CHAPTER 2.1.6.

HEARTWATER

SUMMARY

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, threatening the American mainland. 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. Hydropsicardium and hydrothorax and lung oedema are commonly associated post-mortem signs. 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 adequate 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. With fast stains, the smears can be fixed and stained in less than 1 minute. The colonies (clusters) 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.

Fresh whole blood collected from suspect animals can be inoculated intravenously into a susceptible sheep or goat. The development of clinical signs and the demonstration of E. ruminantium in the brain of the inoculated animal during the febrile reaction are diagnostic for heartwater. Due to animal welfare concerns, this method should be avoided.

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 eosin–methylene blue or by immunofluorescence or immunoperoxidase techniques using a specific antiserum.

DNA probes and especially the more sensitive polymerase chain reaction (PCR) techniques are available to reveal the presence of E. ruminantium in the blood of animals with clinical signs, and in the tick vectors, to a lesser extent in the blood or bone marrow of carrier animals. Apart from diagnosis, PCR is widely used for research on the E. ruminantium genome and for epidemiological studies.
Serological tests: Serological tests available include indirect fluorescent antibody tests, enzyme-linked immunosorbent assays (ELISAs) and Western blot. However, when the whole E. ruminantium is used as antigen, cross-reactions with Ehrlichia spp. occur in all of these tests. Serology has limited diagnostic applications.

One recently developed ELISA uses a recombinant antigen expressed as a partial fragment of the recombinant major antigenic protein 1 (MAP1) antigens – the MAP1-B ELISA. This test has shown a dramatic improvement in specificity compared with previous tests. Although this test is more specific, it still detects cross-reacting antibodies to other Ehrlichial organisms. Hence, definitive proof of heartwater must rely on epidemiological evidence and additional molecular testing indicating the presence of the organism. This ELISA has made the interpretation of serological results more reliable in regions where Ehrlichia infections occur in ruminants. It 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 they recover from the infection.

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; in addition repeated PCR testing should be carried out to demonstrate that the pathogenic agent is not present in the herd.

Requirements for vaccines and diagnostic biologicals: The 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 Montanide ISA 50 adjuvant has given promising results in experimentally controlled conditions and has demonstrated significant protection in the field. An additional isolate, Welgevonden, has been attenuated and shown to confer good protection, and significant protection has also been obtained using DNA vaccination. However, none of these new experimental vaccines has been fully validated under field conditions. Field trials have revealed that 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 (3, 8, 36). It is also known by the synonyms malkopsiekte (Afrikaans), péricardite exsudative infectieuse (French), hidocarditis infecciosa (Portuguese), idropericardite dei ruminanti (Italian), and a variety of names in different African languages (5). Ehrlichia ruminantium is classified in the order Rickettsiales and in the family Anaplasmataceae, together with the genera Anaplasma. Although ruminants remain the primary target of the pathogen, in South Africa a possible canine E. ruminantium infection has been reported (1), and, more recently, E. ruminantium has been strongly suspected in several cases of rapidly fatal encephalitis in humans (14). 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.

Heartwater occurs in nearly all the sub-Saharan countries of Africa 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 the Caribbean (Guadeloupe, Marie-Galante and Antigua) (34), from where it threatens the American mainland. 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 (36).

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 (4), 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 (4), petechiae on the epicardium and endocardium, congestion of the brain, and moderate splenomegaly.

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

#### 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 (42) 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 and harmlessly 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 (5).

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, stained with Giemsa diluted with Sörensen buffer (2.54 g KH$_2$PO$_4$; 8.55 g Na$_2$HPO$_4$·H$_2$O; q.s. to 5 litres with distilled water), pH 7.2, and washed with tap water. Fast Giemsa stains (DiffQuick, RAL555, Field’s stain, CAM’s Quick stain) give quicker results, but the colour contrast is usually poorer. Some ‘fast’ stains do provide excellent contrast, e.g. Hema 3 stain.

The slides are examined under a microscope at a low magnification (×10 objective) to locate the cerebral capillaries. An oil-immersion lens with a magnification of at least ×50 is useful for identifying the colonies of rickettsiae. Experience is required *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), etc. The specificity of the reading can be improved by staining formalin-fixed brain sections using immunoperoxidase techniques.

*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.

