Chapter 13
Ehrlichia ruminantium: The Causal Agent of Heartwater

Isabel Marcelino, Philippe Holzmuller, Frédéric Stachurski, Valérie Rodrigues, and Nathalie Vachiéry

1 Introduction

The *Rickettsiales Ehrlichia ruminantium* causes heartwater, an infectious, non-contagious tropical disease of ruminants. Heartwater (also called cowdriosis) is one of the most important tick-borne diseases of livestock in Africa (Vachiéry et al. 2014;), being a notifiable disease listed by the World Organization for Animal Health (Allsopp 2015).

Heartwater occurs wherever the *Amblyomma* spp. ticks are present, i.e. in nearly all the sub-Saharan countries of Africa and in the surrounding islands Madagascar, La Réunion, Mauritius, Zanzibar, the Comoros Islands, The Cap Vert and São Tomé. The disease is also reported in the Caribbean (Guadeloupe and Antigua), from where it threatens the American mainland (Vachiery et al. 2008a; Molia et al. 2008; Kasari et al. 2010; Vachiéry et al. 2013). It belongs to the 12th most important animal transboundary diseases listed by the US Homeland Security department for American mainland (Roth et al. 2013).

All domestic and wild ruminants can be infected, but the former appears to be the most susceptible. Non-indigenous ruminants that are moved into affected areas are particularly sensitive to heartwater, and mortality rates up to 90 % are observed. In enzootic areas, indigenous cattle, less infested by the vector ticks than introduced animals, have developed resistance to heartwater (Minjauw and McLeod 2003). For instance, in heartwater enzootic areas in southern Africa, it is estimated that mortalities due to the disease are more than double to those due to

I. Marcelino (✉) • N. Vachiéry
CIRAD, UMR CMAEE, 97170 Petit-Bourg, Guadeloupe, France

INRA, UMR1309 CMAEE, 34398 Montpellier, France
e-mail: isabel_m31@hotmail.com

P. Holzmuller • F. Stachurski • V. Rodrigues
CIRAD, UMR CMAEE, 34398 Montpellier, France

S. Thomas (ed.), *Rickettsiales*, DOI 10.1007/978-3-319-46859-4_13

other tick borne diseases such as bovine babesiosis (mostly caused by *Babesia bovis* and *B. bigemina*) and bovine anaplasmosis (caused by the *Rickettsiales Anaplasma marginale*). In 2005, human and canine cases potentially due to *E. ruminantium* were reported in South Africa (Allsopp and Allsopp 2001; Allsopp et al. 2005b), and since then *E. ruminantium* infection has been considered as a potential zoonosis (Chitanga et al. 2014) although any other human case has been reported until now.

The average natural incubation period is 2–3 weeks, but it can vary from 10 days to 1 month. In most cases, heartwater is an acute febrile disease, with a sudden rise in body temperature. The most common macroscopic lesions are hydropericardium, hydrothorax and pulmonary oedema, giving the name "heartwater" to the disease. A clinical diagnosis of the disease is based on the presence of *Amblyomma* spp. ticks on the animals or in the environment, nervous signs, and presence of transudates in the pericardium and thorax on post-mortem examination. Nowadays, efficient molecular diagnostics are available for a reliable diagnosis.

Heartwater control can be achieved using several strategies such as vector chemical control, treatment of animals, chemoprophylaxis and vaccination. Currently, four vaccine strategies against heartwater have been developed: the "infection and treatment method" using live bacteria followed by antibiotherapy, immunization by infecting animals with in vitro attenuated bacteria (Jongejan et al. 1993; Jongejan 1991; Faburay et al. 2007; Zweygarth et al. 2005), immunization with inactivated in vitro grown bacteria (Martinez et al. 1994; Vachiery et al. 2006; Maass and Dalhoff 1995; Marcelino et al. 2007, 2015a) and recombinant vaccines (Simbi et al. 2006; Sebatjane et al. 2010; Pretorius et al. 2002). Nevertheless, the problems caused by high genetic and phenotypic diversity shown in restricted areas still remain, hampering the development of a fully effective vaccine for widespread application (Allsopp and Allsopp 2007; Barbet et al. 2009). Moreover, the knowledge of immune response modulation during heartwater is still limited.

To develop improved therapeutics, it is thus fundamental to increase knowledge on *E. ruminantium* biology and pathogenesis. The complete genome sequences of three *E. ruminantium* strains are currently available (Frutos et al. 2006b; Collins et al. 2005), but at the moment there is no method available for the genetic manipulation of this bacterium and little is currently known on whether specific genes are actually expressed in living organisms. For this reason, additional research on heartwater disease is essential. Currently, global and integrative high-throughput approaches such as functional genomics including transcriptomics and proteomics are being used to increase the knowledge on *E. ruminantium* biology in the frame of bacteria–host–vector interactions.

