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Vaccine 25 (2007) 7939–7947
Vaccine
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# Immunisation of sheep against heartwater in The Gambia using inactivated and attenuated *Ehrlichia ruminantium* vaccines

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Received 10 May 2007; received in revised form 29 August 2007; accepted 2 September 2007
Available online 20 September 2007

**Abstract**

Heartwater (cowdriosis) is a disease of ruminants caused by a rickettsial pathogen *Ehrlichia ruminantium* and transmitted by ticks of the genus *Amblyomma*. The purpose of this work was to evaluate the protective efficacies of inactivated and attenuated vaccines to protect sheep against heartwater in The Gambia. An inactivated vaccine, prepared from *E. ruminantium* (Gardel stock), and a live attenuated vaccine from *E. ruminantium* (Senegal stock), were evaluated in two independent on-station trials. A local stock of *E. ruminantium* (Kerr Seringe) was used as challenge material. Inactivated and live attenuated vaccines provided 43% and 100% protection, respectively, against virulent needle challenge. In a subsequent field trial, the attenuated vaccine protected 75% of sheep against virulent tick challenge, which was fatal for all control sheep. Quantification by real-time PCR showed that an immunising dose of approximately 23,000 attenuated *E. ruminantium* organisms was sufficient. Moreover, restriction fragment length polymorphism (RFLP) analysis indicated that the local Kerr Seringe genotype caused mortality amongst control sheep, whereas fatalities in the vaccinated group could be attributed to a different genotype.


*Keywords:* Heartwater; Sheep; Inactivated vaccine; Attenuated vaccine; The Gambia

## 1. Introduction

Heartwater, caused by *Ehrlichia ruminantium* and transmitted by ticks of the genus *Amblyomma*, is a disease of major economic importance affecting cattle and small ruminants, in sub-Saharan Africa and the Caribbean [1–3]. Recent surveys in West Africa indicated the existence in some areas of high levels of heartwater disease risk for susceptible livestock introduced from non-endemic areas [4,5]. The disease can be controlled by a combination of acaricides [6], chemotherapy and immunophylaxis using an infection and treatment method [7]. The latter method is based on the virulent Ball 3 stock employed in South Africa, where it does not provide a broad protection against field isolates. The use of chemotherapy is constrained by the acute nature of the disease, which

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doi:10.1016/j.vaccine.2007.09.002

often does not allow timely intervention to prevent a fatal outcome. The application of acaricides by spraying or dipping of livestock can be effective, but carries the risk of contaminating the food chain. This is further complicated by the ability of ticks to develop resistance to the acaricides [8]. Hence, immunisation using a safe, effective and affordable vaccine appears to be the most desirable and sustainable control option.

Several attempts which have been made include the development of inactivated vaccines, attenuated vaccines and also DNA vaccines [9,10]. Although progress has been made, the antigenic diversity identified amongst different stocks of *E. ruminantium* resulting in a lack of protection between heterologous stocks, has been identified as a major obstacle in vaccine development. Results of field trials on the efficacy of inactivated vaccines in sub-Saharan Africa vary [10]. For instance, an inactivated vaccine prepared from the Mbizi stock of *E. ruminantium* provided protection to field challenge at specific locations [11]; trials in West Africa with inactivated *E. ruminantium* (Gardel) vaccine have been less satisfactory (A. Gueye, personal communication).

Attempts to develop a live attenuated vaccine were first reported using the Senegal stock of *E. ruminantium*, which became attenuated after 11 passages *in vitro* [12]. Although this stock provided complete protection against homologous challenge [12], it did not provide efficient cross-protection against virulent heterologous challenge on station and in the field [13,14]. However, in a recent report, the South African Welgevonden stock of *E. ruminantium* was successfully attenuated *in vitro* [9] and was able to protect sheep against challenge with four virulent heterologous stocks [15].

In this study, the protective efficacy of inactivated and live attenuated *E. ruminantium* vaccines against heartwater was evaluated in Sahalian sheep in The Gambia under a smallholder traditional husbandry system.

