Author's response to reviews

Title: Characterization of n-Hexane fraction of Bridelia micrantha (Berth) and its antimycobacterium activity.

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Version: 3 Date: 25 February 2011

Author's response to reviews: see over
POSTGRADUATE RESEARCH PROPOSAL

PROJECT IDENTIFICATION

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1.0 PROJECT SUMMARY

*Ehrlichia (Cowdria) ruminantium* is a tick-borne obligate intracellular parasite that causes heartwater, an economically important disease affecting domestic ruminants throughout sub-Saharan Africa. Heartwater (also called Cowdriosis), transmitted by members of the three-host tick genus *Amblyomma*, is a serious constraint to livestock production and improvement programmes throughout sub-Saharan Africa. The disease develops within 10 – 30 days after an infectious tick bite and usually the first symptom is a sudden rise in temperature. The course of the disease may range from peracute to mild depending on age, immune status, breed, and virulence of *E. ruminantium* strain. Strains of *E. ruminantium* are very diverse; while some strains are highly virulent, others appear to be non-pathogenic. *E. ruminantium* has a high level of genomic plasticity with gene segments often deleted or inserted, and possible gene disruptions. Several different genotypes can co-exist in a geographic area, and may recombine to form new strains. Ticks of the genus *Amblyomma* are the only proven vectors of *E. ruminantium*. Of these, *A. variegatum* is the main vector species throughout most of sub-Saharan Africa and the only vector so far found in Cameroon.

Serological and microscopical evidence of the presence of heartwater in domestic ruminants have been reported in some localities in Cameroon, however, the genetic diversity of isolates and the epidemiology of the disease in the country remain unknown. Our hypotheses are that the prevalence of heartwater is identical and correlates with the distribution of the vector in different regions of Cameroon and secondly strains of *E. ruminantium* from cattle and ticks are identical. The long term goal of this project is to isolate and characterise the causative agent of heartwater (*E. ruminantium*), determine the epidemiology of the disease, and obtain a geographic area specific marker for easy detection. In order to achieve our objectives, two sampling approaches will be used. In the first approach, point prevalence survey, blood and ticks will be collected from ruminants (cattle, goats and sheep) from selected sites. Epidemiological data will be collected for each animal. The participation of each animal in the study will depend on a combination of factors including owner cooperation, age, and accessibility. Samples for point prevalence survey, collected on a single occasion, will be used to confirm exposure of these animals to *E. ruminantium* and report incidence and prevalence of heartwater in ruminants and ticks. In the second approach, the longitudinal survey, blood will be collected (twice a week) from 30 tagged cattle from SODEPA Dumbu Ranch in the North West Region of Cameroon for a period of twelve months. A combination of serological (Enzyme-linked immunosorbent assay (ELISA)) and molecular (Polymerase chain reaction (PCR) and Sequencing) assays will be used to determine the incidence and
prevalence of heartwater in ruminants and ticks. Isolation of *E. ruminantium* from PCR positive samples will be attempted. The incidence of heartwater in relation to vector distribution will be investigated. These results will give a clear picture of heartwater incidence and prevalence of Cameroon in the selected regions in Cameroon, and by extension in sub-Saharan Africa and will also generate data needed by the Ministry in charge of animals to develop quick and lasting treatment / control strategies specific to the locality.

2.0 Introduction

*Ehrlichia (previously Cowdria) ruminantium* is an obligately intracellular, tick-transmitted bacterial pathogen belonging to the rickettsial family Anaplasmataceae. This bacterium causes heartwater or cowdriosis, a disease that affects cattle, sheep and goats and also some wild ruminants and is transmitted by ticks of the genus *Amblyomma* (Byrom et al., 1991; Heerden et al., 2004; Faburay et al., 2005). Prevention of the disease is by tick control and immunisation. Heartwater has been reported to cause considerable economic losses through death, decreased meat and milk production, unthriftness, decreased draught power and manure, together with high cost of control measures. It is a major constraint to the upgrading of indigenous breeds of cattle, sheep, and goats and to the introduction of more productive exotic breeds.

Heartwater is considered to be the most important tick-borne disease of cattle in Southern Africa (Allsopp et al., 2007). In Eastern Africa, it is ranked second in importance to East Coast fever; however, its economic importance in domestic ruminants in the remainder of sub-Saharan Africa is recognised but not yet well documented (Norval et al., 1991). Heartwater causes high mortality (20% to 90%) in susceptible animals with small ruminants being at greater risk from the disease (Uilenberg et al., 1993; Faburay et al., 2008). In Cameroon, losses due to heartwater disease are not well known because the incidence/prevalence has not been well studied or documented.

3.0 Literature Review

The most recent classification places *E. ruminantium* in the genus *Ehrlichia* (Dumler et al., 2001). The genus *Ehrlichia* was established in 1945 in honour of the German microbiologist Paul Ehrlich (Walker and Dumler, 1996; Ndip et al., 2005).

