CHAPTER 3.1.9.

HEARTWATER

SUMMARY

Description and importance of the disease: *Heartwater (also known as cowdriosis) is an acute, fatal, non-contagious, infectious, tick-borne rickettsial disease of ruminants caused by* Ehrlichia ruminantium *(formerly* Cowdria ruminantium*) and transmitted by* Amblyomma *ticks. It occurs in nearly all sub-Saharan countries of Africa, in its neighbouring islands, and also in the Caribbean. The disease can cause high mortality (up to 90%) in susceptible domestic ruminants. Goats and sheep are more susceptible than cattle, and European breeds are generally more susceptible than indigenous African breeds.*

Clinically, the disease most commonly presents as an acute form characterised by a sudden high fever, depressed demeanour, nervous signs and a high mortality. Hydropericardium and hydrothorax and lung oedema are commonly associated post-mortem lesions. Acute and peracute clinical forms of the disease occur: in the former, there are high death rates without many clinical manifestations and, in the latter, there is a higher recovery rate.

Recovered animals become carriers of infection. Certain wild animals can play a role as reservoir; Rusa deer, white-tailed deer, and springbok are susceptible to this infection and can experience high mortality.

Identification of the agent: *The specific diagnosis of heartwater is based on the observation of colonies of* E. ruminantium *in capillary endothelial cells of the brain. In the absence of molecular diagnostic tools, a piece of cerebellum can easily be removed with a curette through the foramen magnum after cutting off the head, while a sample of cerebral cortex can be obtained through a hole made in the skull with a hammer and a large nail. Brain smears are prepared by crushing to a paste and spreading thinly a small piece of cerebral or cerebellar cortex between two microscope slides. The capillaries are spread out in a single cell layer by drawing one slide across the other. The smears are air-dried, fixed with methanol and stained with Giemsa. The colonies (clusters) of* E. ruminantium *are reddish-purple to blue, and very often close to the nucleus of the infected endothelial cell. They can be scanty and difficult to find, particularly in peracute cases, but they are always present in the brain of a ruminant that died from heartwater, if not treated with drugs. Colonies are not likely to be detected in animals that were treated with antibiotics. The colonies are still visible 2 days after death in a brain that has been stored at room temperature (20–25°C) and up to 34 days in a brain that has been stored in a refrigerator at 4°C.*

Ehrlichia ruminantium *can be isolated from the blood of an infected host using cultivation on ruminant endothelial cells. When a cytopathic effect consisting of plaques of cell lysis appears, the presence of characteristic morulae is confirmed by staining the cell monolayer with Giemsa or RAL555 or by immunofluorescence.*

Molecular tools such as nested polymerase chain reaction (PCR) and real-time PCR targeting E. ruminantium*-specific genes are currently available for the detection of the presence of* E. ruminantium *in the blood of animals with clinical signs, in organs from dead animals (confirming clinical cases of heartwater), and also in the tick vectors. They could not however, detect* E. ruminantium *in asymptomatic carriers. Two multi-pathogen methods, including* E. ruminantium *detection, have been developed allowing differential diagnosis for tick-borne diseases to be undertaken. Apart from diagnosis, molecular methods are widely used for research on the* E. ruminantium *genome and for epidemiological studies, including* E. ruminantium *tick prevalence.* No commercial molecular kits targeting *E. ruminantium* are currently available.

Serological tests: *Two enzyme-linked immunosorbent assays (ELISAs) have been evaluated: an indirect ELISA and a competitive ELISA targeting major antigenic protein 1 (MAP1) antibodies.*

The current indirect ELISA uses a recombinant antigen expressed as a partial fragment of the MAP1 antigens – the MAP1-B ELISA – which gives improved specificity over earlier methods. However the assay still detects cross-reacting antibodies to other Ehrlichial organisms including Ehrlichia chaffeensis, E. canis *and Panola Mountain* Ehrlichia. *Hence, definitive proof of heartwater must rely on epidemiological evidence and additional molecular testing indicating the presence of the organism. This ELISA can help to monitor experimental infections and to measure the immune response of immunised animals, whose pre-immunisation serological history is known. Serology has very limited diagnostic use as clinically infected animals remain sero-negative during the febrile reaction and sero-convert after recovery.*

Serology is also not an effective import test. Prior to importation of animals from a heartwater endemic region, it is important to study the epidemiological data to try to establish that the herd and the resident ticks are not infected. Repeated serology on the herds and PCR on tick samples in targeted herds can be carried out to demonstrate that they are free of E. ruminantium.

