Safety and efficacy of an attenuated heartwater (*Ehrlichia ruminantium*) vaccine administered by the intramuscular route in cattle, sheep and Angora goats

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Abstract

Heartwater is an economically important tick-borne disease of ruminants in Africa. The current commercial vaccine uses live *Ehrlichia ruminantium* from blood of infected sheep, requires antibiotic treatment during infection, needs to be administered intravenously and does not protect against all South African isolates. An attenuated tissue culture vaccine not requiring antibiotic treatment and effective against different field strains in small groups of goats and sheep was reported previously. The objective of the present study was to test safety and efficacy of this vaccine administered by intramuscular (i.m.) inoculation in larger groups of sheep, Angora goats and cattle. Animals were vaccinated via intravenous (i.v.) and i.m. routes and received *E. ruminantium* homologous challenge by feeding of infected ticks or by i.v. inoculation of infected blood. For vaccine titration in sheep and goats, the optimum safe and efficacious dose was determined using 2 ml equivalent of 10^2–10^5 culture-derived live elementary bodies (EBs). Similarly, the vaccine was titrated in cattle using 5 ml containing 10^5–10^7 EBs. Seventy percent of i.v. vaccinated and 9.7% of i.m. vaccinated Angora goats receiving 10^5 EBs, developed severe reactions to vaccination and were treated. These treated animals and the remaining 90.3% of i.m.- vaccinated goats showed 100% protection against i.v. or tick challenge. Sheep and Angora goats vaccinated i.m. with 10^4 EBs had no vaccination reactions and were fully protected against i.v. or tick challenge. Similarly, vaccinated cattle (dose 10^6 EBs) did not react to vaccine inoculation and were fully protected against i.v. or tick homologous challenge. Control non-vaccinated animals reacted severely to challenge and required oxytetracycline treatment. This successfully demonstrated that Angora goats, sheep and cattle can be safely vaccinated with the attenuated *E. ruminantium* Welgevonden vaccine via the i.m. route, with no clinical reactions to vaccination and 100% protection against virulent i.v. and homologous tick challenge.

Keywords: Heartwater; *Ehrlichia ruminantium;* Attenuated vaccine; Cattle; Sheep; Goats

1. Introduction

Heartwater is a tick-borne disease, caused by the intracellular rickettsial agent *Ehrlichia ruminantium*, affecting cattle, sheep, goats, and some antelope species such as blesbok (*Damaliscus albifrons*), black wildebeest (*Connochaetes gnu*) and springbok (*Antidorcas marsupialis*) [1]. In South Africa, Angora goats are particularly susceptible to heartwater which places a severe economic constraint on mohair producers of the Eastern Cape Province. The vectors responsible for the transmission of *E. ruminantium* belong to the genus *Amblyomma* with the most important species in southern Africa being *Amblyomma hebraeum* and *Amblyomma variegatum* [2]. It is generally considered as one of the most important tick-borne diseases in Africa, and in South Africa, mortalities from heartwater are three times greater than from other tick-borne diseases such as babesiosis and anaplasmosis [1]. It is estimated to cause up to R975 million (US$ 48.8 million) in financial losses in Africa, with South African farmers losing R220 million (US$ 11 million) annually [3]. In South Africa, goats are especially threatened, and in some parts of the rural farming sector up to 30% of goats become infected with heartwater annually [4]. An economic model for the impact of heartwater under various farming systems was developed as a result of a 10-year study in Zimbabwe [5]. Farmers control the disease using antibiotics to treat or block-treat the herd during disease outbreaks. Most farmers rely on tick control with acaricides. These methods are expensive and unsustainable. The only sustainable method of control is by vaccination.

The current commercially available vaccine against heartwater in ruminants in South Africa is an infection and treatment method of immunization. The observation that survival from a virulent challenge of heartwater gives protective immunity against a homologous challenge led to the development of this vaccine in the 1950s [6], [7]. The vaccine uses live *E. ruminantium* organisms from blood of infected sheep and requires subsequent treatment of the animals with antibiotics at specific times during the course of infection. While this method has helped in the control of the disease in some regions of the country, there are several inherent problems preventing the wide use of this live blood vaccine. The vaccine must be administered intravenously and may cause anaphylactic shock while technically competent staff must attend to the inoculation procedure; these are perhaps the greatest limiting factors to its use [7]. A wide genetic diversity of *E. ruminantium* organisms circulate in animals and ticks in different geographical regions resulting in different immunogenic types. This limits the wide use of the current commercial blood vaccine as it does not protect against all the South African isolates. In cross immunity trials in sheep using South African *E. ruminantium* stocks, Ball 3 immunized sheep were completely susceptible to heterologous challenge with Welgevonden and provided only partial protection against two stocks, Blaauwkrans and Mara 87/7 [8].

Considerable research work has been done to develop inactivated heartwater vaccines as alternatives to live virulent blood vaccines in terms of safety and efficacy. Some of the studies have shown that domestic ruminants immunized with inactivated vaccines were found to be protected if exposed to virulent homologous heartwater challenge [9], [10], [11], [12]. In field trials, the levels of protection against heterologous challenge provided limited cross protection [13]**.** The mortality rate in vaccinated Angora goats with the *E. ruminantium* Mbizi inactivated vaccine in South Africa was 72% [14]. Other drawbacks of the inactivated vaccine are that they require several injections spread over a period of weeks during which the animals must be kept tick-free and that the inactivated vaccines, while reducing mortality, do not prevent clinical disease in vaccinated animals following experimental challenge or field exposure, which could also make them unattractive to the farmers [9], [15]. Therefore,

due to the limited protection against natural tick challenge, further development will be required before inactivated heartwater vaccines could become a commercially viable option [16].

