Chapter 1

General introduction
Ehrlichia (Cowdria) ruminantium is a tick-borne rickettsial agent that causes heartwater, an economically important disease affecting both domestic and wild ruminants in sub-Saharan Africa and on certain Caribbean Islands (Uilenberg, 1983). The disease is transmitted transstadially by ticks of the genus Amblyomma and constitutes a major obstacle to livestock production in Africa (Uilenberg and Camus, 1993). Control measures include prevention of transmission through intensive acaricide application, or maintenance of endemic stability through a combination of strategic tick control, natural exposure of very young animals during the period of innate resistance, and vaccination (Uilenberg, 1996). Four different vaccine strategies against heartwater have been developed; the infection and treatment method using live bacteria, infection with in vitro attenuated bacteria, infection with inactivated in vitro grown bacteria and recombinant DNA (Mahan et al., 1999). This chapter reviews current knowledge on heartwater with special reference to molecular characterization and detection.

The disease

Vertebrate hosts become infected with E. ruminantium organisms through the saliva and/or by regurgitated gut contents of feeding ticks (Bezuidenhout, 1987; Kocan and Bezuidenhout, 1987). Heartwater develops within 10 to 30 days after an infectious tick bite and usually the first symptom is a sudden rise in body temperature. The course of the disease may range from peracute to mild depending on age, immune status, breed and virulence of E. ruminantium stock. Death usually follows in susceptible animals without administration of antibiotics. In typical cases, animals show nervous symptoms such as rapid blinking of the eyes, hypersensitivity to touch, and once recumbent they exhibit pedaling movements and recovery is rare (Camus et al., 1996). At necropsy, hydro-thorax and hydro-pericardium (“heartwater”) are found with edema in the lungs, but such lesions are not always present. Histopathological examination of cerebral cortex reveals colonies of E. ruminantium organisms in the cytoplasm of endothelial cells lining the capillaries. Tetracyclines are effective when administered directly after the onset of fever (Van Amstel and Oberem, 1987) and tetracycline resistance has not been reported. In general, indigenous cattle in heartwater endemic areas are resistant, whereas this is less clear for sheep and goats. Heartwater becomes manifest when attempts are made to upgrade local, resistant, breeds with susceptible exotic breeds or when local breeds are translocated from heartwater-free areas to heartwater-endemic areas. An example of the latter is described in chapter 3.

The infectious agent

The American rickettsiologist E.V. Cowdry identified the causative agent in tissue of infected animals (Cowdry, 1925) and infected ticks (Cowdry, 1925), and described
the organism as *Rickettsia ruminantium*, the first description of a rickettsial disease affecting domestic animals. In 1947, the organism was assigned to the new genus *Cowdria*, and renamed *Cowdria ruminantium* in honor of Cowdry (Moshkovski, 1947). Recently the organism was reclassified as *Ehrlichia ruminantium* (chapter 5) and transferred to the genus *Ehrlichia*. Together with the genera *Anaplasma*, *Neorickettsia* and *Wolbachia* they form the family *Anaplasmataceae* in the order *Rickettsiales* (Fig. 1).

*E. ruminantium* is a pleomorphic organism and colonies containing varying numbers of bacteria are primarily found in the cytoplasm of vascular endothelial cells (Cowdry, 1926), and to a lesser extent in neutrophils (Logan et al., 1987; Jongejan et al., 1989). They are gram-negative organisms, which grow by binary fission in membrane-lined cytoplasmic vacuoles. A growing colony is contained within its own vacuole, which does not fuse with other vacuoles or with lysosomes. Characterization of *E. ruminantium* was virtually impossible before 1985, when the first *in vitro* cultivation of the organism in a calf endothelial cell line was described (Bezuidenhout et al., 1985). Since this discovery, many endothelial cell lines as well as media and media components have been examined to improve and standardize the *in vitro* cultivation (Yunker, 1995). Cultivation of *E. ruminantium* under serum-free conditions and in chemically defined media has been shown possible (Zweygarth et al., 1997; Zweygarth et al., 1998; Zweygarth and Joms, 2001) and *in vitro* cloning of *E. ruminantium* from one infected endothelial cell has been reported (Perez et al., 1997). Next to endothelial cells continuous *in vitro* propagation of the Welgevonden isolate of *E. ruminantium* has been described in a canine macrophage-monocyte cell line (Zweygarth and Joms, 2001). Another breakthrough has been the *in vitro* cultivation of *E. ruminantium* in a non-vector (*Ixodes ricinus*) tick cell line (Bell Sakyi et al., 2000) and in the vector (*Amblyomma variegatum*) tick cell line AVL/CTVM13 (Bell-Sakyi et al., 2000).