Fresh whole blood collected from suspect animals can be inoculated intravenously into a susceptible sheep or goat. The development of clinical signs and the demonstration of *E. ruminantium* in the brain of the inoculated ruminant are diagnostic for heartwater.
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 (39). 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.

a) Isolation of *Ehrlichia ruminantium* using in-vitro culture

Although numerous cell lines have been shown to support growth of *E. ruminantium*, isolation is not the first choice of test for a rapid diagnosis of cowdriosis as isolation is a labour intensive and time-consuming laboratory procedure. For a rapid diagnosis, polymerase chain reaction (PCR)/molecular diagnosis is preferable. 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 (45). 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.

- **Isolation procedure**
  - i) The blood of the reacting animal (optimal time for detection of the organism is the second or third day of febrile reaction) is collected in anticoagulant (heparin or sodium citrate, not ethylene diamine tetraacetic acid) and diluted 1/2 in the culture medium consisting of Glasgow minimal essential medium (MEM) supplemented with 10% inactivated fetal bovine serum, 2.95 mg/ml tryptose phosphate broth, 200 mM L-glutamine, and antibiotics if necessary (penicillin 100 international units/ml, streptomycin 100 µg/ml).
  - ii) The culture medium is poured off the endothelial cell monolayer, and infective blood (approximately 2 ml for a 25 cm² flask) is added. The flask is incubated at 37°C 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 is added and the flask is incubated at 37°C. The medium is changed twice weekly.
    (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:
    - • Seed 4 ml of plasma (smaller inoculum can be used if there is a limited amount of plasma available) onto a susceptible endothelial cell culture and incubate for 1 hour at 37°C on a rocking platform.
    - • Wash off plasma with growth medium and then add 5 ml of growth medium (per 25 cm² 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 eosin/methylene blue or Giemsa or DiffQuick and examined microscopically for the presence of *E. ruminantium* morulae. Alternatively, cells can be stained by an indirect fluorescent antibody (IFA) test or an immunoperoxidase test using an *E. ruminantium*-specific antiserum; the immunoperoxidase test is not commonly used.

b) Isolation of *Ehrlichia ruminantium* using in-vivo culture

It is feasible to assess the presence of heartwater in a herd, a region or a country, or to isolate a strain of *E. ruminantium* by inoculating blood or tick homogenate into a susceptible animal. However, due to the animal welfare concerns, this method is not recommended. Blood from individual animals, or pooled blood, is injected slowly at a dose of 10–100 ml intravenously into a susceptible sheep or goat. Blood as an inoculum, to determine infection status of herd, will be infective if there are clinically infected donor animals present; however, the method will rarely detect infection in carrier/recovered animals. Another method consists of collecting and homogenising adult *Amblyomma* ticks, and after centrifuging the homogenate and then inoculating the resulting supernatant into susceptible hosts. This method can be more sensitive than blood from suspect animals (especially if blood is from recovered animals) because the concentration of *E. ruminantium* is higher in the tick than in the blood. However, the tick infection rate in the field is variable and sometimes as low as 1% (6). In this case, to detect an infection as many ticks as possible should be used; at least 100 ticks are needed. In both cases, the inoculum, to which 10% dimethyl sulphoxide (final concentration) has been added, can be stored in liquid nitrogen for several years. Note that inoculation of tick homogenates into susceptible animals may cause anaphylaxis, which can be prevented by the simultaneous administration of adrenaline. The development of clinical signs and the detection of circulating rickettsiae by molecular methods and/or the demonstration of *E. ruminantium* in the brain of the inoculated...
ruminant, on the second or third day of fever, are diagnostic for heartwater. In addition, confirmation could be accomplished by in-vitro isolation on endothelial cells using plasma from the inoculated animals.

2. Molecular methods

a) Detection of *Ehrlichia ruminantium* using DNA probes

A genomic DNA fragment pCS20 specific for *E. ruminantium* has been cloned and used as a nucleic acid probe (24, 50). It recognises all strains of *E. ruminantium* tested to date. This probe, designated pCS20, readily detects infection in clinically ill animals and experimentally infected Amblyomma ticks (18, 20, 24, 51). However, it is not sufficiently sensitive to detect most carrier animals or low level infections in ticks (35, 36).