Advances in the fields of molecular biology, chemistry and immunology are now used in the development of new generation vaccines which, if successfully identified and tested to provide near 100% protection would be ideal since they are cheaper to manufacture, easier to store than conventional vaccines and easy to administer [16]. In a previous trial a nucleic acid vaccine consisting of four *E. ruminantium* genes was used to immunize sheep, which were shown to be completely protected against a subsequent lethal needle challenge with both homologous and heterologous *E. ruminantium*-infected blood [8]. 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 [17]. Recently, a new formulation of DNA vaccine has improved the protective efficiency to protect 60% (3/5 animals in the group) of the sheep against tick challenge [18]. The *E. ruminantium* immunodominant major antigenic protein 1 (MAP1) is a membrane protein and represents a good target for vaccine development. A recombinant *E. ruminantium* MAP1 protein (rMAP1) produced in insect cells exhibited, for the first time, a distinct glycosylation profile of the protein; and vaccination of sheep with the subunit vaccine formulation induced both antibody and Th1 type cellular responses, which are critical to controlling intracellular pathogens, including *E. ruminantium* [19]. These results suggest that a glycosylated rMAP1 subunit vaccine could be efficacious against virulent heartwater challenge. However, this potential vaccine candidate awaits animal vaccination efficacy trials. Tick-borne diseases such as theileriosis, babesiosis, heartwater and anaplasmosis are major animal health and management problems of cattle and small ruminants in Africa and other parts of the world. Currently, there are many difficulties encountered in the development of recombinant vaccines for these pathogens. However, there are effective live attenuated vaccines for the control of Tropical theileriosis (*Theileria annulata*), *Babesia bovis*, and *Babesia bigemina* [20]. The mild *Anaplasma centrale* is used as a live blood vaccine which protects against the virulent bovine anaplasmosis, *Anaplasma marginale* [21]. However, this vaccine suffers from some of the same drawbacks as the commercial heartwater live blood vaccine. The attenuated vaccines are widely used in South Africa, Israel, Australia and countries in South America.

The first heartwater organism to be attenuated was *E. ruminantium* Senegal which provided good protection against infection with the virulent homologous strain [22]. This attenuated vaccine strain was evaluated in a field trial in The Gambia, where it protected it protected 75% of sheep against virulent tick challenge which was fatal for all control sheep [13]. In South Africa, the virulent *E. ruminantium* Welgevonden strain has been successfully attenuated in canine monocyte-macrophage cell line DH82, and the organisms were then adapted to grow in BA 866 cells, in which they were passaged 64 times [23]. The process of propagating the organisms for 64 tissue culture passages in bovine cells, led neither to a reversion to virulence nor to a loss of immunogenicity [23]. When inoculated intravenously into Merino sheep or Boer goats, the attenuated culture-derived organisms did not produce disease and the animals were subsequently found to be fully protected against a lethal needle challenge with the homologous strain or four different heterologous strains [23]. Therefore, these encouraging preliminary results led us to investigate, firstly, the safety and efficacy of the *E. ruminantium* Welgevonden strain in larger groups of sheep, Angora goats and cattle. Secondly, the attenuated heartwater vaccine would only be ideal if the intramuscular route as compared to the intravenous route of administration proved to be efficacious. The third objective of our study was to demonstrate the efficacy of the attenuated *E. ruminantium*

Welgevonden vaccine to protect animals against natural infection by tick transmission as compared to the artificial intravenous challenge with virulent blood stabilate.

2. Materials and methods

2.1. Experimental vaccine preparation

The Welgevonden strain was attenuated in a canine macrophage-monocyte cell line (DH82) [24], re-adapted to grow in a bovine endothelial cell line (BA 886) [23], then harvested and cryopreserved in liquid nitrogen. Briefly, cultures were scraped off, mixed by syringing and clarified at 800 × g for 15 min at 4 °C. The recovered supernatant was centrifuged at 20 000 × g for 15 min at 4 °C. The resulting pellets were resuspended in SPG containing 20% foetal calf serum (cryoprotectant), aliquoted in cryovials and stored in liquid nitrogen. Aliquots of each stabilate were thawed and returned to endothelial cell culture in order to determine the number of infective organisms present [23], which was at 10^7 EBs per ml. For animal immunization the vaccine doses were extrapolated from the stored stabilate titre, and stabilates were diluted to a determined standard number of infective organisms per vaccine dose. Different stabilates, between generation 58 and 79 in BA 886 cells, were used for immunizations in different trials.

2.2. *In vitro* Ehrlichia ruminantium *quantification*

Ehrlichia ruminantium bacteria viability was measured both by flow cytometry and by microscopy and were processed according to the manufacturer's instructions and tested by flow cytometry to detect the percentage of viable EBs. The EBs were separated from the bovine endothelial cells by centrifugation at 1500 × g for 10 min. The supernatant was then diluted 1:10 or 1:100 in SPG, and the EBs were pelleted at 21,000 × g for 20 min at 4 °C. The pellets were resuspended in a final volume of 970 µl of 0.85% SPG buffer and passed through a 26–28 gauge needle several times to break up agglomerates in the elementary body suspension. The EBs were stained with 7 µl PI dye; 7 µl SYPRO9 dye and 10 µl calibrator beads (also diluted 1/10 times). This cell-dye-bead mixture was incubated for 15–20 min at 37 °C after which the samples were run on the Beckman Coulter FX500 flow cytometer using detectors for red (dead EBs) and green fluorescence (viable EBs) (Fig. 1). The number of viable EBs/ml was calculated using the method recommended by the manufacturer.

The method to quantify elementary bodies [23] was used for quantification by microscopy. Briefly, a suspension of EBs was used to infect an endothelial cell layer in 25 cm^2 cell culture flasks and incubated for two days. Slides were then cut out from each flask, allowed to dry, fixed with methanol and stained with Kyro-Quick stain (Kyron Laboratories [Pty] Ltd, Benrose, South Africa). The estimated number of infective EBs was calculated from the percentage of infected cells counted on the slide and the average number of cells in uninfected control cultures.

