A user-friendly and scalable process to prepare a ready-to-use inactivated vaccine: The example of heartwater in ruminants under tropical conditions

Isabel Marcelino a,*, Thierry Lefrançois b, Dominique Martinez a, Ken Giraud-Girard a, Rosalie Aprelon a, Nathalie Mandonnet c, Jérôme Gaucherón d, François Bertrand d, Nathalie Vachiéry a

a CIRAD, UMR CMAEE, F-97170 Petit-Bourg, Guadeloupe, France
b CIRAD, UMR CMAEE, F-34398 Montpellier, France
c INRA-URZ, Domaine Ducloux, 97170 Petit-Bourg, Guadeloupe, France
d SEPPIC, 127 Chemin de la Poudrerie, 81100 Castres, France

A R T I C L E   I N F O

Article history:
Received 30 September 2014
Received in revised form 26 November 2014
Accepted 30 November 2014
Available online xxx

Keywords:
Ready-to-use inactivated vaccine
Vaccine quality control
Protection
Field delivery
Thermostability

A B S T R A C T

The use of cheap and thermoresistant vaccines in poor tropical countries for the control of animal diseases is a key issue. Our work aimed at designing and validating a process for the large-scale production of a ready-to-use inactivated vaccine for ruminants. Our model was heartwater caused by the obligate intracellular bacterium Ehrlichia ruminantium (ER). The conventional inactivated vaccine against heartwater (based on whole bacteria inactivated with sodium azide) is prepared immediately before injection, using a syringe-extrusion method with Montanide ISA50. This is a fastidious time-consuming process and it limits the number of vaccine doses available. To overcome these issues, we tested three different techniques (syringe, vortex and homogenizer) and three Montanide ISA adjuvants (50, 70 and 70M). High-speed homogenizer was the optimal method to emulsify ER antigens with both ISA70 and 70M adjuvants. The emulsions displayed a good homogeneity (particle size below 1 μm and low phase separation), conductivity below 10 μS/cm and low antigen degradation at 4 °C for up to 1 year. The efficacy of the different formulations was then evaluated during vaccination trials on goats. The inactivated ER antigens emulsified with ISA70 and ISA70M in a homogenizer resulted in 80% and 100% survival rates, respectively. A cold-chain rupture assay using ISA70+ER was performed to mimic possible field conditions exposing the vaccine at 37 °C for 4 days before delivery. Surprisingly, the animal survival rate was still high (80%). We also observed that the MAP-1B antibody response was very similar between animals vaccinated with ISA70+ER and ISA70M+ER emulsions, suggesting a more homogenous antigen distribution and presentation in these emulsions. Our work demonstrated that the combination of ISA70 or ISA70M and homogenizer is optimal for the production of an effective ready-to-use inactivated vaccine against heartwater, which could easily be produced on an industrial scale.

© 2014 Elsevier Ltd. All rights reserved.

1. Introduction

Animal diseases have tremendous impact on food supply and economic development of low income tropical countries. Therefore, the availability of cheap and stable vaccines is a major challenge. Many vaccines prototypes have been developed but only few of them have undergone real field use. This is the case for heartwater, caused by the obligate intracellular bacterium Ehrlichia ruminantium (ER). Heartwater is one of the most important tick-borne diseases of ruminants, causing mortality rates up to 90%, in sub-Saharan Africa, adjacent Indian Ocean islands and in some Caribbean islands, from which it threatens to spread to the American mainland [1, 2]. This disease has a strong economic impact (up to 46 million dollars per year for the Southern African Development Community) due to the combined cost of acaricide applications, antibiotic treatments, loss of production and loss of animals [1, 2].

Abbreviations: ER, Ehrlichia ruminantium; ISA50, Montanide Incomplete Seppic Adjuvant 50 V2; ISA70, Montanide Incomplete Seppic Adjuvant 70VG; ISA70M, Montanide Incomplete Seppic Adjuvant 70MVG; W/O, water-in-oil; DCS, daily clinical score; TCS, total clinical score; MAP1, major antigenic protein 1; MAP1–6, major antigenic protein 1–6.

