.95	0.29	13.64	36.35	0.24	58.03	5.14	0.06	0.08
	Standard deviation		±0.49	±11.8	±1.48	±0.03	±2.33	±9.70	±0.33	±8.85	±3.36	±0.24	±0.32
	Range		39.6-41.3	66-112	12.97-22.33	0.21 - 0.36	9.46 - 18.33	22-56	0-2	42-72	2-14	0-1	0-1.3
Control	control F	18-Jul		96	18.17	0.3	17.71	21	0	70	2	6	1
	control F	18-Jul		106	19.81	0.33	16.88	58	0	40	2	0	0
	control F	18-Jul		104	19.61	0.33	19.88	34	0	60	4	2	0
	control F	18-Jul		95	20.39	0.31	14.34	34	0	34	2	0	0
	control F	18-Jul		93	18.79	0.29	18.01	47	0	48	3	1	1
	Control M	18-Jul		102	20.99	0.33	14.69	29.3	0	64.9	3.8	1	0.9
	Control M	18-Jul		106	19.16	0.32	20.28	26.5	0	57.6	3.9	0.9	1.1
	Control M	18-Jul		103	16.9	0.32	13.23	26	0	75	2	0	0
	Control M	18-Jul		90	17.73	0.28	17.81	48	0	52	0	0	0
	Control M	18-Jul		104	20.71	0.34	16.35	52	0	42	4	2	0
	Mean			99.9	19.23	0.32	16.92	37.58	0	54.35	2.67	1.29	0.4
	Stand			±5.	±1.3	±0.0	±2.3	±12.68	0.00	±13.	±1.3	±1.83	±0.5

	ard deviation			84	4	2	1			51	1		2
	Range			90-106	16.9 - 20.99	0.28 - 0.34	13.23- 20.28	21-58	0	34-75	0-4	0-6	0-1.1

Appendix 4

Blood counts results from goats one month after the end of the trial period (18th October 2011)

	Goat No.	Hb (g/l)	RCC	Ht	WCC	Neuts(mat)	Neuts (immat)	Lymph	Mono	Eosinophil	Basophil
		g/l	*x 10e12/l*	*l/l*	*x 10e9/l*	*%*	*%*	*%*	*%*	*%*	*%*
IV immunisation	2584	80	14.84	0.22	16.53	33.2	0	61.4	3.6	1	0.7
	2585	90	15.53	0.25	10.74	26.5	0	70	1.4	0.39	1
	2587	91	19.24	0.26	14.01	39.4	0	58.3	0.4	1.2	0.7
	2589	97	16.85	0.27	17.95	20.2	0	77.3	0.6	0.8	1.1
	2592	92	16.69	0.26	17.02	43.3	0	53.6	1	1	0.9
	2593	105	18.75	0.3	15.25	22.9	0	74.9	0.2	1.1	0.8
	2598	99	18.41	0.28	12.04	41.9	0	54.7	0.5	1.9	0.8
	2599	101	17.96	0.28	19.83	32.6	0	65.2	0.3	0.8	1.1
	2603	96	18.55	0.27	13.38	37.1	0	60.8	0.3	1	0.6
	Mean	*94.56*	*17.42*	*0.27*	*15.19*	*33.01*	*0*	*64.02*	*0.92*	*1.02*	*0.86*
	Standard deviation	*±7.33*	*±1.53*	*±0.02*	*±2.93*	*±8.3*	*0*	*±8.49*	*±1.08*	*±0.4*	*±0.18*
	Range	*80-105*	*14.84-19.24*	*0.22-0.3*	*10.74-19.83*	*20.2-43.3*	*0*	*53.6-77.3*	*0.2-3.6*	*0.39-1.9*	*0.6-1.1*
IM immunisation	2605	89	18.23	0.25	10.52	39.7	0	57.5	0.6	1.5	0.7
	2606	73	14.03	0.21	8.03	28.4	0	68.5	0.8	1.4	0.8
	2609	76	14.9	0.21	13.63	23.7	0	74.1	0.3	0.9	0.9
	2610	98	17.06	0.28	15.71	30.7	0	67.1	0.2	1.2	0.8
	2611	90	17.04	0.25	11.69	41.9	0	55.3	0.5	1.5	0.7
	2612	86	16.2	0.24	15.03	48.9	0	49.3	0.5	0.4	0.7
	2613	101	18.55	0.29	14.23	30.1	0	66.9	0.4	1.6	0.9
	2618	90	16.22	0.26	15.3	32.1	0	64.6	0.5	1.6	1
	2619	102	17.15	0.29	17.15	28.4	0	69.1	0.4	1	1
	2620	86	15.74	0.25	15.8	44	0	53.5	0.3	1.2	0.9
	2622	115	19.24	0.33	13.23	34.6	0	61.7	0.2	2.2	1.2
	2624	100	17.63	0.29	17.43	21.1	0	75.4	1.4	0.8	1.2
	2626	98	17.4	0.28	19.53	33.1	0	63.4	0.4	1.9	1.2
	2628	85	16.45	0.24	16.66	56.2	0	39.4	0.6	2.6	1
	2629	87	16.05	0.25	16.37	28.3	0	69.4	1.2	0.4	0.6
	2631	102	19.29	0.29	18.14	43.9	0	53.5	0.4	1.1	1.1
	2633	88	17.67	0.25	16.88	58.6	0	38.1	0.3	2.4	0.6
	2636	99	15.28	0.28	15.71	33.6	0	63.9	0.6	0.8	1
	2637	101	17.48	0.28	15.94	32.2	0	65.3	0.1	1.1	1.2
	2639	102	17.46	0.28	15.52	29.2	0	67	0.4	2.2	1.1
	2640	79	15.49	0.22	18.83	44.8	0	52.3	0.7	1.2	0.7
	2641	92	16.61	0.26	13.56	27.6	0	69.6	0.2	1.6	0.8
	2645	110	18.39	0.31	18.5	24.9	0	72.2	0.5	1	1.3
	2649	110	17.85	0.31	12.98	33.7	0	62.4	0.6	2.3	0.9
	2650	103	18.58	0.29	17.46	30.7	0	64.7	1.6	1.6	1.2
	2652	101	18.91	0.28	12.49	32.9	0	63.5	0.2	2.5	0.8
	2655	90	14.78	0.25	12.7	32.3	0	65.1	0.6	0.9	1
	2656	90	17.22	0.25	15.44	23.2	0	73	0.3	2.5	1