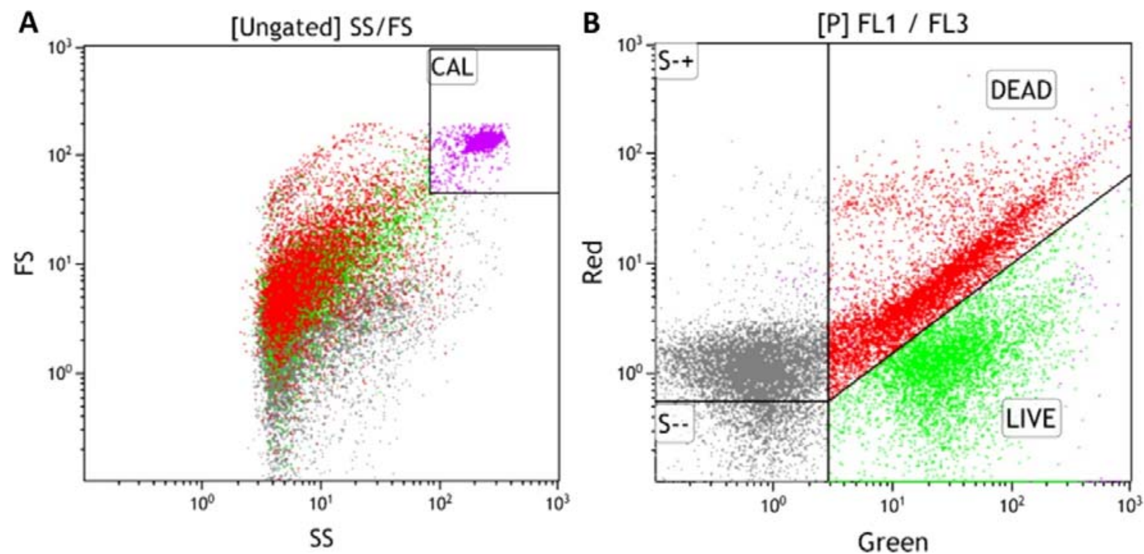

Fig. 1. Quantification of live and dead *Ehrlichia ruminantium* with the LIVE/DEAD® BacLight™ Bacterial Viability and Counting Kit (Invitrogen) using the Beckmann FC500 flow cytometer. Samples of *E. ruminantium* were prepared, stained and analysed as outlined in the text. SYTO9® and propidium iodide (PI) were used to discriminate live vs dead *E. ruminantium* elementary bodies (EBs). (A) A two-parameter comparison of the Forward Scatter (FS) vs Side Scatter (SS) was used to discriminate the stained population, while the purple population represents calibration beads used to determine relative EBs counts. (B) Populations of bacteria were discriminated as two regions of the log-integrated red fluorescence (dead, PI) versus log-integrated green fluorescence (Live; SYTO9®) dot plot, and the number of stained EBs found within these regions were used to estimate the percentage of viable organisms in the population.

2.3. Determination of the vaccination dose

In order to calculate the number of live attenuated *E. ruminantium*/ml in cell cultures, data obtained from two methods were combined: counting infected stained cells under a microscope and counting the live bacterial cells using the LIVE/DEAD® BacLightTM Bacterial Viability Assay (Invitrogen). All counts were made within one hour from fresh culture before freezing or from thawed cultures. Once the number of the live attenuated Welgevonden vaccine bacteria was determined, the vaccine was diluted to the required dose concentration in SPG (0.22 M sucrose, 0.01 M potassium phosphate pH 7.0, 0.5 mM potassium glutamate) and fetal calf serum.

2.4. Origin, maintenance and monitoring of experimental animals

The Angora goats, Merino sheep and Holstein Friesian cattle were purchased from an area within the Free State Province of South Africa that is known to be *Amblyomma*- and heartwater-free [4]. The animals were housed in well-fenced, tick-free stables with cement floors at the Agricultural Research Council-Onderstepoort Veterinary Research (ARC-OVR). The stables were cleaned daily and food and clean water (*ad libitum*) were provided daily. The stables allowed the animals to interact with each other. On arrival, animals were screened for *E. ruminantium* infection by the immunofluorescent antibody test (IFAT) [25] to confirm that animals were negative before the start of the vaccination trials. The IFAT test was also performed on all experimental animals on Day 0 before inoculation and post immunization as a monitoring procedure. The animals were housed at the ARC-Onderstepoort Veterinary Research (ARC-OVR) animal facility for a period of two months before experimentation commenced for adaptation to the new environment, food and daily handling. In addition,

animals received treatment with Tramisol® Plus (rafoxanide + levamisole hydrochloride [Afrivet]) and Sulfazine (sulphadimidine sodium) to ensure they were free from internal parasites before proceeding with inoculations. They were also treated topically with a pyrethroid-based acaricide.

Daily morning rectal temperatures and clinical signs of all animals were monitored for the duration of immunization and challenge. Clinical reactions in an individual animal were assessed by recording the body temperature reaction together with associated clinical signs. Clinical reactions were classified as no reactions, mild reactions (transient fever of 40 °C for 1–2 days, no clinical signs and no treatment), moderate (3 days temperature of 40 °C to 40.5 °C with no clinical signs and no treatment) or progressed to severe reactions (1 day temperature of 42 °C or 2-days temperature of 41 °C; animals showed clinical signs, were treated or euthanized). Clinical signs included: fever, inappetence, incoordination, respiratory distress, nervous symptoms and an animal received treatment, using oxytetracycline or was euthanized if not responding to treatment. The animals were housed in well-fenced, tick-free stables with cement floors at the ARC-OVR. The stables were cleaned daily and food and clean water (*ad libitum*) were provided daily. The stables allowed the animals to interact with each other.