* Corresponding author. Tel.: +590 590255995.
E-mail address: isabel.marcelino@cirad.fr (I. Marcelino).

http://dx.doi.org/10.1016/j.vaccine.2014.11.059
0264-410X/© 2014 Elsevier Ltd. All rights reserved.

Please cite this article in press as: Marcelino I, et al. A user-friendly and scalable process to prepare a ready-to-use inactivated vaccine: The example of heartwater in ruminants under tropical conditions. Vaccine (2014), http://dx.doi.org/10.1016/j.vaccine.2014.11.059
Several experimental vaccines (recombinant, attenuated or inactivated) are under development [1] but none is fully effective due to the high antigenic diversity of ER strains [3–7]. The inactivated vaccine (based on chemically inactivated ER elementary bodies) is a good candidate (despite inducing animal morbidity) as it can protect against homologous and some heterologous challenge [8–10] and it can include a cocktail of strains according to the geographical regions to improve vaccine efficacy [11]. This vaccine has other advantages including antigen storage conditions adapted to the field (−20°C or 4°C), easy administration (subcutaneously) and a cost-effective industrial process for antigen production and purification is also available [12,13]. The remaining step for the large-scale distribution of this vaccine is the “emulsion step”.

Indeed, this inactivated vaccine is currently prepared immediately before injection (in the laboratory or even on the field) as a water-in-oil (W/O) emulsion with Montanide ISA50 adjuvant using the syringe-extrusion method [13,14]. This method is probably the most commonly used technique to create W/O emulsions on a small scale [15–17] but it limits the number of doses to be produced. It also has other drawbacks such as the loss of antigens in the syringe, difficulty of mixing, time-consuming and lack of reproducibility. Vortexing and homogenization are two alternative ways to prepare large volumes of vaccine emulsion and have been both successfully used in clinical trials [15,18–20].

Herein, we aimed at developing a process for the large-scale production of a stable ready-to-use inactivated vaccine that could ease vaccine storage and delivery. For this, we evaluated the ability of syringe, vortexing and homogenization to obtain stable W/O emulsions using Montanide ISA 50, 70 and 70M adjuvants. To validate the optimal adjuvant/preparation method, a vaccination trial was performed on goats. We also evaluated the effect of a 4-days cold chain rupture on vaccine stability and efficacy.

2. Materials and methods

2.1. Preparation of emulsions

Inactivated ER elementary bodies (Gardel strain) were prepared as described elsewhere [10,13,21] and resuspended in NaCl (150 mM). The antigens were emulsified with three Montanide ISA adjuvants: ISA50, ISA70 or ISA70M (all from SEPPIC, France). These adjuvants are based on a high grade injectable mineral oil and anhydromannitol octadecenoate (AO) ether surfactant. ISA70M is an improved version of ISA70 and allows increased vaccine stability during stress conditions such as high storage/shipment temperature. Emulsions were prepared at a 50:50 (v:v) ratio with ISA50 and at a 70:30 (w:w) ratio with ISA70 and ISA70M, to obtain a final protein concentration of 35 µg of ER antigen per vaccinal dose in 2 ml emulsion [21].

The W/O emulsions were prepared using (i) the syringe-extrusion method: the adjuvant and ER antigens (ISA+ER) were passed 10 times through a reinforced 22-gauge connector using rubber-free syringes, (ii) vortex: ISA+ER were vortexed in a conical 15-ml tube for 1 h at a maximum speed and (iii) a homogenizer: ISA+ER were mixed at 20,000 rpm with a Ultra Turrax Type T50 (IKA, Germany).

Several ER antigen batches were used for in vitro optimization assays (each experiment was repeated at least twice). Only one ER antigen batch was prepared for the vaccination trial.

2.2. Characterization of emulsions

To ensure the preparation of a stable W/O emulsion, several physico-chemical parameters were analyzed: (i) particle size (using a microscope [22] or a laser scattering granulometer), (ii) conductivity (using a conductivity meter; below 10 µS/cm), the emulsion is W/O) and (iii) “drop test” (which defines that a drop of a well-prepared Montanide ISA emulsion should not disperse when dropped into a beaker of water [15,18]). To analyze the effect of temperature on the stability of emulsions (phase separation), each ISA+ER emulsion was divided in two equal volumes and left undisturbed at 4°C and 37°C during 35 days. The percentage of phase separation was calculated using the following formula = [height of the oil phase/(total height of emulsion sample × initial fraction of oil in emulsion)] × 100 (Fig. 1A) [15].

