Molecular Detection and Characterization

of *Ehrlichia ruminantium*

Moleculaire detectie en karakterisering

van *Ehrlichia ruminantium*

(met een samenvatting in het Nederlands)

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CONTENTS

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 Josemans, 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 Josemans, 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).

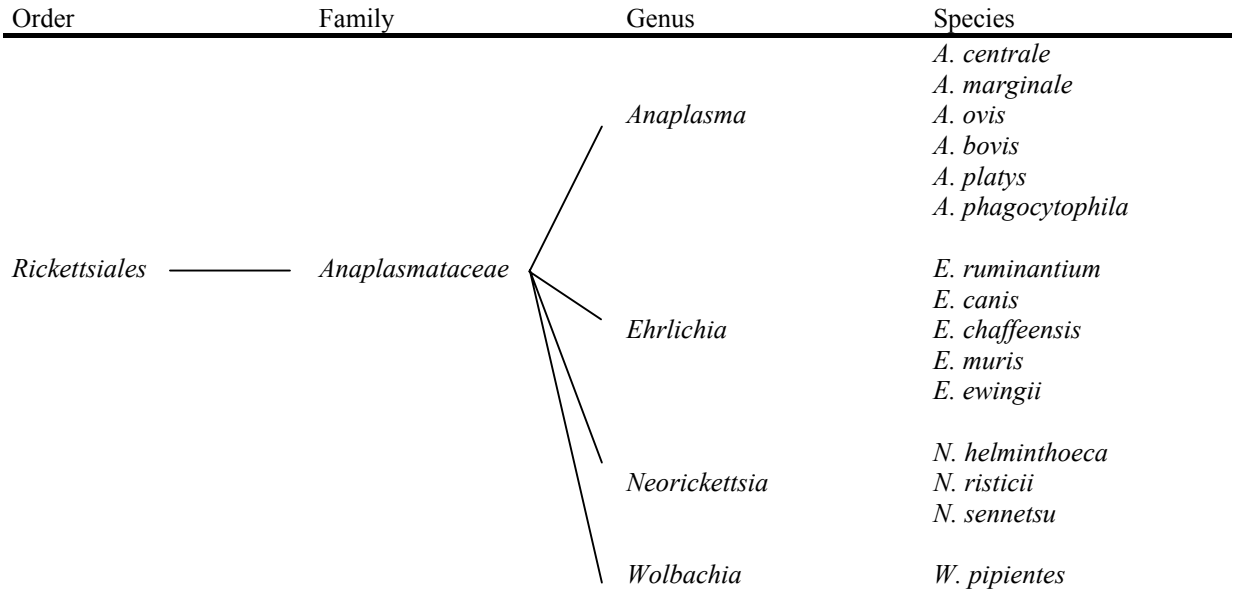

Figure 1. Current classification of the family *Anaplasmataceae* in the order *Rickettsiales*

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.

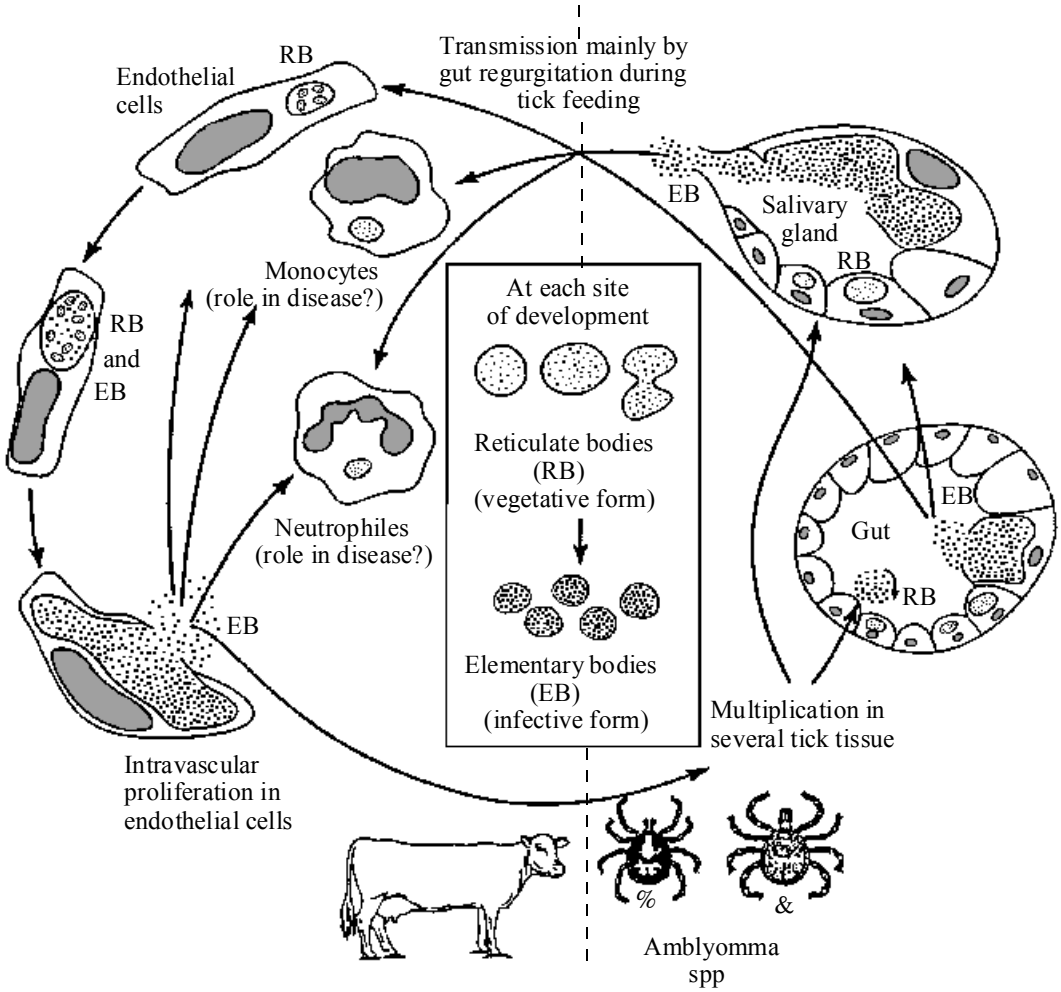

Figure 2. Life cycle of *Ehrlichia ruminantium*. Adopted from the poster accompanying Parasitology Today 15(7), 1999.

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, *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 *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 *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 *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. *E. ruminantium* organisms have been demonstrated in neutrophils, vascular-endothelial cells, and macrophages/monocytes. For instance during the febrile response *E. ruminantium* can be detected in circulating neutrophils (Logan et al., 1987). When maintained *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 *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 *E. ruminantium* are pathognomonic for the disease. Definite diagnosis is usually made after death by demonstrating the presence of *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 *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 *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 *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 *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 MAP-1 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 c**hapter 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**.

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Chapter 2

Validation of the indirect MAP1-B enzyme-linked immunosorbent assay for diagnosis of experimental *Cowdria ruminantium* infection in small ruminants

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Validation of the Indirect MAP1-B Enzyme-Linked Immunosorbent Assay for Diagnosis of Experimental *Cowdria ruminantium* Infection in Small Ruminants

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The major antigenic protein 1 fragment B (MAP1-B) enzyme-linked immunosorbent assay (ELISA) for the diagnosis of *Cowdria ruminantium* infections was validated to determine cutoff values and evaluate its diagnostic performance with sheep and goat sera. *Cowdria*-infected populations consisted of 48 sheep and 44 goats, while the noninfected populations consisted of 64 sheep and 107 goats. Cutoff values were determined by two-graph receiver-operating characteristic (TG-ROC) curves. The cutoff value was set at 31 and 26.6% of the positive control reference samples for sheep and goat sera, respectively. The test's diagnostic performance was evaluated with measurements of the area under the concentration-time curve (AUC) of the ROC curves and by the valid range proportion (VRP). The AUCs were 0.978 for sheep sera and 0.989 for goat sera. The VRP for both sheep and goat sera was approximately 1.0. The intermediate range (IR), which defines results that are neither positive nor negative, was 0 for goat sera and 2.81 for sheep sera. In an ideal test, the AUC and VRP would be 1.0 and the IR would be 0. In this study these parameters were close to those of an ideal test. It is concluded that the MAP1-B ELISA is a useful test for the diagnosis of *C. ruminantium* infection in small ruminants.

Cowdriosis (or heartwater) is a tick-borne disease of ruminants caused by the rickettsia *Cowdria ruminantium* and is transmitted by ticks of the genus *Amblyomma*. The disease is endemic in sub-Saharan Africa and the Caribbean and is a main obstacle to livestock development in the tropics (7, 30). Clinical signs and macroscopic postmortem changes are not pathognomonic for the disease, and diagnosis is based on the detection of rickettsial organisms in the cytoplasms of endothelial cells in brain capillaries. Antemortem tests for detecting *C. ruminantium* include animal subinoculation, cell culture isolation, serodiagnostic tests, DNA hybridization, and PCR. Serodiagnostic methods, such as the indirect fluorescent antibody test, immunoblotting, and enzyme-linked immunosorbent assays (ELISA), have been hampered by cross-reactions with *Ehrlichia* species (18, 22, 24). However, the use of recombinant major antigenic protein 1 (MAP1) of *C. ruminantium* has been recently introduced, and an indirect ELISA based on a specific fragment of this protein (fragment B, referred to herein as MAP1-B) has been developed (32). Cross-reactions were dramatically reduced, although sera from dogs infected with *Ehrlichia canis* and sera from human patients infected with *Ehrlichia chaffeensis* were also positive in this ELISA. Another recent study, using a monoclonal antibody-based ELISA for detecting MAP1, confirmed cross-reactions with *E. canis*, *E. chaffeensis*, and a newly discovered *Ehrlichia*-like organism from white-tailed deer (21). Furthermore, it has also been shown that *E. chaffeensis* can experimentally infect wild ruminants such as white-tailed deer (5). A preliminary validation of the MAP1-B ELISA was done by studying antibody profiles of *C. ruminantium* infections in domestic ruminants (25, 32).

Central to any serological assay is the determination of the diagnostic cutoff value. It is common practice to determine cutoff values for (i) reactions of a noninfected reference population with the addition of 2 or 3 standard deviations to the mean value or (ii) the doubling of the mean optical density readings of the negative reference sera on each ELISA plate (26). The first method is assumed to lead to a specificity of 97.5% (2); however, this assumption holds true only for normally distributed test variables (12), and the second method seems to have no statistical grounds. A cutoff value has to differentiate two subpopulations of infected and noninfected controls with defined operating characteristics (13). Recently a new approach to defining test cutoff values and performance had been proposed (13). The new approach utilizes the conventional receiver-operating characteristic (ROC) principle, modified in such a way that the test sensitivity and specificity can be read directly from these plots, unlike the conventional ROC plots. The modified ROC plot is known as a two-graph ROC (TG-ROC). TG-ROC was developed as a template within a standard spreadsheet computer program, and it provides a clear and comprehensible approach to the problems of selecting cutoff values and identifying intermediate results in ELISA tests (10). TG-ROC analysis also provides other indices, such as efficiency (9), Youden's index (35), and likelihood ratio (LR) (28), for further cutoff value optimization. These indices are useful measures for minimizing the number of false positives and false negatives.

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The aims of this study were (i) to calculate cutoff values for the MAP1-B ELISA for the diagnosis of cowdriosis with TG-ROC, (ii) to compare these values to those determined by conventional methods with sheep and goat serum samples, and (iii) to compare the performance of the MAP1-B ELISA for the diagnosis of cowdriosis in experimentally infected sheep and goats.

MATERIALS AND METHODS

***C. ruminantium* isolates.** The following *C. ruminantium* isolates (from the following locations) were used in this study: Senegal (Senegal), Lutale (Zambia) (19), Umpala (Mozambique) (1), Gardel (Guadeloupe) (31), and Crystal Springs (Zimbabwe) (4) and Ball 3 (14), Kümm (8), Kwanyanga (23), and Welgevonden (6) (all from South Africa).

Experimental animals. Forty-eight adult female Tesselaar sheep, all nonpregnant and 12 to 18 months old, were used as the infected reference sheep population. The animals were challenged with different *C. ruminantium* isolates by needle infection 1 month after vaccination with an attenuated *Cowdria* isolate originating from Senegal (17, 20). Twenty-four sheep were challenged with the Senegal isolate, four with Welgevonden, and five sheep each with the Umpala, Lutale, Gardel, and Ball 3 isolates. The sera used in this study were collected between 4 and 8 weeks postchallenge. The infected reference population of Saanen goats was composed of 44 goats, of both sexes and 12 to 18 months old, experimentally infected by needle challenge with one of several isolates of *C. ruminantium*: Gardel ($n = 1$), Senegal ($n = 16$), Lutale ($n = 1$), Ball 3 ($n = 5$), Kwanyanga ($n = 5$), Kümm ($n = 8$), Crystal Springs ($n = 3$), and Welgevonden ($n = 5$). The infective dose of the different isolates was previously determined in experimental animals. All animals were tested serologically prior to infection and were shown to be negative. The animals had never been exposed to ticks and were born and bred in The Netherlands. The noninfected reference population of sheep consisted of 64 adult Tesselaar sheep, and the noninfected reference population of goats consisted of 107 Saanen goats. As with the infected reference population, the noninfected animals had never been exposed to ticks and were born and bred in The Netherlands.

Recombinant MAP1-B antigen. The immunogenic region of the MAP1 protein (MAP1-B) was cloned and expressed in *Escherichia coli* with expression vector pQE9, as a fusion protein with six histidine residues added at the N terminus (32). Recombinant MAP1-B was purified with Ni^{2+}-nitrilotriacetic acid agarose under denaturing conditions as described by the manufacturer (Qiagen Inc., Chatsworth, Calif.).

ELISA. One hundred microliters per well was used in all the steps described below. MAP1-B antigen was diluted (1.4 μg/ml) in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$ [pH 9.6]) and immobilized onto 96-well ELISA plates (Microlon Multibind immunoassay plates; Greiner Labortechnik, Alphen aan den Rijn, The Netherlands) by incubation for 1 h at 37°C and then stored overnight at 4°C. Plates were incubated for 15 min at 37°C with blocking buffer (phosphate-buffered saline [PBS], pH 7.3, supplemented with 0.1% Tween 20 and 1% nonfat dry milk [PBSTM]) (Protifar; Nutricia, Zoetermeer, The Netherlands). Plates were washed three times with PBS supplemented with 0.1% Tween 20 (PBST) and subsequently incubated with sera (diluted 1:200) in PBSTM for 1 h at 37°C. All samples were analyzed in duplicate on the same plate. Plates were washed three times with PBST and incubated for 1 h at 37°C with rabbit anti-goat or rabbit anti-sheep antibodies conjugated with horseradish peroxidase (RαG/IgG[H+L]/PO or RαSh/IgG[H+L]/PO; Nordic, Tilburg, The Netherlands) diluted in PBSTM (rabbit anti-goat antibodies, 1:1,500; rabbit anti-sheep antibodies, 1:1,750). ELISA plates were washed three times with PBST, and freshly prepared ABTS [2,2′-azinobis (3-ethylbenzthiazoline sulfonic acid)] substrate was added.

Color development was allowed for 30 min in the dark, and absorbance was measured at 405 nm with an ELISA reader (Ceres UV 900 C; Biotek Instruments BV, Abcoude, The Netherlands). Each plate contained one positive and one negative reference serum sample. The means of the duplicate measurements were calculated, and the optical density was expressed as a percentage positive (PP) value of the reference positive control.

ROC plots. ROC plots were constructed by using the vector of 1-Sp_j, where Sp_j is the test specificity at a cutoff value (d_j) on the x axis and the vector of corresponding sensitivity (Se_j) values on the y axis.

The performance of the test was evaluated by calculating the area under the concentration-time curve (AUC) of the ROC plot. The AUC is given by the test statistic U of the Mann-Whitney U test (15) and is determined by the following equation: $AUC = U/n_p \cdot n_n$, where $U = n_p \cdot n_n + [n_n \cdot n_n + 1)/2] - R$, R is the rank sum of the noninfected sample, and n_p and n_n are the numbers of infected and noninfected animals, respectively.

TG-ROC analysis. TG-ROC analysis was carried out with the template described by Greiner et al. (13). For the construction of TG-ROC plots, the measurement range (MR), as observed for each pair of reference populations, was evenly divided into 250 intervals, with the resulting limits termed cutoff values (d_j). Se_j and Sp_j were calculated for each threshold d_j value obtained. The resulting matrix of d_j and the corresponding percentages of Se_j and Sp_j were plotted to represent the two observed parameters over the specified range of PP values. The intersection of the sensitivity curve with the specificity curve is at (d_0,θ_0) and is called the "point of equivalence," whereby cutoff value d_0 yields equivalent test parameters ($Se_j = Sp_j = \theta_0$). Two alternative cutoff values, which are the lower and upper limits of the intermediate range (IR), are defined at an accuracy level of 95%. In this study, the IR upper and lower limits were nonparametrically defined as the 95th and 5th percentile of the noninfected and infected reference populations, respectively. The valid range proportion (VRP) was determined as (MR− IR)/MR, where MR is calculated by $PP_{max} - PP_{min}$, with PP_{max} and PP_{min} being the highest and lowest ELISA PP values in the combined populations of infected and noninfected animals for each species.

The results for the noninfected reference populations of sheep and goats were used to calculate cutoff values (means ± 2 or 3 standard deviations). The negative reference sample on the ELISA test plates was used to determine cutoff values by the method of twice the negative.

Efficiency, Youden's index, and LRs. Three indices were calculated for further cutoff value optimization. Efficiency (at cutoff value d_j) was calculated as follows: $Ef_j = P \cdot S_e + (1 - P) \cdot Sp_j$, where P denotes the proportion of the reference infected sample. Youden's index was determined by the following equation: $J_j = (ad - bc)/(a + b)(c + d)$, where the sum of a and b is the number of infected animals (a is the number of correctly diagnosed infected animals and b is the number of false negatives) and the sum of c and d is the number of noninfected animals (d is the number of correctly diagnosed animals and c is the number of false positives) at cutoff value d_j. Positive and negative LRs (LR+ and LR−, respectively) for each cutoff value (d_j) were calculated by the following equations: $LR+ = Se_j/(1 - Sp_j)$ and $LR- = (1 - Se_j)/Sp_j$. The ratios were logarithmically transformed to give a symmetry, with a log(LR+) of 0 and a log(LR−) of 0 for a test yielding no information and a log(LR+) of ∞ and a log(LR−) of $-\infty$ for an ideal test. The values of the indices were then plotted against the cutoff value.

TABLE 1. Descriptive indices for the results of the MAP1-B ELISA for infected and noninfected reference populations of sheep and goats[a]

Measurement	% of sera positive from:			
	Infected		Noninfected	
	Sheep (48)	Goats (44)	Sheep (64)	Goats (107)
Mean	77.1	83.4	18.8	11.5
Median	81.6	81.1	18.9	9.2
SD	21.2	35.6	8.8	7.7
Minimum	18.2	10.3	8.1	3.3
Maximum	115.2	148.5	54.5	37.6

[a] Results are expressed as percentages of an internal positive control. The number of animals in each group is indicated in parentheses.

RESULTS

PP values for the infected and noninfected reference sheep and goat populations were tested for normality and showed significant skewness ($P < 0.05$). Therefore, the nonparametric option of the TG-ROC analysis was used (13). Table 1 summarizes the results of the MAP1-B ELISA for the populations of sheep and goats. The cutoff values resulting in equal sensitivity and specificity, as well as two alternative cutoff values for definition of the IR, were read directly from the TG-ROC plot in Fig. 1 and are shown in Table 2. The VRP was approximately 1.0 for sheep as well as for goats. The IR for goat sera was zero, because at cutoff value d_0, sensitivity and specificity are both greater than 95%. The sensitivity and specificity measures of the test at cutoff value d_0 are shown in Table 3. Calculated cutoff values for sheep and goats varied considerably according to the different methods shown in Table 3. The cutoff values calculated by TG-ROC analysis for sheep ($d_0 = 31.0$) and for goats ($d_0 = 26.6$) were close to those calculated as the mean plus twice the standard deviation (assuming normal distribution of the data). The performance of the test as measured by the AUC of the ROC plots was very close to 1: 0.978 for sheep and 0.989 for goats (Fig. 2). The LRs in Table 3 were calculated from the TG-ROC analysis and are displayed in Fig. 3, which shows graphs of the LRs over the entire range

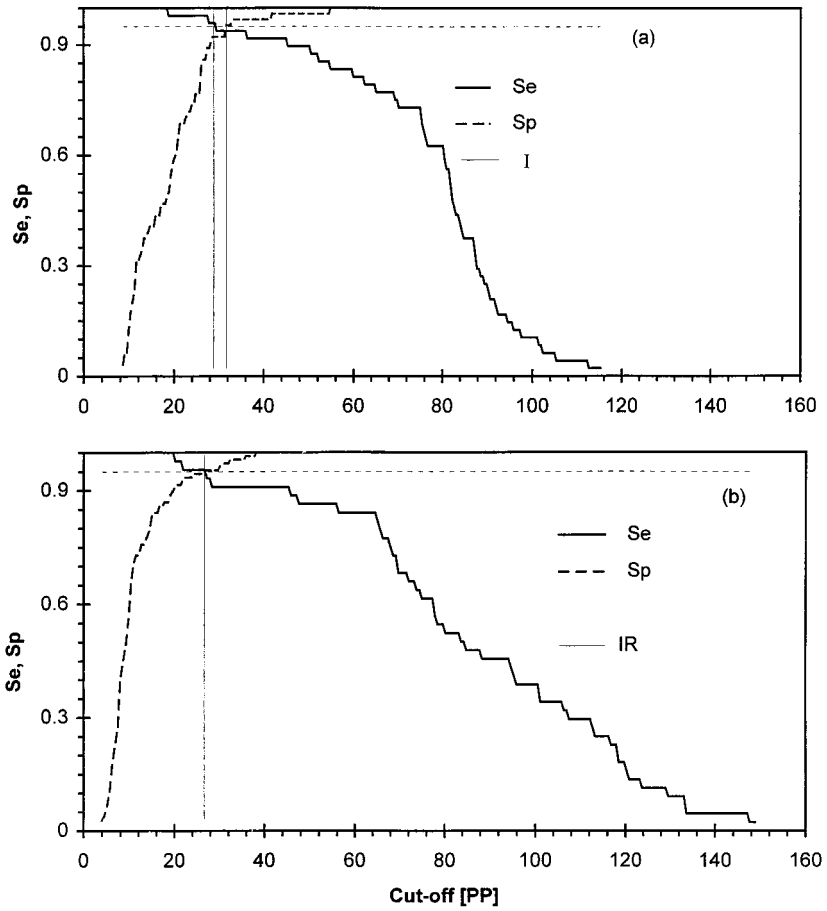

FIG. 1. TG-ROC analysis of MAP1-B ELISA results for sheep (a) and goat (b) sera. The IR is determined by using one cutoff value at 95% sensitivity (Se) and another at 95% specificity (Sp).

of possible cutoff values within the measurement range. The efficiency of the test, measured by efficiency and Youden's index, is shown in Fig. 4. The closer to 1 the indices are, the better the test's performance at a given cutoff value, d_j.

DISCUSSION

The aims of this study were to determine cutoff values by TG-ROC analysis, to compare them to those obtained by previously used methods, and to evaluate the performance of the MAP1-B ELISA for the diagnosis of *C. ruminantium* infections in sheep and goats.

TABLE 2. Results of TG-ROC analysis[a]

Measurement	Result of TG-ROC analysis (CI) on sera from:	
	Sheep	Goats
θ_0	94.5	95.4
d_0	31.0 (27–50)	26.6 (20–36)
IR	2.8 (0–36)	0
Upper limit	31.7 (27–54)	26.2 (20–36)
Lower limit	28.8 (18–45)	26.5 (19–45)
VRP	0.97 (0.662–1)	1

[a] θ_0, point of equivalence where specificity is equal to sensitivity at cutoff value d_0; lower limit, 5th percentile of the percent positivity of the infected population; upper limit, 95th percentile of the percent positivity of the noninfected population. The 95% confidence internal (CI) is shown where appropriate.

The establishment of a reliable cutoff value is essential for a serological test to be useful in differentiating infected and noninfected animals. The assembly of the reference population used for the calculation of a cutoff value is a critical procedure: the sample size has to be large enough to provide the desired statistical power, and moreover, the reference population has to be representative of the target population (12). In order to compensate for various factors that may influence the diagnostic sensitivity and specificity, R. Jacobson (16) suggested the use of at least 300 known positive and 1,000 known negative samples, which numbers are very difficult to obtain under experimental conditions. We conducted this study with the minimal number of positive samples required for a meaningful analysis. We did not have any further positive samples in stock (sera from 48 sheep and 44 goats), but a significant number of noninfected samples (sera from 64 sheep and 107 goats) were available. The latter precondition is very difficult to realize for ELISA tests designed for the screening of tropical infectious diseases: control sera from animals in regions where heartwater is not endemic are guaranteed to be disease free but might not be representative of the target population; on the other hand, negative sera from animals in regions where heartwater is endemic might not be guaranteed to be disease free (33, 34). Previously, determination of cutoff values for the MAP1-B

TABLE 3. Cutoff values determined by different methods with their corresponding sensitivities, specificities, and LRs[a]

Result	Source of sera	Mean + 2 SDs	Mean + 3 SDs	2× negative	TG-ROC
Cutoff	S	36.34	45.11	17.54	31.00
	G	26.90	31.26	13.48	26.60
Sensitivity (%)	S	94	87	100	94.50
	G	90	90	100	95.40
Specificity (%)	S	97	98	50	94.50
	G	95.40	98	80	95.40
LR+	S	4.48	6.05	1.22	2.27
	G	3.47	4.48	1.65	3.32
LR−	S	0.30	0.37	0.00	0.37
	G	0.29	0.37	0.00	0.37

[a] S, sheep sera; G, goat sera; SD, standard deviation for the noninfected reference population; mean, mean value of the negative reference population; 2× negative, twice the value of the negative reference population; LR+, positive LR; LR−, negative LR. The sensitivities, specificities, and LRs were calculated by TG-ROC analysis.

ELISA has mainly been done by doubling the PP value of a reference noninfected sample included on each plate. As shown in Table 3, doubling the PP value of the reference noninfected serum results in a very low specificity and low positive LR for the test. Hence, a single reference sample can serve as an internal test control but can hardly be considered an adequate representation of a noninfected population. Mondry et al. (25) based their cutoff values for the MAP1-B ELISA on the frequency distribution of PP values for a noninfected population in the Caribbean. In their study, cutoff values were determined graphically on the basis of an acceptable number of false-positive results. The authors, however, did not explain how an acceptable number of false positives was defined. The values obtained were fixed at 50% positive for sheep and goats. An overall specificity of 99.4% was reported, but the effect of the cutoff value on the test sensitivity was not investigated in a large enough population of known infected animals.

TG-ROC plots are graphs that show the relationship between the sensitivity and specificity of a test wherein the definition of a positive test is modified over the entire range of obtained values. ROC curves make it possible to compare the quality of the tests with the quality of other quantitative tests and allow a systematic and objective choice of optimal cutoff values (29). Reporting only one value for sensitivity and specificity provides a possibly misleading and even hazardous oversimplification of accuracy. Similarly, calculating just a few sensitivity and specificity pairs provides only a glimpse of a test's real diagnostic abilities (36). The TG-ROC method was originally tested on data obtained with an ELISA for the detection of antibodies to *Borrelia burgdorferi* (13) and also was used to evaluate another ELISA test for the diagnosis of maedi-visna virus (3), giving encouraging results.

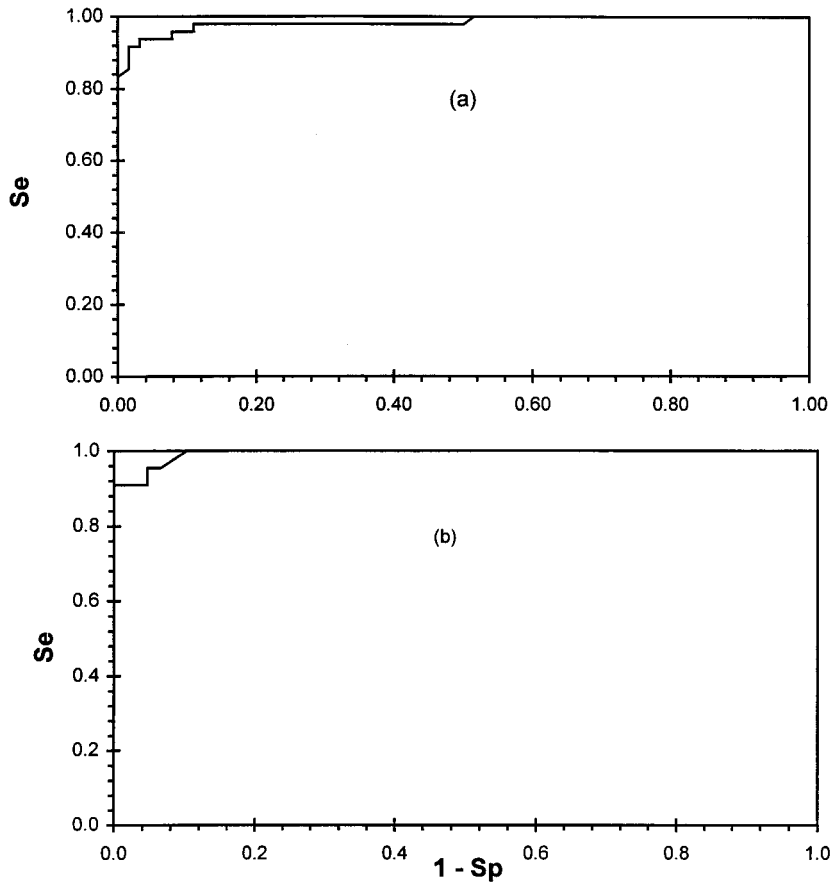

FIG. 2. ROC plots of MAP1-B ELISA results for sheep (a) and goat (b) sera. The AUCs are 0.978 and 0.989, respectively (maximum AUC = 1.0).