According to Walker and Dumler (1996), the emergence of ehrlichiae began in 1910, when Theiler described *Anaplasma marginale*, the causative agent of an economically important severe worldwide disease of cattle. Since then, the contributions of veterinary
medical science in the understanding of ehrlichiae have continued. These contributions include the description of *Cowdria* (now called *Ehrlichia*) *ruminantium* by Cowdry in 1925 (Walker and Dumler, 1996).

*E. ruminantium* is a pleomorphic, gram-negative organism which grows by binary fission in membrane-lined cytoplasmic vacuoles. These vacuoles do not fuse with other vacuoles nor with lysozomes (Bekker, 2002). This bacterium has predilection for endothelial cells and neutrophils. The first *in vitro* cultivation of the organism in a calf endothelial cell line was described in 1985 by Bezuidenhout *et al.*, and since then many cell lines as well as media and media components have been examined to improve and standardize the *in vitro* cultivation of *E. ruminantium* (Yunker, 1995; Bell-Sakyi, 2004). Numerous geographically and antigenically diverse *E. ruminantium* isolates have been cultivated *in vitro* using cell lines from a wide variety of ruminants and non-ruminant mammals (Smith *et al.*, 1998; Zweygarth and Josemans, 2001b; Zweygarth and Josemans, 2003; Bell-Sakyi, 2004). Considerable advances have also been made in the *in vitro* cultivation of *E. ruminantium* using tick cell lines (Bell-Sakyi, 2004; Zweygarth, 2006; Bell-Sakyi, 2007).

Ultrastructural morphology, revealed by electron microscopy, shows *E. ruminantium* organisms surrounded by two unit membranes, and occurs in colonies or morulae with membrane-bound vacuoles in the host cytoplasm. Two types of organism are described: larger, reticulate bodies (vegetative form) and smaller, electron-dense (infective form) which reveal that the organism has a Chlamydia-like developmental cycle (Jongejan *et al.*, 1991; Zweygarth, 2006).

Several wild animal species have been implicated as host of *E. ruminantium* and they include some antelope species, buffalo, giraffe and wild rodents all of which can be infected without developing clinical signs (Peter *et al.*, 2002). When *E. ruminantium* infects domestic ruminants (cattle, sheep and goats) following the bite of an infected *Amblyomma* tick, heartwater disease develops. Heartwater can occur in susceptible ruminants through this natural route or artificial transmission by inoculation (usually intravenous) of infected blood, tissue homogenate, ground-up tick suspension or ruminant endothelial cell culture (Camus *et al.*, 1996). The nature and severity of clinical signs and the mortality rate depends on the species, breed and age of the ruminant host, the route of infection (tick transmitted or needle inoculated), size of the inoculum and the pathogenicity of the *E. ruminantium* isolate or strain (Bell-Sakyi, 2004). Heartwater develops within 10 to 30 days after an infectious tick bite and usually the first symptom is a sudden rise in body temperature. The course of the disease may
range from peracute, acute, subacute to mild (Bekker, 2002). In typical cases of heartwater, animals show nervous symptoms such as rapid blinking of the eyes, hypersensitivity to touch, pedalling movements with rare recovery rate (Camus et al., 1996; Bekker, 2002).

Heartwater was first recognized as a disease entity in South Africa in the later part of the 19th century and from the early 20th century onwards, heartwater was reported from other sub-Saharan African countries (Camus et al., 1996; Bell-Sakyi, 2004). It was first diagnosed in Northern Ghana in 1933 (Bell-Sakyi, 2004) and in South Africa alone, it was estimated that 40 – 50 % of all livestock deaths within the endemic areas were caused by heartwater (FAO, 1996). This disease, transmitted by several species of Amblyomma ticks (Ilemobade and Leeflang, 1977) is limited to the geographical distribution of these ticks (Ndi et al., 1998).

3.1 Diagnosis of heartwater:

Detection of *E. ruminantium* in dead animals is usually done by the identification of the bacteria in endothelial cells of brain capillaries of diseased animals using brain crush smears. Detection of infection in animals that are still alive is either based on the detection of specific antibodies against immunogenic proteins, based on the detection of bacterial DNA using PCR techniques and/or in vitro cultivation of the parasite in an appropriate cell line.