The pCS20 probe proved nevertheless to be more sensitive than 16S and MAP1 (major antigenic protein 1) probes for the detection of *E. ruminantium* in ticks when hybridised on a PCR-amplified product of the homologous DNA fragment (2).

b) Detection of *Ehrlichia ruminantium* using PCR and nested PCR

Two primers – AB128 (5’-ACT-AGT-AGA-AAT-TGC-ACA-ATC-TAT-3’) and AB129 (5’-TGA-TAA-CTT-GGT-GCG-GGA-AAT-CCT-T-3’) – have been designed from the DNA sequence of the pCS20 probe (24) for use in a PCR. These primers amplify a 279 base pair DNA fragment which is specific only for *E. ruminantium*. Hybridisation of the amplified PCR products to a labelled pCS20 probe, as an additional step, resulted in a 350-fold more sensitive assay than using the nucleic acid probe to detect *E. ruminantium* directly in DNA extracted from ticks. Low levels of infection in animals and in ticks fed on carrier animals are detected by PCR, while a hybridisation reaction with the pCS20 probe alone (without PCR first) usually remains negative (37).

Experimentally, the detection limit of the conventional PCR assay was found to be between 10 and 10^2 organisms, whereas it was between 1 and 10 organisms after PCR/hybridisation. The PCR/hybridisation has been shown to detect 37 strains from all endemic areas with a specificity of 98%. However, the sensitivity of the PCR assay is variable, ranging from 88 to 97% with tick samples containing 10^3 to 10^6 organisms, and dropping to 61% and 28% with samples containing 10^3 and 10^2 organisms, respectively (35). Consequently, the rate of 86% of ticks testing positive when fed on a clinically reacting animal dropped to 21% when fed on carrier animals due to a lower rickettsemia in such animals. The PCR/hybridisation assay has been used widely to define the epidemiology of heartwater in southern Africa.

Two nested PCR assays have been developed to enhance detection of low levels of rickettsemia and to do away with the hybridisation step (30, 43). 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 and AB 129 for the nested reaction. The sensitivity of detection of this assay is one gene copy of the pCS20 fragment or 1 organism. The other nested PCR assay (30) uses a pair of external primers comprises the AB128 sense primer together with an anti-sense primer called AB130. These amplify a 413 bp fragment used as a template in a second round PCR using AB128 and AB129 as internal primers. The use of AB128 and AB129 primers avoids the need to repeat a full evaluation of the test specificity. The nested PCR shows a hundred-fold improvement in sensitivity compared with a simple PCR, and an average detection limit of 6 organisms. The direct implication of this was an increase in the detection rate in wild ticks of from 1.7% to 36% in an epidemiological study in the Caribbean. The detection limit is comparable to that of the PCR/hybridisation method, which is nevertheless much more complex and time-consuming to perform. The pCS20 nested PCR allowed regular detection of *E. ruminantium* organisms from ticks, blood, brain and lungs from infected animals, whether the samples were processed fresh, or after freezing or preservation in 70% ethanol. One drawback of the nested PCR is that extreme care needs to be exercised to prevent introduction of contamination due to repeated opening of the tubes containing the first PCR reaction when conducting the nested reaction.

A nested PCR targeting the entire map1 polymorphic gene has been developed in parallel in order to type the strains by restriction fragment length polymorphism or sequencing of the amplification fragment directly from the pathological samples testing positive in the pCS20 nested PCR (30). An additional nested PCR targets the polymorphic map1 gene and can be used to type circulating heartwater strains for vaccine selection and disease management. PCR amplicons are analysed by restriction fragment length polymorphisms or sequencing. A high genetic diversity of *E. ruminantium* is observed in the field may influence the formulation of vaccines and needs to be further investigated. The map1 nested PCR performs well although with a slightly lower sensitivity than the pCS20 nested PCR. Its detection limit was evaluated at around 60 organisms and only 91% of samples testing positive in the pCS20 nested PCR also tested positive in the map1 nested PCR; some positives of low intensity found using the pCS20 nested PCR were negative in the map1 PCR.

Primers 32F1 and 32R1 designed from the sequence of the MAP1 gene of *E. ruminantium* as well as additional primer sets designed to target the MAP1, MAP2, gltA, and 16SrDNA genes of *E. ruminantium*...
have been used in PCR to detect the pathogen in tick, blood and bone marrow of carrier sheep and wild African ungulates, but these methods has not been widely evaluated and used.