## 2. Materials and methods

### 2.1. In vitro cultivation of E. ruminantium (Kerr Seringe): challenge material

The *E. ruminantium* (Kerr Seringe) isolate was made from a naturally infected West African dwarf goat (#1946) reared at the International Trypanotolerance Centre (ITC), Kerr Seringe, The Gambia. Whole blood collected into vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA) as anticoagulant (Becton-Dickson®), just before death due to heartwater (confirmed by identification of *E. ruminantium* in Giemsa-stained brain smears), was cryopreserved at −80 °C in 2 ml aliquots with 10% dimethyl sulfoxide (DMSO) as cryoprotectant. One of the aliquots was used to inoculate a Saanen goat (#4639) at the Institute of Tropical Medicine in Antwerp, Belgium, and a blood stabilate prepared from this goat during the febrile response was used to infect a naïve female Texelaar sheep (#229) at the Faculty of Veterinary Medicine, Utrecht University, The Netherlands. Aliquots of blood collected into EDTA from sheep #229 on the second day of the febrile response, with 10% DMSO as cryoprotectant, were snapfrozen into liquid nitrogen for subsequent use in a vaccination experiments carried out in The Gambia. Other ampoules were used to infect bovine aorta endothelial (BAE) cell cultures in 25 $cm^2$ flasks and the infected culture was maintained at 37 °C in *E. ruminantium* growth medium, Glasgow minimal essential medium (GMEM), supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μl/ml streptomycin, 2.6 g/l tryptose phosphate broth, HEPES buffer (20 mM) pH 7.0–7.2 and 10% foetal calf serum [12]. When the monolayer showed signs of infection 3 weeks later, Giemsa-stained cytocentrifuge smears were prepared from the supernate to confirm the presence of *E. ruminantium*. Subsequently, the culture medium containing elementary bodies was centrifuged for 15 min at 4 °C and 15,000 × *g*. The resulting pellet was resuspended in sucrose–phosphate–glutamate (SPG) buffer and ampoules of 2 ml were filled and stored in liquid nitrogen until further used in vaccination trials. A sample of the infected culture tested by PCR gave specific amplicons using *E. ruminantium map1* and pCS20 primers [20].

### 2.2. In vitro production of attenuated E. ruminantium (Senegal stock)

The attenuated stock was derived from the virulent parent, the Senegal stock, after serial *in vitro* cell culture passages [12]. Briefly, cultures containing extracellular elementary bodies were used to passage *E. ruminantium* onto other bovine umbilical endothelial (BUE) cell cultures with an average interval of 13.9 days between passages. BUE culture supernatant (20 ml) heavily infected with elementary bodies of *E. ruminantium* of passage nos. 11, 14 and 16 proved highly immunogenic in goats and sheep without any clinical signs [12]. *E. ruminantium* (attenuated Senegal) at 72nd passage was grown in BUE cell cultures as described previously [12]. Bacterial growth was monitored by microscopic examination of Giemsa-stained cytocentrifuge smears. When *E. ruminantium*-infected BUE cultures showed about 90% of cytolysis due to infection, the remaining adherent cells were scraped from the bottom of the culture flasks and the cultures centrifuged at 15,000 × *g* for 20 min at 4 °C. The resultant pellet containing both infected cells and free *E. ruminantium* organisms was resuspended in SPG and snapfrozen into liquid nitrogen until used in the vaccination experiments.

### 2.3. Origin, maintenance and monitoring of experimental animals

Sheep used in all vaccination trials were of Sahelian breed aged between 18 and 30 months and were obtained from an *Amblyomma* and heartwater-free area (Lingerr) in northern Senegal [16]. All animals were tested for *E. rumi-*

*nantium* infection by indirect MAP1-B ELISA [17,18] and pCS20 PCR [19] using a nested approach [20] prior to the start of each trial. Animals were dewormed *per os* using Albendazole®, and quarantined for 2 weeks to allow clinical manifestation of possible latent infections and were vaccinated against endemic diseases (Peste de Petit Ruminants (PPR) and Pasteurellosis) at least 2 weeks prior to the start of each trial. On station, animals were maintained on groundnut hay with occasional supplementation with rice bran and watered twice daily. The sheep were maintained tick-free in an insect and tick-proof stable or on tick-proof concrete raised platform under a shed according to the protocol of each experiment with daily cleaning of the housing. In the field trial, exposure to tick challenge was accomplished by allowing animals to graze during the day in the bush in the perimeter and outside the perimeter of ITC, Kerr Seringe, where heartwater is known to occur [5] and at night they were housed in pens.

For all experiments, rectal temperatures were recorded daily and a temperature of 40 °C or above was considered to be fever. The incubation period, time to death and number of fatal cases were recorded for each group of sheep. Serum samples were obtained from the sheep prior to vaccination and thereafter weekly to determine seroconversion and monitor *E. ruminantium* antibody responses by MAP1-B ELISA. All deaths were confirmed for heartwater by *post mortem* examination for lesions typical of heartwater (hydropericardium, hydrothorax and ascites), Giemsa staining of brain-crushed smears for identification of *E. ruminantium* colonies inside brain capillary endothelial cells [21] and detection of *E. ruminantium*-specific pCS20 sequences [22] using nested PCR in brain tissue DNA extracts (for attenuated vaccine trials) as described above. The trials ended 30 or 35 days after challenge infection for the inactivated and attenuated vaccination trials, respectively; and 150 days of field exposure in the attenuated vaccine field trial.