	2661	101	17.94	0.28	16.73	28	0	69.5	0.3	1.3	0.9
	2663	92	17.07	0.27	24.99	52.1	0	45.7	0.6	0.7	0.8
	Mean	*94.53*	*17.06*	*0.27*	*15.54*	*34.96*	*0*	*62.03*	*0.52*	*1.45*	*0.93*
	Standard deviation	*±9.98*	*±1.35*	*±0.03*	*±3.12*	*±9.73*	*0*	*±9.73*	*±0.35*	*±0.63*	*±0.2*
	Range	*73-115*	*14.03-19.29*	*0.21-0.33*	*8.03-24.99*	*21.1-58.6*	*0*	*38.1-75.4*	*0.1-1.6*	*0.4-2.6*	*0.6-1.3*
Control	2665	85	16.55	0.24	14.61	21.1	0	76.9	0.5	0.6	0.8
	2666	95	16.85	0.26	11.89	45.3	0	50.7	0.4	2.5	1
	2671	84	14.29	0.24	12.6	33.8	0	61.5	0.3	3.2	1
	2672	87	14.46	0.25	19.77	21.8	0	75.9	0.4	0.9	1
	2673	85	12.59	0.24	14.88	15	0	83.1	0.2	0.5	1.1
	2677	97	17.26	0.27	14.72	21.7	0	76	0.2	1.3	0.8
	2680	82	12.9	0.23	13.33	25.5	0	72.6	0.4	0.6	0.9
	2683	95	16.47	0.27	12.81	25.3	0	72.5	0.2	1.1	0.8
	2685	92	15.79	0.26	14.11	29.34	0	68.5	0.4	1	0.6
	2692	110	19.56	0.31	14.67	27.3	0	69	0.4	2.1	1.2
	2693	104	18.63	0.29	9.84	40.2	0	57	0.5	1.3	0.9
	2700	83	14.44	0.23	12.87	36.5	0	61.8	0.4	0.6	0.6
	Mean	*91.58*	*15.82*	*0.26*	*13.84*	*28.57*	*0*	*68.79*	*0.36*	*1.31*	*0.89*
	Standard deviation	*±8.93*	*±2.15*	*±0.02*	*±2.37*	*±8.85*	*0*	*±9.39*	*±0.11*	*±0.86*	*±0.18*
	Range	*82-110*	*12.59-19.56*	*0.23-0.31*	*9.84-19.77*	*15-45.3*	*0*	*50.7-83.1*	*0.2-0.5*	*0.5-3.2*	*0.6-1.2*

Appendix 5

Packed Cell Volume (%) of all goats during the experimental period
Immunisation = 6th July
Challenge = 17th August