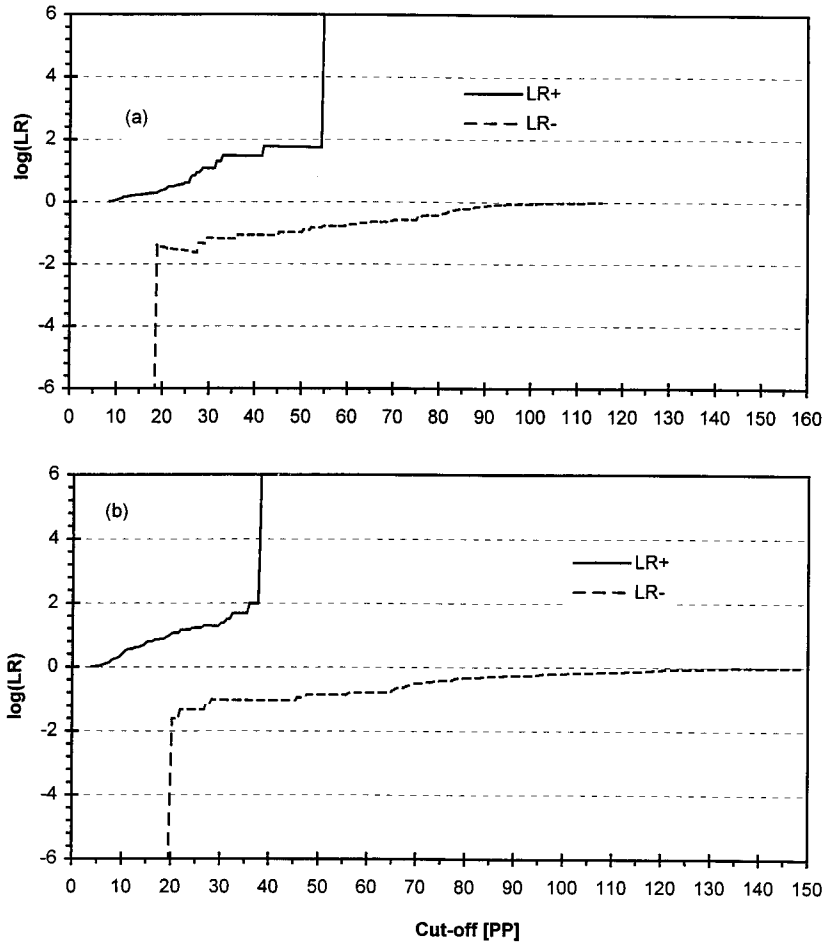

FIG. 3. Logarithm of negative (LR−) and positive (LR+) LRs plotted as a function of the selected cutoff value with sheep (a) and goat (b) sera.

Given the difficulties of obtaining animals not exposed to *Cowdria* or ticks in areas where cowdriosis is endemic, we recommend that the cutoff values for these areas be those given by the mean plus 2 standard deviations, if negative samples from regions of endemicity are available, or to use cutoff d_0 (31.0 and 26.6% positive for sheep and goats, respectively [Tables 2 and 3]), if no such sera are available. The method using the mean plus 2 standard deviations gave results that are quite similar to those obtained by the TG-ROC method. However, a cutoff value should be determined with defined diagnostic accuracy (13), which is not the case with the conventional methods. In this study, the values of d_0 correspond to the efficiency and Youden's index's highest values (Fig. 4). In TG-ROC plots (Fig. 1) an option is given such that the cutoff values can be chosen to suit the required level of accuracy and the effect of the selected cutoff value on the sensitivity and specificity of the test can be read directly from the plots. Likewise, the efficiency, Youden's index, and LRs for the MAP1-B ELISA can be read directly from the plots in Fig. 3 and 4 for a selected cutoff value. Youden's index has a value of 0 whenever a diagnostic test gives the same proportion of positives for both infected and noninfected groups (35).

The IR used to describe nonpositive and nonnegative test results was 2.81 for sheep and 0 for goats. The IR is 0 in cases where the θ_0 is greater than 95%, because the lower limit of the IR is greater than the upper limit. In this study, the IR for goats was 0; hence, over 95% of the goats were correctly diagnosed. The interpretation of intermediate test results depends on the specific diagnostic purpose of the test. Because of the ambiguity of borderline results, it is appropriate to consider only one cutoff value and indicate the test parameters (Se, Sp, and LR) for a given cutoff value selected for an epidemiological situation. In clinical diagnosis, the values that fall between the IR limits would require testing by a confirmatory assay or retesting for detection of seroconversion (16, 27).

The VRP and θ_0 are independent of any selected cutoff value and are, therefore, good measures for test comparison (13). In this study the VRP and θ_0 were reasonably high (Table 2) for both sheeps and goats, indicating the high performance of this ELISA in classifying the animals according to their true health status. It can be concluded that 95% of individual test results are valid, because the VRP was close to 1.0 for both species.

Another convenient way to quantify the diagnostic accuracy of a test is to express its performance by AUC measurements of ROC plots. This is a quantitative, descriptive expression of how close the ROC curve is to the perfect one (AUC = 1.0) (36). AUCs in ROC curves provide an index of accuracy by demonstrating the limits of a test's ability to discriminate between the alternative state of health and the complete spectrum of operating conditions, unlike in TG-ROC plots, where the VRP is limited to 95% accuracy. The MAP1-B ELISA

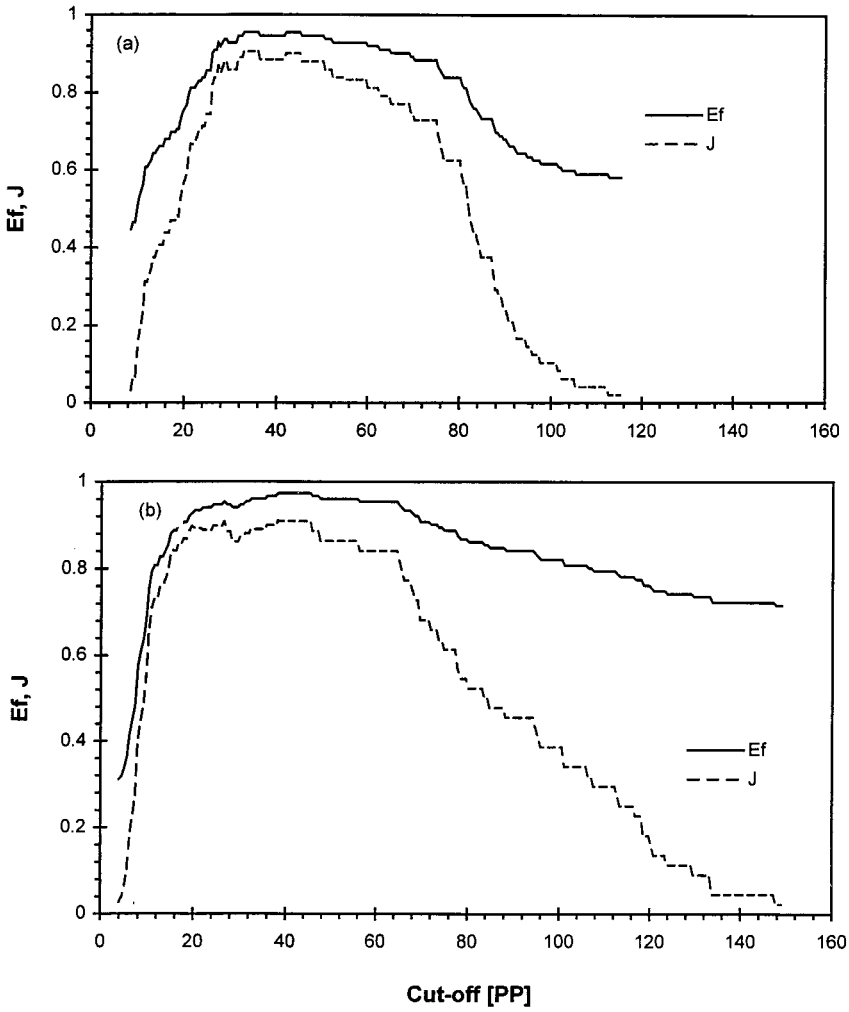

FIG. 4. Youden's index (J) and efficiency (Ef) of the MAP1-B ELISA as a function of the selected cutoff value for sheep (a) and goat (b) sera.

showed high performance because the index AUCs were 0.978 and 0.989 for sheep and goat sera, respectively. From these results (VRP, AUC, and IR), the test appears to have no differences in its diagnostic performance for sheep and goats.

Decisions regarding cutoff values for this ELISA should be reviewed as more data become available, since experimental infections sometimes produce an overoptimistic estimate of accuracy. Analysis similar to that done with sheep and goat sera needs to be done for bovine samples, and work on this has already been started in our laboratory. The effect of the cutoff values on the antibody profiles of ruminants should also be investigated for further cutoff value optimization.

Studies have shown that age is positively correlated with seropositivity but not with the detection of the parasite when a *Trypanosoma* antibody-detecting ELISA was used in an area in Uganda where trypansomiasis is endemic (11). In addition to age, many other factors (such as sex, breed, state of pregnancy, nutritional state, previous chemotherapy, passive immunization, and self-cured infections) may also influence the cutoff value. Further validation of the test precision needs to be done according to the ISO 5725-1986 international procedure, with interlaboratory comparisons of the ELISA results.

In this study we have attempted to calculate cutoff values by using known positive and negative experimental sera. It will be of great interest to repeat this study using samples from animals exposed to infected *Amblyomma* ticks under field conditions to check whether our cutoff values (determined with experimental animals) are also applicable to the situation in the field.

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Chapter 3

Heartwater (*Cowdria ruminantium* infection) as a cause of postrestocking mortality of goats in Mozambique

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Heartwater (*Cowdria ruminantium* Infection) as a Cause of Postrestocking Mortality of Goats in Mozambique

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A serological survey in Mozambique to detect antibodies to *Cowdria ruminantium*, the etiologic agent of heartwater, revealed a seroprevalence of 8.1% ($n = 332$) for goats in the northern province of Tete and of 65.6% ($n = 326$) for goats in the southern provinces. Translocation of 10 serologically negative goats from Tete to farms in the south resulted in two clinical cases of heartwater that were fatal. In addition, four goats seroconverted within the study period of 5 weeks. One goat showed no symptoms. Two goats died of other causes, whereas the remaining goat went missing after 1 week. Experimental needle infections of goats and sheep were conducted to confirm results and to isolate different strains of *C. ruminantium*. These data indicate that translocation of goats from the north to the south of Mozambique bears a high risk of *C. ruminantium* infection, which can cause fatal disease.

Heartwater (or cowdriosis) is a tick-borne disease affecting domestic ruminants throughout sub-Saharan Africa (10). The disease is caused by *Cowdria ruminantium*, a rickettsial agent transmitted by ticks of the genus *Amblyomma*, and constitutes a major constraint to livestock development in the affected areas (11). In Mozambique heartwater has been reported to occur throughout the country and mainly during the rainy season (3, 14). Following restocking of animals from the north to the south of the country, the incidence of disease and animal mortality have been extremely high: an estimated 50% of animals died within 1 year after translocation (7). It is suspected that tick-borne diseases, especially heartwater, contribute to this devastating outcome (7).

To investigate if *C. ruminantium* plays a role in the mortality after translocation, we conducted a serological survey in goats to estimate the prevalence of *C. ruminantium* exposure and a small-scale translocation experiment. Collected blood samples derived from different geographical areas were tested for the presence of *C. ruminantium* antibodies with the MAP1-B enzyme-linked immunosorbent assay (ELISA) (8, 13). The ELISA revealed a seroprevalence of 8.1% for animals kept in the northern part and a high seroprevalence of 65.6% in the southern part of Mozambique (Table 1). These data, which are consistent with a smaller immunofluorescence-based survey (3), suggest a considerable difference in exposure to *C. ruminantium* between animals kept in different parts of the country. The presence of antibodies is likely a good parameter of infection, as small ruminants remain seropositive for several years once they survive a *C. ruminantium* infection. The high seroprevalence in the south indicates that animals which are introduced in this area are at high risk of becoming infected with *C. ruminantium*.

To ascertain that the observed apparent difference in exposure was caused by contact with the agent, 10 serologically negative goats (indigenous Landim breed) were translocated from Tete province to the Veterinary Faculty in Maputo in southern Mozambique. After a 10-day quarantine period at the Veterinary Faculty in Maputo, the goats were subdivided into two sentinel herds and placed at two farms south of Maputo (Porto Henrique and Bom Pastor) and were herded together with the local free-ranging goats. The goats were not treated with any acaricides during the trial. Blood samples were collected on a weekly basis for serological monitoring and at the occurrence of a persistent fever (rectal body temperature above 41.0°C) or nervous symptoms. Of the five animals that were translocated to Porto Henrique (Fig. 1), one goat died 10 days after arrival with severe nervous symptoms. Examination of Giemsa-stained brain crushed smears from this animal for the presence of rickettsial inclusion bodies in endothelial cells confirmed the presence of *C. ruminantium*. MAP-1B ELISA of collected blood samples indicated that three clinically healthy goats at Porto Henrique seroconverted within 35 days. The fifth goat remained seronegative for the entire duration of the study (5 weeks) (Table 2). At the second farm at Bom Pastor (Fig. 1), one goat died 22 days after field exposure with clinical symptoms of heartwater, but this diagnosis could not be confirmed as necropsy could not be performed. A second goat seroconverted without obvious clinical symptoms. Of the remaining three goats one went missing after 1 week and the other two died of other causes (Table 2).

In order to definitively confirm that *C. ruminantium* was the

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TABLE 1. MAP1-B ELISA results for serum samples collected from goats in Mozambique

Location	No. of goats	Seroprevalence (%)	Period
North			
Tete Province			
Marara	131	6.8	September 1997
Kapanga	38	5.3	July 1998
Villa Ulóngue	29	6.9	July 1998
Provincial abattoir	134	10.4	June–September 1997
Total	332	8.1	June–September 1997
South			
Inhambane Province			
Inharrime	35	74.3	January 1998
Gaza Province			
Chibuto	61	50.8	February 1998
Guijá 1	50	76.0	April 1998
Guijá 2	36	86.1	April 1998
Maputo Province			
Bom Pastor	20	90.0	January 1998
Bela Vista	30	46.7	February 1998
Palmeira	29	58.6	February 1998
Boane	14	7.1	February 1998
Porto Henrique	32	75.0	February 1998
Moamba	19	73.7	April 1998
Total	326	65.6	January–April 1998

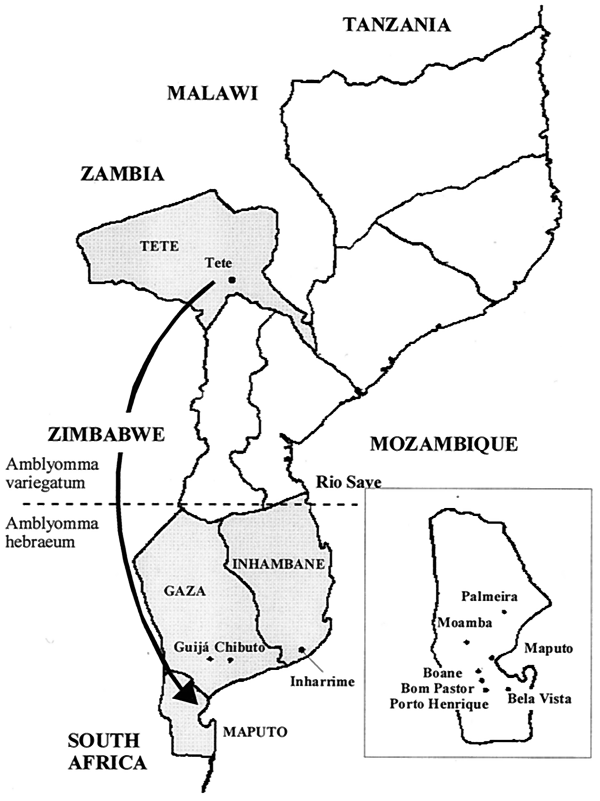

FIG. 1. Map of Mozambique showing the locations where the research was conducted. Arrow, translocation of sentinel goats. Inset, enlargement of Maputo province.

etiologic agent of the diseased animals and to determine if different isolates were present, DNA was extracted from the blood samples of translocated goats showing clinical signs as well as from animals with cases of heartwater encountered in the south during the field study using the method described by Moreira (9). Part of the 16S rRNA gene (rDNA) was amplified using primer 16SF1 (gggctgcagAGTTTGATCCTGGCTCAG) (15) and primer 16SR8 (GGTTCACCTACAGCTACCTT) and was sequenced using primer 16SF1 on a ABI PRISM 310 sequencer. The entire 16S sequences of three samples were determined elsewhere (Baseclear, Leiden, The Netherlands). Blastn searches (National Center for Biotechnology Information website http://ncbi.nlm.nih.gov) using the obtained sequences revealed that samples from Porto Henrique (goat 1) and Bela Vista (field case of heartwater) were homologous to the *C. ruminantium* Crystal Springs isolate (4) (Table 3). The sequence of a sample from Bom Pastor (goat 6) was homologous to *Ehrlichia* sp. strain Omatjenne (1). The sequence of a second sample from Bela Vista was homologous, but not identical, to *Anaplasma marginale* (Table 4). Since this sample originated from a goat, we believe that it might be *Anaplasma ovis*, which has been reported to occur in sheep and goats in Mozambique (2). These data show that *C. ruminantium* and other *Ehrlichia* sp. were present simultaneously in goats, but it is not clear whether the goats were already infected with the *Ehrlichia* and *Anaplasma* species prior to translocation as they were only examined for antibodies against *C. ruminantium*.

Blood samples from suspected heartwater cases encountered in the south (Bela Vista) during the field study were used for experimental infection of Landim goats in Maputo to confirm the presence of *C. ruminantium*. Three goats died shortly after infection, and heartwater could be confirmed. A fourth goat seroconverted after a second inoculation since it was treated with antibiotics 2 days after the first inoculation. In The Netherlands three seronegative Texelaar sheep were subinoculated with 4 ml of blood (no. 172 blood from goat 1 at Porto Henrique, no. 173 blood from a field case at Bela Vista, and

TABLE 2. Outcome of experimental translocation of goats from Tete to Maputo province

Goat	Outcome
Porto Henrique	
1	Died after 10 days of field exposure; heartwater confirmed
2	Seroconversion to *C. ruminantium*
3	Seroconversion to *C. ruminantium*
4	Seroconversion to *C. ruminantium*
5	No reaction, remained seronegative
Bom Pastor	
6	Died after 22 days of field exposure; heartwater suspected
7	Died after 20 days of field exposure with pulmonary distress; heartwater could not be confirmed
8	Died of other causes
9	Missing after 1 week
10	Seroconversion to *C. ruminantium*

TABLE 3. Nucleotide differences between *C. ruminantium* isolates from a field case and from translocated goats in Mozambique and those present in GenBank in a fragment of 1,448 nucleotides of the 16S rDNA sequence[a]

Isolate[b] (sheep no.)	Nucleotide at position:									
	49	58	60	63	67	349	553	793	1129	1143
GenBank										
Senegal	A	C	T	C	A	C	A	T	A	C
Mara 87/7[c]	·	·	C	·	G	·	·	·	T	T
Crystal Springs[d]	·	T	C	·	G	·	·	·	·	·
Omatjenne[c]	G	·	C	T	·	·	G	·	·	·
Ball 3[c]	·	T	C	·	·	—	·	·	·	·
Mozambique										
Bela Vista (173)	·	T	C	·	G	·	·	·	·	·
Porto Henrique (172)	·	·	C	·	G	·	·	A	·	·
Bom Pastor (179)	·	·	C	·	G	·	·	ND[e]	ND	ND

[a] Numbering is based on the 16S rDNA sequence of the Senegal isolate (12). Dots, identical nucleotides; dash, gap (compared to the sequence of the Senegal isolate).
[b] The accession numbers for the different isolates are as follows: X62432, Senegal; AF069758, Mara 87/7; X61659, Crystal Springs; U03776, Omatjenne; U03777, Ball 3.
[c] Isolate originating from South Africa (5, 6).
[d] Isolate originating from Zimbabwe (16).
[e] ND, not determined.

no. 174 blood from goat 6 at Bom Pastor) to confirm earlier findings and to start in vitro cultures. All three sheep developed fever and were treated with oxytetracyclines. They recovered, and seroconversion was demonstrated by the MAP1-B ELISA (Fig. 2). Despite the fact that sheep 174 seroconverted after treatment, no *C. ruminantium* 16S sequence homology was found for the sample used to infect this animal. To further investigate if *C. ruminantium* was present in this blood sample, a fourth sheep was infected (sheep 179). After the animal had succumbed, DNA was extracted from the brain of this animal; rickettsial inclusions were detected in the capillary endothelial cells. A hydropericardium was also detected in this animal, clearly indicating a fatal heartwater case. The 16S sequence obtained from this DNA was identical to the previously obtained sequence of the *C. ruminantium* Porto Henrique isolate (Table 3), which suggests that the original blood sample collected from goat 6 contained two bacterial species. To confirm this, the 16S PCR product obtained from the DNA extracted from the original sample was cloned and transformed to *Escherichia coli*. Ten clones were sequenced; 4 were homologous to *C. ruminantium*, and 6 were homologous to *Ehrlichia* sp. strain Omatjenne. Whether the presence of *Ehrlichia* played a role in the outcome of the disease remains to be investigated.

TABLE 4. Nucleotide differences between an *Anaplasma* sp. isolate from a goat in Bela Vista and the *A. marginale* 16S rDNA sequence present in GenBank[a]

Isolate	Nucleotide at position:								
	69	70	127	139	179	202	221	247	256
GenBank, *A. marginale*	T	A	A	G	—[b]	T	—	T	G
Mozambique, *Anaplasma* sp.	C	G	G	A	C	C	T	C	T

[a] Numbering is based on the *A. marginale* sequence.
[b] —, gap introduced to optimize alignment.

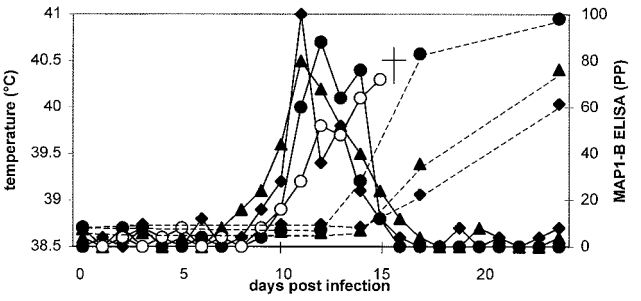

FIG. 2. Rectal body temperature (solid lines) and MAP1-B ELISA results (dashed lines; PP, percentage positive) for four experimentally infected sheep. ◆, sheep 172 (Porto Henrique); ▲, sheep 173 (Bela Vista); ●, sheep 174 (Bom Pastor); ○, sheep 179 (Bom Pastor). All reacting animals, except sheep 179, were treated with oxytetracycline at the second day of fever.

These data demonstrate that there is a risk of transmission of *C. ruminantium* to translocated goats if precautions are not taken and that transmission of *C. ruminantium* may cause fatal disease. Vaccination prior to translocation and proper tick control might be essential components of a successful restocking program in Mozambique.

Nucleotide sequence accession numbers. The entire 16S sequences of three samples were deposited in GenBank under accession no. AF318021 (*C. ruminantium* Porto Henrique), AF318022 (*C. ruminantium* Bela Vista), and AF318023 (*Ehrlichia* sp. Bom Pastor).

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Chapter 4

Transcriptional analysis of the major antigenic protein 1 multigene family of *Cowdria ruminantium*

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Transcriptional analysis of the major antigenic protein 1 multigene family of *Cowdria ruminantium*

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Abstract

The major antigenic protein 1 (MAP1) of the tick-borne rickettsial pathogen *Cowdria ruminantium* is encoded by a multigene family containing conserved and variable genes. The part of a locus containing the *map1* multigene family that was characterized contained three homologous, but non-identical *map1* genes, designated *map1-2*, *map1-1*, and *map1*. Reverse transcriptase-polymerase chain reaction was used to study the transcriptional activity of these genes in isolates of *C. ruminantium* grown in bovine endothelial cells, in two different tick cell lines, and in *Amblyomma variegatum* ticks. The *map1* gene was always transcribed, whereas transcription of *map1-2* was not detected under any of the tested conditions. The *map1-1* gene transcript was detected in *A. variegatum* ticks, but was not found in virulent *C. ruminantium* Senegal grown in bovine endothelial cells at 30 or 37°C. Interestingly, transcripts of *map1-1* were also found in different passages of the in vitro attenuated Senegal isolate grown in bovine endothelial cells, as well as in the Gardel isolate grown in two tick cell lines. When transcribed, *map1-1* was present on a polycistronic messenger together with *map1*. © 2002 Published by Elsevier Science B.V.

Keywords: Differential transcription; *Amblyomma variegatum*; Ticks; Tick cell lines

1. Introduction

Cowdriosis (or heartwater) is a tick-borne disease caused by the rickettsia *Cowdria ruminantium* and transmitted by ticks of the genus *Amblyomma*. The disease affects both domestic and wild ruminants in sub-Saharan Africa and on certain Caribbean islands (Uilenberg, 1983). *C. ruminantium* and members of the related genera *Ehrlichia* and *Anaplasma* usually cause persistent infections in their natural hosts (Andrew and Norval, 1989; Gale et al., 1996; Breitschwerdt et al., 1998). Regulation of surface antigenicity may be an important mechanism for the establishment of such persistent infections in the host. Multigene families encoding for antigenic proteins are present in *C. ruminantium* (*map1*) (Sulsona et al., 1999), *Anaplasma marginale* (*msp-2*) (Palmer et al., 1994, 1998), *Ehrlichia canis* and *Ehrlichia chaffeensis* (*p28*) (Ohashi et al., 1998a; Reddy et al., 1998). The *map1* multigene family found in *C. ruminantium* is more closely related to the *p28* multigene family than to the *msp-2* multigene family, both in sequence similarity and in gene organization. The *map1* and *p28* genes are both located in a single cluster, whereas *msp-2* genes are dispersed over the genome. Recently, the complete *p28* multigene locus of *E. chaffeensis* containing 21 homologous *p28* genes was characterized (Yu et al., 2000), and the presence of a conserved, transcriptionally active *p28* multigene locus of *E. canis* (McBride et al., 2000) has been reported. In both studies only monocistronic transcripts were found even when two neighboring genes were transcribed simultaneously. However, in a study

Abbreviations: RT-PCR, reverse transcriptase-polymerase chain reaction; BEC, Bovine endothelial cells; MAP, major antigenic protein; DNase, deoxyribonuclease; RNase, ribonuclease; cDNA, DNA complementary to RNA

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wherein both gene clusters were compared it was found that all 22 paralogs of *E. canis* were transcriptionally active in monocyte cultures, and that paralogs with short intergenic spaces were co-transcribed (Ohashi et al., 2001).

Gene *p28-19* of the *E. chaffeensis* multigene family and *map1* of *C. ruminantium* encode surface-exposed proteins. The respective genes are largely conserved among different isolates except for three hypervariable regions (Reddy et al., 1996; Ohashi et al., 1998b). Studies using monoclonal antibodies have demonstrated diversity among *E. chaffeensis* isolates in the expressed P28 proteins (Yu et al., 1993) and this was shown to be related to diversity in the *p28* gene (Yu et al., 1999). However, complete conservation of a *p28* paralog in geographically different isolates of *E. canis* has also been reported (McBride et al., 1999). These findings show that both conserved and variable genes are present in the *p28* gene families of *E. canis* and *E. chaffeensis*, a situation that has also been reported for the *C. ruminantium map1* multigene family (Sulsona et al., 1999). Gaining more knowledge about these gene families is important as recombinant *E. chaffeensis* P28 and a DNA vaccine containing *map1* of *C. ruminantium* appeared to provide protection against an otherwise lethal challenge with the homologous isolate in mice (Nyika et al., 1998; Ohashi et al., 1998b). Furthermore, in a recent study it was shown that outer membrane protein-specific monoclonal antibodies protected SCID mice from fatal infection by *E. chaffeensis* (Li et al., 2001).

In this study, we used RT-PCR to examine if there were differences in the transcriptional activity of three homologous, non-identical genes in the *map1* multigene family of *C. ruminantium* under different growth conditions. Conditions included in vitro in tick and bovine endothelial cells and in vivo in *Amblyomma variegatum* ticks. Finally, a comparison was made between transcription in different passages of virulent and attenuated *C. ruminantium* in endothelial cell cultures.

2. Materials and methods

2.1. Cowdria ruminantium isolates and endothelial cell culture conditions

The *C. ruminantium* isolates used in this study were: Senegal (virulent and attenuated) from Senegal, Welgevonden from South Africa, Sankat 430 from Ghana, and Gardel from Guadeloupe. Bovine endothelial cells (BEC) derived from umbilical cord were used to cultivate *C. ruminantium* isolates. To determine if the lower temperature used for the tick cell cultures (30°C instead of 37°C) influenced transcription of *map1* genes, infected BUE cultures were also incubated and passaged at 30°C. After three successful passages, total RNA was extracted from these cultures.

C. ruminantium-infected BEC cultures were regularly examined by preparation of Giemsa stained cytospin smears of culture supernate. When the cultures started showing signs of cell damage due to the infection, cells were scraped from the bottom of the culture flasks and pelleted by centrifugation for 10 min at $300 \times g$ and total RNA was extracted.

2.2. Infected tick cell cultures

The *Ixodes scapularis* cell line IDE8 was maintained at 30 ± 2°C in L-15B medium supplemented with 10% tryptose phosphate broth, 5% foetal calf serum (FCS), 0.1% bovine lipoprotein, 2 mM L-glutamine, and 100 units penicillin and 100 μg streptomycin per ml. A *Rhipicephalus appendiculatus* cell line designated RAN/CTVM3, comprising mainly haemocytes, fibroblast-like, and epithelial-like cells, was established from moulting nymphal ticks. RAN/CTVM3 cells were maintained at 28°C in H-Lac medium (Hanks BSS supplemented with 0.5% lactalbumin hydrolysate, 20% FCS, and L-glutamine and antibiotics as above).

IDE8 cells at passage 65–67 were infected with *C. ruminantium* (Gardel) and maintained as described previously (Bell Sakyi et al., 2000). Cell-free supernate from infected IDE8 cultures, obtained by centrifugation at $1000 \times g$ for 10 min, was used to infect RAN/CTVM3 cultures at passage 56–58, which were incubated thereafter at 30 ± 2°C with weekly medium changes.

C. ruminantium-infected tick cell cultures were examined by preparation of Giemsa stained cytospin smears of suspended cells. When at least 10% of the cells were infected, the cultures were harvested by pipetting off adherent cells and centrifuging the resultant cell suspension for 10 min at $300 \times g$. The cell pellets were thereafter used for total RNA extraction.

2.3. Infected Amblyomma variegatum ticks

Female sheep (Texelaar) between 6 and 12 months of age were inoculated intravenously using cryopreserved tissue culture (BEC) derived *C. ruminantium* stabilate (Senegal isolate). The sheep were treated with oxytetracycline (5 mg/kg) starting on the 3rd day of fever ($T > 40.0$°C) for three consecutive days to ensure recovery. Treatment with oxytetracycline does not clear the infection and animals become carriers. At 60 days post infection, approximately 100 uninfected *A. variegatum* nymphs were applied to the back of each sheep. The ticks originated from Burkina Faso and were maintained in a colony at Utrecht University. Engorged nymphs were collected and allowed to moult to the adult stage at 27°C and 95% relative humidity.