*E. ruminantium* infected ruminants develop antibody responses to several immunogenic proteins, some of which are cell surface proteins like the major antigenic proteins 1 and 2 designated MAP 1 and MAP 2 respectively. Serodiagnosis of *E. ruminantium* infection in domestic animals has utilized a range of immunoassay methods including indirect enzyme-linked immunosorbent assays (ELISA), and a competitive ELISA involving monoclonal or polyclonal antibody reactive with the *E. ruminantium* major antigenic protein 1 (MAP 1). The immunodominant major outer membrane proteins MAP 1 and MAP 2 have been identified and their encoding genes cloned, sequenced and expressed. MAP 1 and MAP 2 are both recognized by antisera and T lymphocytes from infected animals and maybe useful in serological diagnostic assays (Mboloi et al., 1999; Mahan et al., 1998b; Mwangi et al., 1998; Barbet et al., 2001). Bell-Sakyi et al. (1996) reported preliminary results obtained with the ELISA technique from a longitudinal survey of *E. ruminantium* incidence in domestic ruminants in Ghana, and a modification of the PC-ELISA was used by Awa (1997) in a survey of antibody prevalence in sheep and goats in North Cameroon.

In order to increase the specificity of heartwater ELISA protocols, fragments of the 32kDa *E. ruminantium* major antigenic protein 1 (MAP1) were produced by recombinant DNA technology and used as ELISA antigen. This recombinant antigen was named MAP1 –
B (Mondry et al., 1998) and the high specificity of the MAP1 –B antigen has been confirmed (Bell-Sakyi et al., 2003; Faburay et al., 2005). Mahan et al. (1998b) used MAP1-B ELISA to report a seroprevalence rate of 33% after analysing cattle sera in Zimbabwe. Heartwater was reported as a cause of postrestocking mortality of goats in Mozambique following a serological survey with MAP 1-B ELISA (Bekker et al., 2001). Using the MAP1-B ELISA, 1,318 serum samples collected from sheep and goats at 28 sites in the five divisions of The Gambia were tested to determine the E. ruminantium seroprevalence rates and to assess the risk of heartwater. At nearly all sites, seroprevalence rates were higher in sheep than in goats and a gradient of increasing heartwater risk for susceptible small ruminants was identified (Faburay et al. 2005). In a study to detect antibodies to E. ruminantium in field sera collected from sheep and cattle in Ghana, MAP1-B ELISA and PC-ELISA were used and the results indicate that both assays are highly sensitive and specific for detection of E. ruminantium exposure in sheep but that the MAP1-B ELISA lacks sensitivity for the detection of antibodies in bovine sera in comparison to the PC-ELISA (Bell-Sakyi et al., 2003). This finding corroborates the the results obtained by Semu et al. (2001) that antibody responses to MAP1-B and other Cowdria ruminantium antigens are down regulated in cattle challenged with tick-transmitted heartwater. A major limitation of all serological assays is the detection of false positives due to cross- reactivity of E. ruminantium antigens with those of other ehrlichiae. Also positive results from serology do not indicate current infection.

DNA-based assays (indicate current infection) have also been employed in the diagnosis of heartwater. Application of the PCR assay is expected to significantly improve the understanding of heartwater epidemiology, particularly through the determination of field tick infection rates (peter et al., 1995). The PCR and DNA probe assays were compared by applying them simultaneously for the detection of low levels of Cowdria ruminantium infection in Amblyomma ticks. Results show that the PCR assay based on the pCS20 region supersedes the DNA probe assay (Peter et al., 1995). PCR assay based on map1 has been used to detect the presence of E. ruminantium in blood and bone marrow samples from clinically normal, free-ranging Zimbabwean ungulates (Kock et al., 1995). Earlier results considered the pCS20 PCR the method of choice for the detection of E.ruminantium infection (Simbi et al., 2003). A nested pCS20 PCR, three PCR-based assays, a nested map1 PCR and a nested reverse line blot (RLB) hybridization assay, were evaluated to determine their ability to detect E. ruminantium infection in A. variegatum ticks in The Gambia and results indicated that the nested pCS20 PCR assay had the highest detection performance with a detection rate of 16.6% (Faburay et al. 2007). In a bid to reduce turn-around time, increase sensitivity and
specificity in the diagnosis of \textit{E. ruminantium} infection, a quantitative real-time PCR assay has been developed, standardized and validated (Doyle \textit{et al.}, 2005; Peixoto \textit{et al.}, 2005; Espy \textit{et al.}, 2006; Steyn \textit{et al.}, 2008). The pCS20 quantitative real-time PCR TaqMan probe was shown to be most sensitive, specific and time-saving when used alongside the pCS20 PCR and PCR/$^{32}$P-probe to detect \textit{E. ruminantium} in field samples and in blood from experimentally infected sheep (Steyn \textit{et al.}, 2008).