2.5. Vaccination of sheep and Angora goats

The inoculation dose determined for sheep and goats was 2 ml containing viable 10^5 EBs which should be efficacious for the intramuscular (i.m.) and intravenous (i.v.) route of administration [23], [26]. A total of 55 Angora goats and 55 sheep, each allocated into 7 groups of 4–20 animals per group (Table 1, Table 2), were used to test and determine the safety and efficacy of the attenuated heartwater vaccine. Four groups were immunized with the attenuated heartwater vaccine using two routes of administration, i.v. (Groups 1 and 2) and i.m. (Groups 3 and 4) route. The i.v. route was included as a standard control procedure. Groups 5 and 6 served as unvaccinated controls for animals challenged with infected blood and infected ticks, while animals in Group 7 served as untreated and unchallenged negative controls. All vaccinated animals and unvaccinated controls received a homologous *E. ruminantium* Welgevonden challenge (Section 2.7) 6 weeks after immunization.

Table 1
Sheep (n = 55) vaccination (dose 10^4 and 10^5 EBs) administered via i.v. or i.m. routes and challenged with homologous *Ehrlichia ruminantium* Welgevonden virulent blood stabilate via i.v. or infected adult ticks (n = 20) 6 weeks after immunization.

Dose	Group (no. of animals)	Immunization/ challenge	Immunization reactions				Challenge reactions			
			No	Mi	Mod	S	No	Mi	Mod	S
10^4	1 (5)	i.v./i.v.	1	2	2	–	5	–	–	–
10^4	2 (5)	i.v./ticks	0	0	5	–	4	–	1	–
10^5	3 (11)	i.m./i.v.	5	4	2	–	10	1	0	–
10^5	4 (20)	i.m./ticks	11	9	–	–	19	1	0	–
	5 (5)	Control/i.v.	–	–	–	–	–	–	–	5
	6 (5)	Control/ticks	–	–	–	–	–	–	–	5
	7 (4)	Neg	–	–	–	–	–	–	–	–

No: No reactions; Mi: Mild reactions (1-day temperature, no treatment); Mod: Moderate reactions (2–3-day temperature with no clinical signs and no treatment);
S: Severe reactions (animals treated or euthanized).
Neg: negative control.

Table 2
Angora goats (n = 55) vaccination (dose 10^4 and 10^5 EBs) administered via i.v. or i.m. routes and challenged with homologous *Ehrlichia ruminantium* Welgevonden virulent blood stabilate via i.v. or infected adult ticks (n = 5) 6 weeks after immunization.

Dose	Group (no. of animals)	Immunization/ challenge	Immunization reactions				Challenge reactions			
			No	Mi	Mod	S	No	Mi	Mod	S
10^4	1 (5)	i.v./i.v.	–	–	3	2	5	–	–	–
10^4	2 (5)	i.v./ticks	–	–	–	5	5	–	–	–
10^5	3 (11)	i.m./i.v.	3	3	4	1	11	–	–	–
10^5	4 (20)	i.m./ticks	2	9	7	2	20	–	–	–
	5 (5)	Control/i.v.	–	–	–	–	–	–	–	5
	6 (4)	Control/ticks	–	–	–	–	–	–	–	4
	7 (4)	Neg	–	–	–	–	–	–	–	–

No: No reactions; Mi: Mild reactions (1-day temperature, no treatment); Mod: Moderate reactions (2–3-day temperature with no clinical signs and no treatment);
S: Severe reactions (animals treated or euthanized).
Neg: negative control.

Groups 1, 3 and 5 received an intravenous challenge dose of 5xLD50 of the virulent *E. ruminantium* Welgevonden blood stabilate (Section 2.7). Groups 2, 4 and 6 were challenged by infected ticks (Section 2.8).

A total of 21 Angora goats and 21 sheep were used for optimization of the vaccine dose (Table 3). Three vaccine doses in sheep and goats comprising 10^2, 10^3 and 10^4 EBs per 2 ml dose were used to immunize. For each dose of the vaccine, a group of 2 animals received the vaccine via the i.v. route and 5 animals via the i.m. route.

Table 3
Vaccination of sheep (n = 21) and Angora goats (n = 21) using different vaccine doses administered i.v. or i.m. and subsequent i.v. challenged with homologous *Ehrlichia ruminantium* Welgevonden virulent blood stabilate 6 weeks after immunization.

Dose (EBs)	Immunization (no. of animals)	Immunization reactions								Challenge reactions							
		Sheep				Goats				Sheep				Goats			
		No	Mi	Mod	S	No	Mi	Mod	S	No	Mi	Mod	S	No	Mi	Mod	S
10^2	i.v. (2)	1	1	–	–	–	–	–	2	2	–	–	–	2	–	–	–
	i.m. (5)	5	–	–	–	5	–	–	–	–	–	–	5	2	–	–	3
10^3	i.v. (2)	1	1	–	–	–	–	–	2	2	–	–	–	2	–	–	–
	i.m. (5)	4	1	–	–	4	1	–	–	–	–	–	5	5	–	–	–
10^4	i.v. (2)	1	1	–	–	–	–	–	2	2	–	–	–	2	–	–	–
	i.m. (5)	5	–	–	–	3	2	–	–	5	–	–	–	5	–	–	–

No: No reactions; Mi: Mild reactions (1-day temperature, no treatment); Mod: Moderate reactions (2–3-day temperature with no clinical signs and no treatment);
S: Severe reactions (animals treated or euthanized).