Although the 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, only limited studies have been done to evaluate their value in healthy carrier ruminants. *Ehrlichia ruminantium* can easily be demonstrated in the blood of infected animals just before the onset of the febrile period and for a few days after recovery (24, 43), but after that period, its detection is sporadic and appears to be dependant on the rickettsemia levels. In one study in Zimbabwe only between 3.3 and 26.7% of cattle, and 23.3% of goats were found to be positive, while data from ticks collected in the same area would suggest that given the age of the cattle or goats, they should have all been exposed or infected with *E. ruminantium* (19). A comparison of the indirect MAP1-B ELISA and the pCS20 PCR/hybridisation assay, to evaluate their respective detection sensitivity levels over a period of 8 weeks (tests performed every 2 weeks), was done on 15 cattle located in Zimbabwe on a heartwater-endemic farm where tick control was minimal and the infection pressure was high (44). The *E. ruminantium* tick infection rate on this farm was between 10 and 12%. The data demonstrated that the pCS20-PCR assay was more reliable in detecting infection in blood of these cattle than detection of antibodies by the indirect MAP1-B ELISA. These cattle were not always PCR positive or positive for antibodies at every testing time and some cattle were negative by PCR throughout the study. These data suggest that the rickettsemia levels fluctuate from high to low, and that the PCR detects infection when the levels are high. Hence detecting carrier/recovered animals is less reliable than detecting clinically infected animals. This highlighted the fact, that for determination of the status of sub-clinical animals, it is advisable to repeatedly test the blood of such animals for *E. ruminantium* by the pCS20-PCR assay. Whether the absence of detection in most carrier animals is due to an insufficient sensitivity of the PCR methods for detecting very low rickettsemia, or is due to an intermittent release of organisms in the circulation, is not fully understood. 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 a pCS20 semi-nested PCR. It is not known whether ticks act simply by concentrating circulating organisms, or also by amplifying their number or even by inducing release of micro-organisms in the circulation during feeding.

c) 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 (3). Primers 16S8F 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*, *E. 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 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 16S8F 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. However, the sensitivity of the assay has not yet been determined and there is a need to further validate the technique in large epidemiological studies.

d) Detection of *Ehrlichia ruminantium* using real-time PCR

Two real-time PCR (QPCR) tests have been described for the detection and quantitative determination of *E. ruminantium* organisms. In a first test, a 182 bp fragment from the non-polymorphic map1-f1 gene was amplified and detection carried out using the SYBR Green method (41). DNA from six different isolates was successfully amplified. The detection limit mentioned was higher than 0.1 organism/µl, but this finding was not subjected to in-depth investigation. Counting *E. ruminantium* under the microscope after Giemsa staining does not give very precise results. The method does not significantly improve the detection sensitivity of a nested PCR, although it does allow organisms to be quantified. In addition to limited laboratory validation, the QPCR method was used in only one study aimed at following the *E. ruminantium* kinetics in the blood of experimentally infected sheep. *Ehrlichia ruminantium* was detected only during the hyperthermia reaction period. QPCR thus does not improve detection of asymptomatic carriers compared with nonquantitative PCR.

A second SYBR Green-based real-time PCR has been described and fully validated for use in the characterisation of *E. ruminantium* replication and release kinetics in endothelial cell cultures and its subsequent use to control the mass production process in bio-reactors (40). The product is an 873 bp
Chapter 2.1.6. - Heartwater

fragment from the map1 gene. The external standard for quantifying E. ruminantium is a pCI-neo plasmid containing one copy of the map1 target sequence, and is a more precise method of quantifying the organisms than the method described previously where the standard is based on the counting of E. ruminantium bodies under the microscope. The dynamic quantitative range allows accurate measurements to be taken in samples containing 10^2 to 10^8 gene copies. The method was successfully applied to four different isolates but has not been validated for use on diagnostic samples.

- Reading the results

As E. ruminantium is an obligate intra-cellular bacteria that cannot be cultivated in acellular media and its isolation is complex and takes several weeks, molecular detection techniques are the best methods for the diagnosis of cowdriosis. PCR proves to be easier to perform and more sensitive than DNA probes. 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. 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. However, despite interesting experimental results in detecting subclinical carriers, there is not enough available information on the reliability of carrier detection by PCR; more extensive field studies need to be conducted to recommend the best protocol of detecting carrier animals. It is nevertheless clear from the Zimbabwe study in cattle that detection of infection in carrier hosts is going to be difficult and will require repeated testing to confirm status of infection (44). The current results obtained with the PCR, the nested PCR, the RLB assay and more recently the QPCR, 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 convenient for routine diagnostic laboratories as it requires the maintenance of tick colonies and the capacity to experimentally infect animals.