Molecular characterization has been used to differentiate between isolates from different geographical regions. The evaluation of strain diversity in the field is essential towards the formulation of an effective vaccine cocktail. The observation of various levels of cross-protection induced between different isolates reveals an antigenic diversity with implications for the development of vaccines (Jongejan and Thielemans, 1991). Molecular characterization of \textit{E. ruminantium} has been based upon a few genes only. Specific probes for the differentiation of \textit{E. ruminantium} isolates based on the 16S rDNA have been reported (Allsopp \textit{et al.}, 1997). Comparison of the map 1 genes from geographically diverse \textit{E. ruminantium} isolates revealed both conserved and variable regions (Reddy \textit{et al.}, 1996; Sulsona \textit{et al.}, 1999; Bell-Sakyi, 2004). Recent advances in technology have allowed complete sequencing of the genomes of two \textit{E. ruminantium} isolates – Welgevonden from South Africa (Collins \textit{et al.}, 2005) and Gardel from Guadeloupe (Frutos \textit{et al.}, 2006). In livestock, prevention of heartwater is by tick control and immunization. The only vaccines for heartwater available at present are either infected blood, or ground suspensions of infected ticks (Byrom \textit{et al.}, 1991). Both of these vaccines must be injected intravenously and animals must be treated with tetracycline during the febrile period. No practical diagnostic test for live animals has yet been developed. In efforts to produce a more suitable vaccine, and to establish a supply of antigen for diagnostic tests, many attempts at cell culture of \textit{E. ruminantium} have been made.

4.0 Project Rationale

The rapid population growth in sub-Saharan Africa necessitates a great increase in animal production. Although livestock farming is not the most energy-efficient way of producing food, meat will remain a significant component of the global diet for the foreseeable future (Campbell \textit{et al.}, 2009). \textit{Ehrlichia ruminantium} is recognized as a significant agricultural biothreat (McBride, 2009). Although heartwater remains one of the major disease problems that delays the cattle industry development in sub-Saharan Africa,
little is known of the presence of heartwater in domestic ruminants in Cameroon. The
distribution of heartwater coincides with the distribution of the tick vector *Amblyomma variegatum* consequently, one would expect endemicity of heartwater in the selected Regions of Cameroon since the presence of *A. variegatum*, main vector of *E. ruminantium* has been reported in theses region (Ndip et al., 2004). It is important to reduce losses due to mortality by regularly detecting and monitoring *E. ruminantium* infection within livestock populations and this pathogen is also suspected to be an emerging human pathogen as cases of lethal *E. ruminantium* infections in humans have been reported in South Africa (Allsopp et al., 2005; ESCMID, 2006; Yu et al., 2007). The detection of *E. ruminantium* in ticks and ruminants is essential for epidemiological studies and for implementing control measures (Steyn et al., 2003). Although epidemiological factors such as presence of *Amblyomma variegatum* ticks (Ndi et al., 1998; Ndip et al., 2004) and records of previous occurrence of heartwater (Post mortem diagnosis from brain smears) have been reported in Cameroon, there is no molecular evidence yet. We intend to fill this knowledge gap by performing polymerase chain reaction, isolation and characterization of isolates. PCR will be used to amplify pCS20 gene in *E. ruminantium* (Peter et al., 2000). Detection of *E. ruminantium* DNA in samples will indicate current infection. Ndi et al. (1998) diagnosed heartwater in cattle in the North West Province of Cameroon by microscopical examination of Giemsa stained brain impression smears of grey matter obtained from postmortem examinations. We intend to diagnose heartwater along the Coastal Region of Cameroon by using a combination of microscopic, serological, molecular and cultural techniques. Because future vaccines may need to incorporate components from several virulent isolates, it is essential to have information on the extent of genetic variation among isolates in sub-Saharan Africa (Allsopp et al., 2005). Awa (1997) reported seroprevalence of heartwater in North Cameroon, and there is need for similar and more elaborate studies to be carried out in other parts of the country. Competitive ELISA was performed using biotin-labelled IgG against common antigenic epitopes to Ball 3 and Welgevonden strains of *E. ruminantium*. In this study, the indirect MAP-1B ELISA for detection of antibodies to *E. ruminantium* in small ruminants and PC – ELISA to detect antibody in cattle will be used. The MAP-1B recombinant antigen is very sensitive and more specific than crude antigens (Mondry et al., 1998; van Vliet, et al., 1995). This increased sensitivity is attributed to the comparatively longer persistence of MAP 1 antibodies (Faburay et al., 2007).
The point prevalence surveillance will give a measure of the proportion of ruminants in the population that have heartwater disease at the time of the study. Point prevalence will be calculated by the formula:

\[
\text{Prevalence} = \frac{\text{Number of existing heartwater cases at time of sampling}}{\text{Number of ruminants sampled}}.
\]

The longitudinal survey will bring out a correlation between heartwater and seasonality following repeated observations of the same tagged cattle over a period of 12 months.

5.0 Study Aims and Specific Objectives:

5.1 Overall Goal

The long term goal of this project is to isolate and characterize the causative agent of heartwater \((E.\ ruminantium)\), determine the epidemiology of the disease, and obtain a geographic area specific marker for easy detection and/or vaccine development. The immediate objectives of this proposal are to investigate the incidence of heartwater in ruminants and identify the tick vector, investigate seasonality of tick infestation and identify high risk zones between the study sites Cameroon.