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**Figure 1.** Current classification of the family *Anaplasmataceae* in the order *Rickettsiales*
The host

Heartwater mainly affects cattle, sheep and goats. Several wild ruminant species can be infected without developing clinical signs. These include several antelope species, buffalo, giraffe and wild rodents (Oberem and Bezuidenhout, 1987; Kock et al., 1995; Peter et al., 1999). In a recent review on heartwater in wild ruminants evidence was provided that 12 African ruminants, three non-African ruminants and two African rodents (Peter et al., 2002) can become infected with *E. ruminantium*, in most cases without clinical signs. Infected wildlife can spread the infection through infected ticks into heartwater-free areas. Although the precise role of wild ruminants in the epidemiology and spread of heartwater remains to be determined, it has been shown that a vector-wildlife cycle of transmission of *E. ruminantium* can be maintained independently of domestic ruminants (Peter et al., 1999). The broad host range of *E. ruminantium* is also reflected by the ability to infect *in vitro* endothelial cells from a range of different species, including African buffalo, bushpig, eland, giraffe, greater kudu and sable antelope (Smith et al., 1998) as well as human endothelial cells (Totté et al., 1993).

The vector

*E. ruminantium* is transmitted by ticks of the genus *Amblyomma*, which are three-host ticks, as each stage (larvae, nymph and adult) takes a blood meal on a different host, after which they detach and spend long periods on the vegetation (Jongejan and Uilenberg, 1994). Infection contracted in the larval stage can be transmitted not only by the nymph, but also by the adult, even if the host for the nymph was not infected (Bezuidenhout, 1987; Andrew and Norval, 1989). In addition, trans-ovarial transmission has also been reported, but only once, and its role in the epidemiology of heartwater remains to be determined (Bezuidenhout and Jacobsz, 1986). Ten African *Amblyomma* species are known vectors of heartwater: *A. variegatum*, *A. hebraeum*, *A. pomposum*, *A. gemma*, *A. lepidum*, *A. tholloni*, *A. sparsum*, *A. astrion*, *A. cohaerens*, and *A. marmoreum* (Uilenberg, 1983; Bezuidenhout, 1987; Peter et al., 2000; Wesonga et al., 2001). *A. hebraeum* is the main vector of heartwater in South-eastern Africa, whereas *A. variegatum* is widely distributed in tropical sub-Saharan Africa. *A. variegatum* is the only African vector of heartwater which has established itself outside Africa, in the caribbean region (Barré et al., 1987). In addition to the African vectors of heartwater, three American species of *Amblyomma* have been shown capable of experimentally transmitting *E. ruminantium*: *A. cajennense* (Uilenberg, 1983), although only transmission from larval to nymphal stages was proven and transmission from nymphs to adults failed, *A. maculatum* (Uilenberg, 1982; Mahan et al., 2000), and *A. dissimile* (Jongejan, 1992). These species are widely distributed in the Western hemisphere (Walker and Olwage, 1987; Jongejan, 1992). Furthermore, the successful establishment of an exotic vector of *E. ruminantium*, *A. marmoreum*, in Florida through the importation of foreign wildlife has been reported (Allan et al., 1998). In another survey of reptiles imported into Florida, *A. sparsum* ticks were found on leopard tortoises imported from Zambia (Burridge et al., 2000). A total of 15
out of 38 adult *A. sparsum* ticks were found to be positive for *E. ruminantium* by PCR, indicating that infected *A. sparsum* ticks may have been imported into Florida (Burridge et al., 2000). Other (mainly American) *Amblyomma* species have been tested with negative results in transmission experiments, these are: *A. americanum*, *A. neumanni*, and *A. imitator* (Uilenberg, 1982; Camus et al., 1996; Allan et al., 1998). The distribution of heartwater in Africa coincides with that of the *Amblyomma* tick vectors. Transmission of the disease appears to be possible by all African *Amblyomma* species that are normally associated with ungulates in the adult stage.