Requirements for vaccines: *Immunisation against heartwater by the 'infection and treatment' method using infected blood is still in use in some countries. A first-generation vaccine consisting of inactivated purified elementary bodies of* E. ruminantium *emulsified in an oil adjuvant has given promising results in experimentally controlled conditions and has demonstrated significant protection in the field. Further improvement of the method to produce the inactivated vaccine using bioreactors, antigen storage conditions, and different adjuvant has demonstrated good efficiency in controlled conditions. An additional isolate, Welgevonden, has been attenuated and shown to confer good protection in controlled conditions, and significant protection has also been obtained using DNA vaccination. However, none of these experimental vaccines has been fully validated under field conditions. Field trials and studies on genetic characterisation of strains have revealed the presence of a high number of* E. ruminantium *strains in restricted areas. Thus, antigenic diversity is important in formulating effective vaccines, and further investigations are critical for the delivery of any vaccine in the field.*

A. INTRODUCTION

Heartwater (cowdriosis) is a rickettsial disease of domestic and wild ruminants caused by *Ehrlichia ruminantium* (formerly *Cowdria ruminantium*) and transmitted by *Amblyomma* ticks (Marcelino *et al.,* 2016). *Ehrlichia ruminantium* is classified in the order Rickettsiales and in the family Anaplasmataceae, together with the genus *Anaplasma*. Although ruminants remain the primary target of the pathogen, in South Africa a possible canine *E. ruminantium* infection has been reported, and *E. ruminantium* has been strongly suspected in several cases of rapidly fatal encephalitis in humans. However, in all cases, evidence of *E. ruminantium* infection was based on molecular detection. Isolation and characterisation of the infectious agent is necessary before *E. ruminantium* can be considered an emerging pathogen in species other than ruminants and especially in humans. Since then, no other clinical human case associated with heartwater has been observed.

Heartwater is an important tick-borne disease of livestock in Africa occurring in nearly all the sub-Saharan countries where *Amblyomma* ticks are present and in the surrounding islands: Madagascar, Reunion, Mauritius, Zanzibar, the Comoros Islands and Sao Tomé. The disease is also reported in three Caribbean islands (Guadeloupe, Marie-Galante and Antigua) (Marcelino *et al.,* 2016). All domestic and wild ruminants can be infected, but the former appear to be the most susceptible. Indigenous domestic ruminants are usually more resistant to the disease. Wild animals could play a role as reservoir, but Rusa deer, white-tailed deer, springbok, chital, timor deer, which are used in wildlife farming, seem to be the main wild ruminant species in which heartwater can have a significant economic impact.

The average natural incubation period is 2–3 weeks, but can vary from 10 days to 1 month. In most cases, heartwater is an acute febrile disease, with a sudden rise in body temperature, which may exceed 41°C within 1–2 days after the onset of fever. It remains high for 4–5 weeks with small fluctuations and drops shortly before death.

Fever is followed by inappetence, sometimes listlessness, diarrhoea, particularly in cattle, and dyspnoea indicative of lung oedema. Nervous signs develop gradually. The animal is restless, walks in circles, makes sucking

movements and stands rigidly with tremors of the superficial muscles. Cattle may push their heads against a wall or present aggressive or anxious behaviour. Finally, the animal falls to the ground, pedalling and exhibiting opisthotonos, nystagmus and chewing movements. The animal usually dies during or following such an attack.

Subacute heartwater with less pronounced signs, and peracute heartwater with sudden death, can also occur, according to the breed of ruminant and the strain of *E. ruminantium* involved.

The most common macroscopic lesions are hydropericardium, hydrothorax, pulmonary oedema, intestinal congestion, oedema of the mediastinal and bronchial lymph nodes, petechiae on the epicardium and endocardium, congestion of the brain, and moderate splenomegaly (Marcelino *et al.,* 2016).

A tentative diagnosis of heartwater is based on the presence of *Amblyomma* vectors, nervous signs, and presence of transudates in the pericardium and thorax on post-mortem examination. When making a diagnosis based on clinical signs, the following other diseases should be considered: bovine cerebral babesiosis and theileriosis, anaplasmosis, botulism, haemonchosis in small ruminants, rabies and poisoning.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for diagnosis of heartwater and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Identification of the agent						
In-vitro bacterium isolation	–	–	–	+	–	–
Real-time PCR	–*	–	–	+++	–	–
Nested PCR	–*	–	–	+++	–	–
Multi-pathogen real-time PCR	–*	–	–	+++	–	–
Detection of immune response						
ELISA	++	+	–	–	+++	++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
*can be used to screen tick populations, in parallel with serology on hosts.
PCR = polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay.

1. Identification of the agent

During the febrile reaction, *E. ruminantium* can be readily isolated in culture from blood or plasma; however, it is difficult to detect these organisms in a blood smear. Typical colonies of *E. ruminantium* can be observed in brain smears made after death and this represents a definitive diagnosis for heartwater.

Opening the cranium is not necessary. An alternative method is to cut off the head in front of the first cervical vertebra. Then, introduce a curette through the foramen magnum, between the medulla and the meninges. The curette is turned over towards the brain and removed with a piece of cerebellum. Another method consists of making a hole in the skull with a hammer and a large nail and aspirating a sample of brain cortex with a needle

attached to a syringe. These methods also lessen the danger to the operator in cases where the nervous signs have been caused by rabies.

In the live animal, a brain biopsy may be obtained aseptically after local anaesthesia, although with difficulty; appropriate restraint must be used especially with large and horned animals. Colonies of *Ehrlichia* are observed during the febrile period. This method is useful for experimental studies, but not suitable for routine diagnosis.

Colonies of *E. ruminantium* are still present 48 hours after death in a brain that has been stored at room temperature (20–25°C) and for up to 34 days in a brain that has been stored in a refrigerator at 4°C.