### 2.4. On-station trial of inactivated vaccine

#### 2.4.1. Preparation of E. ruminantium inactivated vaccine

The inactivated vaccine was bulk-produced from endothelial cell culture at the Institute for Biological and Experimental Technology (IBET), Lisbon, Portugal. The purified antigen was obtained using a multistep centrifugation process [23]. In brief, *E. ruminantium* (Gardel)-infected endothelial cell suspension was harvested when 80–90% of the cell monolayer was lysed, and passed through a syringe and 26 gauge needle to disrupt the cell clumps and free the intracellular bacteria. The cellular debris was removed by centrifugation at $1800 \times g$ for 15 min at 4 °C. The resulting supernatant was subjected to high-speed centrifugation ($20{,}000 \times g$) for 30 min at 4 °C to pellet the bacteria and the pellet was resuspended in PBS (pH 7.4). The protein content of the *E. ruminantium* suspension was measured with a MicroBCA kit 23235 (Pierce, USA) using the 96-well protocol according to the manufacturer's instructions. The inactivated vaccine stock was obtained by overnight incubation of the *E. ruminantium* suspension at 4 °C with 0.1% sodium azide and was stored at −20 °C until used. The vaccine was emulsified in an equal volume of adjuvant (Montanide ISA50; Seppic, Paris, France) immediately before use.

#### 2.4.2. Animals, immunisation and challenge inoculations

Thirteen sheep were randomly assigned to two groups. On day 0, seven sheep were inoculated subcutaneously in the scapular region with 2 ml of inactivated *E. ruminantium* vaccine with adjuvant prepared as above; six sheep received a placebo consisting of adjuvant in PBS. Thirty days after the first vaccination, each sheep in group 1 was given a second vaccination of 2 ml of inactivated vaccine with adjuvant prepared as above. All sheep received a heterologous challenge of 2 ml of *E. ruminantium* (Kerr Seringe) blood stabilate intravenously 3 weeks after the booster vaccination. Buffy coat samples were prepared from 5 randomly chosen animals (#1838, #1827, #1835, #1828, #1834) during the febrile reaction and were tested by nested pCS20 PCR to confirm *E. ruminantium* infection status. The experimental parameters were recorded as described above.

### 2.5. On-station trial of attenuated vaccine

Twelve naïve sheep were randomly assigned to two groups of six. Group 1 sheep, composed of 6 sheep, was inoculated intravenously with 1 ml of cryopreserved BUE-culture-derived *E. ruminantium* (attenuated Senegal) at the 72nd passage [12] diluted after thawing 1:30 in SPG buffer; sheep in group 2 each received 1 ml SPG buffer administered intravenously. Six weeks post vaccination all sheep were challenged by intravenous inoculation of 1 ml of culture-derived *E. ruminantium* (Kerr Seringe, passage 2) containing $1.4 \times 10^7$ organisms. Monitoring of experimental parameters was carried out as described above.

### 2.6. Field trial of attenuated vaccine

Twenty four naïve sheep were randomly assigned to two groups of 12 each. Group 1 sheep were each inoculated intravenously with 1 ml of *E. ruminantium* (attenuated Senegal) as above except that the culture stabilate was diluted 1:200 in SPG buffer to an immunising inoculum of approximately 23,000 organisms. Four weeks post immunisation all sheep were exposed to natural field tick challenge for 5 months (January to May); and were examined individually fortnightly to confirm infestation by *A. variegatum* ticks. All fatalities were confirmed as decribed above. At *post mortem*, the number of *A. variegatum* ticks on each sheep was counted.

### 2.7. Quantification of the attenuated vaccine inoculum by real-time PCR

The *map1-1* gene (GenBank accession no. AY652746) was selected as the target to quantify the estimated number of *E. ruminantium* (attenuated Senegal) organisms contained in the vaccine dilutions; 1:30 used on-station and 1:200 used for the field trial. The real-time PCR [24] was conducted as recently described by Postigo et al. [25] using MyiQ™ (Bio-Rad). Primers specific for *map1-1* were used to amplify a fragment of 182 bp from the test DNA sample and from the positive control plasmid, PBAD/Myc-His (Invitrogen), containing the *map1-1* gene. Briefly, the reaction mixture used, in a 25-μl volume, was as follows: 12.5 μl of SYBR Green supermix (Bio-Rad), 1 μl each of the forward and reverse primers and 5 μl of template. The cycling programme consisted of: 95 °C for 15 min; and cycles of denaturing, 95 °C for 30 s; annealing, 55 °C for 30 s; and extension, 72 °C for 30 s, repeated 40 times. Ct values were determined and melting graphs analysed accordingly using the iCycler software.