2.4. Cloning of map1 genes

C. ruminantium (Senegal) was purified from BEC cultures and genomic DNA was purified as previously described (van Vliet et al., 1992, 1994). A genomic library was constructed in pUC19 by ligating *Hin*dIII digested *C. ruminantium* DNA with *Hin*dIII digested, dephosphorylated pUC19. The ligation mix was used to transform *Escherichia*

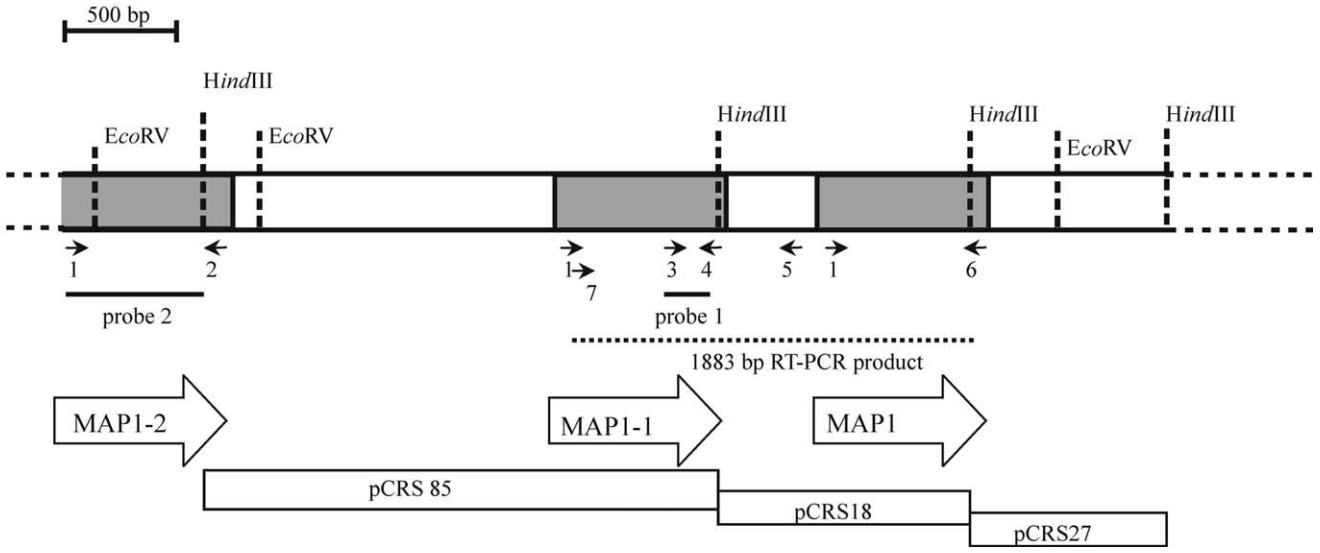

Fig. 1. Schematic representation of the *C. ruminantium* (Senegal isolate) *map1* gene locus (5128 bp) indicating the genomic orientation, primers and probes. The boxes indicated with pCRS represent the different clones obtained from the genomic *Hin*dIII library. The region amplified by RT-PCR to demonstrate the presence of a polycistronic transcript is indicated by the dotted line. The probes used to screen the genomic library are indicated with solid lines. Primers indicated and numbered were as follows; 1, map1g-for; 2, map1-2rev; 3, f5; 4, map1-1rev; 5, r-50; 6, map1-rev; and 7, map1-1for.

coli DH5α (Gibco) to ampicillin resistance. The cloning of *map1* from this library has been described previously (van Vliet et al., 1994). A primer specific for the upstream region of *map1*, designated r-50 (5′-AAA GCA AGC TAT AAT GTA AGT-3′), was used together with primer f5 (5′-GCG CAA AAT ACA TGC CAA CTG CAT C-3′) to amplify the region upstream of *map1*. The resulting PCR product was digested with *Hin*dIII and the 5′ part was used as a probe to screen the genomic library (Fig. 1, probe 1). One of the clones, designated pCRS85, was sequenced and shown to contain the 5′ part of *map1-1* and the 3′ part of the *map1-2* gene (Fig. 1). The partial *map1-2* gene was further characterized by sequencing a PCR product obtained after amplification of genomic DNA with primer map1-2rev, specific for the *map1-2* gene, and a general *map1* primer designated map1g-for (Table 1).

2.5. DNA sequencing

DNA was sequenced on an ABI Prism 310 (Perkin Elmer Applied Biosystems, Foster City, CA) or sent for sequencing to BaseClear (Leiden, The Netherlands)

2.6. Total RNA isolation from infected cells

Total RNA was isolated from *C. ruminantium*-infected BEC grown at either 30 or 37°C and from infected tick cells using a RNeasy mini kit (Qiagen). Total RNA was treated before elution with RNase-free DNase I (10 U). Following elution, the RNA was quantified in a spectrophotometer at A_{260}, while purity was confirmed by a spectrophotometric A_{260}/A_{280} ratio of over 1.8.

Table 1
Primers for cloning and gene-specific RT-PCR amplification

Name	Sequence of (f) forward and reverse (r) primers	Nucleotides[a]
Cloning		
f5	(f) GCG CAA AAT ACA TGC CAA CTG GAT C	602–626
r-50	(r) AAA GCA AGC TAT AAT GTA AGT	409–429
RT-PCR		
map1g-for	(f) TAA T(A/G)T CAT TA(A/G) TGT CAT TTT TAC C	515–539
map1g-rev	(r) A(A/T)(A/C/G/T) (C/T)AA A(C/T)C TT(A/C) (C/T)TC CAA (G/T)TT C	79–100, 1309–1330
map1-rev	(r) TGG ACT AAC AGC ACT ACT GGC	1246–1266
map1-1for	(f) CCA AGC ATA CCA CAT TTC AGA	This study
map1-1rev	(r) TGA AGC GGA AGT GCT TTG AGG	28–48
map1-2rev	(r) TAT TGC AGA TGT TAC TAA TGG GGA T	This study

[a] Numbers based on accession number X74250 (van Vliet et al., 1992).

2.7. Total RNA isolation from infected ticks and determination of infection status

Total RNA was isolated from unfed adult *A. variegatum* ticks, several weeks after moulting, using a total RNA isolation kit (totally RNA™, Ambion). Ticks were cut in half using sterile scalpel blades and one half was incubated in 1 ml of denaturation buffer until all internal organs were completely denatured. Total RNA was subsequently digested for 30 min at 37°C with RQ1 RNase-free DNase (10 U) (Promega). After the addition of 20 mM ethylene glycol-bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), DNase was inactivated by incubation for 10 min at 65°C. RNA was used without any further treatment in RT-PCR reactions.

DNA was extracted from the other half of each tick by boiling it for 10 min in PBS; subsequently SDS was added (1% final concentration) and the sample was spun for 5 min at full speed. DNA was phenol-extracted from the supernatant and subsequently ethanol-precipitated and resuspended in TE. Extracted DNA was used in a PCR using primers EHR-F (5′-ggaattcAGAGTTGGATCMTGGYT-CAG-3′) and primer EHR-R (5′-cgggatccCGAGTTTG-CCGGGACTTYTTCT-3′) to demonstrate the presence of *C. ruminantium*.

2.8. Reverse transcriptase PCR (RT-PCR)

RNA (1 μg) from in vitro cultured *C. ruminantium*-infected cells was used to generate cDNA using a first strand cDNA synthesis system (SUPERSCRIPT™, Life Technologies) and random hexamer primers, according to the manufacturer's instructions. Primer pairs (0.4 μM), one general (g-for) and one specific for each *map1* gene were used to amplify each *map1* gene in a PCR reaction using the same cDNA batch as template (Table 1). PCR parameters were as follows: amplification for 30 cycles at 94°C for 30 s, 50°C for 1 min, and 72°C for 1 min; and a final elongation step at 72°C for 5 min. RNA from *C. ruminantium*-infected ticks was amplified using an Access RT-PCR system (Promega) using gene specific primers for first strand cDNA synthesis. The thermal cycling profile consisted of reverse transcription at 42°C for 50 min followed by 10 min at 94°C. The subsequent PCR step was as described above. A negative control that included all reagents except the reverse transcriptase was included in every test to confirm that genomic DNA was not present in the RNA preparations. Amplified (RT-)PCR products were sequenced to verify the specificity of the primers.

2.9. Nucleotide sequence accession number

The complete sequence of the pCRS85 clone has been submitted to the DDBJ/EMBL/GenBank database under accession number AF319940.

3. Results

3.1. Cloning and sequencing of map1 genes of C. ruminantium (Senegal)

A study by van Vliet et al. (1994) identified an incomplete open reading frame (ORF) adjacent to the *map1* gene of the Senegal isolate that showed homology with 32% similarity to the 3′ end of the *map1* gene. Previous attempts to identify *map1* homologous genes using part of the *map1* gene as a probe had failed and it was originally thought that *map1* was a single copy gene (Reddy et al., 1996). In an attempt to clone the entire gene, reverse primer r-50 specific for the intergenic upstream region of *map1* and forward primers that target regions within the *map1* gene, were used for PCR amplification. Whereas all other primer combinations failed we obtained a PCR product with primer f5. Apparently primer f5 was also able to hybridize to a region upstream of *map1*. The 5′ *Hin*dIII part of the resulting PCR product was used as a probe to screen a *C. ruminantium* (Senegal) *Hin*dIII library (Fig. 1, probe 1). Several positive clones were lifted from the library and digested with *Hin*dIII to determine the insert size. Clone pCRS85, which contained an insert of approximately 2.4 kb, was selected and sequenced. The clone contained the unidentified part of the gene which was named *map1-1*. Upstream of this *map1-1* gene an additional partial *map1* homologue was identified (designated *map1-2*) separated from *map1-1* by a 1385 bp intergenic region (Fig. 1) In order to clone the missing 5′ part of this gene, primer map1-2rev specific for the newly identified gene, and primer map1-gfor, which targets a conserved region at the 5′ end of known *map1* paralogs, were used for PCR amplification. A 0.8 kb PCR product was amplified and sequenced and shown to contain one continuous open reading frame. Use of the 5′ *Hin*dIII fragment of this PCR product as a probe (Fig. 1, probe 2) to screen the *C. ruminantium Hin*dIII library did not reveal any positive clones.

3.2. Amino acid homology

The deduced amino acid sequences of MAP1 and of MAP1-1 and the partial MAP1-2 proteins of the Senegal isolate were aligned using the ClustalV method (Fig. 2). The amino acid homology ranged from 32.8 to 46.7% among the *C. ruminantium* proteins when the entire ORFs of *map1* and *map1-1* and the partial ORF of *map1-2* were compared (Table 2). Higher amino acid homologies were observed between *C. ruminantium* MAP1 and homologous proteins from *E. canis* and *E. chaffeensis* (Table 2); the same was observed for MAP1-1 and MAP1-2. Clear conserved and hypervariable domains such as those found in MAP1 were not detected in MAP1-1 or MAP1-2 (Fig. 3). When MAP1 of the Senegal isolate was aligned with MAP1-1 and MAP1-2 of the same isolate neither clear conserved nor hypervariable domains could be detected (Fig. 2). There-

Fig. 2. Alignment of *C. ruminantium* MAP1, MAP1-1, and MAP1-2 of the Senegal isolates. The *map1* gene and its two paralogs *map1-1* and *map1-2* were translated and aligned using the ClustalV method (Lasergene 5.0 package DNAstar Inc.). The complete amino-acid sequence of MAP1 is presented. Dots indicate identity with MAP1 and dashes were introduced to obtain the best alignment.

fore, ambiguous bases had to be incorporated in the map1-gfor and map1-grev primers (Table 1). All primers were tested on genomic DNA from the isolates used and shown to amplify the expected genes, except for the *map1-2* primer which did not give a product when the Gardel isolate was used (Fig. 5A).

3.3. Transcriptional analysis of map1 paralogs of C. ruminantium isolates grown in bovine endothelial cells

To study the transcriptional activity total RNA was extracted from *C. ruminantium* grown in bovine endothelial cells and cDNA was prepared using random hexamer primers. Amplification of the three gene transcripts from cDNA prepared from *C. ruminantium* (Senegal, Welgevonden, Sankat 430)-infected endothelial cells grown at 37°C was performed using gene specific reverse primers and primer map1g-for (Table 1). RNA transcripts were detected only for the *map1* gene in the three *C. ruminantium* isolates (Fig. 4A). To determine if the lower temperature in ticks compared to mammals could have an effect on transcription, cultures infected with the Senegal, Welgevonden, or Sankat 430 isolate were incubated at 30°C and total RNA was extracted after three passages (23 days). Again RNA transcripts were detected only for the *map1* gene (Fig. 4A). All RT-PCR products were sequenced and confirmed to be *map1* gene sequences, indicating specificity of the primer pairs for *map1*.

In vitro attenuated *C. ruminantium* Senegal isolate (passage 32 or 37) grown in endothelial cells at 30°C or 37°C was also tested using RT-PCR to determine if the same transcription pattern was present as in virulent passages, from which the attenuated culture was derived. As in a virulent passage a RNA transcript was found for *map1* and no transcript was found for *map1-2* (Fig. 4B). However, in contrast to the virulent passage an additional

Table 2
Percentage identity (upper triangle) and divergence (lower triangle) of amino acid sequences of *C. ruminantium* MAP1 and *E. canis* (Eca) and *E. chaffeensis* (Ech) P28 proteins calculated using the MegAlign option of the Lasergene 5.0 package (DNAstar Inc.)

	1	2	3	4	5	6	7	8	9
1 MAP1		46.7	32.8	39.4	46.4	65.5	38.6	47.0	67.2
2 MAP1-1	88.5		35.1	40.3	79.7	49.6	39.4	78.0	47.8
3 MAP1-2	135.5	115.2		57.4	35.7	35.4	56.5	34.5	33.3
4 Eca P28-1	126.7	110.3	56.9		40.9	40.6	69.3	40.0	38.0
5 Eca P28-2	88.5	28.5	110.9	106.4		47.5	39.7	83.2	48.7
6 Eca P28-8	50.7	78.3	119.6	118.4	86.2		40.0	48.7	73.9
7 Ech P28-11	125.6	109.5	56.6	44.9	107.0	118.0		39.7	38.6
8 Ech P28-14	87.9	32.9	116.9	110.3	22.8	81.9	106.7		49.3
9 Ech P28-19	45.9	80.9	124.7	126.4	78.3	35.6	119.5	76.3	

RNA transcript was detected for *map1-1* in the attenuated passage (Fig. 4B). Transcripts from the genes were analyzed for the presence of polycistronic messages with RT-PCR using the same cDNA and the gene-specific *map1-1* forward primer (map1-1for) and the gene specific *map1* reverse primer (map1-rev) (Table 1, Fig. 1). An approximately 1900 bp amplicon was detected in the attenuated passage demonstrating the presence of a polycistronic mRNA (Fig. 4B).

3.4. Transcriptional analysis of map1 paralogs of C. ruminantium grown in tick cells

To investigate possible differences in transcription of genes between tick and mammalian stages, total RNA was extracted from *C. ruminantium*-infected tick cell cultures grown at 30 ± 2°C. Amplification of gene transcripts from *C. ruminantium* (Gardel) infected IDE8 or RAN/CTVM3 cells was performed. RNA transcripts were detected for

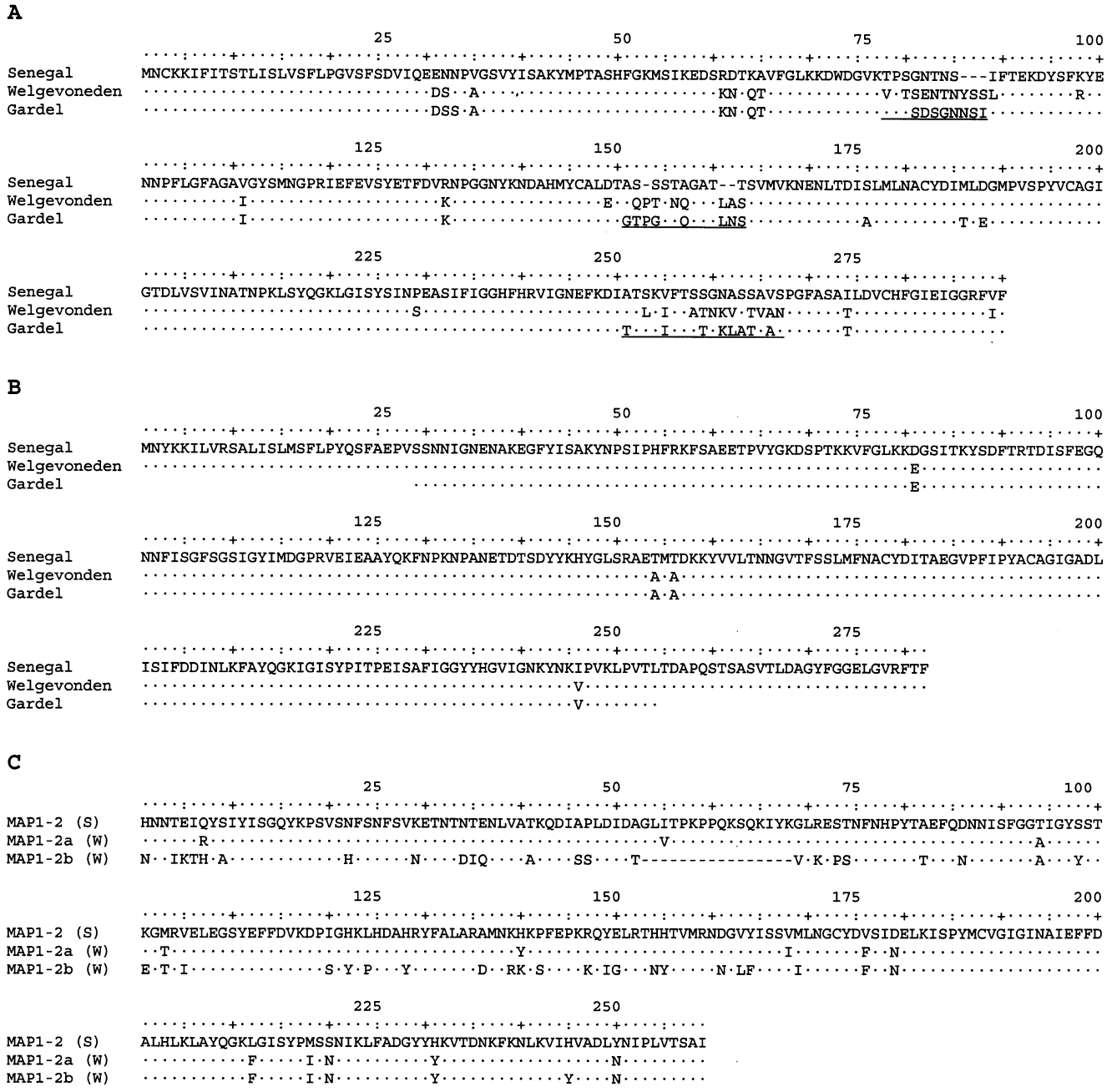

Fig. 3. Alignment of *C. ruminantium* MAP1 (A); MAP1-1 (B); and MAP1-2 (C); from the Senegal (S), Welgevonden (W) and Gardel isolates. The *map1* gene and its two paralogs *map1-1* and *map1-2* were translated and aligned using the ClustalV method (Lasergene 5.0 package DNAstar Inc.). The complete amino-acid sequence MAP1, MAP1-1 and MAP1-2 of the Senegal isolate is presented. Dots indicate identity with MAP1 and dashes were introduced to obtain the best alignment. The hypervariable regions in MAP1 are underlined.

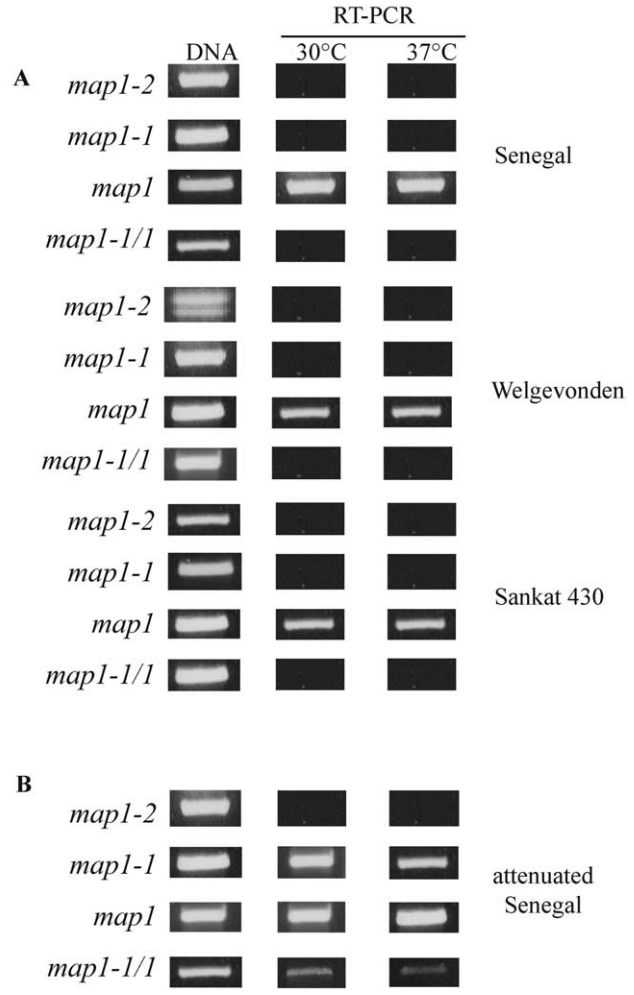

Fig. 4. Transcriptional analysis of the *map1* gene locus of *C. ruminantium* isolates grown in bovine endothelial cells at 30 and 37°C using RT-PCR. Three virulent isolates (A); and one attenuated isolate (B). Reverse primers specific for each gene (Table 1) were used to amplify *map1-2*, *map1-1* and *map1*, a forward primer specific for *map1-1* was used in combination with map1-rev to show the presence of a polycistronic messenger (map1-1/1 row). A PCR control on genomic DNA (first column) is included to show proper primer function.

the *map1* and *map1-1* gene in both cultures by RT-PCR (Fig. 5A). A *map1-1/map1* polycistronic mRNA was detected in both *C. ruminantium*/tick cell line combinations. The same results were observed for attenuated Gardel in both IDE8 and RAN/CTVM3 and for Sankat 430 in RAN/CTVM3 (data not shown).

3.5. Transcriptional analysis of map1 genes of C. ruminantium from infected ticks

In order to determine if the in vitro tick cell culture results were indicative of the in vivo situation, infected *A. variegatum* adults, which were obtained by feeding nymphs on *C. ruminantium* (Senegal) infected sheep, were tested. RNA transcripts were detected for *map1* and *map1-1* in these ticks, and a *map1-1/map1* polycistronic messenger was also detected (Fig. 5B) as was demonstrated in vitro for the Gardel isolate in two tick cell cultures.

4. Discussion

We report here the differential transcription of *C. ruminantium map1* paralogs under different in vitro conditions. It was found that in BEC cultures only the *map1* gene of several virulent *C. ruminantium* isolates was transcribed both at 37 and 30°C. However, both *map1* and *map1-1* were transcribed as a polycistronic messenger in attenuated passages of the Senegal isolate grown in endothelial cells, and in passages of the Gardel isolate grown in two different tick cell lines. Furthermore, the transcription of both *map1* and *map1-1* was also found in the virulent Senegal isolate in vivo in *A. variegatum* ticks.

The basis for our findings was that the Senegal isolate contained at least three *map1* paralogs. These paralogs were found to be located in a head-to-tail orientation within a single cluster. Findings suggest a similar organization in the Welgevonden isolate of *C. ruminantium* (Sulsona et al., 1999). Using a *map1-2* PCR amplified product as a probe (Fig. 1, probe 2) we were not able to detect any *Hin*dIII clone containing the 5′ part of the *map1-2* gene in our library. The genomic *Hin*dIII fragment containing the 5′ part of the *map1-2* gene may have been too large for the pUC19 vector. It is also possible that *C. ruminantium* DNA

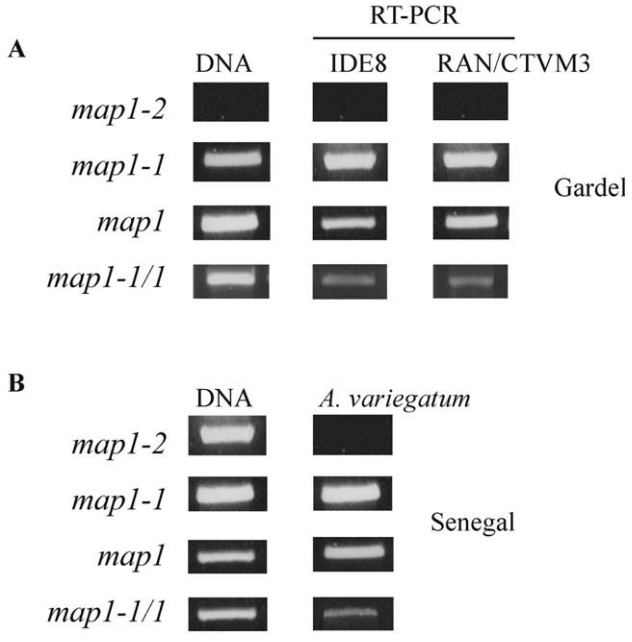

Fig. 5. Transcriptional analysis of the *map1* gene locus of *C. ruminantium* (Gardel isolate) grown in vitro in IDE8 and RAN/CTVM3 tick cells (A); or *C. ruminantium* (Senegal isolate) grown in vivo in *A. variegatum* ticks (B). Reverse primers specific for each gene (Table 1) were used to amplify *map1-2*, *map1-1* and *map1*, a forward primer specific for *map1-1* was used in combination with map1-rev to show the presence of a polycistronic messenger (map1-1/1 row). A PCR control on genomic DNA (first column) is included to show proper primer function.

was unstable as shown before in other vector systems (Brayton et al., 1999). Using the map1-2rev primer in combination with the general forward primer we found different results depending on the isolate used. The Senegal and Sankat 430 isolate each yielded one product, whereas the Welgevonden isolate showed two products, and no products were observed with the Gardel isolate (Figs. 4 and 5). These results indicate that the sequences of the MAP1 paralogs show variation between different isolates of *C. ruminantium.* While MAP1 from different isolates shows sequence variation particularly located in three hypervariable domains, the sequence variation between MAP1-1 proteins of different isolates was quite limited or non-existent (Fig. 3B) as has been reported before (Sulsona et al., 1999). The sequence variation between MAP1-2 of the Senegal isolate and MAP1-2a of the Welgevonden isolate (Fig. 3C) was also quite limited. Additional sequence data from other isolates is required to determine the extent of variation between different MAP1-2 proteins. The results also show that the *map1-2* specific primer needs further improvement for it to be useful for all isolates. The fact that no transcripts of *map1-2* were found in any of the tested systems could be due to improper function of this primer in RT-PCR.

The protein sequence identities of MAP1, MAP1-1, and MAP1-2 of the Senegal isolate were lower (32.8–46.7%) when compared to each other than to orthologs in *E. canis* and *E. chaffeensis* (Table 2). The highest percentage of identity of MAP1-2 is found with *E. canis* P28-1 (57.4%) and *E. chaffeensis* P28-11 (56.5%), MAP1-1 with *E. canis* P28-2 (79.7%) and *E. chaffeensis* P28-14 (78.0%), and MAP1 with *E. canis* P28-8 (56.5%) and *E. chaffeenis* P28-19 (67.2%). Furthermore, it has been shown that the *E. chaffeensis p28-19* gene is divergent among different isolates (Yu et al., 1999), as is the case for *map1* of *C. ruminantium* (Reddy et al., 1996). The limited homology between the different MAP1 paralogs within a single isolate (Fig. 2) provided the scaffolding to analyze the transcription of the corresponding genes under various conditions using specific primers.

To our knowledge our data provide the first evidence of differential transcription of *map1* paralogs between bovine endothelial and tick cell lines. The finding that several genes can be transcribed simultaneously could explain the occurrence of multiple bands in the 28–30 kDa range observed in immunoblots for *Cowdria* (Rossouw et al., 1990; Jongejan, 1991) and for *E. canis* and *E. chaffeensis* (Yu et al., 1993, 1999; Rikihisa et al., 1994). Transcription analysis of the *p28* multigene families in *E. canis* and *E. chaffensis* has been described for in vitro cultures in the canine macrophage cell line DH82 (Reddy et al., 1998; McBride et al., 2000; Yu et al., 2000; Ohashi et al., 2001). It was shown by Reddy et al. (1998) that only one *E. chaffeensis p28* gene was transcribed and four other identified genes were silent. However, when the complete p28 multigene family in *E. chaffeensis* became known, it was shown that six out of 10 *p28* genes were transcribed (Yu et al., 2000), including two which were studied by Reddy et al. (1998). This discrepancy could be explained by the use of different primers as the authors mention, or by differences in the culture techniques used. Transcriptional analysis of *p28* genes in *E. canis* showed that all five genes studied were transcribed (McBride et al., 2000). In all three studies monocistronic transcripts were reported. However, Ohashi et al. (2001) showed that in the *omp* cluster of *E. canis*, paralogs with short intergenic spaces (5′-end half of cluster) were co-transcribed, whereas transcripts for the adjacent genes connected by long intergenic spaces in the 3′-end half of the cluster were undetectable or detectable only at low levels relative to the DNA control. For *C. ruminantium* we found a polycistronic messenger for two adjacent genes (*map1-1* and *map1*) connected by a long intergenic space.

The detection of a polycistronic messenger in tick cells and in ticks may be due to the use of a different promoter under those circumstances. The same promoter could be active in the attenuated Senegal culture thus explaining the detection of *map1-1* transcripts in that culture. Since nothing is known yet about promoter sequences in *C. ruminantium*, more research is required to confirm the presence of different promoters and their specific activity. Active transcription of genes in the tick vector has been described for *Anaplasma marginale* in *Dermacentor* ticks (Rurangirwa et al., 1999), in which a restricted repertoire of major surface protein 2 (MSP2) variants of *A. marginale* were expressed in the salivary gland of infected male *D. andersoni* ticks.

As only a limited number of passages were tested and a non-quantitative detection method was used, low levels of *map1-1* messenger could have gone undetected in virulent passages. Results obtained with the attenuated Gardel isolate grown in endothelial cells, although using different primers and RT-PCR conditions, showed that a polycistronic messenger (*map1-1* and *map1*) seemed to be present in lower quantities in passage 53 (virulent) than in passage 224 (attenuated) (Bensaid and Martinez, unpublished data). Whether the in vitro transcription of *map1-1* in attenuated cultures has any connection with attenuation per se needs to be further investigated. If so, this could be used as a marker for in vitro attenuation of *C. ruminantium.*

Further studies, especially on in vivo transcription in the ruminant host and tick vector, are essential to select valuable vaccine candidate genes which will become available with the ongoing *C. ruminantium* genome sequencing project (www.sanger.ac.uk). Furthermore, clarification of the regulation of transcription and expression of genes in the *C. ruminantium map1* gene family will aid our understanding of the role of these genes in host-parasite interactions and possible immune evasion.