Table 4
Holstein Friesian cattle vaccination using different vaccine doses administered i.v. or i.m. and subsequent i.v. challenged with homologous *Ehrlichia ruminantium* Welgevonden virulent blood stabilate 45 days after immunization.

Group	Number of animals	Dose (EBs)	Time post thawing (mins)	Route	Immunization reactions				Challenge reactions			
					No	Mi	Mod	S	No	Mi	Mod	S
1	5	10^5	60	i.m.	5	–	–	–	3	1	1	–
2	5	5×10^5	60	i.m.	5	–	–	–	2	3	0	–
3	5	10^6	60	i.m.	5	–	–	–	3	1	1	–
4	5	10^6	60	i.v.	5	–	–	–	1	4	–	–
5	6	10^6	30	i.m.	6	–	–	–	5	1	–	–
6	10	10^6	30	i.v.	10	–	–	–	10	0	–	–
7	5	10^7	30	i.m.	5	–	–	–	5	0	–	–
8	5	10^7	60	i.m.	5	–	–	–	3	2	–	–
9	5	Neg			–	–	–	–	–	–	1	4

No: No reactions; Mi: Mild reactions (1-day temperature, no treatment); Mod: Moderate reactions (2–3-day temperature with no clinical signs and no treatment);
S: Severe reactions (animals treated or euthanized).
Neg: negative control.

2.6. Vaccination of cattle

A total of 71 cattle was used; 51 of 5–10 animals per group in the vaccine dose titration study and 20 of 10 animals per group to determine the safety and effectiveness of the optimal vaccine dose against tick transmission. For a vaccine dose titration, 4 doses of vaccine (10^5, 5×10^5, 10^6, 10^7 EBs) were used at two different post thawing times (30 and 60 min) (Table 4). Animals were assigned to 9 Groups; 8 groups of 5–10 animals were immunized via the i.v. or i.m. routes and challenged, while Group 9 (n = 5) served as an unvaccinated challenge control. Cattle in the various groups were challenged i.v. with 5 ml of virulent Welgevonden

blood stabilate 45 days after vaccination (Section 2.7). The vaccine dose which corresponded to 10^6 EBs, was then taken as optimal dose to vaccinate a group of 10 cattle via the i.m. route; these animals were subsequently challenged by feeding infected *A. hebraeum* adults along with 10 non-vaccinated control cattle (Table 5).

Table 5
Holstein Friesian cattle vaccination (dose 10^6 EBs) administered via i.m. and challenged with homologous *Ehrlichia ruminantium* Welgevonden infected adult ticks (n = 17) 30 days after immunization.

Group	Number animals	Immunization reactions				Challenge reactions			
		No	Mi	Mod	S	No	Mi	Mod	S
Vaccinated	10	10	–	–	–	10	–	–	–
Controls	10	–	–	–	–	2	–	–	8

No: No reactions; Mi: Mild reactions (1-day temperature, no treatment); Mod: Moderate reactions (2–3-day temperature with no clinical signs and no treatment); S: Severe reactions (animals treated or euthanized).

2.7. Challenge of animals using infected blood

The animals were challenged with the virulent *E. ruminantium* Welgevonden. Blood stabilates were prepared from an *E. ruminantium* Welgevonden infected sheep as described previously [27]. Briefly, the infected sheep blood was collected on day 2 of temperature reaction, diluted in SPG and stored in liquid nitrogen. The infected blood stabilate 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 i.v. route. The challenge material for cattle was derived from a cow infected by adult *A. hebraeum* ticks. The infected blood was collected during the reaction period and diluted and titrated as for sheep. Animals were challenged using 5 ml via the i.v. route.

2.8. Challenge of animals using infected ticks

Uninfected ticks from an *A. hebraeum* colony maintained in the acaridarium (tick room maintained at 28 °C, 80% RH) of the ARC-OVR were used to feed on an infected animal. A blood stabilate of the virulent Welgevonden strain was used to infect a susceptible sheep by the i.v. route (10 ml). On Day 8, *A. hebraeum* nymphs (n = 300) were applied and allowed to feed on the back of the animal; nymphs were contained in linen bags glued to the skin and secured by rubber bands. Only nymphs that had fully engorged and dropped off the host during the febrile reaction were used for tick challenge. The engorged nymphs were kept in the acaridarium and the emerging adult ticks were used (30 days after moult) for challenge. Five males and 5 females were randomly selected from 3 different containers (n = 30) 45 days post detachment and were tested for infection with *E. ruminantium* Welgevonden using the pCS20 quantitative real-time TaqMan assay (qPCR) as described previously [28].

Five infected *A. hebraeum* (3 males/2 females) were applied to feed on each Angora goat and 10 males and 10 females to feed on each sheep. The males were placed in linen bags fitted on the backs of animals and allowed to feed for three days before the females were placed in the linen bag. 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.

Seventeen infected *A. hebraeum* (7 males/10 females) were used to challenge each animal in the two groups of cattle (Table 5). The male ticks were applied to the linen bags fitted on the backs of animals and allowed to feed for 3 days before applying the females. This allows faster attachment of females to males in response to the released attachment-aggregation-pheromone secreted by feeding males. Ticks were allowed to feed to engorgement.

2.9. Indirect fluorescent antibody test (IFAT)

Serum samples of all sheep, Angora goats and cattle were taken on their arrival to the laboratory and on Day 42 post immunization (pre-challenge). Serum samples were tested using the (IFAT) which detects anti-*Ehrlichia* antibodies [23], [25], [29]. The application of the IFAT with minor modifications was previously described [23]. Negative control sera were prepared from blood samples from a disease-free herd of cattle from the ARC-OVR. Positive control bovine sera were prepared from an animal that was infected with, and reacted to, *E. ruminantium* Welgevonden strain, 35 days after treatment and recovery. The antigen was prepared from infected BA 886 cells with Welgevonden organisms. The prepared antigen slides were then tested by the IFA using positive and negative control sera. The cut-off titres 1/40 dilution was used to determine positive results. The test slides were examined under fluorescent microscope using a 50x objective lens.