### 2.8. Restriction fragment length polymorphism (RFLP) analysis of genotypes

To examine whether the *E. ruminantium* stocks that caused mortality in the study animals were genetically different, we carried out a restriction enzyme profile analysis targeting *map1* using DNA extracts from brains of the sheep that were shown to have died of heartwater. The *map1* gene is conserved in all known stocks of *E. ruminantium* [26–29], exhibits a high degree of sequence polymorphism among different isolates [26,30], and is therefore considered suitable for characterization of genotypic diversity [31,32]. In addition, the attenuated Senegal and the Kerr Seringe stocks were also subjected to RFLP analysis to demonstrate possible genotypic differences between them. A *map1* nested PCR was initially carried out as described previously [20]. PCR amplification products were subjected to RFLP analysis according to the protocol described by Faburay et al. [33]. Briefly, PCR amplification products obtained from nested *map1* PCR were subjected to *Alu*I restriction enzyme analysis as recommended by the manufacturer (New England Biolabs®). Four microlitres of the digested sample was mixed with 2 μl of loading buffer and loaded onto a 10% polyacrylamide gel. A 100 bp ladder was included to determine the fragment size. DNA fragments were separated by horizontal electrophoresis in 1× TBE buffer at 100 V for 2 h 40 min. The gel was subjected to SYBR® green (Cambrex Bio science Rockland Inc.) staining according to the manufacturer's instructions and visualized under ultraviolet illumination. The gels were photographed using a digital camera (NikonE4500, Nikon Corp.) fitted with a green filter.

### 2.9. Statistical analysis

The mortality/survival rate of the vaccinated and control animals in each vaccination trial were compared by applying Fisher's exact test. To determine differences in incubation period and time to death between the different groups, a two-sample *t*-test with equal variances, was used.

Kaplan–Meier survival estimate, by group, was used to analyse the comparative survival of sheep in the vaccinated and control groups exposed to field tick challenge.

## 3. Results

### 3.1. In vitro isolation and cultivation of E. ruminantium (Kerr Seringe)

Microscopic examination of Giemsa-stained smears of culture supernatant prepared on day 22 after inoculation revealed the presence of *E. ruminantium* and confirmed the successful isolation of the organism in cell culture (data not shown). The *map1* and pCS20 PCRs also amplified target sequences and gave specific amplicons (data not shown). The *map1* coding sequence of the Kerr Seringe stock, named after

Table 1
Clinical reactions of sheep immunised with inactivated *E. ruminantium* (Gardel) vaccine to heterologous needle challenge with *E. ruminantium* (Kerr Seringe)

| Sheep number | Group | Incubation period (days) | Maximum temperature (°C) | Time to death (days) | Outcome |
|---|---|---|---|---|---|
| 1827 | V | 10 | 41.3 | – | Survived |
| 1828 | V | 10 | 41.1 | 19 | Fatal heartwater |
| 1829 | V | 16 | 41.4 | 26 | Fatal heartwater |
| 1830 | V | 10 | 41.0 | 20 | Fatal heartwater |
| 1835 | V | 10 | 40.3 | – | Survived |
| 1838 | V | 11 | 40.9 | 18 | Fatal heartwater |
| 1841 | V | 12 | 41.3 | – | Survived |
| 1826 | C | 10 | 41.5 | 24 | Fatal heartwater |
| 1831 | C | 14 | 41.5 | 23 | Fatal heartwater |
| 1832 | C | 14 | 41.2 | 24 | Fatal heartwater |
| 1834 | C | 10 | 41.4 | 21 | Fatal heartwater |
| 1836 | C | 8 | 40.8 | 22 | Fatal heartwater |
| 1840 | C | 14 | 40.5 | 22 | Fatal heartwater |

V: vaccinated; C: control.

Table 2
Clinical reactions of sheep to immunisation with attenuated *E. ruminantium* (Senegal) vaccine and heterologous needle challenge with virulent *E. ruminantium* (Kerr Seringe)

| Sheep no. | Group | Immunisation | | | Challenge | | | Outcome |
|---|---|---|---|---|---|---|---|---|
| | | Incubation period (days) | $t_{max}$ (°C) | Duration of fever (days) | Incubation period (days) | $t_{max}$ (°C) | Duration of fever[a] (days) | |
| 1251 | C | – | – | – | 18 | 41.3 | 1 | Fatal hw[b] |
| 1252 | C | – | – | – | 16 | 40.1 | 1 | Fatal hw |
| 1253 | C | – | – | – | 12 | 41.7 | 5 | Fatal hw |
| 1255 | C | – | – | – | 11 | 41.3 | 2(2)5 | Fatal hw |
| 1267 | C | – | – | – | 11 | 41.9 | 6 | Fatal hw |
| 1269 | C | – | – | – | 14 | 41.1 | 2 | Fatal hw |
| 1259 | V | 5 | 41.4 | 4 | – | – | – | Survived |
| 1260 | V | 6 | 41.1 | 2 | – | – | – | Survived |
| 1262 | V | 7 | 41.2 | 5 | – | – | – | Survived |
| 1268 | V | negative | – | – | – | – | – | Survived |
| 1270 | V | negative | – | – | – | – | – | Survived |
| 1271 | V | negative | – | – | – | – | – | Survived |

C: control; V: vaccinated.
[a] Numbers in brackets indicate fever-free periods.
[b] Heartwater.

the location of origin, was cloned, sequenced and submitted to GenBank and assigned accession no. DQ333230 [5].