This book chapter aims at providing a state-of-the-art in the epidemiology, development of vaccine and immunology of heartwater and to give updated insights in the biology of the bacterium *E. ruminantium*.

2 The Etiologic Agent of Heartwater: *Ehrlichia ruminantium*

2.1 Classification

The causal organism of heartwater is an obligate intracellular bacterium, previously known as *Cowdria ruminantium* (Cowdry 1925, 1926). In 2001, Dumler and co-workers defined after *16S ribosomal DNA* and *groESL heat shock operon* genes comparisons that all members of the tribes *Ehrlichieae* and *Wolbachieae* had to be transferred to the family *Anaplasmataceae* and that the family *Rickettsiaceae* had to be eliminated (Dumler et al. 2001). Molecular evidence led to reclassification of several organisms in the order *Rickettsiales*, the causal agent of heartwater being now classified as *Ehrlichia ruminantium* (Dumler et al. 2001). In 2013, the order *Rickettsiales* was reorganized through 16S and 23S gene comparisons and now it includes the families *Rickettsiaceae*, *Anaplasmataceae* and *Midichloriaceae* (Ferla et al. 2013). The *Anaplasmataceae* family still includes the four genera *Ehrlichia*, *Anaplasma*, *Wolbachia* and *Neorickettsia*. The genus *Ehrlichia* includes *E. ruminantium*, *E. chaffeensis, E. canis* and *E. muris* while the genus *Anaplasma* contains *Anaplasma bovis* (formerly *E. bovis*), *A. marginale*, *A. centrale, A. platys* (formerly *E. platys*) and *A. phagocytophilum* (formerly *E. phagocytophila*), and the genus *Neorickettsia* includes now the formerly named *E. sennetsu* and *E. risticii* (Dunning Hotopp et al. 2006; Dumler et al. 2001; Rikihisa 2010). The genus *Wolbachia* includes *Wolbachia pipientis* (Fig. 13.1).

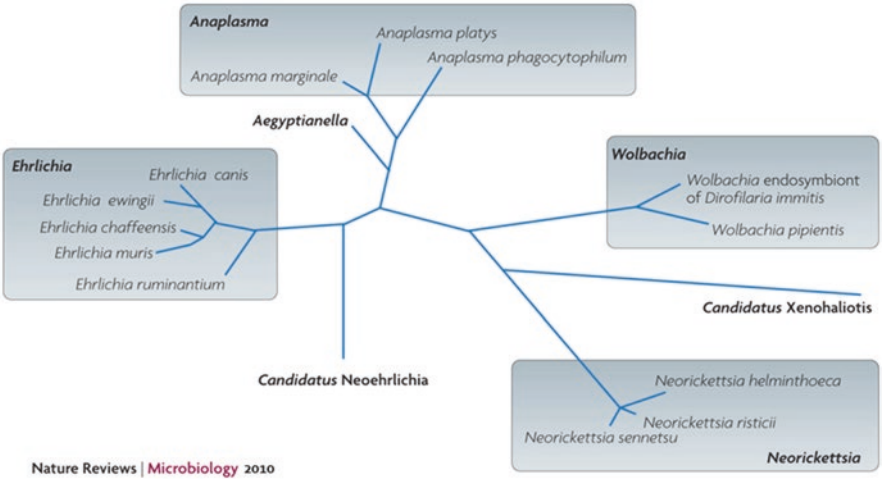

Fig. 13.1 Phylogram of the Family *Anaplasmataceae*. This phylogram is constructed based on 16S rRNA sequences of these species. Family *Anaplasmataceae* contains four genera: *Ehrlichia, Anaplasma, Neorickettsia*, and *Wolbachia* according to Dumler and co-workers (Dumler et al. 2001) and as reviewed by Rikihisa (2010))

2.2 Ehrlichia ruminantium *Colonies Morphology In Vivo and In Vitro*

Histopathological examination of brain smears reveals variable numbers of *E. ruminantium* colonies discernible in the cytoplasm of capillary endothelial cells after Giemsa staining (Fig. 13.2A); they can also be observed in lung smears (Van de Pypekamp and Prozesky 1987; Prozesky 1987b; Van Amstel et al. 1987).