3. Serological tests

Various serological tests for diagnosing heartwater have been described: an IFA test with E.-ruminantium-infected endothelial cell culture as antigen (CIFA test), indirect ELISA, a competitive ELISA (C-ELISA), and a Western blot. The IFA test using E.-ruminantium-infected mouse peritoneal macrophages (MIFA) (10) is no longer used.

The major drawback of all of these tests is the detection of false-positive reactions due to common antigenic determinants between the E. ruminantium MAP1 (11) and similar proteins in several Ehrlichia species (23, 43). Almost all of these tests are no longer used to study the epidemiology or for diagnosis. The CIFA test is still used in some places, but care must be taken when interpreting the results because of the problem of false-positive reactions.

To minimise the problem of cross-reactions with Ehrlichia, two 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) (49). The second is a competitive ELISA that uses the MAP1 gene cloned in a baculovirus and monoclonal antibodies (MAbs) raised against the MAP1 protein (MAP1 C-ELISA) (12). Both tests have dramatically improved specificity, but they still show some reactivity with high titre sera against E. canis, E. chaffeensis and an unclassified white-tailed deer agent. The MAP 1-B ELISA has been the most extensively used and will be described in detail. The MAP1-B ELISA does detect antibodies to E. muris (Mahan S.M., pers. comm.) an Ehrlichial agent that is very closely related to E. ruminantium; this agent is found in white tailed deer in Georgia USA and is transmitted by Amblyomma americanum ticks (15). Hence serology as a diagnostic tool for detecting of individual animals exposed specifically to E. ruminantium is unreliable. Serology should be considered at a herd level taking into consideration the epidemiological environment and, if necessary, be complemented by molecular techniques.

a) Indirect fluorescent antibody test with infected endothelial cell tissue culture as antigen (CIFA test) (29)

To prepare the antigen, an E. ruminantium strain is cultivated in ruminant endothelial cell cultures. When most cells are lysed, the remaining adherent cells are scraped and mixed with the supernate. The cells are centrifuged three times with phosphate buffered saline (PBS) at 200 g for 10 minutes. Of the washed cell suspension, 10 µl are placed in every well of an immunofluorescence slide. The antigen slides are dried, fixed in acetone and stored at –20°C.

- Test procedure

i) The sera to be tested are diluted 1/20 or a higher dilution in PBS, added to the antigen wells and incubated for 30 minutes in a humid chamber at 37°C.
ii) The slides are then washed in PBS buffer for 15 minutes.

iii) The appropriate anti-species conjugate, usually diluted 1/60, is added to cover the wells. The slides are incubated again for 30 minutes at 37°C.

iv) After a second washing, the slides are mounted in glycerine buffer under a cover-slip and examined under a fluorescence microscope.

v) Control positive and negative sera are included on each slide.

b) MAP1-B enzyme-linked immunosorbent assay (43, 49)

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 Ni²⁺-NTA agarose (nitritotriacetic 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 on to 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, 43).

• Test procedure

i) Plates are blocked for 30 minutes by adding 100 µl per well of 0.1 M PBS, pH 7.2, supplemented with 0.1% Tween 20 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% H₂O₂.

vii) The reaction is stopped after 30 minutes of incubation at room temperature (20–25°C) by adding 50 µl of 2 N H₂SO₄. Absorbance is read at 495 nm. Positive and negative controls are included in each plate.

c) MAP1 competitive enzyme-linked immunosorbent assay (32)

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 ascites 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.

• 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% H₂O₂ 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 H₂SO₄ and the absorbance is read at 495 nm. Positive and negative controls are included in each plate.

**Reading the results**

All serological tests based on non-recombinant *E. ruminantium* antigens, such as CIFA, ELISAs, and Western blot, are still used for experimental studies but are no longer used for sero-epidemiological studies. The tests have been compared and applied to known positive and negative sera to *E. ruminantium* (9). No false-positive reactions were observed with any of the tests against known negative sera. There is good correlation among tests, but the specificity of all five tests is low because cross-reactions occur with certain *Ehrlichia* spp.