![Life cycle of *Ehrlichia ruminantium*.](image)

**Figure 2.** Life cycle of *Ehrlichia ruminantium*. Adopted from the poster accompanying Parasitology Today 15(7), 1999.

**Life cycle**

*Amblyomma* larvae and nymphs become infected while feeding on *E. ruminantium* infected domestic and wild ruminants (Fig. 2). Nymphs or adult ticks can transmit *E. ruminantium* to susceptible hosts, whereby nymphs retain their infectivity after feeding whereas adults do not. After the tick has taken an infected blood meal, the initial replication of organisms takes place in the intestinal epithelium of the tick and
eventually the salivary glands also become infected (Kocan et al., 1987; Hart et al., 1991). At both sites reticulate bodies are the predominant type of organism although electron-dense elementary bodies have also been observed. In addition to intestinal epithelium and salivary gland cells, \emph{E. ruminantium} organisms have also been detected in tick haemocytes and malpighian tubules (Du Plessis, 1985; Kocan and Bezuidenhout, 1987). The presence of colonies of \emph{E. ruminantium} in salivary glands of feeding ticks, along with the demonstration of different morphological forms of the organism, suggests a developmental cycle also occurring in its invertebrate host (Fig. 2). Transmission of the organism to the vertebrate host probably takes place either by regurgitation of the gut contents and/or through the saliva of the tick while feeding. The minimum period required for \emph{E. ruminantium} to be transmitted after ticks have attached to susceptible animals is between 27 and 38 hours in nymphs and between 51 and 75 hours in adults (Bezuidenhout, 1988).

Once the vertebrate host becomes infected it is not clear how \emph{E. ruminantium} disseminates from the feeding lesion to other host tissues. Initial replication appears to take place in reticulo-endothelial cells of the regional lymph nodes (Du Plessis, 1970), from where they disseminate to invade endothelial cells of blood vessels of various organs and tissues. \emph{E. ruminantium} organisms have been demonstrated in neutrophils, vascular-endothelial cells, and macrophages/monocytes. For instance during the febrile response \emph{E. ruminantium} can be detected in circulating neutrophils (Logan et al., 1987). When maintained \emph{in vitro}, organisms in neutrophils were frequently observed undergoing binary fission within enlarged phagosomal vacuoles (Jongejan et al., 1989). Experimentally infected monocytes have been shown to stimulate T-cell responses in immunized cattle (Totté et al., 1997; Mwangi et al., 1998; Mwangi et al., 1998). The role of both neutrophils and monocytes in the pathogenesis of the disease is not clear. It has been hypothesized that infected endothelial cells and monocytes present \emph{E. ruminantium} antigens to specific lymphocytes during infection and thereby play a role in the immune response to the pathogen (Mwangi et al., 1998).