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Chapter 5

Reorganization of genera in the families *Rickettsiaceae* and *Anaplasmataceae* in the order *Rickettsiales*: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, description of six new species combinations and designation of *Ehrlichia equi* and 'HGE agent' as subjective synonyms of *Ehrlichia phagocytophila*

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The genera *Anaplasma*, *Ehrlichia*, *Cowdria*, *Neorickettsia* and *Wolbachia* encompass a group of obligate intracellular bacteria that reside in vacuoles of eukaryotic cells and were previously placed in taxa based upon morphological, ecological, epidemiological and clinical characteristics. Recent genetic analyses of 16S rRNA genes, *groESL* and surface protein genes have indicated that the existing taxa designations are flawed. All 16S rRNA gene and *groESL* sequences deposited in GenBank prior to 2000 and selected sequences deposited thereafter were aligned and phylogenetic trees and bootstrap values were calculated using the neighbour-joining method and compared with trees generated with maximum-probability, maximum-likelihood, majority-rule consensus and parsimony methods. Supported by bootstrap probabilities of at least 54%, 16S rRNA gene comparisons consistently clustered to yield four distinct clades characterized roughly as *Anaplasma* (including the *Ehrlichia phagocytophila* group, *Ehrlichia platys* and *Ehrlichia bovis*) with a minimum of 96·1% similarity, *Ehrlichia* (including *Cowdria ruminantium*) with a minimum of 97·7% similarity, *Wolbachia* with a minimum of 95·6% similarity and *Neorickettsia* (including *Ehrlichia sennetsu* and *Ehrlichia risticii*) with a minimum of 94·9% similarity. Maximum similarity between clades ranged from 87·1 to 94·9%. Insufficient differences existed among *E. phagocytophila*, *Ehrlichia equi* and the human granulocytic ehrlichiosis (HGE) agent to support separate species designations, and this group was at least 98·2% similar to any *Anaplasma* species. These 16S rRNA gene analyses are strongly supported by similar *groESL* clades, as well as biological and antigenic characteristics. It is proposed that all members of the tribes *Ehrlichieae* and *Wolbachieae* be transferred to the family *Anaplasmataceae* and that the tribe structure of the family *Rickettsiaceae* be eliminated. The genus *Anaplasma* should be emended to include *Anaplasma* (*Ehrlichia*) *phagocytophila* comb. nov. (which also encompasses the former *E. equi* and the HGE agent), *Anaplasma* (*Ehrlichia*) *bovis* comb. nov. and *Anaplasma* (*Ehrlichia*) *platys* comb. nov., the genus *Ehrlichia* should be emended to include *Ehrlichia* (*Cowdria*) *ruminantium* comb. nov. and the genus *Neorickettsia* should be emended to include *Neorickettsia* (*Ehrlichia*) *risticii* comb. nov. and *Neorickettsia* (*Ehrlichia*) *sennetsu* comb. nov.

Keywords: *Anaplasmataceae*, *Ehrlichia*, *Anaplasma*, *Neorickettsia*, *Cowdria*

Abbreviation: HGE, human granulocytic ehrlichiosis.

Details of the similarity values used in construction of the trees are available in IJSEM Online at http://ijs.sgmjournals.org/

INTRODUCTION

Recent improvements in molecular technologies have significantly advanced our abilities to conduct genetic analyses and, for the first time, clearly indicated the proper phylogenetic positions of most of the fastidious bacterial species in the families *Rickettsiaceae*, *Bartonellaceae* and *Anaplasmataceae* in the order *Rickettsiales* (Woese *et al*., 1990; Weisburg *et al*., 1989; Brenner *et al*., 1993; Birtles *et al*., 1995). By 16S rRNA sequencing, Weisburg *et al*. (1989) demonstrated that *Coxiella burnetii* and *Wolbachia persica* belonged to the *γ-Proteobacteria*, while the remaining members of the order *Rickettsiales* that they examined (three species of *Rickettsia* and *Ehrlichia risticii*) formed a tight monophyletic cluster within the *α-Proteobacteria*. In fact, *Wolbachia persica* and related tick symbionts are most closely related to species of *Francisella* (Forsman *et al*., 1994; Noda *et al*., 1997; Niebylski *et al*., 1997). Subsequently, *Anaplasma marginale* and *Cowdria ruminantium* were also found to be closely related to *Rickettsia* and *Ehrlichia* (Weisburg *et al*., 1991; van Vliet *et al*., 1992; Dame *et al*., 1992). The second major reorganization of the order *Rickettsiales* came with the removal of the *Bartonellaceae* from the order and with the unification of the genera *Grahamella* and *Rochalimaea* in the genus *Bartonella* (Brenner *et al*., 1993; Birtles *et al*., 1995). Subsequently, additional species have been removed from the order *Rickettsiales* as their 16S rRNA sequences were determined. *Rickettsiella grylli* was found to be closely related to *Coxiella* and *Legionella* (Roux *et al*., 1997), while the genera *Haemobartonella* and *Eperythrozoon* were unified in the order *Mollicutes* (Neimark & Kocan, 1997; Rikihisa *et al*., 1997). *Wolbachia* was found to be polyphyletic, as *Wolbachia pipientis* belongs to the cluster of rickettsial species in the *α-Proteobacteria* (O'Neill *et al*., 1992) while *Wolbachia melophagi* is actually a species of *Bartonella* (R. J. Birtles and D. H. Molyneux, unpublished GenBank accession no. X89110).

We propose here a reorganization of the remaining members of the order *Rickettsiales* in the families *Rickettsiaceae* and *Anaplasmataceae*. We emend the order by elimination of the tribes *Rickettsieae*, *Ehrlichieae*, *Wolbachieae* and *Anaplasmataceae* because (i) many of the genera contained in each tribe have no phylogenetic affinities and have already been removed from the order and (ii), as described further below, the remaining species previously placed in the tribes *Ehrlichiaeae*, *Wolbachieae* and *Anaplasmataceae* have molecular and phenotypic affinities that are more appropriate to recognition at the family level. We propose that the family *Rickettsiaceae* be composed of the closely related genera *Rickettsia* and *Orientia*, which was recently split from *Rickettsia* (Tamura *et al*., 1995). All of the species in the family *Rickettsiaceae* are obligate intracellular bacteria that grow freely in the cytoplasm of their eukaryotic host cells.

We retain the family *Anaplasmataceae*, but broaden it to include all species of the *α-Proteobacteria* presently contained in the genera *Ehrlichia*, *Anaplasma*, *Cowdria*, *Wolbachia* and *Neorickettsia*, as described below. *Aegyptianella* is also retained provisionally in the *Anaplasmataceae*, but designated as *genus incertae sedis*, since its 16S rRNA and other gene sequences have not been determined but it has strong phenotypic similarities to the species of *Anaplasma*. All members of the family *Anaplasmataceae* are obligate intracellular bacteria that replicate while enclosed in a eukaryotic host cell membrane-derived vacuole (Rikihisa, 1991a). Except for the genus *Wolbachia*, each species can replicate in vertebrate hosts, usually within cells derived from mesodermal structures, in particular, mature and immature haematopoietic cells (Rikihisa, 1991a; Barbet, 1995; Logan *et al*., 1987). Moreover, for each species of these genera for which sufficient study has been accomplished, an invertebrate vector host has been identified, predominantly ticks or trematodes (Rikihisa, 1991a), except for *Wolbachia* species, which are highly promiscuous for diverse invertebrate hosts and are also found in a variety of helminths (Werren, 1997; Zhou *et al*., 1998).

The data generated by 16S rRNA gene sequencing studies support the prior classification of the species and genera in the newly constituted family *Anaplasmataceae* (Weisburg *et al*., 1989; van Vliet *et al*., 1992; Dame *et al*., 1992). Based upon 16S rRNA gene and *groESL* operon sequence results (Sumner *et al*., 1997; Zhang *et al*., 1997) and antigenic analyses (Zhang *et al*., 1997), the data suggest strongly that an accurate reorganization of these taxa would require the reorganization of most members of the existing genera *Anaplasma*, *Cowdria*, *Neorickettsia*, *Wolbachia* and *Ehrlichia* into four distinct genetic groups. Consistent with these genetic groups, which also have parallel differences in phenotype, we propose the following: (i) that the present genus *Anaplasma* be expanded to include *Ehrlichia phagocytophila*, *Ehrlichia bovis* and *Ehrlichia platys* and that *Anaplasma phagocytophila* comb. nov. will include the subjective synonyms *Ehrlichia equi* and *Ehrlichia* 'HGE agent'; (ii) that the species *Cowdria ruminantium* be placed in the genus *Ehrlichia as Ehrlichia ruminantium* comb. nov. with the existing species *Ehrlichia canis*, *Ehrlichia chaffeensis*, *Ehrlichia ewingii* and *Ehrlichia muris*; (iii) that the genus *Neorickettsia* be expanded to include the species *Ehrlichia risticii* and *Ehrlichia sennetsu*; and (iv) that the species *Wolbachia pipientis* be provisionally retained as the sole member of the genus *Wolbachia*. Molecular and biological data supporting this taxonomic reorganization of species and genera in the family *Anaplasmataceae* are presented here.

METHODS

The literature on species in the family *Anaplasmataceae*, including analysis of nucleic acid sequences, antigenic properties, their ecology and geographical distribution and pathogenicity, was reviewed in order to determine the most

scientifically supported scheme for classification. Due to the subjective nature of the clinical and non-microbial phenotypic parameters used in previous taxonomic associations, accepted standards of phylogenetic analysis based upon identified gene nucleic acid sequences or protein amino acid sequences of ehrlichiae have been given greater weight in the final determination of the positions of proposed taxa. Sequence analyses were conducted by obtaining all 16S rRNA gene and *groESL* sequences deposited in GenBank that could be retrieved with a key word search for *Ehrlichia*, *Anaplasma*, *Cowdria*, *Wolbachia* or *Neorickettsia* (Tables 1 and 2). Because of a paucity of sequences available for *Anaplasma* species and the absence of sequence data for *Ehrlichia ovina*, additional 16S rRNA gene sequences were determined by participating authors, submitted for inclusion in GenBank and included in the final analyses. The methods and details of these sequences will be presented elsewhere. The 16S rRNA gene sequences of *Escherichia coli*, *Rickettsia* species, *Chlamydia trachomatis* and a variety of other bacteria with arthropod associations were included for comparison. Sequences were aligned using CLUSTAL X version 1.8 (Thompson *et al*., 1997) and then corrected by hand to preserve codon alignment and conserved protein motifs, where relevant. Sites containing gaps or having ambiguous alignment were removed prior to phylogenetic analysis.

Phylogenetic trees were inferred from nucleotide sequences using PAUP* (Swofford, 2000). Trees were constructed using the maximum-parsimony, minimum-evolution and maximum-likelihood criteria as implemented in PAUP*. The most parsimonious tree was sought using a heuristic search procedure with 100 random addition sequence replicates and tree bisection–reconnection branch swapping. For distance-based methods, the HKY85 two-parameter model of sequence evolution was applied, with empirical estimation of transition/transversion ratio and base frequency. The minimum-evolution tree was used as the starting tree for maximum-likelihood analyses. Internal node support was verified using the bootstrap method (Felsenstein, 1985) with 1000 replicates.

RESULTS AND DISCUSSION

Multiple analyses and alignments of the 16S rRNA gene sequences of these organisms have revealed four distinct clusters, regardless of method. This phenomenon was also confirmed by comparing the nucleotide sequences of the *groESL* operon for organisms where those sequences have been described (van Vliet *et al*., 1992; Dame *et al*., 1992; Rikihisa *et al*., 1997; Zhang *et al*., 1997; Roux & Raoult, 1995, 1999; Drancourt & Raoult, 1994; Anderson *et al*., 1991; Chen *et al*., 1994a; Wen *et al*., 1995a, b; Sumner *et al*., 1997). In the genetic analyses, full-length sequences were not available for many 16S rRNA gene entries. Therefore, analysis was performed using the largest fragment that was available for most taxa. Thus, a 1292 nt fragment (after gap-stripping) including 87 taxa was used to validate subsequent comparisons using a smaller fragment so that the remaining taxa could also be assessed. This smaller fragment included the first 455 nt of the larger fragment, representing 138 taxa. Four groups were consistently identified (Fig. 1; details of the similarity values are available as Additional Table 1 in IJSEM Online at http://ijs.sgmjournals.org/cgi/content/full/51/6/2145/DC1) in both the large and small fragment comparisons, with 16S rRNA gene sequence similarities between 82·2 and 100 %, but generally greater than 91·0 % (mean 90·9 %). These analyses also revealed the genus *Rickettsia* to be at least 80·2 % but not more than 86·1 % similar to any member in the genus *Ehrlichia*, *Neorickettsia helminthoeca*, *A. marginale*, *C. ruminantium* and *W. pipientis*. In the dendrograms, *E. phagocytophila*, *E. equi*, the human granulocytic ehrlichiosis (HGE) agent, *E. platys* and *A. marginale* (*E. phagocytophila* group) clustered to obtain at least 96·1 % similarity, but were at most 94·9 % similar to the next closest grouping, which included *E. canis*, *E. chaffeensis*, *E. ewingii*, *E. muris* and *C. ruminantium* (*E. canis* group). Likewise, members of the *E. canis* group clustered to obtain at least 97·7 % similarity. In contrast, the group defined by *E. sennetsu* (including *E. sennetsu*, *E. risticii*, *Neorickettsia helminthoeca* and the SF agent) was less than 88·3 % similar to any member of the *E. canis* or *E. phagocytophila* groups or to *W. pipientis*. *W. pipientis* is an obligate intracellular bacterium that is transmitted vertically (maternally) in arthropod and helminth hosts. This species seems to occupy an intermediate phylogenetic position, between 82·3 and 90·0 % similar to each of the other three genetic clusters. The legitimacy of this grouping analysis was confirmed, as very similar results were obtained with nucleotide sequence alignments of *groESL* (Fig. 2; details of the similarity values are available as Additional Table 2 in IJSEM Online at http://ijs.sgmjournals.org/cgi/content/full/51/6/2145/DC2) and comprehensive analyses of the outer-membrane protein genes that are shared among the *E. phagocytophila* and *E. canis* groups and with members of the genus *Wolbachia* but not among *E. sennetsu*, *E. risticii* or *N. helminthoeca* (Sumner *et al*., 1997; Zhang *et al*., 1997; Yu *et al*., 1999a; Ohashi *et al*., 1998a, b; Murphy *et al*., 1998; Dawson *et al*., 1996a; Lally *et al*., 1995).

With the 16S rRNA gene and *groESL* alignments used as an initial starting template for a genetically based taxonomic classification system, further evidence of validity was sought by evaluation of other objective phenotypic characteristics, especially analyses of the amino acid or nucleotide sequences of outer-membrane protein genes, antigenic analyses, biological characteristics including infected host cell type, potential vectors, mammalian hosts with and without clinically evident signs of infection and clinical signs in infected hosts. Progressively less weight was attributed to these characteristics as objectivity decreased.

The *E. phagocytophila/Anaplasma* group

Within the *E. phagocytophila*/*Anaplasma* group cluster, three organisms share at least 99·1 % nucleotide

Table 1. 16S rRNA sequences used in the phylogenetic analyses and associated information

Accession no.	Location	Source	Designation	Prior taxonomic classification
AF283007	Japan	Bovine	Japan	*Anaplasma centrale*
AF318944	South Africa	Ovine	NA	*Anaplasma centrale*
AF309866	Virginia, USA	Bovine	Virginia	*Anaplasma marginale*
AF309867	Florida, USA	Bovine	Florida	*Anaplasma marginale*
AF309868	Idaho, USA	Bovine	South Idaho	*Anaplasma marginale*
AF309869	Israel	Bovine	Israel	*Anaplasma marginale*
AF311303	Virginia, USA	Bovine	Virginia	*Anaplasma marginale*
M60313	NA	Bovine	NA	*Anaplasma marginale*
AF309865	NA	Ovine	South Africa	*Anaplasma ovis*
AF318945	NA	Ovine	NA	*Anaplasma ovis*
NKIT36586	South Africa	Ovine	Sheep 3573/7	*Anaplasma ovis*
AB001521	Africa	*Ornithodoros moubata* tick	Symbiote A	Argasid tick 'symbiote A'
AB001522	Africa	*Ornithodoros moubata* tick	Symbiote B	Argasid tick 'symbiote B'
AE001345	NA	Human	D/UW-3/CX	*Chlamydia trachomatis*
AF069758	South Africa	Ruminant	Mara 87/7	*Cowdria ruminantium*
U03776	South Africa	Ruminant	Omatjenne	*Cowdria ruminantium*
U03777	South Africa	Ruminant	Ball3	*Cowdria ruminantium*
X61659	Zimbabwe	Ruminant	Crystal Springs	*Cowdria ruminantium*
X62432	Senegal	Ruminant	Senegal	*Cowdria ruminantium*
D84559	NA	*Rhipicephalus sanguineus* tick	NA	*Coxiella* sp.
U03775	South Africa	Bovine	NA	*Ehrlichia bovis*
AF162860	Guangzhou, China	Dog	Gzh982	*Ehrlichia canis*
M73221	Oklahoma, USA	Dog	Oklahoma^T	*Ehrlichia canis*
M73226	Florida, USA	Dog	Florida	*Ehrlichia canis*
U26740	Israel	Dog	611	*Ehrlichia canis*
AF147752	China	*Amblyomma testudinarium* tick	NA	*Ehrlichia chaffeensis*
M73222	Arkansas, USA	Human	Arkansas^T	*Ehrlichia chaffeensis*
U23503	Arkansas, USA	Human	91HE17	*Ehrlichia chaffeensis*
U60476	Oklahoma, USA	Human	Sapulpa	*Ehrlichia chaffeensis*
U86664	Florida, USA	Human	Jax	*Ehrlichia chaffeensis*
U86665	Florida/Georgia, USA	Human	St Vincent	*Ehrlichia chaffeensis*
AF036645	California, USA	Horse	Alice	*Ehrlichia equi*
AF036646	California, USA	*Ixodes pacificus* tick/horse	Atempo	*Ehrlichia equi*
AF036647	California, USA	Horse	Meretricious	*Ehrlichia equi*
AF172164	California, USA	Horse	CASOLJ	*Ehrlichia equi*
AF172165	California, USA	Horse	CAMEBS	*Ehrlichia equi*
AF172166	California, USA	Horse	CASITL	*Ehrlichia equi*
AF172167	California, USA	Horse	CAMAWI	*Ehrlichia equi*
M73223	North America	Horse	NA	*Ehrlichia equi*
M73227	Oklahoma, USA	Dog	Stillwater^T	*Ehrlichia ewingii*
U96436	North Carolina/Virginia, USA	Dog	95E9-TS	*Ehrlichia ewingii*
AB013008	Japan	*Apodemus speciosus*	I268	*Ehrlichia muris*
AB013009	Japan	*Haemaphysalis flava* tick	NA1	*Ehrlichia muris*
U15527	Japan	*Eothenomys kageus*	AS145^T	*Ehrlichia muris*
AF318946	Turkey	Ovine	NA	*Ehrlichia ovina*
M73220	Scotland, UK	Sheep	Old Sourhope	*Ehrlichia phagocytophila*
M73224	Scotland, UK	Goat	Feral goat	*Ehrlichia phagocytophila*
AF156784	Guangzhou, China	Dog	Gzh981	*Ehrlichia platys*
M82801	North America	Dog	NA	*Ehrlichia platys*
AF036648	Oregon, USA	Horse	Buck	*Ehrlichia risticii*
AF036649	Oregon, USA	Horse	Bunn	*Ehrlichia risticii*
AF036650	Oregon, USA	Horse	Danny	*Ehrlichia risticii*
AF036651	California, USA	*Juga* spp. (snail)	None	*Ehrlichia risticii*
AF036652	California, USA	*Juga* spp. (snail)	DrPepper	*Ehrlichia risticii*
AF036653	Pennsylvania, USA	Horse	Eclipse	*Ehrlichia risticii*
AF036654	California, USA	*Juga* spp. (snail)	Juga/snail	*Ehrlichia risticii*
AF036655	Oregon, USA	*Juga* spp. (snail)	Stagnicola	*Ehrlichia risticii*
AF036656	Michigan, USA	Horse	MostlyMemories	*Ehrlichia risticii*
AF036657	California, USA	*Juga* spp. (snail)	MsAnnie	*Ehrlichia risticii*
AF036658	Oregon, USA	*Juga* spp. (snail)	Tate	*Ehrlichia risticii*
AF036659	Oregon, USA	*Juga* spp. (snail)	Thorenberg	*Ehrlichia risticii*
AF037210	California, USA	*Juga* spp. (snail)	SHSN-1	*Ehrlichia risticii*
AF037211	California, USA	*Juga* spp. (snail)	SHSN-2	*Ehrlichia risticii*
AF170727	California, USA	Coyote	CATE	*Ehrlichia risticii*
AF170729	California, USA	Coyote	CAPL	*Ehrlichia risticii*
M21290	Maryland, USA	Horse	Illinois^T	*Ehrlichia risticii*
M73219	Japan	Human	Miyayama^T	*Ehrlichia sennetsu*
M73225	NA	Human	11908	*Ehrlichia sennetsu*
AF012528	France	*Ixodes ricinus* tick	EHR62	*Ehrlichia* sp.
AF057707	Switzerland	Horse	NA	*Ehrlichia* sp.
AF069062	California, USA	*Haliotis cracherodii* (abalone)	WSA	*Ehrlichia* sp.
AF084907	Switzerland	*Ixodes ricinus* tick	NA	*Ehrlichia* sp.
AF104680	Netherlands	*Ixodes ricinus* tick	Schotti variant	*Ehrlichia* sp.
AF136712	Germany	*Ixodes ricinus* tick	Frankonia 2	*Ehrlichia* sp.
AF136713	Germany	*Ixodes ricinus* tick	Frankonia 1	*Ehrlichia* sp.

Table 1 (*cont.*)

Accession no.	Location	Source	Designation	Prior taxonomic classification
AF136714	Germany	*Ixodes ricinus* tick	Baden	*Ehrlichia* sp.
AF170728	California, USA	Coyote	CASC	*Ehrlichia* sp.
AF241532	California, USA	Llama	NA	*Ehrlichia* sp.
U02521	Wisconsin, USA	Human	NA	*Ehrlichia* sp.
U10873	Sweden	Dog	Rosa	*Ehrlichia* sp.
U27101	Oklahoma, USA	*Odocoileus virginianus* (white-tailed deer)	OK3	*Ehrlichia* sp.
U27102	Oklahoma, USA	*Odocoileus virginianus* (white-tailed deer)	OK1	*Ehrlichia* sp.
U27103	Georgia, USA	*Odocoileus virginianus* (white-tailed deer)	GA2	*Ehrlichia* sp.
U27104	Georgia, USA	*Odocoileus virginianus* (white-tailed deer)	GA4	*Ehrlichia* sp.
U34280	Japan	*Stellantchasmus falcatus* (fluke)	SF agent	*Ehrlichia* sp.
U52514	Missouri, USA	*Amblyomma americanum* tick	NA	*Ehrlichia* sp.
U54805	South Africa	Sheep	Germishuys	*Ehrlichia* sp.
U54806	South Africa	Bovine	Omatjenne	*Ehrlichia* sp.
U72878	Minnesota, USA	*Peromyscus leucopus* (white-footed mouse)	PL1559	*Ehrlichia* sp.
U72879	Minnesota, USA	*Peromyscus leucopus* (white-footed mouse)	PL505	*Ehrlichia* sp.
U77389	Switzerland	Horse	Swiss horse 1	*Ehrlichia* sp.
AF093788	California, USA	Human	CAHU-HGE1	*Ehrlichia* sp. HGE agent
AF093789	California, USA	Human	CAHU-HGE2	*Ehrlichia* sp. HGE agent
AF189153	Minnesota, USA	*Peromyscus leucopus* (white-footed mouse)	PL59	*Ehrlichia* sp. HGE agent
AJ242785	Sweden	*Ixodes ricinus* tick	NA	*Ehrlichia* sp. type Ia
AJ242783	Sweden	*Ixodes ricinus* tick	NA	*Ehrlichia* sp. type Ib
AJ242784	Sweden	*Ixodes ricinus* tick	NA	*Ehrlichia* sp. type IIb
U88565	NA	University of Illinois, Urbana, USA	Illinois	*Eperythrozoon suis*
AF016546	NA	*Bos taurus*	NA	*Eperythrozoon wenyonii*
J01859	NA	NA	NA	*Escherichia coli*
U95297	NA	*Cat*	NA	*Haemobartonella felis*
U82963	Japan	*Apodemus argentus*	Shizuoka	*Haemobartonella muris*
AB001519	NA	*Haemaphysalis longicornis* tick	NA	Ixodid tick 'symbiote A'
U12457	NA	*Nanophyetus salmincola* in dog	NA	*Neorickettsia helminthoeca*
D38622	Japan	Human	Gilliam	*Orientia tsutsugamushi*
L36217	NA	Human	R strain	*Rickettsia rickettsii*
D84558	NA	*Ixodes scapularis* tick	NA	*Rickettsia* sp.
U12463	North Carolina, USA	Human	Wilmington^{T}	*Rickettsia typhi*
X89110	NA	*Melophagus ovinus*	MO6	*Wolbachia melophagi*
M21292	NA	NA	NA	*Wolbachia persica*
AF179630	NA	*Folsomia candida*	NA	*Wolbachia pipientis*
U23709	NA	*Culex pipiens*	NA	*Wolbachia pipientis*
X61768	Champaign, IL, USA	*Culex pipiens*	NA	*Wolbachia pipientis*
AB025965	NA	*Callosobruchus chinensis*	jC strain	*Wolbachia* sp.
AF035160	Guangzhou, China	*Sitophilus oryzae*	Ch	*Wolbachia* sp.
AF220604	Korea	*Thecodiplosis japonensis*	NA	*Wolbachia* sp.
AJ010275	NA	*Brugia malayi*	NA	*Wolbachia* sp.
AJ010276	NA	*Onchocerca ochengi*	NA	*Wolbachia* sp.
AJ012646	NA	*Brugia pahangi*	NA	*Wolbachia* sp.
L02882	NA	*Muscidifurax uniraptor*	NA	*Wolbachia* sp.
L02883	Spain	*Trichogramma cordubensis*	Spain	*Wolbachia* sp.
L02884	Texas, USA	*Trichogramma deion*	Texas	*Wolbachia* sp.
L02887	NA	*Trichogramma deion*	Bautista Canyon	*Wolbachia* sp.
L02888	South Dakota, USA	*Trichogramma deion*	South Dakota	*Wolbachia* sp.
U17059	NA	*Drosophila sechellia*	NA	*Wolbachia* sp.
U17060	NA	*Drosophila mauritiana*	NA	*Wolbachia* sp.
U80584	NA	*Phlebotomus papatasi*	NA	*Wolbachia* sp.
U83090	Urbana, IL, USA	*Gryllus pennsylvanicus*	NA	*Wolbachia* sp.
U83091	NA	*Gryllus assimilis*	NA	*Wolbachia* sp.
U83092	Gainesville, FL, USA	*Gryllus rubens*	NA	*Wolbachia* sp.
U83093	Gainesville, FL, USA	*Gryllus ovisopis*	NA	*Wolbachia* sp.
U83094	Austin, TX, USA	*Gryllus integer*	NA	*Wolbachia* sp.
U83095	Davis, CA, USA	*Gryllus integer*	NA	*Wolbachia* sp.
U83096	Humboldt Co., NV, USA	*Gryllus integer*	NA	*Wolbachia* sp.
U83097	Wayne Co., UT, USA	*Gryllus integer*	NA	*Wolbachia* sp.
U83098	Urbana, IL, USA	*Diabrotica virgifera virgifera*	NA	*Wolbachia* sp.
Z49261	NA	*Dirofilaria immitis*	NA	*Wolbachia* sp.
AF069068	NA	*Litomosoides sigmodontis*	NA	*Wolbachia*-like endobacterium

NA, Not available or none assigned.

sequence similarity in their 16S rRNA genes and have identical GroEL amino acid sequences (van Vliet *et al*., 1992; Sumner *et al*., 1997; Zhang *et al*., 1997; Roux & Raoult, 1999; Drancourt & Raoult, 1994; Anderson *et al*., 1991; Chen *et al*., 1994a; Wen *et al*., 1995a, b; Dawson *et al*., 1996a). Each of *E. phagocytophila*, *E. equi* and the HGE agent is also closely related on the basis of antigenic analyses by indirect fluorescent antibody tests (Dumler *et al*., 1995). Protein immunoblots and cloned recombinant proteins indicate the presence of several outer-membrane protein antigens in each of these species, including an immunodominant antigen of variable molecular size (mean 44 kDa) (Dumler *et al*., 1995; Asanovich *et al*., 1997; Zhi *et al*.,

Table 2. *groESL* operon sequences used in the phylogenetic analyses and associated information

Accession no.	Location	Source	Designation	Prior taxonomic classification
AF165812	North America	Bovine	NA	*Anaplasma marginale*
M98257	South America	Human	ATCC 35685	*Bartonella bacilliformis*
AF008210	NA	*Schizaphis graminum*	NA	*Buchnera aphidicola*
AE001285	NA	Human	D/UW-3/CX	*Chlamydia trachomatis*
U13638	South Africa	Bovine	Welgevonden^T	*Cowdria ruminantium*
U96731	Florida, USA	Dog	Florida	*Ehrlichia canis*
L10917	Arkansas, USA	Human	Arkansas^T	*Ehrlichia chaffeensis*
AF172158	California, USA	Horse	CASOLJ	*Ehrlichia equi*
AF172160	California, USA	Horse	CAMAWI	*Ehrlichia equi*
AF172162	California, USA	Horse	CASITL	*Ehrlichia equi*
U96727	California, USA	Horse	California horse	*Ehrlichia equi*
U96729	Scotland, UK	Goat	Feral goat	*Ehrlichia phagocytophila*
U96730	Scotland, UK	Sheep	Old Sourhope	*Ehrlichia phagocytophila*
U96735	Switzerland	Horse	Swiss horse	*Ehrlichia phagocytophila*
U24396	NA	Horse	90-12	*Ehrlichia risticii*
U96732	Maryland, USA	Horse	Illinois^T	*Ehrlichia risticii*
U88092	Japan	Human	Japan	*Ehrlichia sennetsu*
AF033101	Slovenia	Human	NA	*Ehrlichia* sp. HGE agent
AF172159	California, USA	Human	CAHU-HGE2	*Ehrlichia* sp. HGE agent
AF172163	California, USA	Human	CAHU-HGE1	*Ehrlichia* sp. HGE agent
U96728	New York, USA	Human	HGE agent	*Ehrlichia* sp. HGE agent
X07850	NA	NA	NA	*Escherichia coli*
U64996	NA	Human	MS11-A	*Neisseria gonorrhoeae*
M31887	NA	Human	Karp	*Orientia tsutsugamushi*
AJ235272	NA	Human	Madrid E	*Rickettsia prowazekii*
U96733	Montana, USA	NA	R	*Rickettsia rickettsii*
AF075440	North Carolina, USA	Human	Wilmington^T	*Rickettsia typhi*
AB002286	NA	*Teleogryllus taiwanemma*	Group B	*Wolbachia* sp.