5.2 Study Aim 1

To investigate the incidence and prevalence of \(E.\ ruminantium\) infection in ruminants and ticks, molecularly characterize \(E.\ ruminantium\) isolates from ruminants and ticks and correlate tick infestation with seasonality.

\textit{Hypothesis: We hypothesize that the prevalence of heartwater in the study Regions of Cameroon is identical and correlates with the distribution of ticks.}

5.2.1 Specific Objectives:

1. Determine sero-prevalence of domestic ruminants to \(E.\ ruminantium\).
2. Determine the incidence of heartwater in domestic ruminants in the study sites in relation to vector distribution.
3. Determine the seasonality of tick infestation and Incidence of \(E.\ ruminantium\) infection in ticks.

5.3 Study Aim 2:
In vitro cultivation of *E. ruminantium* isolates and sequencing of target genes to determine genetic diversity

**Hypothesis:** We hypothesize that strains of *E. ruminantium* from cattle and ticks are identical.

5.3.1 **Specific Objectives:**
1. Make at least one isolate of *E. ruminantium* from each sampling site.
2. Characterize the *E. ruminantium* isolates by Sequencing and comparing target genes.

6.0 **Materials and Methods**
6.1 **Project and Sampling sites**

This work will be carried out in the Laboratory For Emerging Infectious Diseases (LEID), University of Buea – Cameroon. All aspects of this research, except sequencing will be hosted by LEID. Sequencing will be performed at UTMB because the LEID does not have a sequencing facility.

Cameroon lies on the west coast of Africa, latitude 2°–3°N and longitude 9°–16°E, with Nigeria to the west, Chad to the north, Central African Republic to the east and Congo, Gabon and Equatorial Guinea to the south. It has an area of 475000 km². The Coastal Region of Cameroon extends 10 to 50 miles (16 to 80 km) inland from the part of the Atlantic Ocean from the Cameroon’s border with Nigeria to Campo at the Cameroon’s border with Equatorial Guinea. Exceedingly hot and humid, the coastal belt includes some of the wettest places on earth. For example, Debundscha, at the base of Mt. Cameroon, has an average annual rainfall of 405 inches or 10,300 mm (National Climatic Data Centre, 2006). The plain is densely forested.

Samples will be collected from two sites in two ecological regions in Cameroon. Samples will be collected from SODEPA Dumbu Ranch (SDR) of the North West Region of Cameroon in the western highland savannah ecological zone. A longitudinal survey (monitoring 30 tagged cattle) will be carried out in SDR over a period of 12 months. Samples will also be collected from Buea of the South West Region of Cameroon in the monomodal humid forest zone. The sample distribution will be as shown in Table 1 below.

Table 1: Distribution of samples by sites for point prevalence survey

<table>
<thead>
<tr>
<th>Site</th>
<th>Number of ruminants per site</th>
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<tbody>
<tr>
<td>SODEPA Dumbu Ranch</td>
<td></td>
</tr>
<tr>
<td>Buea</td>
<td></td>
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10
<table>
<thead>
<tr>
<th></th>
<th>Cattle</th>
<th>Goat</th>
<th>Sheep</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDR</td>
<td>200</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Buea</td>
<td>200</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>400</strong></td>
<td><strong>100</strong></td>
<td><strong>100</strong></td>
</tr>
</tbody>
</table>

Authorisation to undertake the study will be obtained from the Ministry of Livestock, Fisheries and Animal Husbandry responsible for animals in the South West Region of Cameroon and also from the Director General of SDR.

### 6.2 Sample Collection

For any animal to be included in the study, it must meet the following case definition:

i) **Inclusion criteria:**
- Animals aged ≥ 6 months
- Consent from animal owner
- Accessibility of sampling site

ii) **Exclusion criteria:**
- Animals less than 6 months of age
- Failure to obtain sample from the animal
- Owner’s unwillingness to cooperate
- Inaccessibility of site

Epidemiological information including breed, clinical status, last antibiotic treatment, tick control method, confinement, sex and tick infestation will be collected from each animal sampled. All this information will be captured in an epidemiological data collection sheet (Table 2). Small “field allowances” (which could be money, or attention) will be provided to animal holders/herdsmen to ensure cooperation.