A small fragment of grey matter (approximately the size of a match head) is placed on a microscope slide, crushed to a paste consistency by another slide and, while maintaining pressure, the slides are drawn over each other lengthwise to produce a single layer of cells. The slides are air-dried, fixed in methanol, and stained with eosin and methylene blue or Giemsa. They are then examined under a microscope at a low magnification (×10 objective) to locate the cerebral capillaries and with a magnification of at least ×50 to identify the colonies of rickettsiae. *Ehrlichia ruminantium* are reddish-purple to blue coccoid organisms in the cytoplasm close to the cell nucleus which is coloured in pink (Marcelino *et al.,* 2016). Experience is required as *E. ruminantium* colonies must be differentiated from other haemoparasites (*Babesia bovis*), certain blood cells (thrombocytes, granulocytes), normal subcellular structures (mitochondria, mast cell granules), or stain artefacts (stain precipitates).

Ehrlichia ruminantium colonies are formed from clusters of granules (0.2–0.5 µm), sometimes arranged in the shape of a ring or a horseshoe (1–3 µm), that are placed close to the nucleus inside the endothelial cell. The granules can be scanty, particularly in peracute cases, but they are always present in the brain of an animal that died from heartwater. However, if the animal has been treated with doxycyclin or oxytetracyclin 48 hours before, the granules of *Ehrlichia* tend to fuse, making the diagnosis very difficult, and sometimes impossible. Transmission electron microscopy has been used to demonstrate that the *E. ruminantium* organisms develop inside a vacuole-like structure, which is surrounded by a membrane in the endothelial cell's cytoplasm. Each organism is enclosed by a double membrane. Within the vacuole-like structure, *E. ruminantium* electron-dense forms (elementary bodies), as well as intermediate reticulate forms, are identified.

1.1. Isolation of *Ehrlichia ruminantium* using *in-vitro* culture

Although numerous cell lines have been shown to support the growth of *E. ruminantium,* isolation is not the test of first choice for rapid diagnosis of heartwater, as it is labour intensive and time-consuming. For a rapid diagnosis, molecular methods are preferred. However, *E. ruminantium* isolation should be encouraged for typing the strains present in a region for the purpose of vaccination programmes. *Ehrlichia ruminantium* can be isolated from the blood of reacting animals by cultivation on ruminant endothelial cells. Endothelial cells from umbilical cord, aorta, or the pulmonary artery of different ruminant species (cattle, goat, sheep) are used most often for isolation, although other endothelial cell types (brain capillaries, circulating endothelial cells, etc.) have been described for the routine culture of the microorganism. Endothelial cell lines from sable, eland, buffalo, kudo and bush pig can also be used to grow *E. ruminantium*. No standard cell line has yet been designated for isolation.

1.1.1. Isolation procedure

i) The blood of the clinically affected animal (optimally on the second or third day of febrile reaction) is collected in anticoagulant (heparin or sodium citrate, not ethylene diamine tetra-acetic acid) and diluted 1/2 in the culture medium consisting of Glasgow minimal essential medium (MEM) supplemented with 10% inactivated fetal bovine serum, 200 mM L-glutamine, and antibiotics if necessary (penicillin 100 U/ml, streptomycin 100 µg/ml).

ii) The culture medium is poured off the endothelial cell monolayer, and sampled blood (approximately 2 ml for a 25 cm^2 flask) is added. The flask is incubated at 37°C (if possible with 5% CO_2) on a rocking platform for 2 hours.

iii) After incubation, the blood is poured off and the monolayer is gently washed three times with culture medium prewarmed at 37°C. Fresh culture medium (5 ml per 25 cm^2 flask) is added and the flask is incubated at 37°C with 5% CO_2. The medium is changed every 2 days.

(The *use of plasma instead of blood is more efficient* when taken from an animal with a febrile reaction >41°C. In this case, steps ii and iii above may be replaced with the following:

a) Seed 4 ml of plasma (smaller inoculum can be used if there is a limited amount of plasma available) on to a susceptible endothelial cell culture and incubate for 1 hour at 37°C on a rocking platform.

b) Wash plasma three times with culture medium prewarmed at 37°C. and then add 5 ml of culture medium (per 25 cm^2 flask) and observe for development of cytopathic effect.

iv) The monolayer is inspected regularly for the appearance of small plaques of cell lysis. The first plaques generally appear after about 2 weeks. Passaging on uninfected cell monolayers is performed when the lysis reaches 80% of the cell layer. The remaining cells are stained with eosine and methylene blue or Giemsa and examined microscopically for the presence of *E. ruminantium* morulae (Marcelino *et al.,* 2016).

2. Molecular methods

2.1. Detection of *Ehrlichia ruminantium* using nested polymerase chain reaction

Two nested polymerase chain reaction (PCR) assays have been developed to enhance detection of low levels of rickettsemia (Martinez *et al.,* 2004; Semu *et al.,* 2001). Both use the *pCS20* region as the target sequence. The Semu *et al.* assay uses two external primers: U24 (5'-TTT-CCC-TAT-GAT-ACA-GAA-GGT-AAC-3') and L24 (5'-AAA-GCA-AGG-ATT-GTG-ATC-TGG-ACC-3'), and then the AB 128 (5'-ACT-AGT-AGA-AAT-TGC-ACA-ATC-TAT-3') and AB 129 (5'-TGA-TAA-CTT-GGT-GCG-GGA-AAT-CCT-T-3') for the nested reaction. The analytical sensitivity of detection of this assay is one organism per reaction. The other nested PCR assay (Martinez *et al.*, 2004) uses a pair of external primers that comprise AB128/AB130 (5'-ACT-AGC-AGC-TTT-CTG-TTC-AGC-TAG 3') followed by a second amplification based on AB128/AB129 primers. The final PCR product is 278 pb long. The nested PCR shows a hundred-fold improvement in sensitivity compared with the simple AB128/AB129 PCR, and an average detection limit of 6 organisms per reaction.