NA, Not available.

1997). The gene encoding this 44 kDa immunodominant protein is one of a multigene family comprising multiple distinct genes (Murphy *et al*., 1998; Zhi *et al*., 1998; IJdo *et al*., 1998) that also encode proteins with significant amino acid similarity to (i) the 36 kDa antigen called major surface protein 2 (MSP2) and the precursor of the 31 kDa antigen of *A. marginale* called MSP4 (Murphy *et al*., 1998; Zhi *et al*., 1998; IJdo *et al*., 1998), (ii) the *C. ruminantium* 28 kDa major antigenic protein 1 (MAP1) (Jongejan & Thielemans, 1989; Jongejan *et al*., 1993; Ohashi *et al*., 1998b; Yu *et al*., 1999a), (iii) the *E. chaffeensis* and *E. canis* P28 and P30 protein families (Yu *et al*., 1999a; Ohashi *et al*., 1998a, b; Reddy *et al*., 1998) and (iv) *Wolbachia* spp. outer-surface protein precursors (Yu *et al*., 1999a; Ohashi *et al*., 1998b). This complex of outer-membrane proteins is encoded in the HGE agent, *A. marginale*, *E. chaffeensis*, *E. canis*, *E. muris*, *C. ruminantium* and potentially in other *Ehrlichia* species by polymorphic multigene families that are suspected to contribute to immune evasion or persistence in reservoir hosts (Reddy *et al*., 1998; Alleman *et al*., 1997; French *et al*., 1998; Reddy & Streck, 1999).

A gene encoding a protein antigen of approximately 150–160 kDa that has repeated ankyrin motifs on the amino terminus, *ankA*, has been cloned from the HGE agent (Storey *et al*., 1998; Caturegli *et al*., 2000). The function of this protein is unknown and it is a unique but relatively minor antigen among the HGE agent, *E. equi* and *E. phagocytophila*. Comparison of the nucleotide sequence of a 444 bp region of the ankyrin repeat region from five Wisconsin strains and one New York strain designated as HGE agent by 16S rRNA gene sequence revealed 100% similarity, whereas the sequence of the MRK strain of *E. equi* is 99·6% similar to that of the HGE agent. Similarly, the sequence of *ankA* of the HGE agent is between 95·5 and 96·8% similar to those of Swedish and Spanish strains of *E. phagocytophila* from cattle and goats, respectively (Caturegli *et al*., 2000). These data are confirmed by full gene sequences of a larger number of *E. phagocytophila*-group organisms from various geographical regions (Massung *et al*., 2000).

Biologically, *A. marginale*, *E. phagocytophila*, *E. equi*, *E. platys*, *E. bovis* and the HGE agent are most often detected in cells in the peripheral blood that are derived

from bone marrow precursors. *E. phagocytophila*, *E. equi* and the HGE agent are capable of growth *in vitro* in undifferentiated HL-60 promyelocytic cells, HL-60 cells differentiated into neutrophil-like cells and potentially in precursors of the myelomonocytic lineage, as well as in embryonic *Ixodes scapularis* tick cell lines (Goodman *et al*., 1996; Heimer *et al*., 1997; Klein *et al*., 1997; Feng, 1997; Munderloh *et al*., 1996a). The HGE agent and *E. equi* do not propagate in HL-60 cells differentiated into mature macrophages. This situation resembles that *in vivo*, since each of these species is detected most often in neutrophils or band neutrophils in the blood of infected animals and humans. *A. marginale* infects predominantly erythrocytes *in vivo* and a suitable equivalent mammalian cell line for propagation has not been identified. *A. marginale* can be grown in embryonic tick cells *in vitro* and short-term propagation in erythrocyte culture and endothelial/erythrocyte co-cultures has also been achieved (Munderloh *et al*., 1996b; Kessler *et al*., 1979; Waghela *et al*., 1997). *E. platys* infects canine platelets *in vivo* and *E. bovis* infects bovine monocytes; neither has been cultivated *in vitro*. Although the host cell ligand is not known for *E. platys* or *E. bovis*, the HGE agent, a member of the *E. phagocytophila* group, adheres to platelet glycoprotein selectin ligand-1 (PGSL-1; Herron *et al*., 2000), a sialic acid-bearing surface protein molecule that shares many chemical characteristics, such as sensitivity to neuraminidase and chymotrypsin, with the erythrocyte ligand of *A. marginale* (McGarey & Allred, 1994).

Ticks transmit all of these species, but transovarial transmission in ticks does not occur for those investigated. *E. phagocytophila*, *E. equi* and the HGE agent are each transmitted by members of the *Ixodes persulcatus* complex, whereas *A. marginale* is transmitted by *Dermacentor* spp. ticks in temperate regions of North America and by *Boophilus* spp. or other genera in other geographical regions (Kuttler, 1984; Eriks *et al*., 1993; Kocan *et al*., 1992; Telford *et al*., 1996; Richter *et al*., 1996; Walls *et al*., 1997; *Gordon et al*., 1932; MacLeod & Gordon, 1933). Except for *E. platys* and *E. bovis*, the life cycles of these agents are partially known. *A. marginale* and the closely related *Anaplasma centrale* and *Anaplasma ovis* are usually maintained by persistent subclinical infection of ruminants, including wild ruminants such as deer (Kuttler, 1984; Eriks *et al*., 1993). A role exists for transmission by male ticks among multiple animals in a single herd and mechanical transmission via biting flies provides a potential alternative transmission vehicle (Kocan *et al*., 1992). The HGE agent is maintained, at least in part, by infection of small mammal species such as the white-footed mouse, *Peromyscus leucopus*, or the dusky-footed wood rat, *Neotoma fuscipes*, in which occasional persistent infections may be detected (Telford *et al*., 1996; Walls *et al*., 1997; Nicholson *et al*., 1999). *E. phagocytophila* may establish persistent infections in ruminants under natural and experimental circumstances (Gordon *et al*., 1932; MacLeod & Gordon, 1933; Hudson, 1950; Foggie, 1951; Foster & Cameron, 1970; McDiarmid, 1965) and mounting evidence suggests that both *E. equi* and the HGE agent establish subclinical persistent infections in domestic and wild ruminants, including deer (Foley *et al*., 1998; Belongia *et al*., 1997; Walls *et al*., 1998; Magnarelli *et al*., 1999). The HGE agent produces disease typical of *E. equi* infection in horses and induces protective immunity to challenge with *E. equi* (Madigan *et al*., 1995; Barlough *et al*., 1995). Likewise, *E. equi*-like bacteria have caused infection in humans that is indistinguishable from HGE (Foley *et al*., 1999). Clinical manifestations, even in typical mammalian hosts, are highly variable for each of *E. phagocytophila*, *E. equi* and the HGE agent; clinical features therefore provide a lower degree of certainty about classification, since these are likely to be at least in part host-dependent (Gordon *et al*., 1932; MacLeod & Gordon, 1933; Hudson, 1950; Foggie, 1951; Madigan, 1993; Reubel *et al*., 1998b; Bakken *et al*., 1994, 1996, 1998; Aguero-Rosenfeld *et al*., 1996).

These common features are expected of organisms with a high degree of relatedness and indicate that these bacteria should be unified within a single genus. Moreover, the data indicate that sufficient similarity exists among *E. phagocytophila*, *E. equi* and the HGE agent for them to be classified as a single species. *A. marginale* is sufficiently divergent to be considered a separate species, but the 16S rRNA gene sequences of strains of *A. marginale*, *A. ovis* and *A. centrale*, excepting a Japanese strain, are nearly identical (minimum 99·1% similarity), suggesting the possibility that these also represent variants of a single species, as denoted initially by Theiler (1911). The existence of a strain of *A. centrale* that has 1·8% nucleotide difference from other phenotypically characterized strains of *A. centrale* indicates the polygenic nature of this designation and casts some doubt upon the classical morphological taxonomic methods for this species and genus. Overall, a close grouping of erythrocytic anaplasmas is supported by other genetic, phenotypic and antigenic characteristics that also indicate a close grouping with *A. marginale* (McGuire *et al*., 1984; Palmer *et al*., 1988, 1998; Visser *et al*., 1992). In fact, all species of *Anaplasma* are known to share antigens that reside on 19, 36 and 105 kDa proteins, data that strengthen the close relationship based upon host cell type and morphological characteristics (Palmer *et al*., 1988; Visser *et al*., 1992).

A large genetic distance (minimum 74·3% similarity) in *groESL* sequences was noted between the *E. phagocytophila* group members and *A. marginale*, which is in part explained by the paucity of *groESL* sequences examined. All members of the *E. phagocytophila* group were at least 98·8% similar and no other sequence representatives (*E. platys*, *E. bovis* etc.) of the *Anaplasma*/*E. phagocytophila* group were available. Similarly large genetic distances (minimum 86·31% similarity) were observed for *groESL* sequences between *E. canis* and *C. ruminantium*, which

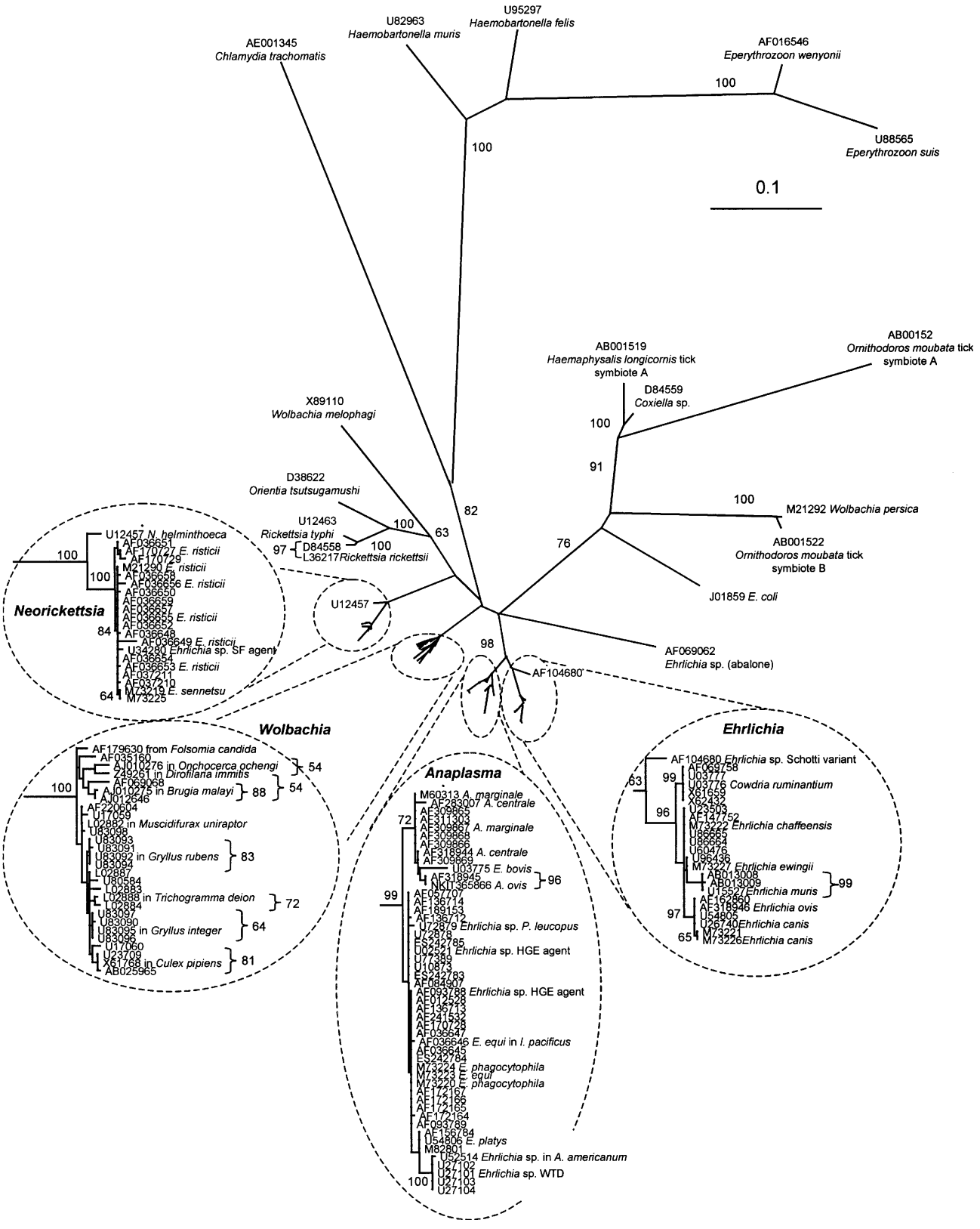

Fig. 1. For legend see facing page.

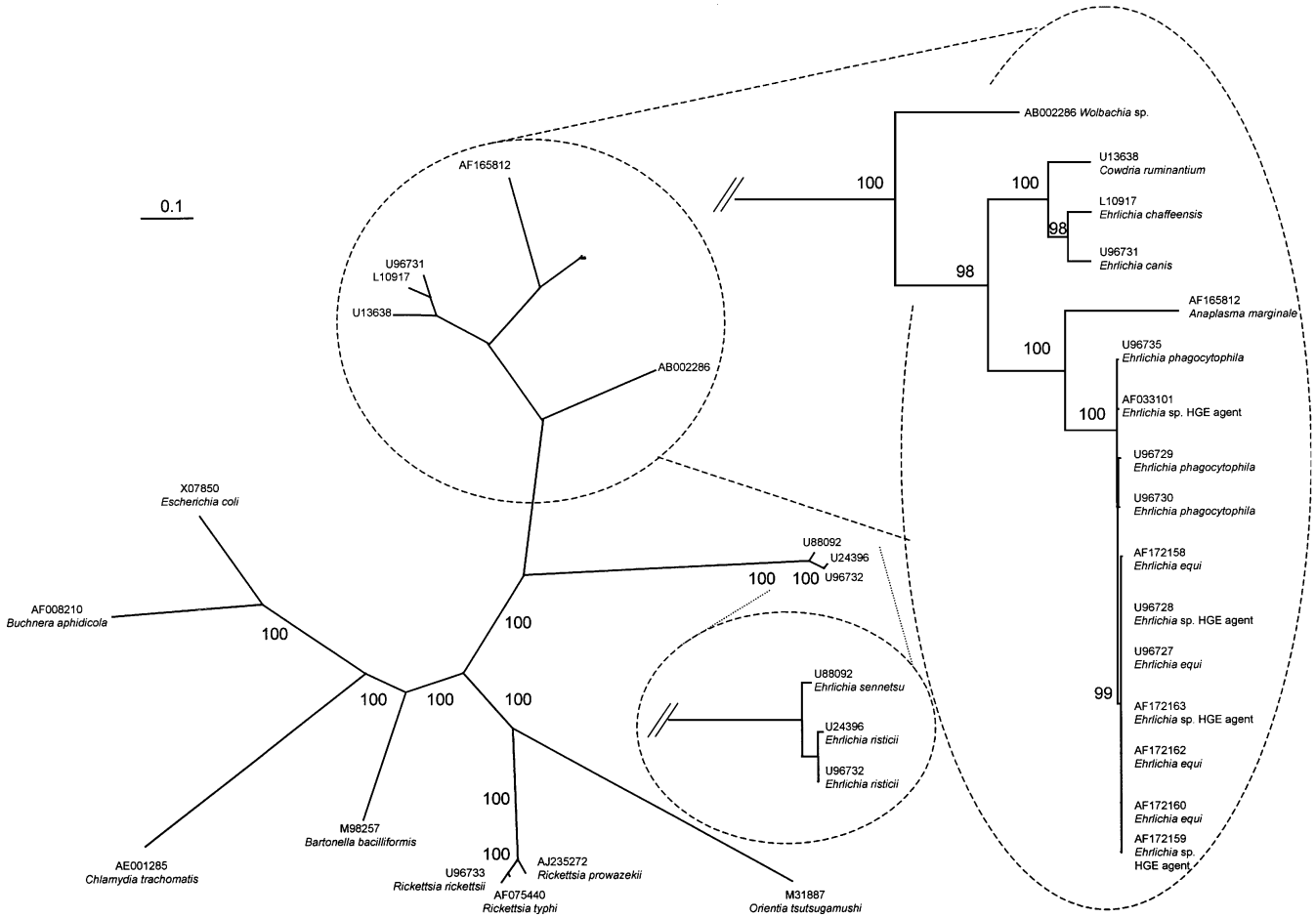

Fig. 2. Phylogenetic tree inferred from *groESL* gene sequences of *Ehrlichia*, *Anaplasma*, *Neorickettsia* and *Wolbachia* species, including 1077 sites after removal of sites containing a gap in any sequence. The sequence from *Chlamydia trachomatis* (accession no. AE001285) was used as an outgroup. Numbers above internal nodes indicate the percentage of 1000 bootstrap replicates that supported the branch. All bootstrap values are included for clades that were consistently observed using the phylogenetic methods applied (maximum parsimony, minimum evolution, maximum likelihood and majority-rule bootstrap analysis of neighbour-joining trees). The maximum-likelihood tree is shown. Bar, estimated number of substitutions per site; scale for the figure and insets are the same.

also appear to be clearly related on the basis of 16S rRNA gene sequences and phenotypic findings. Overall, the *groESL* sequences support the divisions as indicated by 16S rRNA gene sequences and provide evidence of polymorphisms that may be random or may represent subtleties of evolutionary selection. Thus, despite these ambiguous differences, insufficient genetic distance and biological differences exist among the *Anaplasma* species, the *E. phagocytophila* group, *E. bovis* and the *E. platys* clade to designate them into separate genera. This is supported further by the lack of bootstrap support for the clear separation of the two major arms of this clade and by the inconsistent presence of *E. bovis* in either the *Anaplasma* or *E. phagocytophila* clades in the various phylogenetic analyses. Additional sequence analyses of conserved and semi-conserved genes (e.g. *gltA*), whole genome analysis, as well as analysis of additional strains may further identify taxonomic divisions or support the current analyses of 16S rRNA and *groESL* genes.

Little is known about the antigenic characteristics of

Fig. 1. Phylogenetic tree inferred from small subunit (16S) rRNA gene sequences of *Ehrlichia*, *Anaplasma*, *Neorickettsia* and *Wolbachia* species, including 455 sites after removal of sites containing a gap in any sequence. The sequence from *Chlamydia trachomatis* (accession no. AE001345) was used as an outgroup. Numbers above internal nodes indicate the percentage of 1000 bootstrap replicates that supported the branch. All bootstrap values are included for clades that were consistently observed using the phylogenetic methods applied (maximum parsimony, minimum evolution, maximum likelihood and majority-rule bootstrap analysis of neighbour-joining trees). The maximum-likelihood tree is shown. Bars, estimated number of substitutions per site; the scale for the figure and insets are the same.

either *E. platys* or *E. bovis*; their taxonomic positions must therefore be assigned on the basis of what is known about their genetic characteristics (Anderson *et al*., 1992). For some previously described agents, such as '*Cytoecetes microti*' (Tyzzer, 1938), no isolates or genetic information are available for analysis and their relationships to other named species cannot be assessed objectively. Of interest is the identification of several 16S rRNA gene sequences from the blood of white-tailed deer (*Odocoileus virginianus*) from Oklahoma and Georgia in the USA (Dawson *et al*., 1996c), from an *Amblyomma americanum* tick in Missouri (USA) and from the blood of sheep in South Africa (Allsopp *et al*., 1997), each of which is most similar to *E. platys*. A definitive bacterial morphology has never been identified for any of these sequences; their taxonomic positions can therefore only be judged on the basis of the 16S rRNA gene sequences.

The *E. canis*/*Cowdria* group

The second genetic cluster includes *E. canis*, *E. chaffeensis*, *E. ewingii*, *E. muris* and *C. ruminantium*, all of which are at least 97·7% similar in 16S rRNA gene sequences (van Vliet *et al*., 1992; Dame *et al*., 1992; Rikihisa *et al*., 1997; Zhang *et al*., 1997; Roux & Raoult, 1995, 1999; Drancourt & Raoult, 1994; Anderson *et al*., 1991; Wen *et al*., 1995a, b; Shibata *et al*., 2000). *E. canis*, *E. chaffeensis* and *E. muris* are detected mostly in macrophages and monocytes *in vivo* and can be propagated *in vitro*, most effectively in macrophage cell lines (Dawson *et al*., 1991a, b; Barnewell & Rikihisa, 1994; Heimer *et al*., 1998). *C. ruminantium* is most often found in endothelial cells, neutrophils or macrophages *in vivo* and can also be propagated in cell lines derived from both endothelial cells and macrophages (Cowdry, 1926; Logan *et al*., 1987; Bezuidenhout *et al*., 1985; Sahu, 1986; Prozesky & Du Plessis, 1987). *E. ewingii* is the exception in that it is detected most frequently in peripheral blood neutrophils and it has not been grown in long-term culture (Ewing *et al*., 1971). *E. canis* is best recognized as a pathogen of canids (Huxsoll, 1976; Woody & Hoskins, 1991), but can infect humans and may infect felines (Perez *et al*., 1996; Bouloy *et al*., 1994), whereas *E. chaffeensis* causes symptomatic infection in humans and subclinical persistent infections in deer and canids (Fishbein *et al*., 1994; Ewing *et al*., 1995, Lockhart *et al*., 1997; Dawson *et al*., 1996b; Dawson & Ewing, 1992). *E. ewingii* causes low-grade infections of canids that are sometimes characterized by lameness due to polyarthritis (Ewing *et al*., 1971) and has recently been implicated as a human pathogen (Buller *et al*., 1999). *C. ruminantium* is best known as the cause of heartwater in African and Caribbean ruminants (Cowdry, 1926; Uilenberg, 1983; Camus *et al*., 1993). Each of these species is known to be transmitted and maintained in a tick vector reservoir, including *Amblyomma* spp. for *C. ruminantium* (Bezuidenhout, 1987), *Amblyomma americanum* for *E. chaffeensis* and *E. ewingii* (Ewing *et al*., 1995; Anziani *et al*., 1990) and *Rhipicephalus sanguineus* for *E. canis* (Groves *et al*., 1975). Transovarial transmission is ineffective for *E. canis* and *C. ruminantium*, the only species studied sufficiently (Bezuidenhout, 1987; Groves *et al*., 1975).

Polyclonal antibodies to these organisms have a high degree of cross-reactivity by immunofluorescence, a result consistent with a close genetic relationship. Low-level antigenic cross-reactivity is also recognized between *C. ruminantium* and *E. phagocytophila* and between *E. phagocytophila*, *E. equi*, the HGE agent and *E. chaffeensis*, *E. canis* or *E. ewingii* (Dumler *et al*., 1995; Dawson *et al*., 1991a; Jongejan *et al*., 1989; Buller *et al*., 1999; Brouqui *et al*., 1992; Rikihisa *et al*., 1992). The antigens of these organisms have been studied in some detail by Western blotting, which reveals the presence of cross-reactive immunodominant antigens of similar molecular size but with a degree of diversity when detected with monoclonal antibodies (Dumler *et al*., 1995; Asanovich *et al*., 1997; Zhi *et al*., 1997; Visser *et al*., 1992; Palmer *et al*., 1985, 1998; Brouqui *et al*., 1992, 1994; Rikihisa *et al*., 1992, 1994; Yu *et al*., 1993; Chen *et al*., 1994b, 1996; Kim & Rikihisa, 1998; Ravyn *et al*., 1999; Adams *et al*., 1986; Vidotto *et al*., 1994; Alleman & Barbet, 1996; Barbet *et al*., 1994; Rossouw *et al*., 1990; Mahan *et al*., 1993, 1994; Bowie *et al*., 1999; Kelly *et al*., 1994). A group of antigens that range between 27 and 32 kDa is common among these organisms and is shared between these different species when analysed by immunoblotting methods (Rikihisa, 1991a; Rikihisa *et al*., 1992, 1994; Iqbal *et al*., 1994; Wen *et al*., 1995a; Ohashi *et al*., 1998a, b; Jongejan *et al*., 1993). Monoclonal antibodies reactive with proteins in this molecular size range that are raised against one isolate do not always react with other isolates (Chen *et al*., 1996, 1997). These proteins are encoded by polymorphic genes and are called MAP1 in *C. ruminantium*, MAP1 homologue, p28 and p30 in *E. canis* and Omp1 or p28 in *E. chaffeensis*, but have yet to be described in *E. ewingii*; a homologous gene has been identified in other *Ehrlichia* species (Ohashi *et al*., 1998a, b; Reddy *et al*., 1998; Yu *et al*., 1999b; McBride *et al*., 1999; van Vliet *et al*., 1994). In fact, a high degree of amino acid similarity exists between these proteins and the MSP4 of *A. marginale*, further clarifying the basis for prior evidence of serological cross-reactions obtained by immunofluorescence studies (Ohashi *et al*., 1998a, b; Yu *et al*., 1999b; McBride *et al*., 1999; van Vliet *et al*., 1994).

The data on the tick-transmitted ehrlichiae in the *Anaplasma*/*E. phagocytophila* and *E. canis*/*Cowdria* groups argue convincingly for the unification of these species within either one or two separate genera. However, the large degree of internal genetic similarity (Fig. 1), the extent of shared amino acid sequences in major outer-membrane proteins, the similarity in host cells and similarity in serological cross-reactions argue for consolidation of the species of the *E. phagocytophila* complex in a genus that contains only *A. marginale*, *E. platys* and *E. bovis*. Moreover, the

repeated genetic clustering of members of the *E. canis*/*Cowdria* group to the exclusion of members of the *E. phagocytophila*/*Anaplasma* group suggests that the establishment of two separate genera for these groups is the best way to emphasize the degree of biological difference between these clades. However, should a large number of apparently ancestral types to both these groups be found, like the 'Schotti variant' (Fig. 1), future consolidation of these two closely related groups may be warranted.

The *E. sennetsu*/*Neorickettsia* group

The third and most divergent genetic cluster of the ehrlichiae includes *E. sennetsu*, *E. risticii* (van Vliet *et al*., 1992; Dame *et al*., 1992; Rikihisa *et al*., 1997; Zhang *et al*., 1997; Roux & Raoult, 1995, 1999; Drancourt & Raoult, 1994; Anderson *et al*., 1991; Chen *et al*., 1994a; Wen *et al*., 1995a, b), *N. helminthoeca* and an ehrlichia-like bacterium present in the metacercarial stage of the fluke *Stellantchasmus falcatus* (SF), all of which exhibit between 94·9 and 100·0 % similarity in 16S rRNA gene sequences (Wen *et al*., 1996; Barlough *et al*., 1998; Pretzman *et al*., 1995; Chaichanasiriwithaya *et al*., 1994). However, individual isolates of *E. risticii* may diverge in 16S rRNA gene sequence by as many as 15 nucleotides (Wen *et al*., 1995b; Barlough *et al*., 1998). These data underscore the phylogenetic heterogeneity of this clade. In spite of these observations, fluorescent antibody and protein immunoblot studies show a high degree of antigenic similarity among *E. sennetsu*, *E. risticii*, *N. helminthoeca* and the SF agent, but not to other species of *Ehrlichia* (Rikihisa, 1991b; Rikihisa *et al*., 1988; Dumler *et al*., 1995; Wen *et al*., 1996; Holland *et al*., 1985a, b; Ristic *et al*., 1986; Shankarappa *et al*., 1992). Each of these species infects predominantly mononuclear phagocytes *in vivo* and can be propagated *in vitro* most efficiently in cell lines derived from macrophages (Zhang *et al*., 1997; Wen *et al*., 1996; Shankarappa *et al*., 1992; Rikihisa *et al*., 1991, 1995). Ticks have never been implicated in transmission of these agents, whereas transmission via infected metacercariae or cercariae of flukes that infest either snails, fish or aquatic insects has been shown for *N. helminthoeca* and *E. risticii* and is strongly suspected for *E. sennetsu* (Rikihisa, 1991a; Barlough *et al*., 1998; Madigan *et al*., 2000). While no naturally existing mammalian infection with the SF agent has been recognized, its presence in flukes and pathogenicity in mice is consistent with the above observations in other *E. sennetsu*-group organisms (Rikihisa, 1991a; Wen *et al*., 1996; Fukuda & Yamamoto, 1981).

E. sennetsu is best known as the agent of sennetsu fever, a mononucleosis-like illness described only in Japan and Malaysia (Misao & Kobayashi, 1955; Rapmund, 1984). Early epidemiological studies suggested that individuals who consumed uncooked fish from certain areas of Japan were at risk (Rikihisa, 1991a; Tachibana *et al*., 1976). Although not proven, this epidemiology has long suggested the possibility of enteral ingestion of fish contaminated with ehrlichia-infected flukes as the mechanism for transmission. *E. sennetsu* causes a fatal infection in mice and produces no clinical signs in horses, but protects horses against challenge by *E. risticii* (Tachibana & Kobayashi, 1975; Rikihisa *et al*., 1988). *E. risticii* causes Potomac horse fever, also known as equine monocytic ehrlichiosis or 'Shasta River crud' (Holland *et al*., 1985a; Rikihisa & Perry, 1985; Madigan *et al*., 1997). Presumably, the agent is either ingested when horses feed upon snail-ridden grasses or by ingestion of infected metacercaria-containing aquatic insects (Reubel *et al*., 1998a; Barlough *et al*., 1998; Madigan *et al*., 2000). The presentation is that of a febrile illness with profuse watery diarrhoea. *N. helminthoeca* is acquired by ingestion of fluke-infested fish by dogs and causes a febrile infection called salmon poisoning disease (Rikihisa, 1991a).

The degree of 16S rRNA gene sequence similarity of the *E. sennetsu* group to those in the *E. phagocytophila* and *E. canis* groups is not more than exists between the *E. sennetsu* group and *Rickettsia* species (Wen *et al*., 1995a, b). Although minor serological cross-reactivity has been described in some studies (Holland *et al*., 1985a; Ristic *et al*., 1981), no firm similarities in outer-membrane protein amino acid sequences have been established and there appear to be no haematophagous arthropod vectors such as ticks involved in the life cycle. However, the common infected host cells are similar to those of other *Ehrlichia* species, although the clinical manifestations of enteric involvement are more pronounced. The significant genetic, antigenic and ecological traits of the species of the *E. sennetsu* group suggest that it is a distinct clade deserving of designation as a separate genus.