#### 6.2.1 Blood for Point prevalence survey

Whole blood will be collected from cattle, goats and sheep of both sexes that meet the inclusion criteria. Blood will be collected on a single occasion by jugular puncture into two sets of tubes. Blood in tubes with anticoagulant (EDTA) will be used for molecular testing while blood in the tube without anticoagulant will be used for serology. The labelling on the sample tubes will indicate site code/study number/animal code and date of collection. For example: SDR/001/CP, 10/01/10 will indicate blood collected for point prevalence survey.
from cattle 001 in SODEPA Dumbu Ranch on the 10th of January 2010. SDR/001/GP,10/01/10 will indicate blood collected for point prevalence survey from goat 001 in SODEPA Dumbu Ranch on the 10th of January 2010. Samples will be transported to the laboratory on ice. Upon arrival in the laboratory, blood in tubes with anticoagulant (EDTA) will be preserved at -20°C until DNA extraction while blood in the tube without anticoagulant will be processed for sera. Other information that will be collected will include breed, clinical status, last antibiotic treatment, tick control method, body temperature and confinement. This information will be taken down clearly and accurately in an epidemiological data sheet.

6.2.2 Blood for Longitudinal survey

Whole blood will be collected every two weeks from 30 tagged cattle from SDR for a period of 12 months. The blood will be collected by jugular puncture, into two sets of tubes. Blood in tubes with anticoagulant (10 mL; Becton Dickinson with EDTA) will be used for molecular detection while blood in the tubes without anticoagulant will be used for sera extraction needed for serological assays. These animals will be over six months of age to avoid interference from maternal antibodies. The labelling on the sample tubes will indicate site code/ study number /longitudinal survey code and date of collection. For example: SDR/001/1L, 10/01/10 will indicate first blood sample collected for longitudinal survey from cattle 001 in SODEPA Dumbu Ranch on the 10th of January 2010. Samples will be transported to the laboratory on ice. Upon arrival in the laboratory, blood in tubes with anticoagulant (EDTA) will be preserved at -20°C until DNA extraction while blood in the tube without anticoagulant will be processed for sera. Other information that will be collected will include breed, clinical status, last antibiotic treatment, tick control method, body temperature and confinement. This information will be taken down clearly and accurately in a sample collection data sheet. At the longitudinal survey site, about 20 mL of blood will be collected (by jugular puncture) in heparinised tubes from the tagged cattle that have a body temperature ≥ 41°C.

6.2.3 Ticks

Ticks will be collected by hand picking from all the animals and put in 15 mL Falcon tubes. Each tube per animal and the tubes labelled accordingly. The ticks will be immersed immediately in 70% alcohol and transported to the laboratory. Upon arrival in the laboratory
the ticks will be washed thrice in 70% alcohol and preserved in the same concentration of alcohol at 4°C until morphological identification and DNA extraction.

7.0 Sample processing

7.1 Separation of serum from blood

Serum will be separated from whole blood by centrifugation in a low-speed centrifuge (Laboufuge, Germany) at 6,000 x g for 10 min. Each serum sample will be harvested and aliquoted (about 1 mL) into two 1.5 mL tubes and stored separately. Large volumes of sera samples will be stored in 1.5 mL tubes because we might want to use these samples for another survey (to test something else) in future. One tube will be stored at -70°C and the other at -20°C until required. Each serum sample will be stored in two tubes to minimise contamination and maintain quality of the samples. Samples at -70°C will be reserved and can only be used if those at -20°C get finished or are contaminated.

7.2 Plasma inoculum

Blood in the heparinised tubes will be centrifuged at 1000 x g for 20 min and plasma harvested. The plasma will be aliquoted into two 4 mL tubes and stored separately. One tube will be stored at -70°C and the other at -20°C until required. Each serum sample will be stored in two tubes to minimise contamination and maintain quality of the samples. Samples at -70°C will be reserved and can only be used if those at -20°C get finished or are contaminated.

7.3 Identification of ticks

Study Aim 1

Specific Objective 3: Determine the seasonality of tick infestation

Ticks will be identified up to the species level using basic taxonomic keys and a stereo microscope. The main features that will be used for identification include shape, size, mouthparts, spurs on coxa 1, anal groove position, ornamentation and eyes. Only ticks of the genus Amblyomma will be considered for further analysis.

7.4 DNA extraction from blood and ticks
DNA will be extracted from the blood samples with anticoagulant, according to standard protocol, and used for polymerase chain reaction. DNA will be purified from 100µL of blood using the DNeasy™ Tissue Kit (Qiagen, Inc., Valencia, USA) according to the manufacturer’s instruction for the isolation of DNA from whole blood. DNA will be extracted from 150 individual ticks (75 males and 75 semi engorged or flat females) using the DNeasy™ Tissue Kit (Qiagen, Inc., Valencia, USA) according to the manufacturer’s instruction for the isolation of DNA from animal tissues.

7.5 Serological Detection of *E. ruminantium*

**Study Aim 1**

**Specific Objective 1**: Determine sero-prevalence of domestic ruminants to *E. ruminantium*.

For the serological analysis we shall perform both PC-ELISA and the MAP-1B ELISA. The PC-ELISA will be performed for bovine sera while MAP-1B ELISA will be performed for small ruminants.