2.3. Stability of antigens

To analyze the effects of temperature and the emulsifying technique on the stability of ER antigens, each ISA+ER emulsion was divided in two equal volumes and left undisturbed at 4°C and 37°C. At 24 h and 3 weeks post-incubation (Fig. 1B), ER antigens were extracted from emulsions using the benzyl alcohol extraction method [19]. Briefly, benzyl alcohol was added to each emulsion to a final concentration of 10% (v/v), vortexed at the maximum speed for 20 min and centrifuged for 10 min at 16,000 × g. The antigen-containing aqueous phase was transferred to an Eppendorf tube and stored in acetone at −20°C until further analysis. ER antigen degradation profiles were analyzed by Western blot, as previously described [23]. Anti-MAP1 mouse monoclonal antibody was used at 1/2000 and anti-MAP1–6 rabbit polyclonal antibody at 1/3000 [23]. This procedure was also used to assess antigen stability of vaccine batches.

2.4. Vaccination and challenge

Five groups of five goats each received two intramuscular injections one month apart (Supplemental Fig. S1). Group I (control) was injected with an ISA70M+NaCl emulsion prepared with the homogenizer. Group II received the conventional ISA50-based vaccine. Group III was injected with ISA70+ER prepared with the homogenizer. Groups IV and V were vaccinated with ISA70M+ER emulsions prepared with the homogenizer; for Group V only, the vaccines were incubated 4 days at 37°C prior to injection. All emulsions, expect for ISA50, were prepared one month before the primary vaccination and stored at 4°C until use to mimic the stability of the emulsion after preparation and storage at 4°C before delivery. Three months post-boost, all animals were injected intravenously with a lethal dose of ER (Gardel strain, 2.8 × 10⁴ live ER per goat) [13,21].

This study was conducted according to internationally approved OIE standards, under authorizations set forth by the director of the veterinary services of Guadeloupe on behalf of the Prefect of Guadeloupe on August 2006 (authorisation number: A-971–18-01).

2.5. Clinical scores

During the challenge period, the intensity of the disease was quantified using a clinical reaction index, assigned to the different clinical symptoms [13,21] (Supplemental Table S1).

Daily clinical score (DCS) values correspond to the sum of clinical reaction indices per animal and per day. The mean DCSs per group are shown in Fig. 2A. For statistical analysis purposes, these DCSs were added up from day of challenge (D0) to the day of measurement (DX) to calculate the total clinical score (TCS). A square root transformation was then applied to each TCS to normalize residual variances (Supplemental material and method 1). Transformed TCSs from day 10 to day 22 post-challenge were compared between Groups I, II, III, IV and V using a repeated model with the MIXED procedure of SAS software version 8.1 (SAS institute Inc., 1999) (Fig. 2B).

Please cite this article in press as: Marcelino L, et al. A user-friendly and scalable process to prepare a ready-to-use inactivated vaccine: The example of heartwater in ruminants under tropical conditions. Vaccine (2014), http://dx.doi.org/10.1016/j.vaccine.2014.11.059
2.6. Antibody response

Evaluation of the antibody titers (Abs) was carried out on sera from all animals at different time points before and after vaccination using an indirect MAP1-B ELISA [13,24]. Titration was performed three times per sample and the mean of antibody titers and standard deviations were presented in Fig. 3.

3. Results

3.1. Quality control of the new vaccine formulations

3.1.1. Stability of W/O emulsions

All the prepared emulsions were thick, viscous and white with conductivity values below 10 μS/cm. They did not disperse when dropped into a beaker of water, even after gentle stirring of the beaker (data not shown). In particular, ISA70+ER and ISA70M+ER emulsions prepared with the homogenizer were homogeneous with particle-size below 1 μm, being stable up to 6 and 12 months, respectively (Table 1). Heterogeneous emulsions with particles ranging from 1 to 20 μm were obtained using the syringe-extrusion method (using ISA70M) and vortex (using ISA50 and 70M).