Wolbachia species

The sole remaining named species of the genus *Wolbachia* is *W. pipientis*, an obligate intracellular bacterium that resides within cytoplasmic vacuoles, predominantly in the ovaries of many species of arthropods and increasingly identified in helminths (Werren, 1997; Popov *et al*., 1998; O'Neill *et al*., 1992; Dobson *et al*., 1992; Bandi *et al*., 1998). Analysis of *ftsZ* gene amplicons of arthropod and filarial wolbachiae indicates the existence of at least two distinct host-associated clades (Bandi *et al*., 1998; Vandekerckhove *et al*., 1999). However, by 16S rRNA gene sequence analysis, *W. pipientis* and the *Wolbachia* spp. occupy a position intermediate between the two tick-transmitted groups (*E. canis*/*C. ruminantium* and *E. phagocytophila*/*Anaplasma*) and the helminth-borne *E. sennetsu*/*Neorickettsia* group (Roux & Raoult, 1995; Wen *et al*., 1995b; O'Neill *et al*., 1992). Deduced amino acid sequences of *Wolbachia* spp. outer-membrane protein genes exhibit similarity to those of the major outer-membrane proteins of *A. marginale*, the *E. phagocytophila* complex, *E. chaffeensis*, *E. canis* and

C. ruminantium, thus corroborating the phylogenetic position of *W. pipientis* (Yu *et al*., 1999a; Ohashi *et al*., 1998b; Zhou *et al*., 1998). However, *W. pipientis* is not recognized as a vertebrate pathogen, since mammalian infection has never been documented.

Although there are significant morphological, genetic and amino acid sequence similarities between *W. pipientis* and the other *Ehrlichia*/*Cowdria*/*Anaplasma* groups, the significant degree of differences in 16S rRNA and *groESL* gene sequences, the lack of a significant vertebrate host phase, the promiscuous invertebrate host associations and its highly efficient transovarial transmission adequately differentiate *W. pipientis* and related organisms from species found in the genera *Ehrlichia*, *Cowdria*, *Anaplasma* and *Neorickettsia*.

Historical precedents

The historical precedent for naming species in the entire group of ehrlichiae is *A. marginale*, which was first described and named by Theiler (1910). The organism currently denoted *C. ruminantium* was described initially by Cowdry (1925) and given the genus designation *Cowdria* by Moshkovski (1947). Gordon first clearly differentiated tick-borne fever from louping ill in goats in 1932 and suggested that the disease was caused by a rickettsia, an assertion that was affirmed in 1940 by Foggie (Gordon *et al*., 1932; Foggie, 1951). The genus designation *Ehrlichia* was first coined in 1945 to honour Paul Ehrlich (Moshkovski, 1945; Silverstein, 1998), 2 years before the designation of *C. ruminantium*; however, the type species, *E. canis*, was first described as *Rickettsia canis* by Donatien & Lestoquard (1935) and, in 1936, the same authors described *E. bovis* as *Rickettsia bovis* (Donatien & Lestoquard, 1936). Hertig first described rickettsia-like organisms in insects in 1936 and these were placed in the genus *Wolbachia* in honour of S. Burt Wolbach, who demonstrated the presence of rickettsiae in pathological lesions in the vasculotropic rickettsioses (Hertig, 1936). The designation '*Cytoecetes microti*' was created to describe a microorganism with morphological features similar to the organism now called *E. phagocytophila* (Tyzzer, 1938); however, original materials and isolates no longer exist for verification of its identity (Ristic & Huxsoll, 1984). Subsequently, other designations were made into the genus *Ehrlichia* (in North America) or '*Cytoecetes*' (in Europe and Asia) for organisms that were recognized to be pathogenic for mammals (Ristic & Huxsoll, 1984; Moshkovski, 1945). *N. helminthoeca* was described in 1953, *E. sennetsu* in 1954, *E. equi* in 1969, *E. platys* in 1982, *E. risticii* in 1984, *E. chaffeensis* in 1991, *E. ewingii* in 1993 and *E. muris* in 1995 (Wen *et al*., 1995a; Anderson *et al*., 1992; Misao & Kobayashi, 1955; Philip *et al*., 1953; Stannard *et al*., 1969; Gribble, 1969).

Convincing phylogenetic data now show that a series of significant flaws exists in the taxonomic structure of the families *Anaplasmataceae* and *Rickettsiaceae* in the order *Rickettsiales*. Similar phylogenetic studies led to a significant taxonomic modification of the former genera *Rochalimaea* and *Grahamella* (Brenner *et al*., 1993; Birtles *et al*., 1995). It is now clear that a distinction between some members of the families *Rickettsiaceae* and *Anaplasmataceae* is not supported. Moreover, some members of the family *Anaplasmataceae*, the genera *Eperythrozoon* and *Haemobartonella*, are clearly not related to the genus *Anaplasma* and should be removed and reassigned within the family *Mycoplasmataceae* (Rikihisa *et al*., 1997). While no classification system fits all criteria perfectly, genetic data have become the objective standards and, when evaluated carefully, often closely predict similar biological and clinical behaviours. Thus, the data compiled here indicate that a sufficient genotypic and phenotypic relationship exists among the genera *Anaplasma*, *Cowdria*, *Wolbachia* and *Ehrlichia*, excluding *N. helminthoeca*, *E. sennetsu* and *E. risticii*, to merit unification into two separate genera. Since the validly published names *Anaplasma* and *A. marginale* and *Ehrlichia* and *E. canis* predate *Cowdria* and *Wolbachia*, *Anaplasma* should be retained for the unified genus that encompasses the existing *Anaplasma* species, the *E. phagocytophila* group, *E. bovis* and *E. platys*, while the genus *Ehrlichia* should be retained and used to describe members of the *Ehrlichia canis* group, including *C. ruminantium*. This change further necessitates accommodation of the members of the *E. sennetsu* group within a single genus, *Neorickettsia*. Thus, a revised classification may be formulated that differentiates organisms in the order *Rickettsiale*s into two families, *Rickettsiaceae*, which contains the rickettsiae (*Rickettsia*, *Orientia*) that occupy an intracytoplasmic compartment, and *Anaplasmataceae*, which contains the ehrlichiae (*Neorickettsia*, *Wolbachia*, *Ehrlichia*, *Anaplasma*) that occupy an intravacuolar compartment within infected host cells. Consequently, new combinations for the multiple genera and species that are involved must also be created.

Emended description of *Rickettsiales* (Gieszczykiewicz 1939) Weiss and Moulder 1984

It is proposed that the tribes *Rickettsieae*, *Ehrlichieae* and *Wolbachieae* should be abolished. Furthermore, all species formerly within the tribes *Ehrlichieae* and *Wolbachieae* are transferred into the family *Anaplasmataceae*.

Emended description of *Rickettsiaceae* (Pinkerton 1936) Weiss and Moulder 1984

It is proposed that the genera *Ehrlichia*, *Cowdria*, *Neorickettsia* and *Wolbachia* be transferred from the family *Rickettsiaceae* to the family *Anaplasmataceae*, a change that results in the elimination of all tribes within the family *Rickettsiaceae*. It is also proposed that the genera *Haemobartonella* and *Eperythrozoon*

should be transferred from the family *Anaplasmataceae* to the order *Mycoplasmatales* and that *Coxiella*, *Rickettsiella*, *Francisella* (*Wolbachia*) *persica* and *Wolbachia melophagi* (Weisburg *et al*., 1989; Roux *et al*., 1997) should be removed from the family *Rickettsiaceae*. This proposal also requires emendation of the description of the family *Rickettsiaceae* to specify that organisms infect host cells within the cytoplasm or nucleus and are not bounded by a vacuole. The family *Rickettsiaceae* includes only the genera *Rickettsia* and *Orientia*.

Emended description of *Anaplasmataceae* (Philip 1957) Ristic and Kreier 1984

It is proposed that the family *Anaplasmataceae* be emended to include species in the genera *Wolbachia*, *Ehrlichia*, *Cowdria* and *Neorickettsia* and to retain species in the genera *Anaplasma* and *Aegyptianella*. This requires emendation of the description of the *Anaplasmataceae* to specify infection within a cytoplasmic vacuole of host cells that include erythrocytes, reticuloendothelial cells, bone marrow-derived phagocytic cells, endothelial cells and cells of insect, helminth and arthropod reproductive tissues. *Aegyptianella* is retained as *genus incertae sedis*.

Emended description of *Anaplasma* (Theiler 1910) Ristic and Kreier 1984

It is proposed that members of the *E. phagocytophila* group, including *E. phagocytophila*, *E. equi*, the HGE agent, as well as *E. bovis* and *E. platys*, should be united with the genus *Anaplasma*. This change requires emendation of the description of the genus *Anaplasma* (Ristic & Kreier, 1984) by integrating it with some descriptions of the genera *Ehrlichia* and new data for *Anaplasma* and *Ehrlichia*, as follows.

Gram-negative, small, often pleomorphic, coccoid to ellipsoidal organisms that reside within cytoplasmic vacuoles, either singly and more often in compact inclusions (morulae) present in mature or immature haematopoietic cells, particularly myeloid cells and neutrophils and including erythrocytes, in peripheral blood or in tissues, usually mononuclear phagocyte organs (spleen, liver, bone marrow) of mammalian hosts. By ultrastructure, two morphological forms are observed, including larger reticulate cells and smaller forms with condensed protoplasm called dense-core forms (Popov *et al*., 1998). Vectors, where known, are ticks. Organisms grow in tick vectors. Non-motile. Not cultivable in cell-free media or chicken embryos. Some species are cultivable in neutrophils, myelomonocytic cell lines, promyelocytic cell lines, erythrocytes and tick cell lines. Aetiological agents of diseases of dogs and other canids, humans and ruminants such as cattle, goats, sheep and llamas. Variably pathogenic or non-pathogenic infections in some ruminants such as cattle, goats, sheep and deer, horses and rodents. The estimated G+C content of the DNA varies between approximately 30 and 56 mol%. The type species is *Anaplasma marginale* (Theiler, 1910).

Emended description of *Ehrlichia* (Moshkovski 1945) Ristic and Huxsoll 1984

Gram-negative, small, often pleomorphic, coccoid to ellipsoidal organisms that reside within cytoplasmic vacuoles, either singly and more often in compact inclusions (morulae) present in mature or immature haematopoietic cells, especially mononuclear phagocytes such as monocytes and macrophages and for some species in myeloid cells such as neutrophils, in peripheral blood or in tissues, usually mononuclear phagocyte organs (spleen, liver, bone marrow, lymph node) of mammalian hosts. By ultrastructure, two morphological forms are observed, including larger reticulate cells and smaller forms with condensed protoplasm (dense-core forms) (Popov *et al*., 1998). Vectors, where known, are ticks. Organisms grow in tick vectors. Non-motile. Not cultivable in cell-free media or chicken embryos. Some species cultivable in blood monocytes, monocytic or macrophage cells lines, myelomonocytic cell lines, endothelial cell lines and tick cell lines. Aetiological agents of diseases of dogs and other canids, rodents and humans. Variably pathogenic or non-pathogenic infections in some ruminants such as deer and some rodents. The G+C content of the DNA varies between approximately 30 and 56 mol%. The type species is *Ehrlichia canis* (Donatien and Lestoquard 1935) Moshkovski 1945.

Emended description of *Neorickettsia* (Philip, Hadlow and Hughes 1953)

It is proposed that some descriptions of the genus *Ehrlichia* be united with the genus *Neorickettsia* (Pretzman *et al*., 1995). This requires emendation of the description of the genus *Neorickettsia* by integration with some descriptions of the genus *Ehrlichia* and new data, as follows.

Small, coccoid, often pleomorphic, intracytoplasmic bacteria that occur primarily in vacuoles of monocytes in the blood and macrophages of lymphoid or other tissues of dogs, horses and humans. Certain tissues of mature fluke vectors, all other fluke stages, eggs, rediae, cercariae and metacercariae have been proven infectious by injection into susceptible vertebrate hosts, as have mature stages of aquatic insects, which confirms that the infectious cycle includes transovarial and *trans*-stadial transmission in the vectors (Reubel *et al*., 1998a; Barlough *et al*., 1998). Gram-negative. Non-motile. Not cultivable in cell-free media or in chicken embryos. Sensitive to tetracycline antibiotics. The G+C content of the DNA is not known. The type species is *Neorickettsia helminthoeca* (Philip *et al*., 1953).

Description of *Anaplasma phagocytophila* comb. nov.

The most recent description of *E. phagocytophila* is that of Ristic & Huxsoll (1984). It is proposed that the species *E. equi* (Ristic & Huxsoll, 1984; Stannard *et al*., 1969) and the unnamed HGE agent (Chen *et al*., 1994a; Bakken *et al*., 1994) be united within the single species designation *E. phagocytophila* and transferred into the genus *Anaplasma*. This requires emendation of the species description for *E. phagocytophila* by integrating portions of the description of the species *E. equi* (Ristic & Huxsoll, 1984) and new data for *E. equi* and the HGE agent as follows.

Gram-negative, coccoid to ellipsoidal, often pleomorphic, intracytoplasmic bacteria that infect cells of mammalian bone marrow derivation, predominantly cells in the myeloid lineage. Two ultrastructural morphologies are observed, including a larger reticulate form and a smaller dense-core form that contains condensed protoplasm. Tick vectors include species of the *Ixodes persulcatus* complex (Telford *et al*., 1996; Richter *et al*., 1996; MacLeod & Gordon, 1933; Foggie, 1951). In mammalian cells, morulae are usually 1·5–2·5 μm in diameter, but may be as large as 6 μm (Popov *et al*., 1998). Individual bacterial cells are of two types, dense-core and reticulate, both present in the same vacuole; both may undergo equal or unequal binary fission. Individual cells may be as large as 2 μm in diameter. Empty vesicles may be present in the vacuolar space, but fibrillar matrix is lacking. Abundant cytoplasmic membrane may be present, forming protrusions into the periplasmic space or invaginations into the bacterial protoplasm. Mitochondria do not contact with or cluster around morulae. Causative agent of tick-borne fever of ruminants (Gordon *et al*., 1932; Hudson, 1950; Foggie, 1951). Equine granulocytic ehrlichiosis (Madigan, 1993; Stannard *et al*., 1969; Gribble, 1969), a type of canine granulocytic ehrlichiosis that lacks lameness as a significant sign (Greig *et al*., 1996; Pusterla *et al*., 1997) and human granulocytic ehrlichiosis (Chen *et al*., 1994a; Goodman *et al*., 1996; Bakken *et al*., 1994) are caused by variants of *A. phagocytophila*, previously known as *E. equi* and the HGE agent, respectively. Tick-borne fever is chiefly reported as a febrile disease of goats, sheep and cattle in the UK, The Netherlands, Scandinavia, Spain, France, Germany and Switzerland. Clinical signs vary from no detectable illness to severe febrile disease associated with opportunistic infections, haemorrhage and abortions. Equine granulocytic ehrlichiosis and a form of canine granulocytic ehrlichiosis have been described broadly across the USA, Canada, Brazil, Venezuela and Northern Europe. Equine and canine diseases are characterized by fever, depression, anorexia, leukopaenia and thrombocytopaenia; equine infection also frequently results in limb oedema and ataxia and may lead to opportunistic infections. Human granulocytic ehrlichiosis has also been described in many of the same geographical areas of California, Wisconsin, Minnesota and the New England states in the USA and in Slovenia, Norway, Switzerland and Sweden in Europe; serological evidence of human infection in the absence of overt human disease has been described in the USA, UK, Switzerland, Norway, Sweden, Denmark, Germany and Bulgaria. Human disease is characterized by fever, headache, myalgia and malaise and by the presence of leukopaenia, thrombocytopaenia and evidence of hepatic injury (Bakken *et al*., 1996; Aguero-Rosenfeld *et al*., 1996). The case fatality rate in humans is less than 1%, but is associated with severe opportunistic infections (Walker & Dumler, 1997). Although *A. phagocytophila* has a broad geographical distribution and all isolates appear to have significant serological cross-reactivity, a minor degree of variation in the nucleotide sequence of up to 5 bp ($>99{\cdot}5\%$ identity) in the 16S rRNA gene and $\geqslant 99{\cdot}0\%$ identity in *groESL* is detected. The organism shares significant antigens with *E. canis*, *E. chaffeensis* and *E.* (*Cowdria*) *ruminantium* comb. nov. The major constitutively produced protein antigens are encoded by a multigene family, vary between 42 and 49 kDa in molecular size and are expressed on the outer membrane (Murphy *et al*., 1998; Dumler *et al*., 1995; Asanovich *et al*., 1997; Zhi *et al*., 1997, 1998). The amino acid sequences of the major outer-membrane proteins are similar to those of *A. marginale*, *E.* (*Cowdria*) *ruminantium*, *E. canis*, *E. chaffeensis* and *Wolbachia* species. The genome size is approximately 1500 kbp (Rydkina *et al*., 1999). The G+C content of the DNA estimated from sequenced genes is 41 mol%. The 16S rRNA gene sequence of the *A. phagocytophila* type strain Webster^{T} is the same as deposited in GenBank under the accession number U02521.

Description of *Anaplasma bovis* comb. nov.

The most recent description of *Ehrlichia bovis* is that of Scott (1994). In addition, *A. bovis* is a Gram-negative, coccoid to coccobacillary and often pleomorphic obligate intravacuolar bacterium that infects cattle and perhaps other mammals. Mononuclear cells are most often infected but are infrequently identified in peripheral blood. African tick vectors include *Hyalomma excavatum*, *Rhipicephalus appendiculatus*, *Amblyomma variegatum* and possibly *Amblyomma cajennense* in Brazil. Serological cross-reactions with *E. ruminantium* have been reported (Du Plessis *et al*., 1987). The 16S rRNA gene sequence of *A. bovis* is deposited in GenBank under the accession number U03775.

Description of *Ehrlichia ruminantium* comb. nov.

The most recent description of *Cowdria ruminantium* is that of van Vliet *et al*. (1992). In addition, *E. ruminantium* is a Gram-negative, coccoid to ellipsoidal, often pleomorphic, intracytoplasmic bacteria that infects cattle, sheep, goats and occasionally murine endothelial cells as well as cells of bone marrow derivation, predominantly cells in the myeloid and monocytic lineages. Various species of wild African ruminants are reservoir hosts (Neitz, 1933, 1935; Peter

et al., 1998, 1999). Tick vectors include at least 10 species of the genus *Amblyomma*. The major constitutionally produced protein antigens (MAP1) are encoded by a multigene family, vary between 31 and 32 kDa in molecular size and are expressed on the outer membrane. The amino acid sequences of this major outer-membrane protein are similar to those of *A. marginale*, *A. phagocytophila*, *E. chaffeensis*, *E. canis* and *W. pipientis*. The 16S rRNA gene sequence of the type strain, Welgevonden^T, is the same as that for the Crystal Springs strain deposited in GenBank under accession no. X61659 (M. T. Allsopp, personal communication).

Emended description of *Ehrlichia canis*

The most recent description of *E. canis* is that of Ristic & Huxsoll (1984). In addition, cells are Gram-negative, coccoid to ellipsoidal, often pleomorphic, intracytoplasmic bacteria that infect canid and perhaps human cells of bone marrow derivation (Perez *et al*., 1996), predominantly cells in the monocytic lineage. The predominant tick vector is *Rhipicephalus sanguineus*. The major constitutionally produced protein antigen varies between 28 and 32 kDa in molecular size and is expressed on the outer membrane (Yu *et al*., 1999a; Ohashi *et al*., 1998b; Reddy *et al*., 1998; McBride *et al*., 1999). The amino acid sequences of the major outer-membrane proteins are similar to those of *E. chaffeensis*, *E. ruminantium*, *A. marginale*, *A. phagocytophila* and *W. pipientis*. The 16S rRNA gene sequence of the type strain Oklahoma^T is deposited in GenBank under accession no. M73221.

Emended description of *Ehrlichia chaffeensis*

With the following additions, the description is the same as that given previously (Anderson *et al*., 1991). The tick vector is *Amblyomma americanum*. The type strain is strain Arkansas^T. The major constitutionally produced protein antigens are encoded by a multigene family, vary between 28 and 32 kDa in molecular size and are expressed on the outer membrane (Yu *et al*., 1999a; Ohashi *et al*., 1998a; Reddy *et al*., 1998). The amino acid sequences of the major outer-membrane proteins are similar to those of *E. canis*, *E. ruminantium*, *A. phagocytophila*, *A. marginale* and *Wolbachia* species. The genome size is approximately 1250 kbp (Rydkina *et al*., 1999). The 16S rRNA gene sequence of the type strain, Arkansas^T, is deposited in GenBank under accession no. M73222.

Emended description of *Ehrlichia ewingii*

With the following additions, the description is the same as that given previously (Anderson *et al*., 1992). The tick vector is *Amblyomma americanum*. The type strain is strain Stillwater^T. Aetiological agent of canine and human disease (Ewing *et al*., 1971; Buller *et al*., 1999). The 16S rRNA gene sequence is deposited in GenBank under accession no. M73227.

Emended description of *Ehrlichia muris*

With the following additions, the description is the same as that given previously (Wen *et al*., 1995a). *Haemaphysalis flava* ticks may be naturally infected, but a role as a vector has not been established (Kawahara *et al*., 1999). The 16S rRNA gene sequence of the type strain, AS145^T, is deposited in GenBank under accession no. U15527.

Description of *Anaplasma platys* comb. nov.

With the following additions, the description is the same as that given previously for *E. platys* (Ristic & Huxsoll, 1984; Anderson *et al*., 1992). A tick vector is suspected, but has not been established. The 16S rRNA gene sequence is deposited in GenBank under accession no. M82801.

Description of *Neorickettsia sennetsu* comb. nov.

With the following additions, the description is the same as that given previously for *E. sennetsu* (Ristic & Huxsoll, 1984). The organism shares antigens with *N.* (*Ehrlichia*) *risticii*. Not pathogenic for the horse but, after infection, horses are protected from infection with *N. risticii*. The mode of transmission is not known, although a fish parasite is suspected. Mice are highly susceptible to infection. The genome size is approximately 880 kbp (Rydkina *et al*., 1999). The type strain is Miyayama^T, for which the 16S rRNA gene sequence is deposited in GenBank under accession no. M73225 (Anderson *et al*., 1991).

Description of *Neorickettsia risticii* comb. nov.

With the following additions, the description is the same as that given previously for *E. risticii* (Holland *et al*., 1985b). Causative agent of Potomac horse fever, also called equine monocytic ehrlichiosis. Transmitted by the ingestion of fresh-water snail species or insects infested with infected trematodes or metacercariae (Reubel *et al*., 1998a; Madigan *et al*., 2000). Shares antigens with *N. sennetsu*, *N. helminthoeca* and the SF agent bacterium. The organism is found to infect peripheral blood monocytes, intestinal epithelial cells and equine mast cells. The 16S rRNA gene may vary by up to 15 bases in nucleic acid sequences. The approximate genome size is 880 kbp (Rydkina *et al*., 1999). The type strain is Illinois^T, for which the 16S rRNA gene sequence is deposited in GenBank under accession no. M21290.

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Chapter 6

Simultaneous detection of *Anaplasma* and *Ehrlichia* species in ruminants and detection of *Ehrlichia ruminantium* in *Amblyomma variegatum* ticks by reverse line blot hybridization

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Simultaneous detection of *Anaplasma* and *Ehrlichia* species in ruminants and detection of *Ehrlichia ruminantium* in *Amblyomma variegatum* ticks by reverse line blot hybridization

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Abstract

The detection of *Anaplasma* and *Ehrlichia* species is usually based on species-specific PCR assays, since no assay is yet available which can detect and identify these species simultaneously. To this end, we developed a reverse line blot (RLB) assay for simultaneous detection and identification of *Anaplasma* and *Ehrlichia* species in domestic ruminants and ticks. In a PCR the hypervariable V1 region of the 16S ribosomal RNA (rRNA) gene was amplified with a set of primers unique for members of the genera *Anaplasma* and *Ehrlichia* [Int. J. Syst. Evol. Microbiol. 51 (2001) 2145]. Amplified PCR products from blood of domestic ruminants or *Amblyomma variegatum* tick samples were hybridized onto a membrane to which eight species-specific oligonucleotide probes and one *Ehrlichia* and *Anaplasma* catch-all oligonucleotide probe were covalently linked. No DNA was amplified from uninfected blood, nor from other hemoparasites such as *Theileria annulata*, or *Babesia bigemina*. The species-specific probes did not cross-react with DNA amplified from other species. *E. ruminantium*, *A. ovis* and another *Ehrlichia* were identified by RLB in blood samples collected from small ruminants in Mozambique. Finally, *A. variegatum* ticks were tested after feeding on *E. ruminantium* infected sheep. *E. ruminantium* could be detected in adult ticks even if feeding of nymphs was carried

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out 3.5 years post-infection. In conclusion, the developed species-specific oligonucleotide probes used in an RLB assay can simultaneously detect and identify several *Ehrlichia* and *Anaplasma* species. However, as no quantitative data for the detection limit are available yet, only positive results are interpretable at this stage.

Keywords: *Ehrlichia* sp.; *Anaplasma* sp.; *Amblyomma variegatum*; Simultaneous detection; Heartwater

1. Introduction

Tick-borne rickettsial diseases (e.g. heartwater and anaplasmosis) pose important economic constraints to livestock management throughout the world (Uilenberg, 1997). Heartwater is limited to sub-Saharan Africa and certain Caribbean Islands (Uilenberg, 1983), whereas other rickettsial diseases are found on all continents. Heartwater is caused by the bacterium *Ehrlichia ruminantium* (Dumler et al., 2001), which is transmitted by ticks of the genus *Amblyomma* and constitutes a major obstacle to livestock production in sub-Saharan Africa (Uilenberg and Camus, 1993). Besides *E. ruminantium*, other tick-borne rickettsial diseases in cattle are caused by *Anaplasma marginale* and *A. centrale* infecting red blood cells (Kuttler, 1966), or *Anaplasma bovis* which infects and multiplies in monocytes (Uilenberg, 1993). Tick-borne rickettsial diseases in sheep and goats are caused by *E. ruminantium*, *A. ovis* (Neitz, 1968a) and *Ehrlichia ovina*. *E. ovina* has the same characteristics as *A. bovis* and was the first monocytophilic rickettsia to be recognized in livestock (Neitz, 1968b). *Anaplasma phagocytophila*, which encompasses the former *Ehrlichia equi* and the agent of human granulocytic ehrlichiosis (HGE) (Dumler et al., 2001), multiplies predominantly in neutrophils and can infect both cattle and small ruminants (Gokce and Woldehiwet, 1999; Pusterla et al., 1999; Lepidi et al., 2000).

Separate PCR assays have been developed for most species mentioned (Sparagano et al., 1999). For instance, for *A. marginale*, *A. centrale* and *A. ovis* PCR-based assays have mainly targeted the MSP5 gene, which is conserved among these *Anaplasma* species (Visser et al., 1992; Ndung'u et al., 1995), or the MSP2 gene (Eriks et al., 1989; Ge et al., 1995; Gale et al., 1996; French et al., 1998). Three different targets have been used for PCR and hybridization assays to detect *E. ruminantium*, pCS20 (Waghela et al., 1991; Peter et al., 1995), the major antigenic protein 1 (MAP1) (Kock et al., 1995) or the 16S ribosomal RNA (rRNA) gene (Allsopp et al., 1997, 1998). Several PCR assays for detection of other *Anaplasma* and *Ehrlichia* species have been described (Sparagano et al., 1999), but there are no specific assays to detect either *A. bovis* or *E. ovina*.

Recently, a PCR-based assay for the simultaneous detection and identification of *Ehrlichia* and *Borrelia burgdorferi* sensu lato using the reverse line blot (RLB) technique was reported (Schouls et al., 1999). RLB has also been used to detect *Streptococcus pyogenes* serotypes (Kaufhold et al., 1994), *Mycobacterium tuberculosis* strains (Kamerbeek et al., 1997), and different *Theileria* and *Babesia* species (Gubbels et al., 1999). This technique allows the analysis of multiple samples against multiple probes at the same time, which is a major advantage.

In this study we used RLB to detect and differentiate *Anaplasma* and *Ehrlichia* species known to occur in ruminants in the (sub)tropics on the basis of their differences in the small-subunit rRNA (srRNA) gene. Conserved domains within the 16S rRNA gene were used to amplify the hypervariable V1 loop by PCR. Species-specific oligonucleotide probes were designed in this V1 loop to allow species-specific detection. The sequence of the 16S rRNA gene for *A. ovis*, *A. centrale* and *E. ovina* were not available and therefore were sequenced. A probe for *Ehrlichia* sp. strain Omatjenne was included, because a 16S sequence identical to this species was detected in goats in Mozambique. The developed assay can simultaneously detect *E. ruminantium*, *A. ovis*, *A. centrale*, *A. marginale*, *A. bovis*, *E. ovina*, *Ehrlichia* sp. strain Omatjenne and *A. phagocytophila*.

2. Material and methods

2.1. GenBank accession numbers of sequences used

The GenBank accession numbers of the 16S sequences used for deducing PCR oligonucleotides and species-specific RLB oligonucleotide probes are as follows: X62432, X61659, U03776, U03777 and AF069758 for *E. ruminantium*; M60313 for *A. marginale*; U03775 for *A. bovis*; U54806 for *Ehrlichia* sp. strain Omatjenne; and M73220, M73223, M73224, U02521, AF036645, AF036646, AF036647, AF057707, AF084907, AF093788, AF093789, AF170728, AF172164 until AF172167, AF189153 and AJ242783 until AJ242785 for *A. phagocytophila*.

2.2. Bacterial stocks

Bacterial stocks previously described are listed in Table 1. *A. phagocytophila* was isolated from a dog in Bilthoven, The Netherlands, in 1997. *E. ruminantium* (Burkina Faso) was obtained in 1998 from an experimental sheep at Utrecht on which adult *Amblyomma variegatum* ticks, collected from a herd of sheep in Burkina Faso, were allowed to feed. *A. bovis* DNA was provided by Dr. T. de Waal.

2.3. Sequencing of 16S genes

The 16S rRNA gene from *A. ovis*, *A. centrale*, and *E. ovina* was PCR amplified using primers previously described for the cloning and sequencing of the *E. ruminantium* 16S rRNA gene (van Vliet et al., 1992). The PCR products were sent for sequencing to Baseclear (Leiden, The Netherlands).