7.6 Polyclonal competitive enzyme linked immunosorbent assay (PC-ELISA)

PC-ELISA will be performed according to Bell-Sakyi *et al.* (2003) without any modification. The antigen coated slides for the PC-ELISA will be ordered from the Centre for Tropical Veterinary Medicine (CTVM), University of Edinburgh, United Kingdom, and stored at -70°C. The plates will be washed manually five times with phosphate-buffered saline (PBS) with 0.05% Tween 20 (PBST). Fifty microlitres of positive control sera and negative control sera, or PBS will be added to each well, followed by 50 µL of biotinylated competitor antibody diluted in PBS with 0.1% Tween 20, and the plates will be incubated on a plate shaker at 37°C for 1 hour. The plates will then be washed as before, 100 µL of Extravidin-peroxidase conjugate (Sigma) diluted 1 in 2,500 in PBST will be added to each well, and the plates incubated as before at 37°C for 30 min and washed as before. Every well then will receive 100 µL of tetramethyl benzidine substrate (Sigma) dissolved in phosphate-citrate buffer (Sigma) with 0.1% hydrogen peroxide (30% solution; Sigma) added just before use. The plates will be incubated at room temperature (22 to 32°C) for 20 min, and the reaction will be stopped by adding 100 µL of 1 M sulfuric acid to every well. The plates will be read on a Bio-Rad iMark™ Microplate reader at 450 nm, and the optical density (OD) values will be obtained after automatic deduction of the OD value of the no-antigen control.
well that will be used to calculate the percent inhibition (PI) for each serum sample as follows:

\[ PI = 100 - \left( \frac{\text{mean OD value for serum} + 100}{\text{mean OD value of PBS controls}} \right) \]

As they are percentage calculations that adjust the data to inbuilt controls, PI values can fall outside the 0-to-100% scale. PI levels of >70 for cattle sera will be considered to indicate positivity for *E. ruminantium*.

### 7.7 MAP1-B ELISA

The antigen coated slides for the MAP1-B-ELISA will be ordered from the Centre for Tropical Veterinary Medicine (CTVM), University of Edinburgh, United Kingdom, and stored at -70°C.

The plates will be removed from the freezer and blocked for 15 min at 37°C with phosphate-buffered saline (pH 7.3) supplemented with 0.1% Tween 20 and 1% non-fat dry milk (PBSTM). The plates will be washed thrice in PBS supplemented with 0.1% Tween 20 (PBST), and sera diluted 1:200 in PBSTM will be applied in duplicate wells and incubated for 1 h at 37°C. The plates will be washed five times with PBST and incubated with animal-specific second-step antibodies conjugated with horseradish peroxidase diluted in PBSTM.

After incubation at 37°C for 1 h, the plates will be washed five times in PBST and substrate added. Colour development will be measured after a 30 min incubation at room temperature by using a BioRad iMark™ Microplate Reader at 405 nm. Each plate will contain one positive and one negative reference serum sample. Optical density will be expressed as a percentage positive (PP) value of the reference positive.

### 7.8 Nested Polymerase Chain Reaction (PCR)

**Study Aim 1**

**Specific Objective 2:** Determine the incidence of heartwater in domestic ruminants in the study sites in relation to vector distribution.
Specific Objective 3: Determine the incidence of E. ruminantium infection in ticks.

Study Aim 2

Specific Objective 2: Characterize the E. ruminantium isolates by Sequencing and comparing target genes.

Five microlitres (5µl) of the purified DNA from cattle, goats, sheep and ticks will be used as template in reactions with primers AB 128 (5’- ACTAGTAGAAATTGCACAATCTAT-3’) and AB129 (5’- TGATAACTTGGTGCGGGAAATCTT-3’) as internal primers and AB129 also used as external reverse primer with ITM 130 (5’TCAATTGCTTAATGAAGCACTAACTCAC-3’) in a nested PCR. Reaction conditions for the first amplification will be: initial denaturating step at 94°C/3 min, 39 cycles of denaturation at 94°C/30 s, annealing 62°C/45 s, elongation 72°C/1 min and a final extension of 72°C/10 min. Reaction conditions for the second amplification will be: initial denaturating step at 94°C/3 min, 25 cycles of denaturation at 94°C/30 s, annealing 58°C/45 s, elongation 72°C/1 min and a final extension of 72°C/10 min. The expected amplicon size is 279-bp. Each set of PCRs will include negative and positive reagent controls. The positive control will be requested from Professor Frans Jongejan of the Utrecht Centre for Tick-borne Diseases, Faculty of Veterinary Medicine, Utrecht University, The Netherlands. The pCS20 PCR assay is presently the most characterised and reliable test for E. ruminantium in ticks and ruminants and thus is highly useful for field and laboratory epidemiological investigations of heartwater. PCR positive products will be sequenced, analysed and compared with known E. ruminantium sequences in the GenBank.