			PCV (%)						
Group No.	**Group**	**Goat No.**	**05-Jul**	**20-Jul**	**03-Aug**	**17-Aug**	**31-Aug**	**14-Sep**	**18-Oct**
1	IV/IV	**2584**	26	18	19	23.5	29	20	22
1	IV/IV	**2585**	25	19	31	22.5	26	23	26
1	IV/IV	**2587**	31	23	25	28	20	24	26
1	IV/IV	**2589**	28	20	22	22	28	23	26
1	IV/IV	**2592**	34	30	26	30	27	25	24
		Mean	*28.8*	*22*	*24.6*	*25.2*	*26*	*23*	*24.8*
		STD	*±3.70*	*±4.85*	*±4.51*	*±3.58*	*±3.54*	*±1.87*	*±1.79*
2	IV/Tick	**2593**	28	26	25	27	21	30	31
2	IV/Tick	**2596**	25	16	21	24	21	25	*
2	IV/Tick	**2598**	34	25	25	27	25	26	29
2	IV/Tick	**2599**	31	25	31	31	20	29	28
2	IV/Tick	**2603**	26	33	30	27	28	29	26
		Mean	*28.8*	*25*	*26.4*	*27.2*	*23*	*27.8*	*28.5*
		STD	*±3.70*	*±6.04*	*±4.10*	*±2.49*	*±3.39*	*±2.17*	*±2.08*
3	IM/IV	**2605**	35	28	27	31	33	27	26
3	IM/IV	**2606**	28	24	22	24	22	21	20
3	IM/IV	**2609**	38	20	22	21	29	22	22
3	IM/IV	**2610**	28	23	23	27	21	25	26
3	IM/IV	**2611**	34	27	31	31	28	25	25
3	IM/IV	**2612**	32	25	26	29	29	25	22
3	IM/IV	**2613**	*	24	25	28	26	25	28
3	IM/IV	**2618**	*	20	25	23	25	25	25
3	IM/IV	**2619**	*	30	32	30	31	25	28
3	IM/IV	**2620**	*	25	34	22	25	26	24
3	IM/IV	**2622**	*	27	32	33	23	30	30
		Mean	*32.50*	*24.82*	*27.18*	*27.18*	*26.55*	*25.09*	*25.09*
		STD	*±3.99*	*±3.12*	*±4.35*	*±4.09*	*±3.80*	*±2.34*	*±2.98*
4	IM/Tick	**2624**	*	23	27	28	27	27	29
4	IM/Tick	**2626**	*	27	30	27	25	31	25
4	IM/Tick	**2628**	*	25	27	22	23	25	24
4	IM/Tick	**2629**	*	25	29	31	25	25	23
4	IM/Tick	**2631**	*	29	34	27	23	27	27
4	IM/Tick	**2633**	32	26	27	27	21	28	23
4	IM/Tick	**2636**	31	28	26	28	23	26	26
4	IM/Tick	**2637**	26	29	28	30	28	33	28
4	IM/Tick	**2639**	*	25	26	27	29	24	25
4	IM/Tick	**2640**	31	22	26	23	27	24	21
4	IM/Tick	**2641**	31	25	27	26	25	26	24
4	IM/Tick	**2644**	*	22	25	24	29	26	*

4	IM/Tick	**2645**	34	27	27	26	23	30	30
4	IM/Tick	**2649**	33	31	27	28	23	30	29
4	IM/Tick	**2650**	29	27	27	31	28	28	26
4	IM/Tick	**2652**	31	34	28	25	30	29	28
4	IM/Tick	**2655**	29	24	23	24	22	21	23
4	IM/Tick	**2656**	28	28	24	25	23	27	24
4	IM/Tick	**2661**	31	23	22	25	22	25	27
4	IM/Tick	**2663**	28	29	25	29	25	27	24
		Mean	*30.31*	*26.45*	*26.75*	*26.65*	*25.05*	*26.95*	*25.58*
		STD	*±2.21*	*±3.09*	*±2.55*	*±2.50*	*±2.72*	*±2.76*	*±2.48*
5	Neg/IV	**2665**	*	28	27	27	20	20	24
5	Neg/IV	**2700**	34	32	29	31	22	22	23
5	Neg/IV	**2669**	*	24	30	29	27	*	*
5	Neg/IV	**2671**	31	27	25	28	27	19	21
5	Neg/IV	**2672**	30	28	28	29	25	25	23
		Mean	*31.67*	*27.80*	*27.80*	*28.80*	*24.20*	*21.50*	*22.75*
		STD	*±2.08*	*±2.86*	*±1.92*	*±1.48*	*±3.11*	*±2.65*	*±1.26*
6	Neg/Tick	**2673**	*	26	27	24	23	19	22
6	Neg/Tick	**2676**	*	32	32	26	30	*	*
6	Neg/Tick	**2677**	34	31	24	25	26	25	25
6	Neg/Tick	**2680**	30	30	24	24	25	19	22
6	Neg/Tick	**2683**	30	32	26	23	31	26	25
		Mean	*31.33*	*30.20*	*26.60*	*24.40*	*27.00*	*22.25*	*23.50*
		STD	*±2.31*	*±2.49*	*±3.29*	*±1.14*	*±3.39*	*±3.77*	*±1.73*
7	Neg/Neg	**2685**	28	28	20	31	28	27	23
7	Neg/Neg	**2692**	35	28	28	31	27	27	32
7	Neg/Neg	**2693**	34	32	28	31	31	30	29
7	Neg/Neg	**2666**	*	27	30	*	23	24	24
		Mean	*32.33*	*28.75*	*26.50*	*31.00*	*27.25*	*27.00*	*27.00*
		STD	*±3.79*	*±2.22*	*±4.43*	*0.00*	*±3.30*	*±2.45*	*±4.24*