2.4. Experimental infections, tick feeding and transmission

Experimental infections with *E. ruminantium* stocks were conducted in 6- to 12-month-old female Texelaar sheep by intravenous inoculation of infected blood stabilates. The sheep were treated with tetracyclines (5 mg/kg) for three consecutive days when the rectal temperature exceeded 40.5 °C for 2 days (Table 2). Serum samples for serodiagnosis and

Table 1
Origin and nature of bacterial and parasite stocks

Species	Stock origin		Material	Reference
	Country	Location or name		
E. ruminantium	South Africa	Welgevonden	Culture	Du Plessis (1985)
	South Africa	Ball 3	GUTS[a]	Haig (1952)
	Senegal	Senegal (Niaye)	Culture	Jongejan et al. (1988)
	Guadeloupe	Gardel	Blood	Uilenberg et al. (1985)
	Ghana	Sankat 430	Culture	Bell-Sakyi et al. (1997)
	Zimbabwe	Crystal Springs	Culture	Yunker et al. (1988)
	Burkina Faso	Burkina Faso	Blood	
	Cameroon	Ngoudere	Culture	Stachurski (1989)
	São Tomé	São Tomé	Brain	Uilenberg et al. (1982)
	Sudan	Um Banein	Culture	Jongejan et al. (1984)
	Kenya	Kiswani	Culture	Kocan et al. (1987)
	Mozambique	Porto Henrique	Culture	Bekker et al. (2001)
	Zambia	Lutale	Culture	Jongejan et al. (1988)
A. marginale	South Africa		DNA	Potgieter et al. (1981)
	USA	South Idaho	DNA	McGuire et al. (1984)
	USA	St. Maries	DNA	Eriks et al. (1994)
	USA	Florida	DNA	Ristic and Carson (1977)
Anaplasma centrale	South Africa		DNA	Potgieter (1979)
	Israel		DNA	
Anaplasma ovis	South Africa		DNA	Visser et al. (1991)
A. bovis	South Africa		DNA	
E. ovina	Turkey		Blood	Jongejan et al. (1993)
Ehrlichia sp. strain Omatjenne	Mozambique	Bom Pastor	Blood	Bekker et al. (2001)
A. phagocytophila	The Netherlands	Bilthoven	Blood	
T. annulata	Turkey	Ankara	Blood	Schein et al. (1975)
B. bigemina	Nigeria	Runka	Blood	Leeflang and Ilemobade (1977)

[a]Ground up tick supernatant.

citrate-blood samples for PCR were collected three times a week and stored at −20 °C. *A. variegatum* ticks originating from Burkina Faso were kept as previously described (Heyne et al., 1987). Approximately, 100 *A. variegatum* nymphs each were applied on the back of sheep #175 to #178, 2 months post-infection (p.i.) with *E. ruminantium* and on the back of sheep #580 3.5 years p.i. (Table 2). Engorged nymphs were collected and stored at 27 °C with a 90% relative humidity and were left to molt to the adult stage. Transmission of *E. ruminantium* by these adult ticks was investigated for three batches (originating from sheep #175, #178 and #580) by applying 30 males, followed 4 days later by 30 females, to the back of uninfected female Texelaar sheep. Uninfected *A. variegatum* ticks were fed on

Table 2
Experimental infections of sheep by *E. ruminantium* and transmission by *A. variegatum*

Sheep no.	Heartwater isolate	Start of treatment (d.p.i.)	RLB[a]	Tick feeding (d.p.i.)	RLB[b]	Percentage of RLB positive ticks[c]	Tick transmission[d]
175	Sankat 430	7	+	60	–	0 (0/20)[e]	No reaction
176	Senegal	15	+	60	–	60 (12/20)	n.d.[f]
177	Welgevonden	10	+	60	–	15 (3/20)	n.d.
178	Gardel	12	+	60	–	55 (11/20)	Fatal outcome
580	Senegal	13	n.d.	3.5 years	–	70 (14/20)	Fatal outcome

[a] Blood samples collected during the clinical phase of infection were tested.
[b] Blood samples collected 2 days prior to and during tick feeding were tested.
[c] Ticks were tested after molting to the adult stage.
[d] Tick transmission of heartwater to susceptible female Texelaar sheep.
[e] Percentage (no. positive/no. tested).
[f] Not determined.

rabbits (larvae and nymphs) or on uninfected sheep (adults) to provide negative control ticks.

2.5. *Processing of samples for PCR*

DNA of the various *E. ruminantium* isolates was extracted as described before (van Vliet et al., 1994) or using the tissue protocol from the QIAamp extraction kit (Westburg, Leusden, The Netherlands). DNA of *E. ovina* and *A. phagocytophila* was extracted from EDTA–blood stored in liquid nitrogen as previously described (d'Oliveira et al., 1995). Briefly, 200 µl samples were washed three times with 0.5 ml of lysis mixture (0.22% NaCl, 1 mM EDTA, 0.015% saponin) by centrifugation at maximum speed for 5 min. Pellets were resuspended in 100 µl of PCR mixture (10 mM Tris–HCl [pH 8.0], 50 mM KCl, 0.5% Tween 20, 100 µg proteinase K/ml), and incubated overnight at 56 °C. Samples were subsequently heated for 10 min at 100 °C and centrifuged at maximum speed for 2 min and used for PCR.

2.6. *Collection and processing of blood samples from Mozambique*

Ten heparin-blood samples were collected during a field study in the south of Mozambique from goats either inoculated intravenously ($n = 3$) with blood stabilate or placed in two herds of goats near Porto Henrique ($n = 4$) or Bom Pastor ($n = 3$) (Bekker et al., 2001). A further two samples were collected from a goat near Chokwe and a sheep near Bela Vista (Table 3). DNA was extracted from these samples as described by Moreira (1998), which uses an agar-embedding method to remove PCR inhibitors. Briefly, cells were collected from 1 ml of blood by centrifugation at 3000 × *g* for 10 min. Cells were included in low melting point agar to a final concentration of 0.8%. Embedded cells were lysed by overnight incubation at 50 °C in lysis buffer. Subsequently, lysis buffer was removed and replaced by an excess volume of TE buffer (10 mM Tris–HCl of pH 8.0 and

Table 3
Blood samples collected from small ruminants in Mozambique

Sample no.[a]	Collected from[b]
1	Translocated goat #1
2	Translocated goat #2
3	Translocated goat #3
4	Translocated goat #4
5	Translocated goat #6
6	Translocated goat #7
7	Translocated goat #10
8	Infected goat #11
9	Infected goat #12
10	Infected goat #15
11	Field collection from a sheep at Bela Vista
12	Field collection from goat at Chokwe

[a] Samples 1–4 were collected at Porto Henrique, samples 5–7 at Bom Pastor and samples 8–10 at Veterinary Faculty in Maputo.

[b] The first seven samples were from experimentally translocated goats (translocated from Tete province (north of Mozambique) and placed in existing herds in the south of Mozambique (Bekker et al., 2001)). Samples 8–10 were from experimental infections using blood samples collected from goats at Bela Vista.

1 mM EDTA). After two extensive washes in TE (5 h under continuous shaking) genomic DNA remained trapped in the agarose matrix. The agarose was used directly for PCR.

2.7. Processing of ticks

Adult *A. variegatum* ticks, which had been feeding as nymphs on infected sheep or rabbits (negative control), were cut in half with a sterile blade (Swann-Morton, Sheffield, UK) inside 2 ml Eppendorf tubes. For each tick a new blade was used. DNA was extracted essentially as described before (d'Oliveira et al., 1997). Shortly, ticks were boiled for 10 min in PBS and subsequently SDS was added and the sample was spun for 5 min at full speed. DNA was phenol extracted, ethanol precipitated and resuspended in TE.

2.8. PCR

One set of primers was used to amplify a 492–498 bp fragment of the 16S rRNA gene spanning the V1 region. The forward primer 16S8FE was previously described (Schouls et al., 1999) and the reverse primer B-GA1B-new (5′-cgggatccCGAGTTTGCCGGGACT-TYTTCT-3′) was modified from the previously described primer B-GA1B (Schouls et al., 1999) to hybridize with the region conserved for *Ehrlichia* and *Anaplasma* species and contained a biotin molecule at the 5′ site. Primer 16S8FE corresponds to nucleotides 70–89 and primer B-GA1-B-new corresponds to nucleotides 520–540 of the *E. ruminantium* (Crystal Springs isolate) 16S rRNA gene (accession no. X61659). Primers were obtained from Isogen (Maarssen, The Netherlands). Reaction conditions in a 50 µl volume were as follows: 1× SuperTaq PCR buffer (Promega, Leiden, The Netherlands), 1.5 mM $MgCl_2$

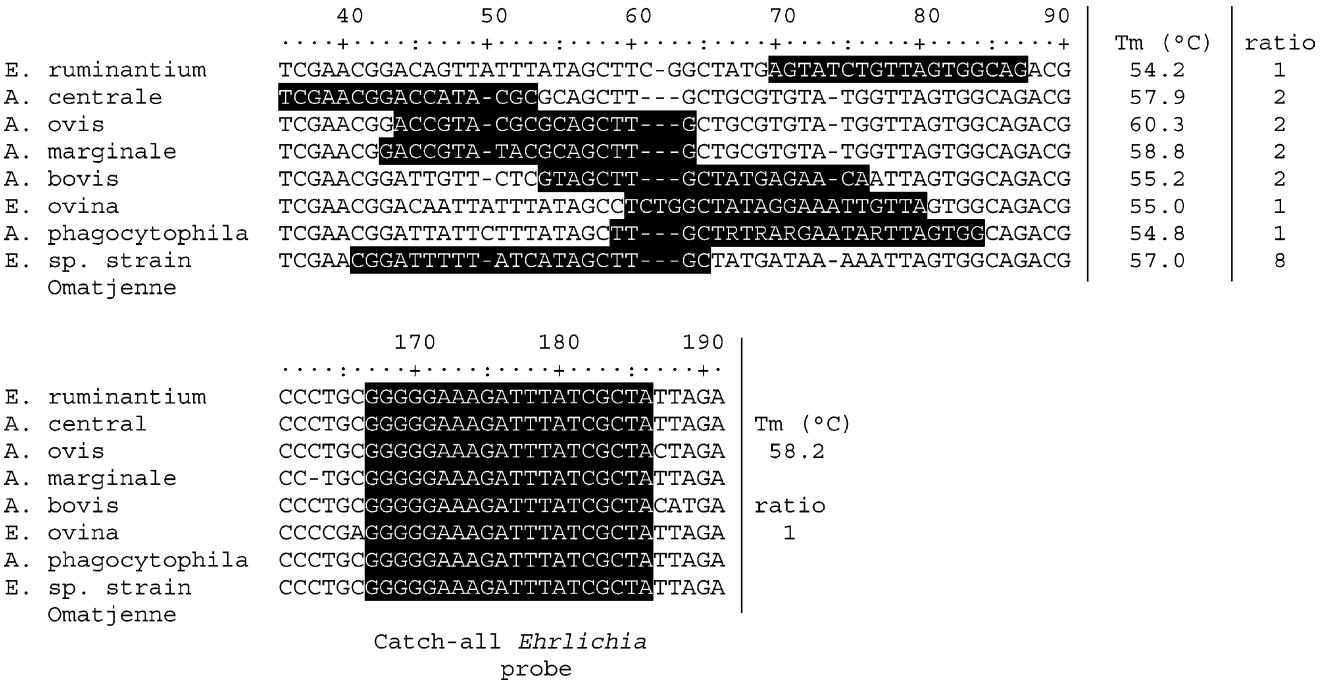

Fig. 1. Alignment showing the locations of the species-specific oligonucleotide probes (shaded) in the 16S rRNA V1 hypervariable region. The indicated melting temperature (T_m) of each probe was calculated with program Oligo 4.1. The ratio of each probe to the catch-all probe (a ratio of 1 corresponds to 100 pmol) is also indicated. The numbering on top correspond to the position of the nucleotides in the 16S sequence of *E. ruminantium* Senegal isolate (accession no. X62432 (van Vliet et al., 1992)). The R at positions 67, 69, 71 and 77 in the *A. phagocytophila* sequence denotes either A or G.

(Promega), 200 and 100 μM of each of the following deoxynucleoside triphosphate (dATP, dCTP, dGTP) and (dTTP, dUTP), respectively (Pharmacia Biotech), 1.25 U of Supertaq polymerase (Promega), 0.2 U of Uracil DNA glycosylase (Life Technologies, Breda, The Netherlands), 50 pmol of each primer, and 10 μl of purified DNA sample. The reactions were performed on an automated DNA thermal cycler (Perkin-Elmer, Foster City, CA) using a three phase program. The first phase consisting of 3 min incubation at 37 °C, to degrade any contamination template from previous PCR amplification by the uracil DNA glycocylase, followed by 10 min at 94 °C to inactivate the uracil DNA glycosylase. The second phase consisted of a touch down program of each time two cycles of 20 s 94 °C, 30 s at the annealing temperature (T_a), 30 s 72 °C, whereby the T_a was lowered from 67 to 57 °C by steps of 2 °C. The third phase consisting of 40 cycles of 20 s 94 °C, 30 s 57 °C and 30 s 72 °C.

2.9. RLB hybridization

All oligonucleotide probes shown in Fig. 1 contained a N-terminal *N*-(trifluoracetamidohexyl-cyanoethyl, *N*,*N*-diisopropyl phosphoramidite [TFA])-C_6 amino linker (Isogen). The preparation and subsequent hybridization of the RLB membrane was done as described before (Gubbels et al., 1999) with the exception that the first 10 min wash step in 125 ml of 2× SSPE–0.5% SDS after addition of the PCR products was done at 52 °C followed by a second 10 min wash at 42 °C. After use, all PCR products were stripped from the membrane by incubation in a 1% SDS solution for twice 30 min at 90 °C. Subsequently, the membrane was rinsed in a 20 mM EDTA (pH 8.0) solutions and stored in a sealed plastic bag containing fresh EDTA solution at 4 °C for reuse.

2.10. Nucleotide sequence accession numbers

The 16S sequences determined in this study for *A. centrale*, *A. ovis*, and *E. ovina* have been deposited in GenBank under accession nos. AF318944, AF318945 and AF318946, respectively.

3. Results

3.1. 16S sequences

The 16S rRNA genes of *A. ovis*, *A. centrale* and *E. ovina*, respectively, were PCR amplified with general 16S primers and sequenced directly from the PCR product. The sequence of *A. ovis* (1435 bp) and *A. centrale* (1435 bp) differed from the sequence of *A. marginale* (accession no. M60313) in 7 and 3 positions, respectively, and the sequence of *A. ovis* differed in five positions from the sequence of *A. centrale*, in the part of the 16S sequence spanned by RLB primers 16S8FE and B-GA1B-new. The 16S sequence from *E. ovina* (1431 bp) differed in two positions from the *E. canis* 16S sequence (accession no. AF162860), but was identical to *E. canis* in the part spanned by the RLB primers.

3.2. RLB specific oligonucleotides

For each species an oligonucleotide was designed specific for the amplified V1 region, with an annealing temperature between 54.2 and 60.3 °C under the conditions used (Fig. 1). One oligonucleotide probe cross-reactive with all species (catch-all probe) was designed in a similar way (Fig. 1). This probe served as a control in case a PCR product did not hybridize to any of the species-specific probes, as may occur with an unknown species or mutated 16S rRNA. The optimal oligonucleotide probe concentration giving specific and sensitive signals was determined empirically and is indicated in Fig. 1. The location of the catch-all probe was conserved among all *Anaplasma* and *Ehrlichia* species tested.

3.3. RLB

RLB-PCR was performed on all species and isolates listed in Table 1. PCR products were hybridized to the membrane and were shown to bind with specific oligonucleotide probes only (Fig. 2). All *E. ruminantium* isolates were detected by the *E. ruminantium* probe and the catch-all probe. All four *A. marginale* isolates, two *A. centrale* isolates, *A. ovis*, *A. bovis*, *E. ovina* and *A. phagocytophila* were detected in the RLB by their specific probe and by the catch-all probe and did not hybridize with any of the other species-specific probes.

RLB-PCR performed on parasite DNA extracted from *Theileria annulata*, *Babesia bigemina* or mammalian DNA did not yield any detectable product on agarose gel (data not shown). When these PCR samples were applied onto the RLB membrane no reaction with any of the oligonucleotide probes was observed (Fig. 2 lanes 25–29). Previous PCR amplification on the *T. annulata* and *B. bigemina* DNA samples had been successful indicating that DNA was present in these samples and could be amplified (Gubbels et al., 1999).

3.4. Detection of artificially mixed Anaplasma and Ehrlichia DNA samples

In order to mimic possible co-infections occurring in the field, two mixtures of DNA samples from species that are known to occur in either cattle or sheep and goats were prepared and subsequently tested in the RLB. The mixture for cattle (mix I) contained *E. ruminantium*, *A. marginale*, *A. centrale*, *A. bovis* and *A. phagocytophila*. The mixture for sheep and goat (mix II) contained *E. ruminantium*, *A. ovis*, *E. ovina*, *Ehrlichia* sp. strain Omatjenne and *A. phagocytophila*. Fig. 3 shows that all species in both mixtures were detected and correctly identified, although a difference in the signal intensity was observed.

3.5. Detection of Anaplasma and Ehrlichia spp. in blood samples from small ruminants in Mozambique

E. ruminantium was detected in all 12 blood samples (Table 3) collected during a study in Mozambique. In addition, four samples also contained *A. ovis* and one sample was positive for both *E. ruminantium* and *Ehrlichia* sp. strain Omatjenne (lane 5, Fig. 4).

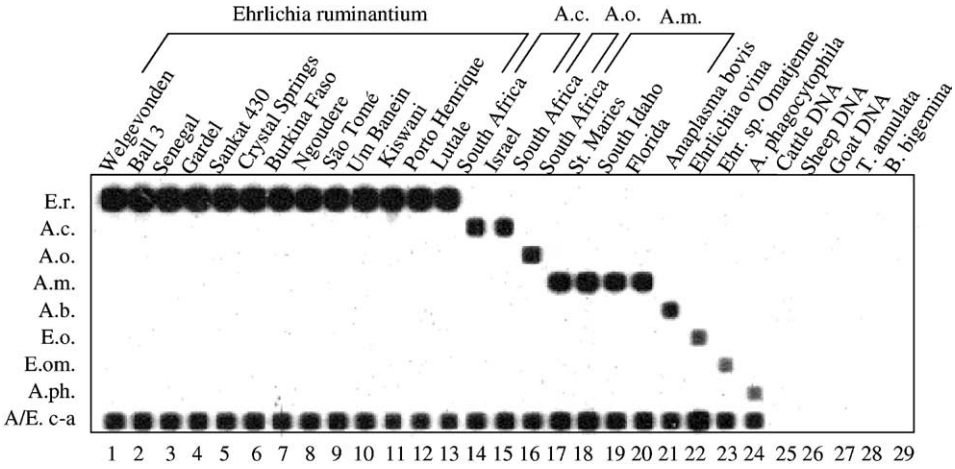

Fig. 2. RLB of PCR products obtained from *Anaplasma*, *Ehrlichia*, and other hemoparasites. Species-specific oligonucleotide probes are applied in horizontal rows. Lanes 1–13 contain *E. ruminantium* isolates from South Africa (lanes 1 and 2), Senegal (lane 3), Guadeloupe (lane 4), Ghana (lane 5), Zimbabwe (lane 6), Burkina Faso (lane 7), Cameroon (lane 8) São Tomé (lane 9), Sudan (lane 10), Kenya (lane 11), Mozambique (Lane 12), and Zambia (lane 13); lanes 14 and 15 contain *A. centrale* isolates from South Africa and Israel, respectively; lane 16, *A. ovis* from South Africa; lanes 17–20, *A. marginale* from South Africa (lane 17), US (lanes 18–20); lane 21, *A. bovis* from South Africa; lane 22, *E. ovina* from Turkey; lane 23, *E.* sp. strain Omatjenne from Mozambique; lane 24, *A. phagocytophila* from The Netherlands; lane 25, bovine DNA; lane 26, ovine DNA; lane 27, caprine DNA; lane 28, *T. annulata*; lane 29, *B. bigemina*. Rows: 1, *E. ruminantium* (E. r.); 2, *A. centrale* (A. c.); 3, *A. ovis* (A. o.); 4, *A. marginale* (A. m.); 5, *A. bovis* (A. b.); 6, *E. ovina* (E. o.); 7, *E.* sp. strain Omatjenne (E. om.); 8, *A. phagocytophila* catch-all (A. ph.); 9, catch-all *Anaplasma* and *Ehrlichia* control probe (A/E. c-a).

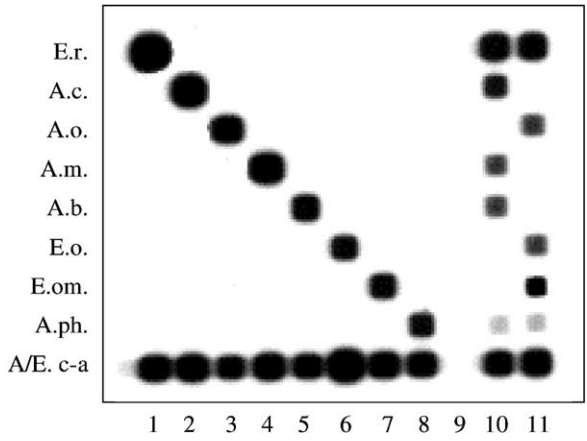

Fig. 3. RLB of mixed DNA samples from *Anaplasma* and *Ehrlichia* likely to occur in cattle and small ruminants. Lanes 1, *E. ruminantium*; lane 2, *A. centrale*; lane 3, *A. ovis*; lane 4 *A. marginale*; lane 5, *A. bovis*; lane 6, *E. ovina*; lane 7, *E.* sp. strain Omatjenne; lane 8, *A. phagocytophila*; lane 9, distilled water control (neg); lane 10, mix I containing *E. ruminantium*, *A. bovis*, *A. marginale*, *A. centrale*, and *A. phagocytophila*; lane 11, mix II containing *E. ruminantium*, *E. ovina*, *A. ovis*, *E.* sp. strain Omatjenne, and *A. phagocytophila*. Rows are identical to those in Fig. 2.

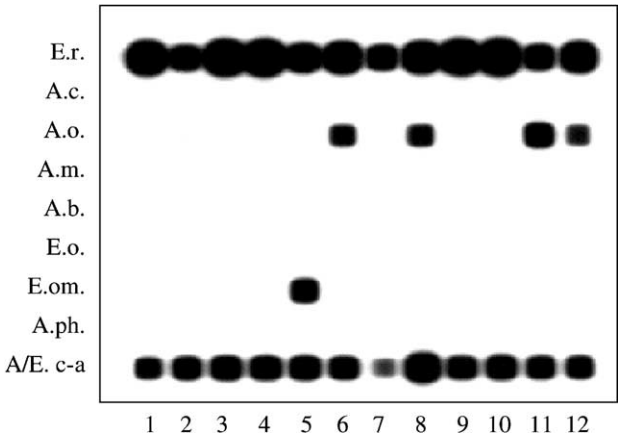

Fig. 4. RLB with PCR products derived from DNA extracted from blood samples from goats and a sheep in Mozambique (Table 3). Rows are identical to those in Fig. 2.

3.6. Detection of E. ruminantium in sheep and transmission by A. variegatum ticks

E. ruminantium could be detected by RLB in blood samples from experimentally infected sheep collected during the clinical phase of infection (Table 2). However, after treatment, *E. ruminantium* could no longer be detected by RLB in the blood of these carrier animals, nor could it be detected during tick feeding on these animals (Table 2). Adult *A. variegatum* ticks, which had been feeding as nymphs on these *E. ruminantium* infected sheep (Table 2) or on rabbits (negative control), were also tested by RLB. *E. ruminantium* could be detected in ticks originating from sheep #176, #177, #178 and #580, but not in ticks originating from sheep #175. Negative control ticks were always negative in the RLB assay (data not shown). The percentage of RLB positive ticks varied between 15 and 70% and is given in Table 2. Ticks from two RLB positive batches transmitted fatal cowdriosis to experimental sheep, whereas a third batch of RLB negative ticks failed to transmit *E. ruminantium* (Table 2).

4. Discussion

We developed an RLB assay based on the 16S rRNA gene of *Anaplasma* and *Ehrlichia* species infecting ruminants in tropical and subtropical regions. All species included were detected by their respective species-specific oligonucleotide probe and cross-reactions were not observed. Neither mammalian DNA nor representatives of *Theileria* and *Babesia* species were detected. The catch-all control probe was included in case an amplified PCR product did not hybridize with any of the species-specific probes. PCR products which only hybridize to the catch-all probe could indicate the presence of a novel *Anaplasma* or *Ehrlichia* species or the presence of 16S rRNA sequence variants of a known species. Unfortunately, we were not yet able to determine the assay sensitivity for the various probes and therefore only positive results at this stage are meaningful.

The 16S rRNA sequences of *A. centrale* and *A. ovis* differed only in one position in the V1 region allowing the development of species-specific probes. While developing and testing our species-specific probes new *Anaplasma* 16S sequences were becoming available via the GenBank database. The *A. centrale* (Israel) sequence (accession no. AF309869) was identical to the sequence determined for *A. centrale* from South Africa. However, *A. centrale* from Japan (accession no. AF283007) differed considerably from the other *A. centrale* and will, based on sequence differences at the probe level, most likely not be recognized by any of the *Anaplasma* probes. However, this *A. centrale* isolate should be recognized by the catch-all probe. Based on new sequences determined from samples that only hybridize to the catch-all probe, it is possible to redesign species-specific probes and adding them onto the membrane without changing the basic setup of the RLB assay.

Although *A. phagocytophila* is a heterogeneous species and variant specific probes have been developed (Schouls et al., 1999), we decided to include just one probe for *A. phagocytophila* because of the limited relevance of this species in the (sub)tropical region. Ambiguous nucleotides were incorporated to allow the detection of all variants for which a 16S sequence was available. This probe requires further testing to determine if it recognizes all isolate/strain known thus far for this species. Sequencing the 16S rRNA gene of *E. ovina* revealed that it was highly similar to *Ehrlichia* sp. strain Germishuys and *E. canis*. In fact, we could use the *E. canis* probe previously described by Schouls et al. (1999) to detect *E. ovina* in the RLB assay.

Fig. 2 shows that all the designed probes are species-specific and that cross-reactions therefore did not occur. It is also clear from this figure that ruminant DNA or DNA from other hemoparasites did not react with any of the probes, due to the fact that no product was amplified from this DNA with primers 16S8FE and B-GA1B-new. The optimal amount of each oligonucleotide probe used on the membrane (Fig. 1) was determined to generate hybridization signals of comparable intensity. Because the optimal concentration was determined for DNA samples that are still contaminated with host DNA, the intensity of the hybridization signals varied between different samples from the same species. Because purified DNA free of contaminating host DNA was not available for most species, we were not able at this time to determine a detection limit for the various probes or to determine if competition is taking place if simultaneous infection occurs, as has been done for the *Theileria/Babesia* RLB (Gubbels et al., 1999).

PCR products amplified from DNAs of species that may occur in the same host (cattle or small ruminants) were mixed, re-amplified and tested in the RLB assay (Fig. 3). All five species combined in mix I were detected correctly, as were all five species in mix II. It was shown that not all species were equally amplified, which could be due to unequal amounts of target DNA for the various species. When 12 blood samples collected in Mozambique from goats and from one sheep, suspected (and in some cases confirmed by post-mortem examination) to be infected with *E. ruminantium* (Bekker et al., 2001) were tested in the RLB assay it was found that some of the animals were infected by more than one species (Fig. 4). All 12 samples contained *E. ruminantium* and in four samples *A. ovis* could also be detected. Presence of *A. ovis* in sheep and goats in Mozambique has been reported before (Arnold and Travassos Santos Dias, 1983). One sample also contained a hybridization signal with the *Ehrlichia* sp. strain Omatjenne probe. A probe

for this recently described species (Allsopp et al., 1997) was included because a recent study indicated the possible presence of this species in Mozambique (Bekker et al., 2001).

The RLB assay was also tested for its ability to detect *E. ruminantium* in experimental infections of sheep and subsequent *A. variegatum* tick transmission. *E. ruminantium* could only be detected in experimentally infected sheep during a short period coinciding with the clinical phase of infection. However, not all samples collected during the clinical phase showed a hybridization signal with the *E. ruminantium* probe and further optimization of the assay is required. After treatment, *E. ruminantium* could no longer be detected in the blood of these carrier animals, most likely due to the limited number of bacteria present in the blood of carrier animals. Four out of five of these animals were confirmed to be carriers as ticks that had been feeding on them were shown to have *E. ruminantium* infection rates between 15 and 70%, whereas the fifth batch was negative. The negative ticks could either be true negatives, or the number of bacteria present in those ticks was too low to be detected by the RLB. This problem can be solved when the assay sensitivity has been determined. The high percentage of positive ticks feeding on sheep #580 showed that, although the infection had taken place 3.5 years earlier, the bacteria present in the animal were highly infective for ticks. Transmission of fatal heartwater infection (xenodiagnosis) using these ticks was demonstrated for two batches of ticks with infection rates of 55 and 70% (Table 2). Transmission of heartwater was not found using a third batch of ticks, which showed no infection with RLB. These results show that there seems to be a correlation between the positive RLB result and the ability of the ticks to transmit heartwater to susceptible animals. More experiments are required to confirm this since a very limited number of animals and isolates was used. Although xenodiagnosis is not very practical, feeding *Amblyomma* nymphs on animals and testing the resulting adults in the RLB assay could clearly demonstrate whether the animal is a carrier of *E. ruminantium*.

An additional advantage of using RLB to screen ticks for the presence of infectious agents is that information is obtained about possible occurring co-infections, which will not be detected by species-specific PCR alone or hybridization with single species-specific probes (e.g. the pCS20 probe used for detecting *E. ruminantium* in ticks (Peter et al., 1995)).

RLB has a major advantage over existing methods in that only one PCR reaction is required for simultaneous detection of several *Anaplasma* and *Ehrlichia* species. The RLB assay is useful in testing ticks for the presence of *E. ruminantium*, but may be less suitable for detection in blood samples from carrier animals. Although this was shown for *E. ruminantium*, it remains to be determined for the other *Anaplasma* and *Ehrlichia* species. *Anaplasma* infections are usually more easy to detect in carrier animals (Eriks et al., 1989; Kieser et al., 1990). Another major advantage is that the RLB assay can be used to detect thus far unrecognized *Ehrlichia* species or variants of known *Ehrlichia* species. Finally, the use of RLB as a versatile tool for integrated epidemiological monitoring of tick-borne pathogens can become a reality as soon as the sensitivity of the assay has been determined, since RLB has already been developed for all protozoan tick-borne hemoparasite species (Gubbels et al., 1999), which can occur simultaneously with *Anaplasma* and *Ehrlichia* species.

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Chapter 7

General Discussion

The research described in this thesis was carried out within the framework of an INCO-DC project entitled, "Integrated Control of Cowdriosis (*Cowdria ruminantium*): development and field assessment of improved vaccines and epidemiological tools." The work focused on two genes, the 16S rRNA gene and the *map1* gene, or more precisely the *map1* multigene family, which were studied to characterize and detect *Ehrlichia ruminantium* (formerly *Cowdria ruminantium*) in vertebrate hosts and vector ticks.