Lack of amplification with conventional *pCS20* nested PCR was observed due to single nucleotide polymorphisms (SNPs) on primer hybridisation areas of the *pCS20* fragment DNA. Thus, AB128/129 and 130 primers modified using universal nucleotides allowed the detection of a wider range of *E. ruminantium* strains: AB128' (5'-ACT-AGT-AGA-AAT-TGC-ACA-ATC-YAT-3'), AB130' (5'-RCT-DGC-WGC-TTT-YTG-TTC-AGC-TAK-3') and AB129' (5'-TGA-TAA-CTT-GGW-GCR-RGD-ART-CCT-T-3'). This *pCS20* nested PCR is used routinely at the WOAH Reference Laboratory for diagnostic purposes on blood samples from clinical cases and for tick screening (Adakal *et al.,* 2009; 2010a; Cangi *et al*., 2016). The *pCS20* nested PCRs allow detection in organs (lung and brain) from infected dead animals, blood from infected animals during hyperthermia, and ticks fresh, frozen or preserved in 70% ethanol. The detection of *E. ruminantium* by nested PCR is possible in the blood of animals 1 or 2 days before hyperthermia and during the hyperthermia period but not on asymptomatic animals.

A nested PCR targeting the entire *map*1 polymorphic gene has been developed in parallel in order to type the strains by restriction fragment length polymorphism (RFLP) or sequencing of the amplification fragment directly from the pathological samples testing positive in the *pCS20* nested PCR (Martinez *et al.*, 2004). The *map*1 nested PCR performs well although with a slightly lower sensitivity than the *pCS20* nested PCR with 60 copies per sample. This tool is useful for genetic characterisation of *E. ruminantium* but not for diagnosis due to the polymorphic property of *map1* targeted genes.

The genetic characterisation and structure of the *E. ruminantium* population at the regional level is essential for the selection of potential vaccinal strains. The genetic typing of strains was previously done using RFLP on the *map1* polymorphic gene after PCR amplification (Adakal *et al.,* 2010b; Faburay *et al.,* 2007). However, multi-locus methods adapted to *E. ruminantium* have been validated such as multi-locus sequence typing (Adakal *et al.,* 2009) and multi-locus variable number of tandem repeat sequence analysis (Pilet *et al.,* 2012). These tools are being used on field tick samples for molecular epidemiological studies to better characterise the genetic structure of *E. ruminantium* strains (Adakal *et al.,* 2010a; Cangi *et al.,* 2016). However, these genetic characterisations are not associated with clusters of protection.

2.2. Detection of *Ehrlichia ruminantium* using real-time PCR

Several real-time PCR tests targeting *map1*, *map1-1* and *pCS20* region genes have been developed for the detection of *E. ruminantium* organisms. SYBR Green real-time PCRs targeting *map1* and *map1-1* were used to detect and quantify *E. ruminantium in vitro* during mass antigen production in a bioreactor and in experimentally infected sheep during the hyperthermia period (Marcelino *et al.,* 2005; 2007; Peixoto *et al.,* 2005; Postigo *et al.,* 2002). They were tested on a limited number of strains (up to six strains) and therefore they are not recommended for diagnostic purposes due to the polymorphic characteristics of *map1* multigenic family.

A real-time PCR assay targeting the *pCS20* region using a fluorescent-labelled probe has been developed to detect *E. ruminantium* in livestock blood and ticks from the field, and has a sensitivity similar to the nested PCR with seven copies per sample. The sequences of primers and probes are: CowF (5'-CAA-AAC-TAG-TAG-AAA-TTG-CAC-A-3'), CowR (5'-TGC-ATC-TTG-TGG-TGG-TAC-3') and Cow probe (5'-FAM-TCC-TCC-ATC-AAG-ATA-TAT-AGC-ACC-TAT-TA-XT-PH-3'). Unfortunately, this assay displayed cross reaction with *E. canis* and *E. chaffeensis.* It successfully detected 15 different *E. ruminantium* strains (Steyn *et al.,* 2008). As shown previously for the *pCS20* nested PCR, the presence of SNPs on hybridisation regions could inhibit strain detection. Testing more strains is necessary to further validate the method.

More recently a new real-time PCR targeting another *pCS20* region has been developed and demonstrated a good reproducibility, sensitivity and specificity with a limit of detection of 6 copies per sample. It can be used with appropriate fluorescent probes. Primers and probes are: Sol1F (ACA-AAT-CTG-GYC-CAG-ATC-AC), Sol1R (CAG-CTT-TCT-GTT-CAG-CTA-GT) and Sol1^{TqM} (6-FAM-ATC-AAT-TCA-CAT-GAA-ACA-TTA-CAT-GCA-ACT-GG-BHQ1). It detects 16 *E. ruminantium* strains from different geographical areas and there is no cross protection with *Anaplasma marginale, A. phagocytophilum, A. platys, Babesia bovis*, *B. bigemina, E. canis*. *E. muris* and *Rickettsia felis* and *parkeri* (Cangi *et al.*, 2017). It has been tested on 700 tick field samples from Mozambique and will be used routinely in the WOAH Reference Laboratory for diagnostic use and tick screening.