STD – Standard Deviation

Appendix 6

Reciprocal titres of IFA test

Group	Goat number	Immunisation route	rIFA titre* pre-immunisation	rIFA titre* post immunisation(Day 14)	rIFA titre* post-immunisation (Day 42)	rIFA titre* 64 days post challenge
1	2584	IV	Neg	≥320	320	ND
1	2585	IV	Neg	≥320	≥320	ND
1	2587	IV	Neg	≥320	≥320	ND
1	2589	IV	Neg	≥320	320	ND
1	2592	IV	Neg	ND	≥320	ND
2	2593	IV	Neg	320	≥320	ND
2	2596	IV	Neg	320	≥320	ND
2	2598	IV	Neg	≥320	≥320	ND
2	2599	IV	Neg	≥320	≥320	ND
2	2603	IV	80	≥320	≥320	ND
3	2605	IM	40	≥320	≥320	ND
3	2606	IM	Neg	Neg	≥320	ND
3	2609	IM	Neg	320	≥320	ND
3	2610	IM	Neg	≥320	≥320	ND
3	2611	IM	≥80	320	≥320	ND
3	2612	IM	Neg	320	≥320	ND
3	2613	IM	Neg	320	≥320	ND
3	2618	IM	Neg	≥320	≥320	ND
3	2619	IM	Neg	≥320	≥320	ND
3	2620	IM	ND	≥320	≥320	ND
3	2622	IM	Neg	≥320	≥320	ND
4	2624	IM	Neg	≥320	≥320	ND
4	2626	IM	40	≥320	≥320	ND
4	2628	IM	Neg	≥320	≥320	ND
4	2629	IM	ND	≥320	≥320	ND
4	2631	IM	Neg	160	≥320	ND
4	2633	IM	Neg	320	≥320	ND
4	2636	IM	Neg	160	≥320	ND
4	2637	IM	Neg	320	≥320	ND
4	2639	IM	Neg	160	≥320	ND
4	2640	IM	Neg	≥320	≥320	ND
4	2641	IM	Neg	≥320	≥320	ND
4	2644	IM	Neg	≥320	≥320	ND
4	2645	IM	Neg	≥320	≥320	ND
4	2649	IM	Neg	≥320	≥320	ND
4	2650	IM	Neg	≥160	≥320	ND
4	2652	IM	Neg	320	≥320	ND
4	2655	IM	Neg	≥320	≥320	ND
4	2656	IM	Neg	≥320	≥320	ND
4	2661	IM	Neg	≥160	≥320	ND
4	2663	IM	Neg	80	≥320	ND

5	2665	None	Neg	Neg	Neg	80
5	2700	None	Neg	Neg	Neg	160
5	2669	None	Neg	Neg	Neg	Died
5	2671	None	Neg	Neg	Neg	160
5	2672	None	Neg	Neg	Neg	320
6	2673	None	Neg	Neg	Neg	160
6	2676	None	Neg	Neg	Neg	Died
6	2677	None	Neg	Neg	Neg	Neg
6	2680	None	Neg	Neg	Neg	160
6	2683	None	Neg	Neg	Neg	320
7	2685	None	Neg	Neg	Neg	ND
7	2692	None	40	Neg	Neg	ND
7	2693	None	Neg	Neg	Neg	ND
7	2666	None	Neg	ND	Neg	ND

Neg: negative; ND not done
* Reciprocal indirect fluorescent antibody titre
Titres higher than 1:40 were considered *Ehrlichia*-positive