2.10. Statistical analysis

The number of heartwater infected engorged *A. hebraeum* females that dropped from the vaccinated group and controlled group were statistically compared. A two-sample *t*-test was used to determine whether there was a statistically significant difference in the mean number of engorged adult ticks fed and dropped between the two groups at 95% confidence intervals.

2.11. 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 experimental procedures and protocols.

3. Results

3.1. Serology: sheep, Angora goats, cattle

All animals were screened on arrival and prior to vaccination for the presence of antibodies to *E. ruminantium* by the IFAT. The tests showed that 2/31 (6.5%) of sheep, 5/55 (9.1%) of Angora goats and 3/20 (15%) of cattle had positive antibody titers. Overall, the percentage of animals with positive titres was 10/106 (9.4%) and with negative titres (96/106), indicating an IFAT specificity of 90.6%. The seropositive animals were randomly distributed between different experimental groups. All animals in the three groups had sero-converted post vaccination.

3.2. Tick infection rate

All the randomly selected adult *A. hebraeum* ticks (n = 30) tested by pCS20 qPCR were found positive for *E. ruminantium.* There was no statistically significant mean difference (p-value 0.3609, at 5% level) in the genomic DNA concentration of *E. ruminantium* detected in *A. hebraeum* females (mean cut-off point “CP” value 17.92) and males (mean CP value 17.00). The lower limit of detection of the pCS20 is CP value 38 [25].

3.3. *In vitro* **Ehrlichia ruminantium** *quantification*

There was a good correlation between the infectivity (counts by microscopy) and viability (counts by flow cytometry) at 1:10 dilution of the undiluted culture (1.51×10^6 and 1.54×10^6, respectively) but higher viability counts were detected at 1:100 dilution. The comparison of bacterial counts by microscopy of the undiluted culture highly correlated with counts by flow cytometry at 1:10 and 1:100 dilutions (1.58×10^6, 1.54×10^6, 1.59×10^6, respectively). Thus, the possibility that some viable EBs may not be infective could not be excluded.

3.4. Sheep vaccination safety and efficacy

Table 1 shows the results of sheep vaccination (dose 10^4 and 10^5 EBs) administered via i.v. or i.m. routes and subsequent i.v. challenge with the virulent homologous Welgevonden strain or infected ticks. Of the i.m inoculated animals, only 2/31 showed moderate post-vaccination reactions but no treatment was required. Of the i.v.-immunized sheep, 7/10 showed moderate post-vaccination reactions but no treatment was required. None of the i.m. immunized sheep (31/31) showed any clinical signs that required treatment after receiving a homologous virulent i.v. challenge or tick challenge. In contrast, all the unvaccinated control animals (n = 10) experienced severe clinical reactions to challenge administered via i.v. or through feeding of infected ticks.

3.5. Angora goats vaccination safety and efficacy

Table 2 shows the results of vaccination (dose 10^4 and 10^5 EBs) of Angora goats challenge with the virulent Welgevonden strain or infected ticks. The incubation period, indicated by a rise in rectal temperatures in vaccinated goats, ranged between 7 and 17 days. This incubation period was similar for both i.v. and i.m. immunization. In the goats vaccinated i.v. (Groups 1 and 2), the highest temperature recorded was 42 °C and a total of 7/10 (70%) received treatment as a result of severe vaccine related reactions. All these goats recovered fully after treatment. In the goats vaccinated i.m. (Groups 3 and 4), the highest temperature recorded was 41.5 °C. This occurred on day 11 and Day 14. A total of 11/31 Angora goats in the i.m. groups showed moderate post-vaccination reactions but recovered without treatment. However, 3/31 (9.7%) showed severe clinical reactions and were treated and recovered. Therefore, the vaccine dose 10^5 protected 91.3% (28/31) of Angora goats.

Animals in Group 1 and Group 2 that were immunized i.v. and challenged with virulent Welgevonden blood or infected ticks, respectively, were all protected with no temperature reactions or any other apparent heartwater associated symptoms (Table 2). Animals in Group 3 (n = 11) and Group 4 (n = 20) which were immunized i.m. and challenged with virulent Welgevonden blood or infected ticks, respectively, were all fully protected with no clinical signs.

All the Angora goats in the unimmunized control Groups (5 and 6) that were challenged with virulent Welgevonden or infected ticks, respectively, developed severe temperature reactions, displayed clinical signs and all required treatment. One animal died notwithstanding treatment.

3.6. Optimization of immunizing dose for sheep and Angora goats

Table 3 shows the results of vaccination of sheep (n = 21) and Angora goats (n = 21), using different vaccine doses administered i.v. or i.m. and subsequent i.v. challenge with a virulent Welgevonden strain. The three groups of sheep immunized i.v. or i.m. with the vaccine doses (10^2, 10^3, 10^4 EBs) did not show any signs of clinical reactions to vaccination. None of the i.v. vaccinated (10^2, 10^3, 10^4 EBs) sheep reacted to challenge (6/6), indicating good protection. However, all animals in the two i.m. vaccinated groups (10^2, 10^3 EBs) reacted severely to challenge, and all required treatment (10/10). Conversely, the group of sheep that received the i.m. vaccine dose of 10^4 EBs, were all protected against the virulent challenge and required no treatment (5/5).