Although nested and real-time PCR methods have proved highly effective in detecting infection in ticks or in animal samples during the clinical phase of the disease or after death, they could not allow detection of *E. ruminantium* in healthy carrier ruminants. A useful technique for confirming the status of a suspected carrier animal, whose blood is PCR negative, is to feed batches of naive ticks on the animal and then test the ticks by *pCS20* nested or real-time PCR. It is not known whether ticks act simply by concentrating circulating organisms, or by amplifying their number or even by inducing release of micro-organisms in the circulation during feeding.

2.3. Detection of *E. ruminantium* using multi-pathogen real-time PCR

A single FRET-real-time PCR has been developed to differentiate eight species in four distinct groups in a single reaction: *E. canis, E. chaffeensis, E. ewingii, E. muris, E. ovina, Ehrlichia* sp. *BOV2010,* Panola Mountain *Ehrlichia* and *E. ruminantium* (Zhang *et al.,* 2015). It is based on 16S recombinant RNA amplification using two fluorescent probes; different dissociation curves are detected depending on the species. *Ehrlichia ruminantium* could be clearly differentiated from other species with the lowest melting temperature. The limit of detection is five copies per sample and simultaneous detection is possible with a mix of 300 copies of each species. However, only four *E. ruminantium* strains were tested. Even if the lack of amplification is limited due to the targeted conserved gene 16S recombinant RNA, validation on more *E. ruminantium* strains is necessary.

On the other hand, Sayler *et al.* developed a dual-plex Taqman real-time PCR targeting the groEL gene of Panola Mountain *Ehrlichia* and *E. ruminantium* (Sayler *et al.,* 2016). This assay allows Panola Mountain *Ehrlichia,* which is endemic in the USA, to be differentiated from *E. ruminantium,* which is currently not present. The limit of detection is 10 copies per sample, and 27 *E. ruminantium* strains from 11 countries were detected. It seems therefore to be a promising method for differential diagnosis between the two species.

2.4. Detection of *Ehrlichia ruminantium* using the reverse line blot technique

The reverse line blot technique (RLB) has been used for the simultaneous detection and identification of *Anaplasma* and *Ehrlichia* species known to occur in ruminants, on the basis of differences in the small subunit rRNA gene (Bekker *et al.,* 2002). Primers 16S8FE and B-GA1B-new were designed from conserved domains and used to amplify a 492–498 bp fragment of the 16S rRNA gene spanning the variable V1 region. Species-specific oligonucleotide probes were designed in this V1 loop to allow species-specific detection of *E. ruminantium*, *E. ovina*, *Ehrlichia* sp. strain Omatjenne, *Anaplasma marginale*, *A. centrale*, *A. bovis*, *A. ovis* and *A. phagocytophilum*. One oligonucleotide probe cross-reactive with all species (catch-all probe) was also designed to serve as a control in case a PCR product does not hybridise to any of the species-specific probes. In the method, the species-specific probes are covalently linked to the hybridisation membrane, which is hybridised with the PCR product obtained using primers 16S8FE and B-GA1B-new. PCR products obtained from all above-mentioned microorganisms were shown to bind with specific oligonucleotide probes only. No PCR product was detected and no hybridisation occurred when the PCR-RLB was applied to *Theileria annulata*, *Babesia bigemina* or mammalian DNA. Similarly, negative control ticks were always negative in the RLB assay whereas it was possible to detect *Ehrlichia ruminantium* infection in 15–70% of ticks fed on experimentally infected or long-term carrier sheep. In Mozambique, *E. ruminantium* could also be detected in the blood of 12 sentinel small ruminants placed in the field with the infected animals; mixed infection was detected in five of the infected sentinel animals, thus demonstrating the usefulness of the method for detecting multiple infections. The RLB has been used recently in several studies in Western Kenya and in Nigeria to evaluate the prevalence of tick-borne diseases in cattle (Lorusso *et al.,* 2016; Njiiri *et al.,* 2015) however, they obtained very low prevalence of *E. ruminantium*. It was suggested by the authors that sequences of the primers and probes of RLB were not adapted to Kenya *E. ruminantium* strains.