A detailed characterization of *E. ruminantium* morphology in mammalian host cells was possible in 1985, when the first in vitro cultivation of the organism in a calf endothelial cell line was described (Bezuidenhout et al. 1985). Electron microscopy reveals two morphologically distinct forms: one that develops within membrane-bound vacuoles reticulate bodies (RB or reticulate cells, RC) forming colonies called morula, and that differentiate into the free infectious forms (elementary bodies, EB) (Jongejan et al. 1991). Morula are arranged in grapefruit and close to the nucleus inside endothelial cells; they are dark purple when coloured with Giemsa whereas elementary bodies are bright pink outside lysed cells (Fig. 13.2B).

Currently, the organism is propagated in vitro most reliably not only in endothelial cells (from cattle, sheep, goats, wild African mammals (Smith et al. 1998), human (Totte et al. 1993) and murine origins) but also in primary neutrophil cultures and macrophage cell lines (Yunker 1995). Several studies also showed that *E. ruminantium* is able to infect tick cell lines (Bell-Sakyi et al. 2000; Bell-Sakyi 2004) as well as cells from non-endothelial origin such as Chinese Hamster Ovary cells (CHO) and Baby Hamster Kidney Cells (BHK) (Zweygarth and Josemans 2001, 2003). These cultures are generally performed in small tissue culture systems but *E. ruminantium* can also be cultured in stirred tanks using microcarriers for large-scale production (Totte et al. 1993; Pedregal et al. 2008; Marcelino et al. 2006). *E. ruminantium* can also be propagated experimentally by inoculating infected blood from reacting animals or infected tick homogenate into a susceptible animal. Blood from the infected animal is then collected during hyperthermia and incubated in vitro on endothelial cells. *E. ruminantium* isolation from blood can take several weeks and several media exchanges must be performed to stimulate bacterial growth (Marcelino et al. 2005, 2006).

3 *Ehrlichia ruminantium* Biology

3.1 Ehrlichia ruminantium *Tick Transmission*

Heartwater has been transmitted experimentally by 12 species of *Amblyomma* ticks: *A. variegatum, A. hebraeum*, *A. pomposum*, *A. gemma*, *A. lepidum*, *A. tholloni*, *A. sparsum*, *A. astrion*, *A. cohaerens*, and *A. marmoreum*, *A. maculatum* and *A. cajennense* (Bezuidenhout and Bigalke 1987). *A. variegatum* is the most important heartwater vector with a worldwide distribution, followed by *A. hebraeum* which is only present in southern Africa. Five species (*A. tholloni*, *A. sparsum*, *A. gemma*,

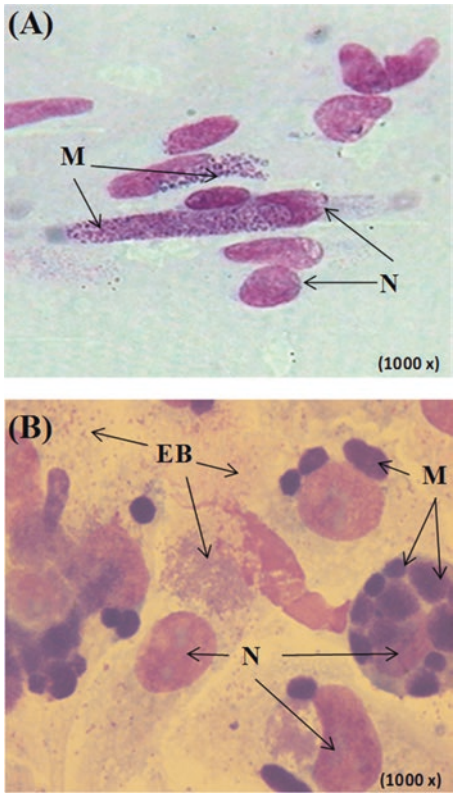

Fig. 13.2 *Ehrlichia ruminantium* colonies morphology (**A**) in vivo (brain smear from heartwater-infected goat stained with Giemsa showing numerous colonies of *E. ruminantium*) and (**B**) in vitro (endothelial cell monolayer stained with RAL 555 showing morula and infectious extracellular bacterium, after host cell lysis). *N* stands for nucleus of endothelial cells, *M* for morula (*E.ruminantium* colonies inside the host cell containing the intracellular form of the bacterium, the reticulate bodies) and *EB* for extracellular infectious *E. ruminantium* elementary bodies. (CIRAD photos: Nathalie Vachiéry and Isabel Marcelino)

A. cohaerens and *A. marmoreum*) have not been implicated in field outbreaks either because they are confined to forest areas or because of their host preference (Martinez 1997). The presence of *A. maculatum* and *A. cajennense* in the Americas threatens the American mainland from the introduction of the disease (Vachiery et al. 2013). In the Caribbean, *A. variegatum* is also known as the Senegalese tick (Barre et al. 1995) and the Antigua gold tick (Pegram et al. 2004).