**Serological detection**

None of the clinical signs observed in animals infected with \emph{E. ruminantium} are pathognomonic for the disease. Definite diagnosis is usually made after death by demonstrating the presence of \emph{E. ruminantium} colonies within endothelial cells of capillaries in brain crush smears stained with Giemsa. For an accurate assessment of the distribution of the disease, a diagnostic method is needed which is convenient, fast, reliable, reproducible, cheap and above all sensitive and specific for \emph{E. ruminantium}. The first workable tests were based on the indirect fluorescent antibody test (IFA) using peritoneal mouse macrophages infected with the Kümm isolate of \emph{E. ruminantium} (Du Plessis, 1981). Other IFA tests were developed thereafter using infected neutrophils (Logan et al., 1987; Jongejan et al., 1989) or endothelial cells cultivated \emph{in vitro} (Martinez et al., 1990; Asselbergs et al., 1993). A competitive ELISA using a monoclonal antibody to a 32 kDa protein (MAP1) conserved between stocks of \emph{E. ruminantium} (Jongejan and Thielemans, 1989) was developed later (Jongejan et al., 1991). Indirect ELISA using semi-purified organisms from endothelial cell cultures as antigen were also extensively
used (Martinez et al., 1993). However, in all these tests false positive reactions were found with sera against several other *Ehrlichia* species (Du Plessis et al., 1993). Subsequently, it was shown by immunoblotting that the 32 kDa protein (MAP1) of *E. ruminantium* was conserved within the genus *Ehrlichia* (Jongejan et al., 1993) and responsible for these false positive reactions (Mahan et al., 1993). In order to overcome this problem, van Vliet et al. (1995) identified an immunogenic region of the MAP1 protein (MAP1-B fragment) that did not give cross-reactions with *A. bovis* and *E. ovina*. Cross-reactions with *E. canis* and *E. chaffeensis* were however still detected in an indirect ELISA (van Vliet et al., 1995). Another approach was used by Katz et al. (1996), who cloned the entire MAP1 gene in baculovirus and developed monoclonal antibodies to the protein for use in a competitive ELISA (Katz et al., 1996). Some cross-reactions with *E. canis* and *E. chaffeensis* remained and also with an unclassified agent responsible for positive reactions in sera of the white-tailed deer. Cross-reactions with *E. canis* antigens were not observed with a monoclonal antibody directed against an approximately 43kDa *E. ruminantium* antigen (Shompole et al., 2000). It was found that the antigen was surface exposed on intact elementary bodies and conserved among eight geographically distinct strains (Shompole et al., 2000).

Both the MAP1-B ELISA and the MAP1 cELISA have been evaluated in the Caribbean and shown improved specificity as compared with an ELISA based on crude antigens (Mondry et al., 1998). Validation and comparison of the indirect ELISA based on purified antigen, the competitive ELISA using full-length recombinant MAP1 and the MAP1-B ELISA was done using approximately 3000 sera of ruminants in the Caribbean (Mondry et al., 1998). Overall specificity was 98.1% for the indirect ELISA, 98.5% for the recombinant MAP1 ELISA and 99.4% for the MAP1-B ELISA. Finally, validation of the MAP1-B ELISA in sheep and goats using two-graph receiver-operating characteristics (TG-ROC) confirmed its usefulness for the diagnosis in small ruminants (Mboloi et al., 1999) (Chapter 2). The usefulness of the test for cattle sera appears to be limited. Rather low sero-prevalence (33%) among cattle from endemic areas were observed in Zimbabwe, whereas sero-prevalence in goats from the same areas was high (>90%) (Mahan et al., 1998). When the underlying causes for this low sero-prevalence in cattle were investigated, it was found that antibody responses to *E. ruminantium* antigens are apparently down regulated in cattle challenged with tick transmitted heartwater (Semu et al., 2001). Therefore, serological responses to *E. ruminantium* antigens in cattle in heartwater endemic areas do not seem to be reliable indicators of *E. ruminantium* exposure and molecular diagnosis seems to be the method of chose for cattle.

**Molecular detection**

Waghela et al. (1991) were the first to describe the use of cloned DNA probes to detect the presence of *E. ruminantium* in *A. variegatum* ticks (Waghela et al., 1991). One of these probes, pCS20, hybridized with all eight heartwater isolates tested and was able to detect *E. ruminantium* DNA prepared from plasma samples from infected sheep before and during the febrile reaction (Mahan et al., 1992). Primers were designed to amplify a fragment of *E. ruminantium* DNA encoded on the pCS20 probe
and it was shown that PCR with these primers was more sensitive than DNA probe hybridization (Peter et al., 1995). The test was used to determine the prevalence of *E. ruminantium* in *A. hebraeum* ticks from heartwater endemic areas in Zimbabwe and found to be between 8.5 and 11.2 % (Peter et al., 1999) and the reliability of the pCS20 PCR was also tested on field ticks (Peter et al., 2000). The sensitivity varied with tick infection intensity and ranged between 28% and 97% with ticks bearing $10^2$ organisms and $10^7$ organisms, respectively. Cloning of the genes encoding two major antigenic proteins of *E. ruminantium*, (MAP1 and MAP2) has been reported (Mahan et al., 1994; van Vliet et al., 1994). PCR assay based on *map1* has been used to detect the presence of *E. ruminantium* in blood and bone marrow samples from clinically normal, free-ranging Zimbabwean ungulates (Kock et al., 1995). Allsopp et al. (1997) used the 16S ribosomal RNA gene to design specific probes for the detection of *E. ruminantium* and proposed four geno-types (Allsopp et al., 1997). An evaluation of the three different probes (16S, *map1*, and pCS20) for the detection of *E. ruminantium* showed that the pCS20 probe was the most sensitive indicator for heartwater isolates except for the Omatjenne isolate (Allsopp et al., 1998).