2.5. Reading the results

As *E. ruminantium* is an obligate intracellular bacterium that cannot be cultivated in acellular media and its isolation is complex and takes several weeks, molecular tools are the best methods for the diagnosis of heartwater. Nested and real-time PCRs prove to be easier to perform and more sensitive than RLB. With all PCRs, however, care must be taken to ensure that no cross-contamination occurs between samples. Negative and positive controls must be included in each test. For each PCR assay, nested or real-time, positive and negative extraction controls (from experimentally infected and uninfected blood or organs, infected or uninfected ticks) should be included allowing detection of a default in the extraction procedures. The absence of inhibitor products in each sample should be proven by targeting a housekeeping gene from the vector or the host such as 16S or 18S ribosomal DNA (for tick screening, Cangi *et al*., 2017). As heartwater serology has several limitations (see Section B.3), the PCR could be used to help confirm if seronegative animals, originating from an endemic area, are not infected, prior to translocating them to a heartwater-free area that has the risk of becoming infected, because of the presence of potential vectors. Screening of ticks by PCR, along with serology on targeted herds over time could be used to establish the herd status before any movement of animals from this endemic area to a free area. However, *E. ruminantium* cannot be detected in asymptomatic carriers by molecular methods. The results obtained with nested PCRs, the RLB assay and real-time PCR, show that the direct detection of *E. ruminantium* in the blood is only reliable during and around the febrile phase of the disease. PCR-based methods appear to be more reliable in detecting infection in ticks, and this could have epidemiological value in determining the geographical distribution of *E. ruminantium*. In addition, when necessary in endemic areas, the inclusion of testing (originally naive) ticks fed on a suspect animal would greatly improve the sensitivity of carrier detection when serology and PCR on blood have failed. The procedure is nevertheless not suitable for routine diagnostic laboratories as it requires the maintenance of tick colonies and the capacity to experimentally infect animals.

3. Serological tests

To minimise the problem of cross-reactions with *Ehrlichia* spp., two enzyme-linked immunosorbent assays (ELISAs) based on a recombinant MAP1 antigen have been developed. The first is an indirect ELISA that uses an immunogenic region of the MAP1 protein (called MAP1-B) and gives far fewer cross-reactions with *Ehrlichia* spp. (MAP1-B ELISA) (Semu *et al.,* 2001). The second is a competitive ELISA that uses the *map*1 gene cloned in a baculovirus and monoclonal antibodies (MAbs) raised against the MAP1 protein (MAP1 C-ELISA) (Mondry *et al.,* 1998). Both tests have dramatically improved specificity, but they still show some reactivity with high titre sera

against *E. canis*, *E. chaffeensis* and Panola Mountain *Ehrlichia*. Cross reaction of serum from Panola Mountain *Ehrlichia*-infected goats has been observed with *E. ruminantium* MAP1-B antigen and, conversely, serum from heartwater-infected sheep with MAP1-B of Panola Mountain *Ehrlichia,* thus preventing their use in the detection of *E. ruminantium* introduction on the American mainland (Sayler *et al.,* 2016). The MAP1-B ELISA has been the most extensively used and will be described in detail. Serology as a diagnostic tool for detecting individual animals exposed specifically to *E. ruminantium* is therefore unreliable. Serology should be considered at the herd level taking into consideration the epidemiological environment and, if necessary, be complemented by molecular techniques.

3.1. MAP1-B enzyme-linked immunosorbent assay (Semu *et al.*, 2001)

Using the vector pQE9, the PCR fragment MAP1-F2R2, which encodes the amino acids 47–152 of the MAP1 protein including the immunogenic region MAP1-B, is expressed in *Escherichia coli* M15[pREP4] as a fusion protein containing six additional histidine residues. The recombinant MAP1-B is purified using Ni^{2+}-NTA agarose (nitrilotriacetic acid agarose) under denaturing conditions as described by the manufacturer. The antigen is preserved at 4°C and each batch is titrated.

The antigen is diluted at 0.5 µg/ml in 0.05 M sodium carbonate buffer, pH 9.6, and immobilised onto polystyrene plates by incubation for 1 hour at 37°C, and stored at 4°C until use. However, in initial trials, an antigen concentration of 2 µg/ml reduced background noise and improved specificity (data not shown: Semu *et al.*, 2001).

3.1.1. Test procedure

i) Plates are blocked for 30 minutes by adding 100 µl per well of 0.1 M phosphate buffered saline (PBS), pH 7.2, supplemented with 0.1% Tween 20 (PBST) and 3% non-fat dry milk (PBSTM).

ii) The plates are washed three times with PBS supplemented with 0.1% Tween 20 (PBST) and twice with distilled water.

iii) 100 µl of test serum diluted 1/100 in PBSTM is added in duplicate to wells, which are then incubated for 1 hour at 37°C.

iv) Plates are washed three times in PBST and twice in distilled water.

v) Horseradish-peroxidase-conjugated anti-species IgG optimally diluted in PBSTM is added at 100 µl per well and the plate is incubated for 1 hour at 37°C.

vi) After washing as in step iv, each well is filled with 100 µl of 0.1 M citrate buffer, pH 5.5, containing 0.5 mg/ml orthophenylene-diamine and 3 µl/ml of 9% H_2O_2.

vii) The reaction is stopped after 30 minutes of incubation at room temperature (20–25°C) by adding 50 µl of 2 N H_2SO_4. Absorbance is read at 495 nm. Positive and negative controls are included in each plate.

3.2. MAP1 competitive enzyme-linked immunosorbent assay (Mondry *et al.*, 1998)

Recombinant MAP1 antigen is prepared as follows: 8-day-old *Trichoplusia ni* insect larvae are infected by a baculovirus expressing the *map1* gene and moribund larvae are homogenised (10% [w/v]) in PBS supplemented with 0.001% (v/v) Triton X-100.