All the Angora goats in the three groups (10^2, 10^3, 10^4 EBs) which were vaccinated i.v. became sick and were treated (6/6). Even so, all were protected against the virulent challenge similar to the existing vaccine's infection and treatment method of immunization. The three groups (10^2, 10^3, 10^4 EBs) of Angora goats which were i.m. vaccinated had no reactions or only mild reactions (3/15) but required no treatment. Only 2/5 Angora goats in the i.m. (10^2) vaccinated group showed protection against challenge, the rest requiring treatment. However, the remaining i.m. (10^3, 10^4) vaccinated Angora goats were all protected against challenge and none required treatment

Therefore, the vaccine dose administered i.m. at 10^4 EBs to both sheep and Angora goats was safe and protected all animals (10/10) against a virulent homologous strain challenge.

3.7. Vaccine titration in cattle

All vaccinated cattle (Groups 1–9; n = 46) receiving doses of 10^5–10^7 EBs and at two times after thawing (30, 60 min) did not show clinical reactions to either i.v. or i.m. routes for vaccine inoculation (Table 4). None of the immunized animals showed any severe reactions to i.v. challenge with the virulent homologous strain. However, one animal in each of the i.m. vaccinated Groups 1, 2 and 3 (10^5, 5×10^5, 10^6 EBs) exhibited moderate clinical signs and recovered without treatment. Four of five challenged control animals (Group 9) reacted severely and required treatment.

3.8. Safety and efficacy of cattle vaccination

Table 5 shows the results of vaccination of Holstein Friesian cattle (dose 10^6 EBs) administered i.m. 30 min after thawing and challenged with infected ticks. The mean (±SD) of female ticks engorged and dropped from vaccinated and control groups was 8.3 ± 1.56 and 7.7 ± 1.16, respectively, and there was no significant difference (p-value = 0.3433) between the two groups. None of the vaccinated cattle (10/10) showed clinical symptoms after i.m. inoculation of the vaccine dose. Similarly, none of the vaccinated animals developed high fever during or after tick challenge. The incubation periods for the unvaccinated control group ranged between 13 and 18 days as shown by a rise in rectal temperatures of up to 41.2 °C and lasted 4 to 8 days. Eight out of 10 unvaccinated control animals developed heartwater disease and required treatment. Seven recovered and one was euthanized due to its failure to respond to treatment. This animal showed classical post-mortem lesions of heartwater: hydrothorax (400 ml), hydropericadium (250 ml) and ascites (800 ml). It was confirmed positive for *E. ruminantium* infection in stained brain crushed smears. The number of ticks which engorged and dropped (6 and 7) from the two control animals which did not

react to the tick challenge, were not statistically significantly different from the group mean (7.7 ± 1.16).

4. Discussion

The first attenuated heartwater vaccine was the *E. ruminantium* Senegal stock and although it provided good protection against challenge with the virulent homologous strain, it was not very effective against heterologous virulent field challenge [13], [22]. The current commercial live blood vaccine against heartwater, the *E. ruminantium* Ball 3 strain in ruminants in South Africa, is essentially an infection and treatment method of immunization. The vaccine has several inherent problems and limitations for wide use [7], [8], [30], [31]. These limitations include intravenous administration which may cause anaphylactic shock in the animal and limited or no protection against local South African isolates such as Welgevonden or exotic strains [8], [32]. A survey conducted in 1994 indicated that only 35% of cattle farmers and 15% of farmers keeping sheep and goats vaccinate their animals against heartwater [31].

South Africa currently produces over 50% of the total world mohair stock [33], making Angora goat farming an economically important enterprise in the country. The reduction in heartwater losses in the national Angora herd is therefore imperative. The Angora goat is the domestic ruminant most susceptible to heartwater with mortality rates of almost 90% [34]. Vaccination using the infection and treatment method of immunization is particularly difficult and hazardous in Angora goats. It was 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 [35]. 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 commercial blood vaccine. Vaccination of Angora goats against heartwater using the inactivated Zimbabwean Mbizi strain provided wide protection against Zimbabwean strains and the local South African Bathurst strain [14]. However, this vaccine did not offer good protection under field conditions in South Africa. All Angora goats that were immunized i.v. and i.m. with the attenuated *E. ruminantium* Welgevonden vaccine in the current study were fully protected against a virulent homologous needle as well as an infected tick feeding challenge. This confirmed the results of a previous preliminary experiment [23]. These authors have shown that all sheep immunized with the attenuated vaccine were subsequently found to be fully protected against a lethal needle challenge with the virulent homologous stock or with one of four different heterologous stocks (Ball 3, Gardel, Mara 87/7, Blaauwkrans). The current study showed that the attenuated *E. ruminantium* Welgevonden vaccine stimulates a protective immune response that protects against natural tick challenge. This is a significant finding as the Welgevonden strain has a broader cross-protection spectrum than the Ball 3 strain [8], [23], [32]. Further work is needed to test the attenuated vaccine for protection against heterologous tick-derived challenge.

The previous vaccine dose of 10^5 EBs had been reported to be safe and effective in Boer goats when administered i.v. or i.m. [26]. Our results show that 70% and 9.7% of Angora goats vaccinated i.v. (10^4) or i.m. (10^5 EBs), respectively, had severe post immunization reactions, which necessitated treatment upon which the animals recovered. This illustrates that the i.m. route of administration is safer than the i.v. route. In the present study we have also shown that the vaccine dose used (10^5 EBs) is not optimal in Angora goats and a dose titration study was performed using the intramuscular route of administration in order to

determine the optimal effective dose to achieve a 100% safety margin rather than the 90.3% obtained. The results of the vaccine titration met this vaccination target and the optimum vaccine dose determined was 10^4 EBs administered i.m. It is significant that the vaccine dose 10^4 EBs administered i.m. was safe and protected all sheep and goats against virulent homologous needle challenge or infected tick challenge. These results show the feasibility of using one commercially produced vaccine for both species.