### 3.2. Inactivated vaccine trial

#### 3.2.1. Antibody responses, rickettsaemia and responses to heterologous challenge

Prior to the start of the experiment, all animals were confirmed seronegative for *E. ruminantium* infection by MAP1-B ELISA. Five out of 7 sheep were seropositive 2 weeks after the first vaccination; sheep #1841 seroconverted 2 weeks after the booster vaccination, while sheep #1829 remained seronegative. All five sheep tested by the nested pCS20 PCR assay during the febrile reaction were positive, indicating development of rickettsaemia (data not shown). A summary of the outcome of the virulent heterologous challenge using the local Kerr Seringe stock is shown in Table 1. All sheep in the vaccinated group reacted with maximum temperatures between 40.3 and 41.4 °C, and all control sheep reacted with maximum temperatures between 40.5 and 41.5 °C. The incubation period did not differ between both groups ($P = 0.7830$); the vaccinated animals showed a mean incubation period of 11.3 days and the controls a mean of 11.8 days. The time to death did not differ significantly between groups ($P = 0.3097$). Similarly, mortality rate in both groups was statistically not significantly different ($P = 0.192$), with four and six (all) deaths in the vaccinated and control groups respectively (Table 1).

### 3.3. Attenuated vaccine trial (on-station)

#### 3.3.1. Antibody profile and responses to heterologous challenge

Each sheep in the vaccinated group was inoculated with approximately 153,000 *E. ruminantium* organisms determined by real time PCR. All vaccinated sheep seroconverted as determined by MAP1-B ELISA within 3 weeks after vaccination. All sheep in the control group remained seronegative. Three sheep in the vaccinated group (#1259, #1260 and #1262) reacted transiently to the immunising inoculum with elevated temperatures for 2–5 days (Table 2). Following challenge with the virulent Kerr Seringe stock, all sheep in the control group showed hyperthermia with maximum temperatures ranging from 40.1 to 41.9 °C (Table 2). The duration of fever varied between 1 and 6 days. None of the vaccinated sheep developed fever during the 5-week observation period. Mortality rates differed significantly between the two groups ($P = 0.002$); all six sheep in the control group succumbed to the infection, whereas none in the vaccinated group died (Table 2). All sheep, which died, exhibited *post mortem* lesions typical of heartwater and were confirmed positive for *E. ruminantium* infection in brain-crush smears and by nested pCS20 PCR on brain tissue DNA extracts.

### 3.4. Attenuated vaccine trial (field trial)

The MAP1-B serology showed that all 12 sheep in the vaccinated group had seroconverted when tested 3 weeks after receiving the vaccine, which consisted of approximately 23,000 *E. ruminantium* organisms. Unlike the dose used in the on-station vaccine trial (153,000 organisms), no apparent clinical or febrile reaction was seen in the vaccinated animals. It was found that all sheep became infested during the first 2 weeks of exposure with ticks, mainly *A. variegatum* nymphs. All 12 sheep in the control group reacted with pyrexia to natural challenge, whereas 7 out of 12 vaccinated sheep showed temperature reactions (Table 3). The mean incubation periods were not significantly different ($P = 0.865$); 16.9 days (S.D. = 1.77) in the vaccinated group and those in the control group had a mean of 16.6 days (S.D. = 2.15). Mortality rates between both groups differed significantly ($P < 0.001$) over the 150-day observation period; 3 out of 12 sheep died