Anti-MAP1 MAb is prepared as follows: spleen cells of BALB/C mice previously inoculated with larval homogenate are fused to SP2/0 cells. Supernatant fluids from hybridoma cell cultures are screened for reactivity with MAP1 by immunoblotting and immunoperoxidase methods. A reactive cell culture is subcloned, isotyped and subsequently used for MAb production.

After a further 1/800 (v/v) dilution in PBS, the antigen is immobilised on to polystyrene plates (Nunc-Immuno Plates PolySorp) by incubation overnight at 4°C, and stored at –70°C.

3.2.1. Test procedure

i) Prior to use, the plates are blocked for 30 minutes by adding 100 µl per well of PBS, pH 7.2, supplemented with 0.05% Tween 20 and 5% nonfat dry milk.

ii) Plates are washed three times with PBS/Tween, 50 µl/well of test serum diluted 1/50 in PBS supplemented with 0.05% Tween 20 and 1% nonfat dry milk (PBSTM) is added in duplicate and the plates are incubated for 30 minutes at 37°C.

iii) Without an intervening washing step, 75 µl/well of the MAb diluted 1/4000 (v/v) in PBSTM is added and the plates are incubated for another 30 minutes at 37°C.

iv) Plates are washed three times in PBS/Tween and horseradish-peroxidase-conjugated anti-mouse IgG optimally diluted in PBSTM is added at 50 µl per well. The plate is incubated for 1 hour at 37°C.

v) After three washings as before, 100 µl of 0.1 M citrate buffer, pH 5.5, containing 0.5 mg/ml O-phenylene diamine and 3 µl/ml of 9% H_2O_2 are added to each well. After 30 minutes of incubation at room temperature in the dark, the reaction is stopped by adding 50 µl of 2 N H_2SO_4 and the absorbance is read at 495 nm. Positive and negative controls are included in each plate.

3.3. Reading the results

Both the MAP1-B ELISA and the MAP1 C-ELISA have shown a high specificity after evaluation in 3000 ruminant sera (goat, sheep and cattle) collected from 14 *A.-variegatum*-infested islands of the Lesser Antilles, among which only three are known to be infected by *E. ruminantium* (Mondry *et al.*, 1998). Overall specificity calculated from the 11 heartwater-free islands was 98.5% and 99.4% for the MAP1 C-ELISA and the MAP1-B ELISA, respectively. In another study undertaken in the Caribbean, ELISA MAP1-B positive samples were found in four of six islands free of heartwater (Kelly *et al.,* 2011). Moreover, high seroprevalence in vector-free areas of Zimbabwe or South Africa has also been reported although not explained (it may be caused by a cross-reacting agent not transmitted by *Amblyomma*) and should be kept in mind when interpreting the results (Kakono *et al.,* 2003).

Evaluating the sensitivity of the tests is more problematic as it would require knowledge of the exact status of a high number of animals sampled in the field. As mentioned before there is currently no simple technique available to confirm if an animal is infected. Experimentally, the sensitivity of the C-ELISA in goats was reported to be 91.6–95.4% for the MAP1-B ELISA, and 96.3–96.9% for the MAP1 C-ELISA (Mondry *et al.,* 1998). However, in another study the sensitivity averaged 95% for cut-off values set at 31% and 26.6% of the positive control serum for sheep and goat sera, respectively (Mboloi *et al.,* 1999). Indeed, calculations are based on a limited number of experimentally inoculated animals in a period of time soon after inoculation, when almost all the animals are still positive. Sensitivity in cattle is even lower and several reports show that after infection most of the animals become seronegative again in less than 6 months and some animals never seroconvert (Mahan *et al.,* 1998b; Semu *et al.,* 2001). This observation is in line with the difference in antibody prevalence observed between small ruminants and cattle in epidemiological surveys that cannot be explained by a lower risk of infection of the latter. For example, in Zimbabwean farms situated in endemic areas, more than 90% of goats presented antibodies in their serum compared with only 33% of cattle maintained in the same conditions (Mahan *et al.,* 1998b). Similar observations were made in the Caribbean.

Serological tests are useful for the assessment of heartwater antibody response in vaccinated animals. The tests should not be used to screen animals for importation into heartwater-free areas. Antibodies are maintained at detectable levels in naturally infected domestic ruminants for a few months only and circulating antibodies disappear more rapidly in cattle than in small ruminants. It is thus possible that serologically negative animals may be carriers of infection. Serology should therefore only be considered as a diagnostic method to be applied at the herd level and not at the individual animal level (Peter *et al.,* 2001). When interpreting diagnostic serology results, other epidemiological parameters must be considered.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

No commercial vaccines are available at present. The only method of immunisation against heartwater remains the "infection and treatment" method using infected blood followed by treatment of reacting animals with tetracycline. This method is still in use in several areas, but it is likely to be replaced by preparations using attenuated or inactivated organisms, which have given promising research results.

1. Inactivated vaccine preparations

Inactivated vaccine based on *E. ruminantium* elementary bodies chemically inactivated or lysed, emulsified in oil adjuvant, conferred good protection against homologous and field challenges (Adakal *et al.,* 2010b; Mahan *et al.,* 1998a; Marcelino *et al.,* 2015a; Martinez *et al.,* 1996). However, it does not prevent vaccinated animals from developing clinical signs, and morbidity is observed after virulent challenge.