Immunization using different vaccine doses (10^2, 10^3, 10^4) inoculated i.v. indicated that Angora goats suffered more than sheep (6/6 vs 0/6), confirming the greater susceptibility of Angora goats to heartwater compared to sheep. In contrast, i.v. challenge of the groups of animals which received 10^2 and 10^3 vaccine doses i.m. resulted in sheep suffering more than goats (10/10 vs 3/10). This suggested that these lower doses did not confer immunity in all sheep but conferred partial (70%) immunity in Angora goats.

Cattle could be safely vaccinated i.m. using a range of vaccine doses (10^5-10^7 EBs) although 2 out of 10 immunized animals receiving doses of 10^5 and 5×10^5 EBs experienced moderate disease reactions after needle challenge and recovered without treatment. However, i.m. and i.v. vaccination of 26 animals with doses of 10^6 at different times after thawing led to a moderate response post challenge in only one animal. Therefore, the vaccine dose given i.m. is the preferred and safer route of application under field conditions. Alternatively, using 10^5 EBs as a vaccine dose may require closer monitoring of animals during natural field challenge. In the simulated field tick challenge, vaccinated cattle were fully protected against the virulent tick transmitted Welgevonden strain. Unexpectedly, 2 out of 10 of the unvaccinated control group did not show any signs of clinical reactions to tick challenge. One animal tested serologically negative before and after tick challenge and the other bovine was serologically positive on arrival and tested negative by day 25 and before receiving the tick challenge. The number of ticks engorged and dropped from these two animals was found not significantly different from the average for the group.

The performance of cattle after vaccination with higher vaccine doses inoculated i.v. in this study have shown a higher resistance to heartwater infection than sheep and goats. Sixty percent (3/5) of sheep that received an i.v. vaccine dose of 10^6 EBs required treatment [26]. Moreover, the inoculation of susceptible cattle with the virulent *E. ruminantium* Welgevonden, which was lethal for sheep and goats, did not produce severe reactions in cattle (Combrink personal observation). In the present study, the challenge material for cattle was obtained from the blood of a clinically reactive cow after challenge using infected ticks. These results support the general belief that Angora goats and sheep are more susceptible than cattle in endemic heartwater areas [36].

The sensitivity of the IFAT enables the detection antibodies in the serum of animals that have been infected with *E. ruminantium*, either via natural infection on the farms or to monitor the course of the disease after artificial infections. The IFAT, however, has its limitations as it was reported to show cross reactions with antibodies against related Ehrlichia spp. and Anaplasma spp., resulting in the common occurrence of false positive results [23], [29], [37]. Non-specific cross-reactivity in the IFAT or by the PC-ELISA was related to cross-reactions with *Rickettisa conorii* or to other Ehrlichia spp. as demonstrated experimentally in cattle [29], [38]. In the present study, the IFAT gave positive results for 6.5%, 9.1% and 15% of sheep, Angora goats and cattle, respectively, in animals that had been obtained from farms from areas non-endemic for heartwater in the Free State, South Africa, considered free of the tick vector [23]. All unvaccinated control sheep and Angora goats challenged with virulent

homologous *E. ruminantium* Welgevonden via i.v.(10/10) or infected adult ticks (9/9) developed severe temperature reactions, displayed clinical signs and all required treatment including the ones which were serologically positive on arrival. Only one bovine, which was serologically positive on arrival to the laboratory, tested negative by Day 25, and before receiving the tick challenge. This animal did not react to the tick challenge and remained seronegative. Overall, of the three species of animals tested positive on arrival (total 10), only one bovine did not react to tick challenge. These results from sheep, Angora goats and cattle confirmed their susceptibility to heartwater infection. Therefore, serologic tests should not be used as the sole method for the establishment of a definitive diagnosis of heartwater in areas endemic for these other pathogens.

5. Conclusions

In conclusion, the attenuated *E. ruminantium* Welgevonden tissue culture vaccine against heartwater proved to be safe and efficacious using the i.m. route of immunization in sheep, Angora goats and cattle. No post vaccination related reactions were observed in these animal species. The optimum vaccine doses have been determined through vaccine titration using the i.m. compared to the i.v. route of administration. In addition, the i.m. route is not only safer but also more convenient to use than the i.v. route. Having to administer the vaccine i.v., is one of the factors that currently limit the commercial use of the blood vaccine across the country. The study also demonstrated for the first time the efficacy of immunization of cattle using the attenuated heartwater vaccine. In addition, the vaccine protected sheep, Angora goats and cattle against homologous challenge by experimental tick transmission, a major impediment in the development and efficacy of subunit vaccines. The attenuated Welgevonden tissue culture vaccine was shown to provide protection by needle challenge against all locally known strains tested and against a virulent exotic strain [23]. Further work is needed to test the attenuated vaccine for protection against heterologous tick-derived challenge. *Amblyomma hebraeum* adults fed as nymphs on sheep previously immunized with the attenuated *E. ruminantium* Welgevonden vaccine were able to transmit the attenuated vaccine to a susceptible sheep, which was found to be protected against a subsequent lethal homologous needle challenge [23]. Under farming conditions, this safe tick transmission from immune to susceptible animals would ensure the development and maintenance of endemic stability in the herd.

Contribution

AAL conceived the study, directed research and wrote the manuscript; HCS performed vaccine trial experiments in sheep and Angora goats, conducted qPCR experiments and wrote the manuscript; AIJ performed cell culture experiments and wrote the manuscript; RDM performed vaccine trial experiments in cattle; AP performed quantification of EBs using flow cytometry; PCT performed cattle based experiments; MPC performed cattle based experiments and designed the experimental approach; LCM was involved in all aspects of the study; AH performed vaccine trial experiments in sheep and Angora goats; SM performed animal based experiments; EZ was involved in experimental design, vaccine trials and writing of the manuscript; BJM was involved in experimental design, supervision of vaccine trials and writing of the manuscript.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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