Table 3
Clinical reactions of sheep immunised with attenuated *E. ruminantium* (Senegal) vaccine and exposed to natural *E. ruminantium* tick challenge

| Sheep. no. | Group | Incubation period (days) | $t_{max}$ (°C) | Duration of fever[a] (days) | Outcome (day of death) |
|---|---|---|---|---|---|
| 626 | C | 17 | 40.9 | 1 | Fatal heartwater (day 19) |
| 629 | C | 19 | 40.4 | 1(4)1(1)2 | Fatal heartwater (day 29) |
| 631 | C | 15 | 41.3 | 3 | Fatal heartwater (day 21) |
| 634 | C | 17 | 41.2 | 4 | Fatal heartwater (day 23) |
| 635 | C | 18 | 41.4 | 1(1)1 | Fatal heartwater (day 27) |
| 637 | C | 14 | 41.5 | 3 | Fatal heartwater (day 23) |
| 638 | C | 18 | 40.5 | 3 | Fatal heartwater (day 24) |
| 640 | C | 15 | 41.3 | 7 | Fatal heartwater (day 23) |
| 641 | C | 12 | 41.1 | 2 | Fatal heartwater (day 14) |
| 642 | C | 19 | 41.4 | 3 | Fatal heartwater (day 23) |
| 644 | C | 17 | 40.8 | 2(1)1 | Fatal heartwater (day 21) |
| 645 | C | 18 | 41.0 | 5 | Fatal heartwater (day 25) |
| 627 | V | 18 | 40.5 | 3(5)1 | Survived |
| 628 | V | 15 | 41.1 | 7 | Survived |
| 630 | V | 19 | 41.2 | 4 | Fatal heartwater (day 24) |
| 632 | V | 15 | 40.7 | 1(2)3 | Fatal heartwater (day 21) |
| 633 | V | 15 | 40.7 | 6 | Survived |
| 636 | V | 18 | 40.1 | 2(1)5 | Survived |
| 639 | V | – | – | – | Survived |
| 643 | V | 18 | 40.1 | 1(6)1(11)1 | Fatal heartwater (day 40) |
| 646 | V | – | – | – | Survived |
| 647 | V | – | – | – | Survived |
| 648 | V | – | – | – | Survived |
| 649 | V | – | – | – | Survived |

C: control; V: vaccinated.
[a] Numbers in brackets indicate fever-free periods.

in the vaccinated group, whereas all 12 sheep in the control group died (Table 3). There was no significant difference in time to death between both groups ($P = 0.214$); sheep in the vaccinated group showed a median of 24 days and an interquartile range of 21 to 40 days, whereas sheep in the control group showed a mean of 23 days (S.D. = 3.82). Survival analysis (Fig. 1) showed that, on average, sheep in the control group survived for a shorter period in the field compared to those in the vaccinated group. In contrast to vaccinated sheep, all sheep in the control group died before the time point of day 29. In the latter group, the first time point of death occurred earlier at day 14, compared with day 19 in the former group, and the last time point of death occurred on day 30. The vaccinated group showed a survival rate of 0.75 at the end of the study (Fig. 1). *Post mortem* examination revealed that all sheep were infested with partly engorged *A. variegatum* nymphs with the level of infestation on individual animals ranging between 12 and 27 ticks. All sheep which died showed *post mortem* lesions typical of heartwater and were confirmed positive for *E. ruminantium* infection in brain-crush smears and/or by nested pCS20 PCR on brain tissue DNA extracts.

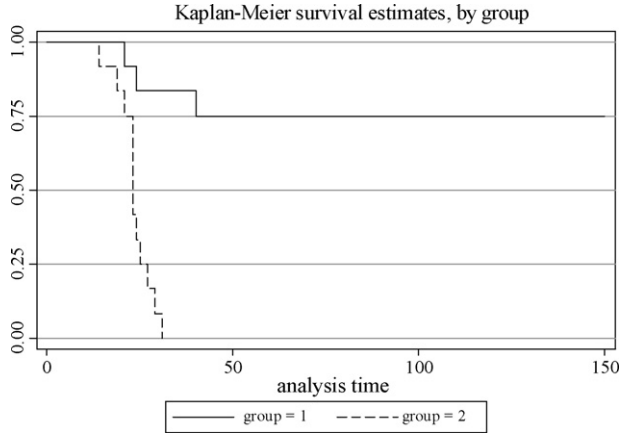


Fig. 1. Comparative survival of vaccinated and control sheep exposed to field tick challenge: Kaplan–Meier survival estimates. *Key*: Group 1, vaccinated; group 2, control.

### 3.5. Restriction fragment length polymorphism of *E. ruminantium* genotypes

Fig. 2 shows the restriction enzyme profiles of the *E. ruminantium map1* genotypes recovered from control and vaccinated sheep, which died in the attenuated vaccine field trial. All profiles from the control sheep (with the possible exception of #642) were similar to the profile of the Kerr Seringe stock, which contained 140 and 230 bp bands, suggesting that the latter may be the primary cause of death in these animals. In comparison, the profile of the attenuated Senegal stock is quite different, since it contains a unique 250 bp band. Restriction profiles from vaccinated sheep (#630, #632 and #643), which died upon challenge, lack the 160 bp band which is present in the control sheep and in the Kerr Seringe stock, which indicates that mortality in the immunised animals was caused by a different genotype (Fig. 2).