The interpretation of results of the various tests applied to field surveys is thus difficult in areas where *E. ruminantium* infections occur in ruminants, which is probably the case in most of the heartwater-endemic regions of Africa. This situation has also been demonstrated in farms without *Amblyomma* but infected with tick species not known to be vectors of *E. ruminantium* (13, 49).

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* (32). 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. Although a few false-positive sera are still found, these tests are likely to solve much of the specificity problems of the earlier serological tests. However, 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.

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 (32). 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 (31). 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 (21, 43). 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 (21). Similar observations were made in the Caribbean. In addition, some areas of Zimbabwe, which was labelled heartwater-free, had a large number of goats positive for MAP1-B antibodies; this further complicates the sero-diagnosis of heartwater (13).

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 regarded as a diagnostic method to be applied at the herd level and not at the individual animal level (38). When interpreting diagnostic serology results, other epidemiological parameters must be considered.

Molecular methods, such as PCR assay, could potentially help in detecting carrier animals, but this approach has still significant drawbacks (see Section B.2 Molecular methods).

**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 (4). This method is still in use in several areas, but it is likely to be replaced soon by preparations using attenuated or inactivated organisms, which have given promising research results.

1. Inactivated vaccine preparations
Vaccination with preparations of inactivated *E. ruminantium* elementary bodies emulsified in oil adjuvants was shown to be possible following the demonstration that susceptible goats can be protected by inactivated *Ehrlichia* in Freund’s adjuvant (27). This vaccine also protected against challenge in sheep (16) using different strains of *E. ruminantium* (17), and in cattle (47) using the same strain as in goats. A first generation vaccine preparation of inactivated *Ehrlichia* in Montanide ISA 50 oil adjuvant was shown to be similarly effective to the Freund’s adjuvant preparation on laboratory challenge of immunised goats and sheep (28).

In initial vaccine trials, animals were immunised with two subcutaneous injections of 250–1000 µg of antigen (depending on trial) emulsified (50/50) in Montanide ISA 50 adjuvant in a volume of 2 ml. It has recently been shown in goats in experimental conditions that the vaccine dose can be lowered to 35 µg of antigen without decreasing the effect on protection (48). From the initial description, this represents a 28-fold reduction in the dose of vaccine from 1 mg to 35 µg of *E. ruminantium* without any modification to the protective effect. The process for the mass production of *E. ruminantium* has been developed in parallel (25). Critical parameters have been determined and optimised for the production of *E. ruminantium* in endothelial cells in stirred-tank bioreactors. Using serum-free medium in such bioreactors, *E. ruminantium* production yields reached a 6.5-fold increase compared with conventional methods. Using 2-litre bioreactors and the estimated efficient 30 µg vaccine dose, the cost estimation for one vaccine dose was around 0.11 euro, which makes it affordable in countries with limited resources. Efficacy trials conducted with vaccine preparations entirely produced using the mass production and purification process followed by preservation of the product in various solutions (NaCl versus PBS) and at different temperatures (–20°C, 4°C) have demonstrated that the efficacy of the vaccine is maintained after the entire mass production and preservation process (26).

In Zimbabwe, field trials of the inactivated vaccine emulsified in ISA 50 adjuvant have also demonstrated protection of sheep against natural tick challenge (17). In larger field trials conducted in East and South 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 (22). 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. Lack of cross-protection between *E. ruminantium* isolates due to disparities of antigenic composition is well established, but the complexity of the *E. ruminantium* population structure in the field has been underestimated. It has recently been demonstrated in large field evaluation trials carried out in several farming systems in West Africa that, in limited geographical areas, more than 10 genotypes with differing cross-protection capacities can be present and have a significant influence on protection with inactive vaccine preparations (unpublished data).

The Mbizi strain inactivated vaccine is being developed commercially by Onderstepoort Biological Products in South Africa (Mahan S.M., pers. comm.). 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 characterization of the extent of strain diversity in a region to be covered by an appropriate formulation of the vaccine. This knowledge will also be essential for new generation vaccines that will be developed in the future.

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 infection and treatment using virulent isolates. Isolates of attenuated virulence that do not necessitate 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. Recently, a third isolate named Welgevonden from South Africa has been attenuated and shown to confer complete protection against four heterologous isolates under experimental conditions (46). 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.

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 (33). 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 (7). No further results have been described since then. Recombinant vaccines will probably not be available in the near future.
REFERENCES


NB: There is an OIE Reference Laboratory for Heartwater (see Table in Part 3 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: www.oie.int).