Phase separation was observed during emulsion storage. When below 10% of the final volume, it was not considered as critical, being reversible by manually shaking the emulsion. Fig. 1A shows that ISA70 and 70M based-emulsions were stable with no detectable phase separation up to 35 days at 4 °C, independent of the emulsifying technique. At 37 °C, a phase separation above 10% was observed at 29 days post-incubation with emulsions prepared

---

Please cite this article in press as: Marcelino I, et al. A user-friendly and scalable process to prepare a ready-to-use inactivated vaccine: The example of heartwater in ruminants under tropical conditions. Vaccine (2014), http://dx.doi.org/10.1016/j.vaccine.2014.11.059
with the syringe and the vortex. The less stable emulsion was ISA50+ER as phase separation above 10% was observed after 6 days incubation at both 4 and 37 °C (Fig. 1A). Moreover it was not possible to formulate a stable ISA50+ER emulsion when using the homogenizer.

3.1.2. Stability of ER antigens

To analyze the effect of the emulsifying techniques, adjuvants and temperature on ER antigen stability, antigens were extracted from emulsions and analyzed by Western blotting (Fig. 1B). When control antigens (ER+NaCl) were stored during 3 weeks at 4 °C, no changes in protein migration pattern were observed; MAP1 protein was detected at 28, 48 and 90 kDa (corresponding to MAP1 monomer, dimers and trimers) and MAP1–6 at 21 and 31 kDa (MAP1–6 monomers and dimers, respectively), as described elsewhere [23]. Apparent antigen degradation (a smear above 50 kDa) was only observed for MAP1 protein after 3 weeks at 37 °C.

The ER antigens extracted from emulsions presented, in general, a different migration pattern compared to control antigens. Moreover, antigens extracted from ISA70+ER emulsions resulted in a completely different protein migration pattern from the other emulsions. This is possibly due to the benzyl alcohol extraction protocol, as previously reported [19,25]. The results showed that for ISA70+ER emulsion, there is no antigen degradation (MAP1–6 and MAP1) at 4 °C independently of the emulsifying method either after 24 h or 3 weeks. For ISA70+ER emulsions stored at 37 °C during 3 weeks, there was a minor antigen degradation visualized for MAP1, with a smear above 50 kDa. For ISA70M+ER emulsions, MAP1 antigen appeared to be stable at 4 °C even after 3 weeks incubation, whereas at 37 °C a smear was observed from high to low MM,
indicating protein degradation. No degradation of MAP1-6 was observed. For ISA50+ER emulsions, both MAP1 and MAP1-6 antigens were stable after 24 h at 4 °C but were degraded after 3 weeks. At 37 °C, the antigens were partly (MAP1-6) or totally degraded (MAP1) after 24 h or 3 weeks incubation.

3.2. Vaccine efficacy and stability

3.2.1. Protectiveness

To evaluate the suitability of ISA70 and/or ISA70M as alternatives to ISA50 adjuvant, a vaccination trial on goats was performed (Supplemental Fig. S1, Table 2). After the lethal challenge, Groups II and IV presented a 100% survival rate while Groups III and V, 80% (Table 2). All control animals (Group I) died from the challenge, with an incubation period similar to natural infections (11 days) (Table 2, Fig. 2A).

During the infectious challenge, all groups showed significant differences in their clinical scores ($P < 0.01$, Fig. 2A and B). Control animals presented the lowest daily clinical scores because the animals died shortly after hyperthermia (Fig. 2A). Group IV and V (both with ISA70M adjuvant) had the longest hyperthermia period with fever of approx. 42 °C for 9–10 days. These 2 groups presented the shortest incubation period, between 6 and 7 days (Fig. 2A, Table 2). Statistical analysis of TCSs revealed that the disease developed...
faster in Group I than in Group II (P < 0.001), while Group II developed faster than in Group V (Fig. 2B). Groups III and IV had an intermediate speed of development. Between days 10–22 post-challenge, the TCSS were higher in Groups III and V than in Groups II and IV (P < 0.5), reflecting the higher mortality rate in Groups III and V compared to complete recovery in Groups II and IV (Fig. 2B).