The *map1* gene

Role in serological detection

Accurate serological and molecular tools are required for the detection of infected ruminants in heartwater endemic areas. The MAP1-B ELISA was already developed before the start of the project and work was carried out to further validate the test as described in chapter 2. The MAP1-B ELISA was developed to detect antibodies against a specific fragment of the MAP1 protein of *E. ruminantium* and was shown to limit cross-reactions with other *Ehrlichia* species (van Vliet et al., 1995). Cross-reactivity was still observed with *E. canis* and *E. chaffeensis,* but it was argued that since those species had never been isolated from domestic ruminants, cross-reactivity would not hamper heartwater serology (van Vliet et al., 1995). However, in chapter 3, it is shown that *E. ovina* is highly similar to both *E. canis* and *Ehrlichia* sp. (Germishuys) on the basis of 16S rRNA and all may even be the same organism capable of infecting several hosts. Furthermore, another *Ehrlichia* species closely related to *E. chaffeensis* was identified in white-tailed deer in the United States and shown to give cross-reactions in heartwater serology (Dawson et al., 1996; Katz et al., 1996; Katz et al., 1997). These findings show the limitation of the existing heartwater ELISA in its specificity and that more specific tests are necessary for future diagnostics.

Validation of the MAP1-B ELISA, as described in chapter 2, showed that the assay works very well in experimentally infected small ruminants, wherein antibodies against the MAP1-B antigen can be detected for prolonged periods. However, the test is less suitable for cattle because antibodies appear to be down regulated in cattle challenged with tick-transmitted heartwater (Semu et al., 2001). Although the MAP1-B antigen contains two of the three hypervariable domains present in the *map1* gene, all tested isolates thus far of *E. ruminantium* have been shown to develop antibodies against MAP1-B. However, it cannot be ruled out that other isolates of *E. ruminantium* in the field may not be detected.

Role in molecular detection

Research conducted on wild ungulates from Zimbabwe, showed that *E. ruminantium* DNA could be amplified in some instances from bone marrow, but not from blood samples using the *map1* gene as a target (Kock et al., 1995). It was also shown that carrier sheep

could be identified in some instances using this technique. Experiments comparing *map1* and the 16S rRNA gene as targets for detection should show if using *map1* can improve the detection sensitivity. Improved detection sensitivity using a *p30* (ortholog of *map1*) based PCR assay for the detection of *E. canis* was shown (Stich et al., 2002). The *p30*-based PCR assay was at least 100-fold more sensitive than a previously reported nested 16S ribosomal DNA-based assay and did not amplify the target amplicon from *E. chaffeensis, E. muris* or *A. phagocytophila*. Perhaps the detection sensitivity could be further improved by targeting the *map1-1* gene in a reverse transcription assay. The advantage of *map1-1* over *map1* is that the gene is highly conserved among different isolates of *E. ruminantium* (Sulsona et al., 1999). RT-PCR instead of PCR has been shown to be more sensitive in the detection of *E. chaffeensis* infection (Felek et al., 2001) and we have shown that *map1-1* is transcribed *in vivo* in ticks (chapter 4). Furthermore, preliminary data suggest that the gene is also transcribed *in vivo* in the mammalian host, at least during the clinical phase of the infection (unpublished results). Future research is required to confirm this and to determine whether *map1-1* is also transcribed in carrier animals.

Role in antigenic variation

The *E. ruminantium* MAP1 protein is encoded by a gene that is part of a multigene family of paralogs clustered together on the genome (Sulsona et al., 1999) (chapter 4). The presence of orthologous multigene families has been reported in the closely related species *E. canis* and *E. chaffeensis* (Ohashi et al., 1998; Ohashi et al., 1998; Reddy et al., 1998; Yu et al., 2000). In *E. chaffeensis* the complete multi-gene family was cloned and sequenced and shown to consist of 21 genes of which at least 6 were actively transcribed *in vitro* (Yu et al., 2000). A later study showed that 22 paralogs are present of which 16 were transcribed *in vitro* (Long et al., 2002). Amino acid sequence analysis indicated that P28-19 was produced *in vitro* in the Arkansas strain of *E. chaffeensis*. Therefore the *p28-19* gene and its promoter region were sequenced from 12 clinical isolates and only three different allele variants were found (Long et al., 2002) indicating that antigenic variations of the *E. chaffeensis* 28-kDa proteins may result from differential expression rather than gene conversion. In another study it was shown that *in vivo* 16 genes were transcribed in blood monocytes of dogs throughout a 56-day infection period (Unver et al., 2002). Although the same number of transcribed genes was found, there were differences in the genes that were transcribed. The *in vivo* study also showed that only one paralog (*omp-1B*) was transcribed by *E. chaffeensis* in three developmental stages of *Amblyomma americanum* ticks before or after *E. chaffeensis* transmission to naïve dogs (Unver et al., 2002). The multigene family of *p30* major outer membrane proteins for *E. canis* consists of 25 paralogs; 22 located on a stretch of 28 kb and a shorter stretch of DNA which contains a duplication of 3 of the *p30* genes (Ohashi et al., 2000). From these, 11 out of 14 were transcribed in blood monocytes of infected dogs during

the 56 days postinoculation period (Unver et al., 2001). As for *E. chaffeensis* in *A. americanum* ticks only one paralog (*p30-10*) was detected by RT-PCR in acquisition fed *Rhipicephalus sanguineus* ticks. Transcription of the same paralog was increased in *E. canis* cultivated in dog monocyte cell line DH82 at 25°C than in *E. canis* cultivated at 37°C (Unver et al., 2001). These results suggest that transcription of these paralogs is induced due to a lower temperature in ticks than in mammals. Interestingly, *omp-1B* (or *p28-14* in chapter 4) in *E. chaffeensis* and *p30-10* (or *p28-2* in chapter 4) in *E. canis* are the paralogs with the highest similarity to *map1-1* in *E. ruminantium*. As for *E. chaffeensis omp-1B* and *E. canis p30-10*, transcription of *map1-1* was found *in vivo* in *A. variegatum* ticks (chapter 4). Contrary to what was found *in vitro* for *E. canis p30-10*, we did not find transcription of *map1-1* when the cultures were incubated at 30°C instead of 37°C. Perhaps decreasing the temperature further to 25°C, as was used for *E. canis* cultures, would induce transcription of *map1-1 in vitro* in endothelial cells. In chapter 4 it was also shown that the *map1-1* gene is also transcribed in attenuated isolates of *E. ruminantium* grown in endothelial cells. It will be important to further study the possible relation between expression and attenuation.

The study of gene transcription for the *map1* gene family may also identify possible candidates for vaccine development as immunization of mice with a *map1* DNA vaccine protected 23-88% of the mice against lethal challenge, which killed 143 out of 144 control mice (Nyika et al., 1998). The same research group showed in a subsequent paper that boosting DNA vaccine-primed mice with recombinant MAP1 protein significantly augmented protection on homologous challenge (Nyika et al., 2002). However, the protective capacity of the *map1* gene will be fully appreciated if these findings can be reproduced in ruminants. Furthermore, cross-protection against heterologous isolates is another prerequisite, because MAP1 is polymorphic. Using *map1-1* instead of *map1* may circumvent this problem since it was shown that this gene is highly conserved among *E. ruminantium* isolates (Sulsona et al., 1999) (chapter 4). Furthermore, data presented in chapter 4 showed that the gene is transcribed *in vitro* in tick cells and *in vivo in A. variegatum* ticks. In addition to its potential as a vaccine *map1-1* could also be exploited as a target for diagnostic purposes.

The 16S rRNA gene

Role in taxonomy

The 16S rRNA sequence for several new *E. ruminantium* isolates (chapter 3), and the sequences determined for *Anaplasma* and *Ehrlichia* species (chapter 6), for which the 16S sequences was not available in Genbank, were used to reorganize the genera in the families *Rickettsiaceae* and *Anaplasmataceae* (chapter 5). Despite the new classification there is still a large number of unclassified/uncharacterized *Ehrlichia* species for which only a 16S rRNA sequence is available. Of particular interest is a species recently identified in a dog in south Africa (Allsopp and Allsopp, 2001). The dog presented with symptoms of canine ehrlichiosis but sequencing of the 16S rRNA gene revealed a sequence more closely related to *E. ruminantium* than to any other *Ehrlichia*. Further studies will have to show whether this species is infective for, or causes heartwater in, ruminants. Other examples of "species" that need further characterization are *Ehrlichia* sp. Bom Pastor (chapter 3) and *E. ovina* (chapter 6).

Ehrlichia sp. Bom Pastor was found to be highly similar to *Ehrlichia* sp. strain Omatjenne and very similar to *A. platys*. Since no further data are available on the target cells of these "species" or the host range we cannot exclude that they are variants of *A. platys*. Ongoing research using the RLB assay has shown hybridization to the *Ehrlichia* sp. Omatjenne probe in cattle in France (Corsica) (M-J. Gubbels, F. Jongejan and G. Uilenberg, unpublished observations, 2000). The fact that this species was not detected before and was isolated from apparently healthy cattle suggests that it is a nonpathogenic species able to infect different hosts.

The determined 16S rRNA sequence of *Ehrlichia ovina* showed a high similarity with *E. canis* and *Ehrlichia* sp. strain Germishuys (chapter 6). Since this "species" was shown to infect monocytes in sheep (Uilenberg, 1993) and *E. canis* infects monocytes in dogs (Ristic and Holland, 1993), *E. ovina* may be the same species as *E. canis*. A similar situation has been described for the *A. phagocytophila* group in which the former species *E. phagocytophila*, *E. equi* and the agent of human granulocytic ehrlichiosis are considered to be the same species occurring in different hosts. Cross protection studies or experimental infection of dogs with *E. ovina* will have to be conducted to confirm this hypothesis.

Role in detection and differentiation of *Anaplasma* and *Ehrlichia* species

When conduction research on heartwater in field situations one has to take into account the effect of other tick-borne infections occurring in the same animal. The translocation experiment described in chapter 3 showed that the experimental animals were infected with two other tick-borne parasites. From this and other research it has become clear that a test which could detect and differentiate various tick-borne infections would be of great value. In

chapter 6 the development of such a test using reverse line blot hybridization is described. It was shown that *E. ruminantium* could only be detected shortly before and during the febrile response, and shortly after the beginning of treatment. *Anaplasma* and other haemoparasites such as *Theileria* and *Babesia* spp., can be detected with the RLB throughout the course of experimental infections (Gubbels et al., 1999). The observed difference is most likely due to the fact that *Anaplasma*, *Theileria* and *Babesia* infect red blood cells, whereas *E. ruminantium* infects mainly endothelial cells and neutrophils. The number of circulating *E. ruminantium*-infected cells is apparently to low in carrier animals to be detected by the RLB. Also another assay, using pCS20 PCR as a tool to detect *E. ruminantium*, was shown to be very useful to detect infected ticks, but not carrier animals (Peter et al., 2000). Detection of *E. ruminantium* carrier animals remains a problem independent of which gene is used as a target as the organism mainly infects endothelial cells. Detection sensitivity in the closely related species *E. chaffeensis* was improved approximately 100 times when a nested reverse transcription (RT)-PCR based on the 16S rRNA gene was compared to a nested PCR for detection of *E. chaffeensis* in infected DH82 cells, experimentally infected dog peripheral blood mononuclear cells, or experimentally infected *Amblyomma americanum* tick samples (Felek et al., 2001). An added advantage of the RT-PCR detection is that positive results imply the presence of viable pathogens. However, as mentioned before, the use of RT-PCR for the detection of *E. ruminantium* can only work if samples can be obtained from carrier animals in which viable organisms are present.

The RLB blot has the advantage that it can simultaneously detect several *Ehrlichia* species in the same sample, as was shown in chapter 6 for the samples collected in Mozambique. A second advantage of the RLB is that it allows detection of uncharacterized/new *Anaplasma* and *Ehrlichia* species by the incorporation of a catchall probe. Perhaps in the future the RLB assay can also be used to determine the host on which ticks have been feeding. Cytochrome b has been used as a target to identify the zoonotic reservoir of Lyme borreliosis by analysis of the blood meal in *Ixodes ricinus* ticks (Kirstein and Gray, 1996). However, before this can be done the RLB has to be tested using field samples and proper controls need to be incorporated. Instead of using DNA from all species for which a probe is present on the blot, it would be more convenient to include one sample containing all species specific 16S DNA sequences (cloned into a vector) flanked by the primers used for amplification of individual samples. In order to be able to test if a sample contains *Ehrlichia* species, DNA will have to be extracted from the sample. It would be advisable to include an extraction control into each sample. To this end we already designed an extraction control in which a part of the 16S rRNA gene (not containing the variable V1 region in which the probes are located) is flanked by both the *Anaplasma/Ehrlichia* and *Babesia/Theileria* RLB primer sets cloned into the pGEMT-easy vector.

Future research

With the genomes of the Welgevonden and Gardel isolate of *E. ruminantium* nearly completely determined (Dominique Martinez and Basil Allsopp, personal communication) a wealth of data will become available for the research on heartwater. As shown for *map1-1* differential transcription of genes between tick and mammalian hosts is occurring. It will be important to study the transcription of *E. ruminantium* genes during the different developmental stages of the organism. Transcriptional differences between virulent and attenuated passages of the same isolate could indicate if certain genes are transcribed in virulent isolates but not after attenuation or vice versa. Indications for this were found for the transcription of *map1-1* (chapter 4). Transcriptional analysis of genes during the carrier status, if possible, could provide vital information on how the organism is able to cause a persistent infection in immuno-competent hosts.

The selection of candidate genes for recombinant vaccine development could also benefit form data on transcriptional analysis. The ultimate test for heartwater vaccines is a tick challenge in the field. Most research on the efficacy of heartwater vaccines have been conducted in animals that were challenged by inoculation of infective material rather than by infected ticks (Mahan et al., 1999). From research conducted in Guadeloupe (Camus and Barré, 1992) and from data based on four infections of sheep using infected ticks (data not published) it was found that the development of disease is often more severe than when sheep were infected with the same stock by using needle infection. One reason to explain this difference could be a difference in the number of bacteria that are used. Another reason could be that there is a difference in the proteins expressed in *E. ruminantium* during the different stages of the live cycle of *E. ruminantium*. It has been shown recently that *E. ruminantium* cultivated in a *Ixodes scapularis* tick cell line are different from those that develop in endothelial cells (Bell Sakyi et al., 2000). As it has now been shown to be possible to cultivate *E. ruminantium* in the vector *A. variegatum* cell line AVL/CTVM13 (Bell-Sakyi et al., 2000). This will allow transcriptional analysis of *E. ruminantium* genes in its invertebrate host without having to work with live ticks.

An interesting example of the difference in efficacy of a vaccine between needle and tick challenge was recently reported for *Borrelia burgdorferi*. Decorin-binding protein A (DbpA), which is expressed by *B. burgdorferi* during the mammalian phase of the infection, was protective in the murine model of Lyme borreliosis when immunized mice were challenged by needle inoculation intradermally or subcutaneously with *in vitro* cultivated spirochetes (Cassatt et al., 1998; Hagman et al., 1998; Hanson et al., 1998; Hagman et al., 2000). However, DbpA was not protective when immunized mice where challenged with *Ixodes scapularis* nymphs harboring virulent *B. burgdorferi*. The lack of protection correlated with the failure to detect DbpA on *B. burgdorferi* in ticks (Hagman et al., 2000).

Furthermore, the effect of tick bites on the immune system should also be considered. Tick feeding stimulates host immune response pathways involving antigen-presenting cells, cytokines, B-cells, T-cells, circulating antibodies, granulocytes, and an array of biological active molecules (Wikel, 1999; Willadsen and Jongejan, 1999). Tick-induced host immunosuppression facilitates blood meal acquisition and is an important factor in the transmission/establishment of for instance *B. burgdorferi* (Zeidner et al., 1996) but also for the establishment of dermatophilosis transmitted by *A. variegatum* ticks (Ambrose et al., 1999). Vaccination with *A. americanum* gut material has been shown to produce protection (Wikel et al., 1987). A vaccine against *Boophilus microplus* is already on the market (Willadsen et al., 1995; De Rose et al., 1999) and other vaccines against other tick species are being developed (Mulenga et al., 2000). Whether vaccination against *A. variegatum* is feasible and whether transmission of *E. ruminantium* may be adversely affected by it, remains to be shown and could be worth testing in future heartwater research.

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Summary

Ehrlichia ruminantium is a bacterium that can only multiple inside cells (obligate intracellular) of the host (small or large ruminants) or in the vector (ticks belonging to the family *Amblyomma*). Research conducted on this bacterium will therefore always have to take in account a role of the host cell the bacterium has infected. *Amblyomma* ticks have four different developmental stages: egg, larva, nymph and adult off which the larva and nymph each consume a blood meal on a host after which they develop into the next stage. After consumption of a blood meal the adult female tick lays eggs from which larvae will develop. The vertebrate host becomes infected when infected ticks (nymphs or adults) feed on it. When the host survives the infection it becomes a carrier. Ticks become infected with *E. ruminantium* when they feed on an infected host. The infection is transmitted from one developmental stage to the other except from adult to eggs for as far as is known now.

E. ruminantium can cause a disease which is known as heartwater. The name of the disease comes from the observation of excess fluid present in the pericard of diseased animals. Heartwater occurs in most African countries located south of the Sahara dessert and on some island in the Caribbean. There is a risk of the disease spreading to the American mainland and very recently the bacterium has been detected in *Amblyomma* ticks present on animals imported into the United States.

Detection of *E. ruminantium* is difficult, especially in carrier animals. Detection of *E. ruminantium* is usually done by the identification of the bacteria in endothelial cells of brain capillaries of diseased animals using brain crush smears. Detection of infection in animals that are still alive is either based on the detection of specific antibodies against immunogenic proteins (e.g. the MAP1-B ELISA) or based on the detection of bacterial DNA using PCR techniques. Both kind of diagnostic test have their limitations. Test based on the detection of antibodies raised against bacterial proteins are not suitable for early detection of infection since the immune response takes some time to develop. An added disadvantage to these tests is that they are not specific enough and give cross-reactions to closely related bacterial species. Test based on the detection of the bacteria itself have thus far not been sensitive enough to be able to consistently detect carrier animals.

Vaccination against heartwater is possible although no commercial vaccine is available at this moment. Vaccines used thus far either use live virulent bacteria, live attenuated bacteria (these bacteria don't kill susceptible animals but do trigger an immune response), or inactivated bacteria. The disadvantage of these vaccines is that they do not induce solid protection against all isolates of *E. ruminantium* but only induce protection against a limited number of isolates. It is clear from all this that new or improved diagnostic tests and vaccines are needed for the battle against heartwater. The purpose of the research described in this thesis was to molecularly characterize *E. ruminantium* and to develop new or improved diagnostic tests for the detection of *E. ruminantium*.

Chapter 2

In this chapter the before mentioned MAP1-B ELISA, which was already available at the start of the project, was further validated. Using a computer program which was specifically developed to validate ELISA test (TG-ROC analysis) a “cutoff ” (the point above which a sample is called positive) with equal sensitivity and specificity was established for sheep and goat using known positive and negative experimental sera. The TG-ROC method was compared with other methods of determining cutoff values that were found to give either higher sensitivity or specificity but never both.

Chapter 3

In Mozambique restocking programs were started after the civil war to repopulate the life stock in the south of the country. These programs included restocking goats from the north of the country to the south. The programs were hampered by high post-restocking mortality. It was suggested that heartwater was one of the causes of this post-restocking mortality. Two serological surveys, one in the north and one in the south, using the MAP1-B ELISA were conducted. These surveys showed a large difference in the sero-prevalence between the two regions suggesting a much higher exposure to *E. ruminantium* in the south. By carrying out a small restocking experiment in Mozambique, in which goats were moved from the north of the country to the south, we were able to confirm that *E. ruminantium* was one of the causes of post-restocking mortality. Next to *E. ruminantium* we also detected *Anaplasma* and another *Ehrlichia* species in these animals but it remains to be determined whether they play a role in the observed mortality.

Chapter 4

During the project it became clear that the *map1* gene, which codes for the protein on which the MAP1-B ELISA is based, might be part of a multigene family. In the closely related species *Ehrlichia canis* and *Ehrlichia chaffeensis* multigene families with homology to *map1* were detected whereas initial experiments in *E. ruminantium* showed that *map1* was a single copy gene. Further research revealed that, as for *E. canis* and *E. chaffeensis,* more *map1* genes (paralogs) were present clustered together on the genome. The presence of a multigene family of immunogenic proteins suggests a function of these proteins in evasion of the immune system of the host. One possibility to evade the immune system is by switching the transcription of these genes on and off in a regulated orderly fashion. Chapter 4 describes research conducted on three *map1* genes in different conditions. It was found that one of these genes was transcribed under certain conditions (in two tick cell lines and in *Amblyomma* ticks) whereas it was not transcribed in other conditions (in an endothelial cell line).

Chapter 5

This chapter describes the reorganization in the taxonomic classification of a number of bacterial species including the reclassification of *Cowdria ruminantium* into *Ehrlichia ruminantium*. The reorganization was necessary as the old classification, which was based upon morphological, ecological, epidemiological and clinical characteristics, were shown to be flawed by recent genetic analysis of 16S rRNA genes, *groESL* and surface protein genes.

Chapter 6

Chapter 6 describes the development of a diagnostic tool that enables the detection of *E. ruminantium* in both the host and the vector. This tool uses the reverse line blot technique that enables the simultaneous detection of several closely related bacterial species in one sample. Unfortunately, this new test, which includes species-specific probes for 8 different species next to a probe that will detect any *Anaplasma* or *Ehrlichia* species, was also not able to detect bacteria in carrier animals. However, it was possible to detect *E. ruminantium* in *Amblyomma* ticks that had been feeding on carrier animals using this new test. As no quantitative data for the detection limit are available yet, only positive results are interpretable at this stage.

Chapter 7

In chapter 7 the obtained results are discussed focusing on the role the *map1* multigene family and the 16S rRNA gene play in the detection and characterization of *E. ruminantium*. This chapter also contains some thoughts on the future of heartwater research.

Samenvatting

Ehrlichia ruminantium is een bacterie die zich alleen kan vermenigvuldigen in cellen van zowel de gastheer (kleine en grote herkauwers) als de vector (teken die behoren tot de familie *Amblyomma*). Onderzoek aan deze bacterie zal dus altijd inhouden dat er ook gekeken moet worden naar de invloed van de cellen waarin de bacterie leeft. De *Amblyomma* teken kennen vier levensstadia: ei, larf, nimf en volwassen waarvan de larf en de nimf elk een bloedmaal gebruiken op een gastheer en daarna overgaan naar het volgende stadium. Na een bloedmaal van de volwassen teek legt het vrouwtje de eitjes waaruit de larven voortkomen. De gastheer raakt geïnfecteerd zodra geïnfecteerde teken (nimf of volwassen) zich voeden op de gastheer. Als de gastheer de infectie overleeft blijft hij drager van de bacterie en infectieus voor teken. Teken op hun beurt raken geïnfecteerd bij het voeden op geïnfecteerde gastheren. Bij het vervellen naar het volgende stadium blijft de teek zijn infectiviteit voor de gastheer behouden behalve bij de overgang van volwassene naar ei / larf.

E. ruminantium is de veroorzaker van de ziekte met de naam heartwater. De ziekte dankt haar naam aan vocht dat aangetroffen wordt in het hartzakje bij dieren die zijn overleden. Heartwater is een ziekte die voorkomt in de meeste Afrikaanse landen ten zuiden van de Sahara en op enkele eilanden in het Caribische gebied. Het gevaar bestaat dat de ziekte zich verspreidt naar het Amerikaanse vasteland. Zeer recentelijk zijn geïnfecteerde *Amblyomma* teken aangetroffen op geïmporteerde dieren in de Verenigde Staten.

Het aantonen van *E. ruminantium* is moeilijk, met name in dragers. Aantonen van *E. ruminantium* gebeurt meestal na de dood door het maken van hersenuitstrijkjes en deze te inspecteren op het aanwezig zijn van bacteriën in endotheelcellen van capillairen. Het aantonen van de infectie in nog levende dieren gebeurt met diagnostische testen die gebaseerd zijn op het aantonen van antilichamen (b.v. de MAP1-B ELISA) of het aantonen van de ziekteverwekker zelf (PCR). Beide soorten diagnostische test hebben hun beperkingen. Het aantonen van antilichamen tegen de bacterie kan alleen nadat een immuunreactie is opgewekt tegen eiwitten van de bacterie en is niet geschikt om het eerste stadium van infectie aan te tonen. Een bijkomend nadeel is dat deze testen niet specifiek genoeg zijn en ook reageren met andere dan *E. ruminantium* antilichamen. Testen die gebaseerd zijn op het aantonen van de ziekteverwekker zelf zijn tot nu toe niet gevoelig genoeg om dragers aan te tonen.

Vaccinatie tegen heartwater is mogelijk maar er is nog geen commercieel vaccine beschikbaar. Vaccinatie is mogelijk door gebruik te maken van levende bacteriën, levende afgezwakte bacteriën (deze bacteriën zijn niet meer dodelijk voor de gastheer maar induceren nog wel een immuunreactie) of geïnactiveerde bacteriën. Het nadeel bij deze vaccines is dat de opgewekte bescherming niet beschermt tegen alle *E. ruminantium* isolaten maar slechts tegen een beperkt aantal. Uit het bovenstaande wordt duidelijk dat nieuwe en betere testen en vaccines nodig zijn om de strijd aan te gaan tegen heartwater. Het doel van het onderzoek

beschreven in dit proefschrift was het moleculair karakteriseren van E. ruminantium en het ontwikkelen van detectie testen.

Hoofdstuk 2

In dit hoofdstuk wordt de verdere validatie van een bij aanvang van het onderzoek reeds bestaande test, de *E. ruminantium* MAP1-B ELISA, beschreven. Door gebruik te maken van een speciaal ontwikkeld computerprogramma (TG-ROC analyse) kon de "cutoff" (het punt waarboven een monster positief is) met gelijke sensitiviteit en specificiteit bepaald worden voor schapen en geiten. Tevens werd een vergelijking gemaakt tussen deze TG-ROC methode en andere methoden om te komen tot het bepalen van de "cutoff ". De andere methoden gaven soms een hogere sensitiviteit of specificiteit maar nooit beide.

Hoofdstuk 3

In Mozambique werd na de burgeroorlog een start gemaakt met het aanvullen van de veestapel in het zuiden van het land, o.a. door geiten vanuit het noorden te verplaatsen naar het zuiden. Eenmaal uitgezet in het zuiden gingen veel dieren dood aan ziekten. Het werd verondersteld dat heartwater één van de veroorzakers was van deze sterfte. Door zelf op kleine schaal in Mozambique geiten uit het noorden van het land te verplaatsen naar het zuiden van het land kon worden aangetoond dat *E. ruminantium* inderdaad een van de veroorzakers was van sterfte onder deze dieren. Tevens werd, gebruik makend van de ELISA beschreven in hoofdstuk 2, epidemiologische onderzoek gedaan in zowel het zuiden als het noorden van het land naar het voorkomen van antilichamen tegen *E. ruminantium*.

Hoofdstuk 4

Tijdens het voortgaande onderzoek bleek dat van het gen dat codeert voor het eiwit waarvan gebruik wordt gemaakt in de ELISA (het *map1* gen), meerdere vormen (ookwel paralogs genoemd) aanwezig zijn op het *E. ruminantium* genoom. Bij verdere bestudering bleken deze genen in een cluster bij elkaar te liggen. Een zelfde organisatie werd ook al gevonden bij twee nauw verwante bacterie soorten. De aanwezigheid van een multigen familie van immunogene eiwitten suggereert een rol bij het omzeilen van het immuun systeem van de gastheer. Een van de mogelijkheden tot het omzeilen van het immuun systeem is het gefaseerd aan- en uitschakelen van het afschrijven (transcriptie) van oppervlakte eiwitten. Hoofdstuk 5 beschrijft onderzoek naar de transcriptie van 3 *map1* paralogs onder verschillende omstandigheden. Hierbij werd gevonden dat één van de onderzochte genen in het ene geval (in een teken cellijn en in teken zelf) wel en in het andere geval (in een endotheel cellijn) niet werd afgeschreven.

Hoofdstuk 5

In dit hoodstuk wordt de herorganisering van de taxonomische classificatie van een aantal bacteria soorten, waaronder de herbenoeming van *Cowdria ruminantium* naar *Ehrlichia ruminantium*, beschreven. Deze herorganisatie was nodig omdat uit recente genetische analyse van 16S rRNA, *groESL* en oppervlakte eiwitten genen bleek dat de oude classificatie, gebaseerd op morphologische, ecologische en klinische kenmerken, niet meer klopte.

Hoofdstuk 6

Hoofdstuk 6 beschrijft de ontwikkeling van een nieuwe diagnostische test die het mogelijk maakt om in zowel de gastheer als de vector *E. ruminantium* aan te tonen. Deze test maakt gebruik van de zogeheten reverse line blot techniek die het mogelijk maakt om tegelijkertijd in één monster de aanwezigheid van verschillende, nauw verwante, bacteriesoorten aan te tonen. Deze nieuwe test bleek helaas niet in staat om dragers van *E. ruminantium* aan te tonen. Met deze test bleek het wel mogelijk om in teken die zich op dragers gevoed hadden de bacterie aan te tonen. Omdat op het moment nog geen kwantitatieve data voor de detectie limit van deze test beschikbaar zijn zeggen alleen positieve resultaten iets.

Hoofdstuk 7

Ter afsluitingen worden in hoofdstuk 7 de verkregen resultaten samengevat en bediscussieerd. Tevens wordt aan het eind een aantal gedachten over de richting van toekomst heartwater onderzoek weergegeven.

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Curriculum Vitae

Cornelis Bekker werd geboren op 28 februari 1972 in Ede. In 1990 werd het VWO diploma behaald aan het Christelijk Streeklyceum te Ede (tegenwoordig Het Streek). In hetzelfde jaar werd begonnen met de studie Medische Biologie aan de Universiteit Utrecht. Het doctoraal examen werd gehaald in april 1996 met als afstudeer vakken virologie (Infectieziekten & Immunologie, Diergeneeskunde, Utrecht) en signaal transductie (Fysiologische Chemie, Geneeskunde, Utrecht). Vanaf juni 1996 was hij aangesteld als assistent in opleiding bij de afdeling bacteriologie en vanaf januari 2001 bij de afdeling Parasitologie en Tropische Diergeneeskunde van de hoofdafdeling Infectieziekten en Immunologie van de Faculteit Diergeneeskunde, Universiteit Utrecht en werd het onderzoek zoals beschreven in dit proefschrift uitgevoerd.

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ik heb het zelf uit zijnen mond gehoord.
'k Vertrouw op God, door gene vrees gestoord,
wat sterv'ling zou mij schenden?
Ik heb beloofd, wanneer G'in mijn ellenden
mij bijstand boodt, en t'onheil af zoudt wenden,
tot U o God, mijn lofzang op te zenden,
door ijver aangespoord.