7.9 Preparation of smears for microscopic examination

Smears will be prepared from the buffy coat of ruminant blood samples. The buffy coat will be obtained thus:

1. The whole blood samples in tubes containing anticoagulant will be centrifuged at 1500-2000 X g for 10-15 min at room temperature.
2. The plasma will be removed carefully with a pipet without disturbing the buffy coat which forms a thin film between the upper plasma layer and the lower layer of packed RBCs.
3. Using the same pipet the exposed WBC layer will be carefully aspirated in a volume of about 0.5 ml or less. Aspirating will be done slowly, using a circular motion, to pull all the visible buffy coat material into the transfer pipet.

The smears will be stained as outlined below:
1. A drop of the buffy coat suspension will be place on a clean slide and spread in a circular manner.
2. The smear will be air-dried
3. Fixed in “Diff Quick” Fixative (or methanol) for 30 secs/drain
4. Stained with “Diff Quick” solution II for 30 secs/drain
5. Counterstained (optional) with “Diff Quick” solution I for 30 secs/drain
6. Rinsed in tap water to remove excess stain
7. Rapidly dehydrated in absolute alcohol
8. Clear and mount

7.10 **In vitro cultivation of E. ruminantium**

**Study Aim 2**

**Specific Objective 1.** Make at least one isolate of *E. ruminantium* from each sampling site along the Coastal Region of Cameroon

Bovine endothelial cells will be requested from Professor Frans Jongejan of the University of Utrecht, Utrecht, The Netherlands.

7.10.1 **In vitro cultivation of *E. ruminantium* from plasma**

The maintenance medium will be decanted from cells and 1-4 mL of inoculum introduced into 25 cm² flasks. Flasks will be incubated on a slowly rocking platform at 37°C for 2 hr. Inoculum will be decanted and 4 mL of maintenance medium added and re-incubated on a rocking platform. Cultures will be monitored for cytopathic effect from the 7th day.

8.0 **Statistical analysis**

We will calculate descriptive statistics for clinical and epidemiological variables. We will calculate the overall incidence rate (probable and confirmed cases) and the seroprevalence rates of heartwater infection. The logistic regression models will be used to compare tick infestivity and heartwater infection. All analysis will be conducted using intercooled STATA v 9.2 (StataCorp, College Station, TX).
9.0 Expected problems and anticipated solutions

There is the likelihood of lack of cooperation from animal owners and holders. To solve this problem, Small “field allowances” (which could be money, or attention) will be provided to animal holders/herdsmen to ensure cooperation. Since SDR is located in an enclaved area there will be a problem with sample transportation from SDR to the laboratory in the University of Buea. To eliminate or reduce this difficulty, a sample transportation arrangement will be made with “Amour Mezam”, a local transport agency that commutes steadily between Buea and SDR. The lack of crunches (restrain facility for cattle) may prohibit blood collection from cattle. To overcome this hurdle, good ropes will be purchased and carried along to the field for physical restrain of cattle if need arises. Due to lack of electricity and the enclaved nature of Dumbu, samples will be transported from SDR to the University of Buea at room temperature. Because of this, culture of E. ruminantium will be attempted only with samples from Buea and not with samples from SDR. Since antibodies are stable at room temperature for up to about seven days, samples from SDR will be good for molecular and serological diagnosis of heartwater but not culture.

10.0 Expected/outcome of results

The expected benefits of this research will be an improved understanding of the exposure of domestic ruminants in the study sites in Cameroon to *Ehrlichia ruminantium* infection as well as adding to the understanding of heartwater epidemiology in sub-Saharan Africa. Data generated may be used by the Ministry incharge of animals to develop quick and lasting treatment / control strategies specific to the locality. A pure culture of *E. ruminantium* could be a good starting material for the development of a practical diagnostic test and vaccine development.
## 11.0 Budget

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12.0 REFERENCES


MboloI, M. M., Bekker, C. P. J., Kruitwagen, C., Greiner, M., Jongejan, F. (1999): Validation of the indirect MAP1-B Enzyme-linked Immunosorbent Assay for Diagnosis of


Collect blood and tick samples from cattle, goats and sheep.

SAMPLE PROCESSING
- Separate serum from blood
- Separate plasma from blood
- Harvest buffy coat from blood
- Identify ticks
- Extract DNA from blood and ticks

Serological diagnosis
- PC-ELISA for bovine sera
- MAP-1B ELISA for small ruminants

Molecular diagnosis
- Polymerase chain reaction (pCS20 target)
- Sequencing

Microscopy:
- Diff-Quik staining of buffy coat smears

Cell culture using bovine endothelial cell lines.
- Plasma as inoculum

4.1 Flow diagram of sample processing