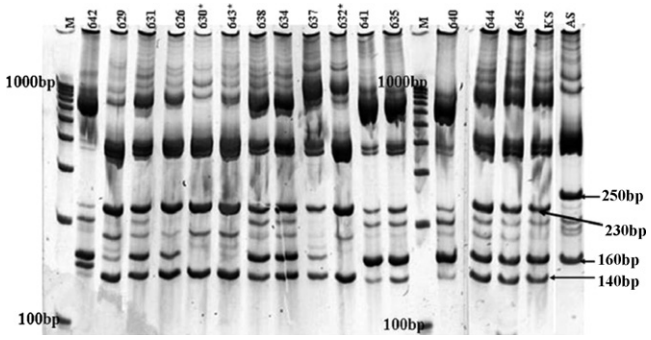


Fig. 2. Restriction fragment length polymorphisms of *E. ruminantium map1* from brains of sheep that died from heartwater in the attenuated vaccine field trial. Number 630*, 643* and 632* correspond to profiles from immunised sheep; the other profiles are from control sheep; KS: profile from *E. ruminantium* (Kerr Seringe) strain, AS: profile from *E. ruminantium* (Attenuated Senegal); M: 100 bp marker. Profiles from the three vaccinated animals (#630, #643, #632) which died upon challenge, lack a 160 bp band. The attenuated vaccine stock (AS) contains a unique 250 bp band, but lacks a band of 140 bp.

## 4. Discussion

Current methods available for evaluation to protect small ruminants in West Africa against heartwater are based on inactivated [11,23,34–36] and live attenuated vaccines [12,15]. In the present study, we evaluated the efficacy of both vaccination methods in laboratory (on-station) and field trials using Sahelian sheep in The Gambia. Prior to the on-station trials, it was necessary to first isolate a local stock of *E. ruminantium* (Kerr Seringe), which was successfully established *in vitro* in bovine endothelial cell culture. Since this stock was found to be virulent for local sheep and the RFLP analysis showed that this genotype is widely distributed in The Gambia [33], it was considered suitable for use as challenge material in the vaccination experiments.

Although a local West African stock such as Kerr Seringe, may have been a more appropriate choice as a basis for an inactivated vaccine, it was not available at the time our study was carried out. Instead, the Gardel stock, which originated from Guadeloupe in the French West Indies [37] was used in the inactivated vaccine trial, which formed part of a wider series of inactivated vaccine studies in West Africa. The data presented here showed that the inactivated Gardel-Montanide ISA50 vaccine provided only partial protection in sheep against heterologous needle challenge with the local Gambian *E. ruminantium* (Kerr Seringe). Although three sheep survived challenge in the vaccinated group (3/7) compared to none in the control group (0/6), none of the clinical parameters measured showed significant differences between groups. All sheep in both groups, including the four animals that survived, reacted clinically to challenge infection accompanied by fever.

Previous vaccination experiments with inactivated Gardel conferred variable levels of protection in goats against homologous needle challenge [34,35], with mortalities between 17% and 50%. In the present experiment, using heterologous needle challenge, we recorded a mortality rate of 57%, which is a significant loss. Moreover, as all vaccinated sheep developed clinical disease following challenge, it is likely that the mortality rate would be even higher in animals maintained under conditions of sub-optimal nutrition and concurrent challenge by other pathogens. The low efficacy of the Gardel stock against the Kerr Seringe stock shown here may have resulted from disparities in antigenic composition between these two *E. ruminantium* isolates. Lack of cross-protection between *E. ruminantium* isolates has been reported previously, and attributed, principally, to antigenic differences [11,38,39].

In the second vaccine trial, the protective efficacy of attenuated *E. ruminantium* (Senegal) stock was evaluated to protect sheep against heartwater induced by needle challenge with *E. ruminantium* (Kerr Seringe). Since the *E. ruminantium* organisms, though attenuated and therefore of low pathogenicity, are alive and therefore potentially tick-transmissible and possibly capable of reverting to virulence, we used a regional local isolate for the immunisation. *In vitro* attenuation has been reported for the Senegal [12], Gardel [40], Welgevonden [9] and Plumtree stocks (D. Mwangi, personal communication); of these only the Senegal stock was appropriate for use in The Gambia, to avoid introduction of geographically exotic *E. ruminantium*. Although initial attempts to develop an attenuated vaccine using the Senegal stock of *E. ruminantium* gave variable results [12,13], our results showed 100% protection against heterologous challenge with none of the immunised sheep reacting clinically, while all sheep in the control group died of heartwater. This suggests that the two *E. ruminantium* stocks are antigenically very closely related.