The development of a large-scale production process and optimisation of storage conditions for the inactivated vaccine has led to a decrease in the cost of a vaccinal dose to 0.11 euros (Marcelino *et al.,* 2007; Peixoto *et al.,* 2005). In 2015, Marcelino *et al.* developed a ready-to-use inactivated vaccine that could be easily used in the field. It was shown that even after breaking the cold-chain of 3 days at 37°C, mimicking field conditions, the vaccine was still efficient (Marcelino *et al.,* 2015a). This study showed the robustness of the vaccine under field conditions.

In Zimbabwe, field trials of the inactivated vaccine emulsified in oil adjuvant have also demonstrated protection of sheep against natural tick challenge (Mahan *et al.,* 1998a). In larger field trials conducted in eastern and southern Africa, a significant reduction in mortality has been achieved in cattle, goats and sheep using either a prototype strain from Zimbabwe (Mbizi strain) or a local strain from the experimental sites (Mahan *et al.,* 2001). However, in three out of four sites, the vaccine prepared from the local isolate was less effective than the prototype Mbizi vaccine, strongly suggesting an inadequate coverage of the antigenic repertoire of isolates present in each site. Vaccination trials in Burkina Faso showed a significant increase in the protective effect of inactivated vaccine when a local strain was added to the Gardel vaccinal strain (Adakal *et al.,* 2010b).

Lack of cross-protection between *E. ruminantium* isolates due to genetic or antigenic diversity is well established, but the complexity of the *E. ruminantium* population structure in the field has been underestimated. A large *E. ruminantium* genetic diversity has been observed throughout Africa, the Caribbean islands and the Indian Ocean, which raises the problem of the protective effect of the vaccinal strain against field strains (Adakal *et al.,* 2010a; Cangi *et al.,* 2016; Raliniaina *et al.,* 2010). Even if genetic characterisation is defined, there is a lack of a genetic marker associated with protection; moreover, it is essential to isolate *in-vitro* field strains to know their capacity for protection against heterologous strains and be able to mix several strains in the inactivated vaccine in order to cover widely the genetic diversity of field strains.

Inactivated vaccine is being developed commercially in South Africa. These inactivated vaccines do not prevent infection but do prevent or reduce death of vaccinated animals when exposed to live virulent challenge. The advantage however is that several field strains can be incorporated to make the vaccine more widely cross-protective.

A major challenge remains the identification of *E. ruminantium* genetic markers associated with protection in order to identify the vaccinal strains to include in the inactivated vaccine adapted to a region.

2. Attenuated vaccine preparations

Infection of ruminants with live *E. ruminantium* strains induces a strong long-lasting protection against an homologous isolate. This is the basis for the "infection-and-treatment" method using virulent isolates. Isolates of attenuated virulence that do not require the treatment of animals would be ideal, but a limited number of such attenuated isolates are available. An attenuated Senegal isolate has been obtained and shown to confer 100% protection against an homologous lethal challenge, but very poor protection against a heterologous challenge. The Gardel isolate, which gives a significant level of cross-protection with several isolates (although far from complete), has also been attenuated (Marcelino *et al.,* 2015b). A third isolate named Welgevonden from South Africa has been attenuated and shown to confer complete protection against four heterologous isolates under experimental conditions (Zweygarth *et al.*, 2005). However, it has not been tested in field conditions. The main drawback of attenuated vaccines is their extreme lability, which necessitates their storage in liquid nitrogen and their distribution in frozen conditions. In addition, they have to be administered intravenously. Moreover, there is

also a possible reversion to virulence and, as it is a live vaccine, it could not be used in heartwater free areas. Despite the recent efforts to understand the mechanism of virulence and attenuation (Marcelino *et al.,* 2015a), these are still largely unknown independently from the strain.

3. Recombinant vaccine preparations

Several reports show partial protection of mice using *map1* DNA vaccination and an improvement of protection by vaccination following a prime (plasmid) – boost (recombinant MAP1) protocol (Nyika *et al.,* 2002). However, protection of ruminants has never been demonstrated using this strategy. In opposition, significant protection of sheep was reported against homologous and heterologous experimental challenge following plasmid vaccination using a cocktail of four ORFs (open reading frames) from the 1H12 locus in the *E. ruminantium* genome (Collins *et al.,* 2003). No further results have been described since then. Recombinant vaccines will probably not be available in the near future. A prime DNA/boost recombinant protein vaccine has been developed (Pretorius *et al.,* 2008). An efficient protective effect was obtained using a cocktail of four open reading frames (ORFs) against homologous challenge, but the vaccine did not give satisfactory results during field tick challenge. Moreover, simple intramuscular immunisation is not sufficient to induce protection. The use of a gene gun is necessary for prime DNA injection, which is not suitable for a large vaccination campaign. A polymorphic gene was identified as an efficient component of a recombinant vaccine against heartwater using the prime/boost method (Pretorius *et al*., 2010). However, as this gene is polymorphic, a recombinant vaccine should include at least three different genotypes.

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NB: There is a WOAH Reference Laboratory for heartwater (please consult the WOAH Web site:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3)
Please contact the WOAH Reference Laboratories for any further information on
diagnostic tests, reagents and vaccines for heartwater

NB: FIRST ADOPTED IN 1990. MOST RECENT UPDATES ADOPTED IN 2018.