3.2.2. Antibody response

Regarding the humoral immune response (Fig. 3), no MAP1-B antibodies were detected in Group I due to early mortality before seroconversion. In vaccinated groups, Abs titers were shown to be very heterogeneous between animals varying from 2 to 6 log10 and 3 to 5.5 log10 in Groups II and V, respectively. Abs titers in Groups III and IV were homogeneous with an average of 4.5 and 4 log10, respectively (Fig. 3).

3.2.3. Vaccine stability

Our results showed that ISA70+ER and ISA70M+ER emulsions were identical to the placebo (ISA70M+NaCl), with a suitable droplet size (below 1 μm), being stable up to 12 months with no critical defects (5% phase separation) at 4 °C (Supplemental Fig. S2). Incubation of the vaccine for one month at 4 °C resulted in some antigens degradation for MAP1, especially at higher MM (Supplemental Fig. S2). At boost injection, MAP1 antigen degradation is more pronounced at all MM. An incubation of the vaccine at 37 °C during 4 days prior to injection drastically accelerated antigen degradation for both MAP1 and MAP1–6. Similar migration patterns were obtained for antigens emulsified in ISA 70 (data not shown).

4. Discussion

The present work aimed at developing and validating a scalable process for the large-scale production of an inactivated vaccine for ruminants using heartwater, as a model. Herein, we present a user-friendly process to produce a high number of stable, ready-to-use inactivated vaccine doses in an attempt to finalize the industrialization of heartwater vaccine.

Montanide ISA50 adjuvant has been successfully used for the preparation of the heartwater vaccine [8,9,13,14,21] and also for foot-and-mouth disease and bovine rotavirus [26,27]. Nevertheless, this vaccine is still prepared immediately before injection using the syringe-extrusion method, being totally inadequate in the context of disease outbreaks or during massive vaccination campaigns. To determine the best combination of emulsifying technique and adjuvant, we first compared the stability of each vaccine preparation (using syringe, vortex or homogenizer) in different adjuvants (ISA50, 70 and 70M). All emulsions presented satisfactory drop test results and conductivity values but particles with 1 μm size (required for emulsion long-term stability) were only obtained for ISA70+ER and ISA70M+ER prepared with the homogenizer. Moreover, these two emulsions were shown to be stable for up to 12 months when stored at 4 °C. This is a crucial result because if the emulsion separates immediately after preparation, the antigens could be released and degraded rapidly, yielding an ineffective vaccine [15]. The two ER antigens MAP1 and MAP1–6 analyzed during this study were shown to be relatively stable (principally MAP1–6) in both ISA70 and ISA70M-based emulsions, especially at 4 °C. By combining the homogenizer with ISA70 and ISA70M adjuvants, we produced 200 vaccine doses in 5 min compared to only 5 doses obtained with ISA50, in 30 min. With the vortexer, it was possible to produce 35 vaccine doses in 1 h, but 1 μm size particles were only stable over time with ISA70 adjuvant.

The vaccination trials showed that the ISA70M+ER vaccine prepared with the homogenizer resulted in a 100% survival rate, as for the conventional ISA50+ER, despite some antigen degradation (Supplemental Fig. S2). When ISA70M+ER vaccine were kept at 37 °C for 4 days to mimic a cold chain rupture during vaccine transportation, a slight decrease in vaccine efficacy (80% survival rate) was obtained. This clearly showed the robustness in using ISA70M adjuvant and the homogenizer for the preparation of a vaccine to be used in the field. ISA70+ER vaccine was also able to induce a good protection with a 80% survival rate. During the vaccination trial (at primary and boost injections), we also observed that ISA70M-based vaccines were easily injected and well tolerated by the animals, with no major side effects, as previously observed [28]. ISA70 and/or ISA70M adjuvants have already proven to induce strong humoral and cellular protective immune responses [28,29].