Moreover, three of the six immunised sheep reacted to the attenuated inoculum with transient hyperthermia lasting 2–5 days. Previous experiments showed an attenuated *E. ruminantium* (Welgevonden) stock provided full protection against lethal needle challenge with four virulent heterologous stocks of *E. ruminantium* [15] and, as in the present study, transient temperature reactions were observed in some sheep upon immunisation. This suggests the need for further reduction or optimization of the number of attenuated *E. ruminantium* in the immunising inoculum.

Several methods for the quantification of *E. ruminantium* for immunisation and challenge have been published recently [23,24,41]. In the present study, real-time PCR [24] was applied to estimate the number of organisms per dose of live attenuated inoculum used to induce immunity in sheep. The technique quantifies the *map1-1* gene, which is a single copy gene [42] and therefore allows a direct estimation of the number of *E. ruminantium* organisms per sample. In the on-station laboratory trial, the quantification showed that sheep were vaccinated with approximately 153,000 attenuated organisms, which caused transient temperature reaction in some animals indicating a need for further reduction in number of attenuated organisms in the immunising inoculum. As a result, in the field trial, sheep were vaccinated

with a lower number of attenuated *E. ruminantium* organisms (approximately 23,000 organisms), which did not evoke any febrile reaction. This number of organisms falls within the estimated range of 3000–500,000 of infective *E. ruminantium* (Welgevonden) organisms shown to induce protective immune response in goats in experiments conducted in South Africa [15]. Further titration of culture material is required to determine the optimal dose which may be lower than used here.

Recent studies in West Africa showed that considerable genetic diversity exists among *E. ruminantium* isolates [32], indicating a significant degree of antigenic diversity in the field, which may jeopardize vaccine development. Therefore we evaluated the protective efficacy of the attenuated Senegal vaccine in sheep exposed to field tick challenge in the same geographic location where the local Kerr Seringe stock originated. The vaccine induced a significant level of protection in sheep against field tick challenge with 75% (9/12) survival of immunised sheep compared to 0% survival of the control sheep.

A further observation made in the field trial concerned the febrile reaction upon exposure to tick challenge in all control sheep, and in the majority of the immunised sheep. Despite this, 75% of the vaccinated animals survived tick challenge. Moreover, all animals had become infested with *A. variegatum* ticks within 2 weeks of exposure in the field. DNA samples obtained from pooled engorged *A. variegatum* nymphs collected from the vaccinated sheep that survived until the end of the study tested positive by nested pCS20 PCR for *E. ruminantium* [20]. This, coupled with the simultaneous febrile response in both vaccinated and control sheep ($P = 0.865$), indicates that all animals were challenged by infected ticks.

Interestingly, 25% (3/12) mortality rate among vaccinated sheep could be attributed to an *E. ruminantium* genotype different from the Kerr Seringe stock (Fig. 2). Eleven out of 12 *map1* restriction profiles obtained from *post mortem* brain samples from the control sheep were similar to the profile of the Kerr Seringe stock (Fig. 2). This suggests that the Kerr Seringe stock is the major cause of mortality in sheep in this area and that the attenuated Senegal stock fully cross-protected against it. However, the outcome of a related field trial in Senegal using the attenuated Senegal isolate at passage 21 was complicated by concurrent infections, ehrlichiosis and anaplasmosis. Of the 30 vaccinated sheep, 13 animals died and *E. ruminantium* was detected only in two sheep, which had previously suffered from ehrlichiosis or anaplasmosis [14].

In conclusion, the present study showed that the inactivated Gardel vaccine provided only partial protection against needle challenge with the local Kerr Seringe stock, possibly due to antigenic differences. Considering the wide diversity of *E. ruminantium* stocks in the field it was considered unlikely that the vaccine would perform well under field challenge and was therefore not further tested. The live attenuated Senegal stock fully cross-protected against heterologous needle challenge, and in a field trial it showed a high level of protection against tick challenge that was lethal for unvaccinated control sheep. The field trial was carried out in small area called Kerr Seringe over a 5-month observation period and should be repeated in other areas of The Gambia.

## Acknowledgements

This work was supported by The European Development Fund, contract no. REG/6061/006, Rothamsted International (UK), The International Foundation for Science (Sweden) in partnership with COMSTECH, Epigenevac project under contract number no. INCO-CT-2005-003713, The Integrated Consortium on Ticks and Tick-borne Diseases (ICTTD-3) financed by the European Union through Coordination Action Project no. 510561, the Utrecht Scholarship Programme (Utrecht University) and The Rural Finance and Community Initiatives Project (IFAD, World Bank), The Gambia. We thank Drs. Dominique Martinez, Nathalie Vachiery and Frederick Stachurski for support in the inactivated vaccine trial. The experiments were approved by the Animal Ethics Committee under number 0303.0803. Bonto Faburay holds a Rothamsted International African Fellowship Award.

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