**Molecular characterization**

Although recently the genome size of *E. ruminantium* was determined at approximately 1576 kb (de Villiers et al., 2000), a full length genomic sequence is not yet available. Molecular characterization of *E. ruminantium* has therefore been based upon a few genes only. One of the genes used to differentiate between isolates is the 16S rRNA gene. Although differences based on 16S ribosomal DNA are relatively small, specific probes for the differentiation of *E. ruminantium* stocks have been reported (Allsopp et al., 1997). Another gene used for characterization is the *map1* gene, which codes for the major antigenic protein 1. Analysis of MAP1 sequence data from seven stocks of *E. ruminantium* revealed conserved and three variable domains (Reddy et al., 1996). A further study including 30 different isolates showed that the variants are not geographically constrained (Allsopp et al., 2001). A second antigenic protein (MAP2) was shown to be highly conserved among geographically distinct isolates showing amino acid substitutions at only three positions (Bowie et al., 1999). Perez et al. (1997) used random amplified polymorphic DNA (RAPD) to generate reproducible fingerprints for six heartwater isolates (Perez et al., 1997). Distinction between isolates was possible using one or two primers, and three amplified fragments could determine a restriction fragment length polymorphism (RFLP) when used as probes on restriction digested genomic DNA. Macro-restriction profile analysis by pulse-field gel electrophoresis (PFGE) was also described to distinguish seven isolates of *E. ruminantium* (de Villiers et al., 2000). A meaningful comparison between the results obtained by the different methods is difficult, because different isolates were used. Furthermore, reported differences between *E. ruminantium* stocks do not appear to correlate with immunological differences determined in cross-protection tests in animals. Possible polymorphic loci used to differentiate other bacterial species and which have been cloned from *E. ruminantium* are the *groESL* heat shock operon, and the
internal transcribed spacer 2 (ITS 2) (Mahan et al., 1994; Lally et al., 1995; van Meer et al., 1999). The groESL operon has been used to differentiate Ehrlichia species by using both groEL gene sequences as well as the intergenic spacer sequence between groES and groEL (Sumner et al., 1997; Sumner et al., 2000) So far, only the groESL operon of the Welgevonden isolate has been sequenced. ITS 2 has been used to differentiate different Wolbachia strains involved in cytoplasmic incompatibility or sex-ratio distortion in arthropods (van Meer et al., 1999).

**Outline of this thesis**

The purpose of this thesis was to develop and validate serological and molecular diagnostic tools for the detection and characterization of *E. ruminantium*. In chapter 2 the validation is described of the MAP1-B ELISA for the detection of antibodies against *E. ruminantium* in experimentally infected small ruminants using two-graph receiver-operating characteristic (TG-ROC) curves. Chapter 3 includes a field study in Mozambique where the MAP1-B ELISA was used to determine the difference in sero-prevalence in goats between the north and the south of the country, and where sentinel goats were used for the isolation of field stocks of *E. ruminantium*. Chapter 4 concerns with the discovery that map1 of *E. ruminantium* is a member of a multigene family encoding both conserved and variable genes. In *vitro* transcription of three genes of this family is described in cell lines derived from cattle and ticks. In chapter 5 reclassification of some rickettsial species, including the reclassification of *Cowdria ruminantium* into *Ehrlichia ruminantium*, is described. As a result the new name appears in chapters 1 and 5 to 7. In chapter 6 the development of a reverse line blot (RLB) assay for simultaneous detection of *Ehrlichia* and *Anaplasma* species is described. Finally, the results of the various chapters are summarized and discussed in chapter 7.

**References**