| Table 1
<table>
<thead>
<tr>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Particle size in each vaccine emulsions.</td>
</tr>
<tr>
<td>Adjuvant</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>ISA 50</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>ISA 70</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>ISA 70M</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

| Table 2
<table>
<thead>
<tr>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Survival ratio and reactions of goats from control and vaccinated groups to ER infectious challenge.</td>
</tr>
<tr>
<td>Group</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>Emulsions</td>
</tr>
<tr>
<td>Survival ratio</td>
</tr>
<tr>
<td>Mean incubation period (days)</td>
</tr>
<tr>
<td>Min–max</td>
</tr>
<tr>
<td>Max temperature (°C)</td>
</tr>
<tr>
<td>Mean duration of fever (days)</td>
</tr>
<tr>
<td>Min–max</td>
</tr>
<tr>
<td>Duration to death (days)</td>
</tr>
<tr>
<td>Min–max</td>
</tr>
</tbody>
</table>

T: vaccine stored 4 days at 37 °C before injection.
Herein, we observed a strong MAP-1 antibody response in animals vaccinated with all adjuvants. In particular, ISA70 and ISA70M-based vaccines stored at 4 °C were able to induce a homogeneous antibody response. This was probably due to an increased W/O ratio (70:30) (which results in increased vaccine viscosity [30]) and to an homogeneous particle size distribution (1 μm) that contributes to good antigen presentation. When ISA70M+ER vaccine was kept at 37 °C before injection, an heterogeneous antibody response was observed; although the reasons for this are not yet clear, this could be due to a decrease in vaccine viscosity, leading to antigen release and its rapid destruction [30,31].

One of the problems associated to the inactivated vaccine against heartwater is the high levels of morbidity during the challenge period [10,13,21]. Herein, we observed that the intensity and duration of clinical signs was longer for animals vaccinated with ISA70+ER or ISA70M+ER compared to the conventional ISA50+ER vaccine. Additionally, a longer period of hyperthermia with high fever (42 °C) was observed for animals vaccinated with ISA70M+ER compared to ISA70+ER. This is a critical result as a longer period of sickness after infection could result in a possible increase of animal morbidity in field conditions. The addition of preservatives or stabilizers could be used to improve ER antigen stability within the ISA70M emulsions, eventually leading to a lower duration of clinical signs and thus lower morbidity. At the moment, as both adjuvants have shown to induce a good protection in the vaccinated animals, the choice of the best adjuvant (ISA70 versus ISA70M) would depend on the herd conditions and the different herd systems.

In conclusion, our work demonstrated the successful association of the 70M and ISA70 adjuvants with high-speed homogenization to produce a stable inactivated vaccine against heartwater for large scale application. We strongly believe that our results could be used by scientists working on other experimental inactivated vaccines for large scale application on the field. Globally, our process allows to: (i) rapidly produce several hundred vaccine doses with minimal effort, (ii) ensure consistency in vaccine batches, (iii) up-scale emulsion production to industrial level, (iv) prepare the vaccines long before vaccination due to their increased stability at 4 °C and (v) minimize the adverse effects of cold chain rupture up to 4 days at 37 °C, as could happen under field.

Herein, the Gardel strain was used as a gold standard for ER. However, considering the issue on wide antigenic diversity, other ER strain (isolated in vitro) that would cross-protect against other regional strains should be included in this inactivated formulation to improve vaccine efficacy.

Author contributions

Conceived and designed of experiments: IM, FB, NV, DM, TL. Performed the experiments: IM, KGG, RA, JG. Analyzed the data: IM, KGG, RA, NM, DM, TL, NV. Wrote the paper: IM, NV. Corrected the paper: DM, TL.

Acknowledgements

The authors are grateful to Christian Sheikboudou for technical support on the purification of antigens, Loïc Jacquet-Cretides for his help with animal handling and blood sampling. Potential conflicts of interest: FB and JG are employees of Seppic. All other authors: no reported conflicts. Financial support: the authors acknowledge the financial support received from European project, EGIPENNEVAC contract number FP6-003713, FEDER 2007–2013, FED 1/1.4-30305, “Risque en santé animale et végétale”. Author Isabel Marcelino acknowledges financial support from post-doc grant SFRH/BPD/45978/2008 from FCT/MCTES [Lisbon, Portugal]. The authors thank Adela Sarahi Oliva Chavez and Emmanuel Albina for the reviewing of the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2014.11.059.

References

A user-friendly and scalable process to prepare a ready-to-use inactivated vaccine: The example of heartwater in ruminants under tropical conditions.