Amblyomma spp. ticks are three-host ticks (one host *per* developmental stage and moulting or egg laying on the ground after engorgement) (Fig. 13.3). There is no trans-ovary transmission of *E. ruminantium*, however there is transstadial transmission. *Amblyomma* ticks become infected during the larval and/or nymphal stages when they feed on heartwater-infected domestic and wild ruminants (Martinez 1997). *Amblyomma* spp. are vividly coloured and decorated ticks, especially the males (Fig. 13.4). Different stages of *A. variegatum* ticks are shown in Fig. 13.4. In tropical regions, the life cycle of *Amblyomma* ticks shows important seasonal variations: adults infest their hosts during the rainy season, with a peak at the beginning of the season; larvae infest their hosts at the end of this rainy season and disappear rapidly from the environment, because of high sensitivity to desiccation, as soon as the rains cease; and nymphs attach to their hosts mainly at the beginning of the dry season. Heartwater is therefore a seasonal disease in these areas, observed mainly at the beginning of the rainy and dry seasons. In regions with equatorial climate, i.e. where the dry

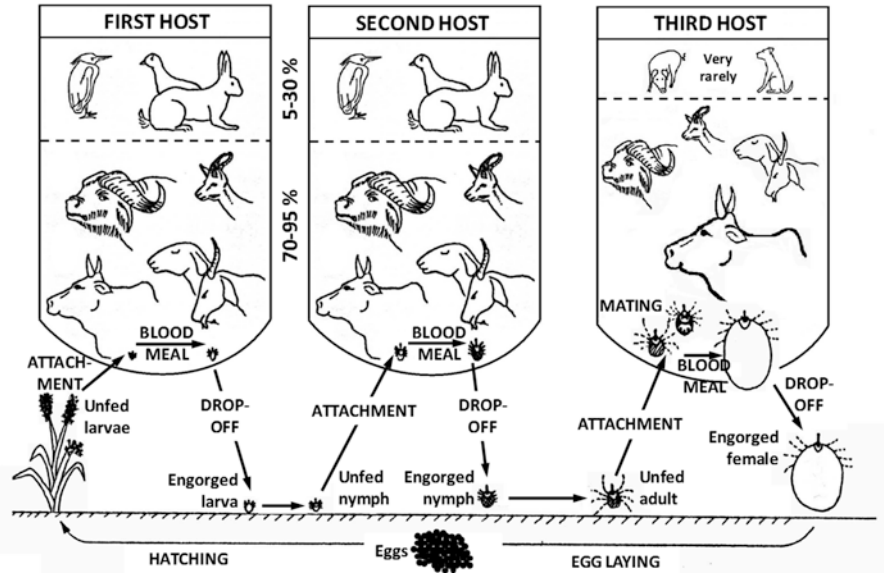

Fig. 13.3 Life cycle of a typical three-host tick (such as *Amblyomma variegatum*) feeding on three separate individual cows (by Frédéric Stachurski)

season is short or nonexistent, nymphs and adults may infest their hosts, and therefore transmit heartwater, all the year round. Such a situation is observed in French West Indies, but also in the humid and coastal areas of African countries and Madagascar.

The minimum period required for transmission of the parasite after tick attachment is between 27 and 38 h in nymphs and 21–75 h in adults (Bezuidenhout 1988), the pathogen being generally transmitted only after 3–4 days of attachment. Almost 100 % larvae or nymphs feeding on a clinically affected ruminant can acquire infection, which does not seem to lead to mortality or reduced survival of the ticks. After natural recovery or treatment, ruminants can become reservoir of *E. ruminantium* for months but the presence of the bacterium in the blood is not permanent; only part of the infesting ticks can pick up the bacterium from these animals.

Unfed nymphs sampled in the environment have an infection rate of 3 % whereas that of unfed adults is 8–20 %, according to different studies and regions (Mahan et al. 1998b). This allows the regular infection of cows and, therefore, the maintenance of enzootic stability when tick control is not drastically implemented. It has actually been demonstrated that regular infection of cows allows the vertical transmission of *E. ruminantium* to calves in utero (Deem et al. 1996). Calves can thus acquire early infection either by their dam, either by rapid infection by nymph or adults ticks, for those born during the adequate infestation periods. These infections, occurring when calves are protected by passive immunity provided by colostrum, enable early development of active immunity and persistence of enzootic stability.

On the contrary, infestation level of small ruminants by adult ticks is too low to enable systematic acquisition of immune protection by lambs and goat kids born during the rainy season. As nymphs infest their hosts only during a few months, at

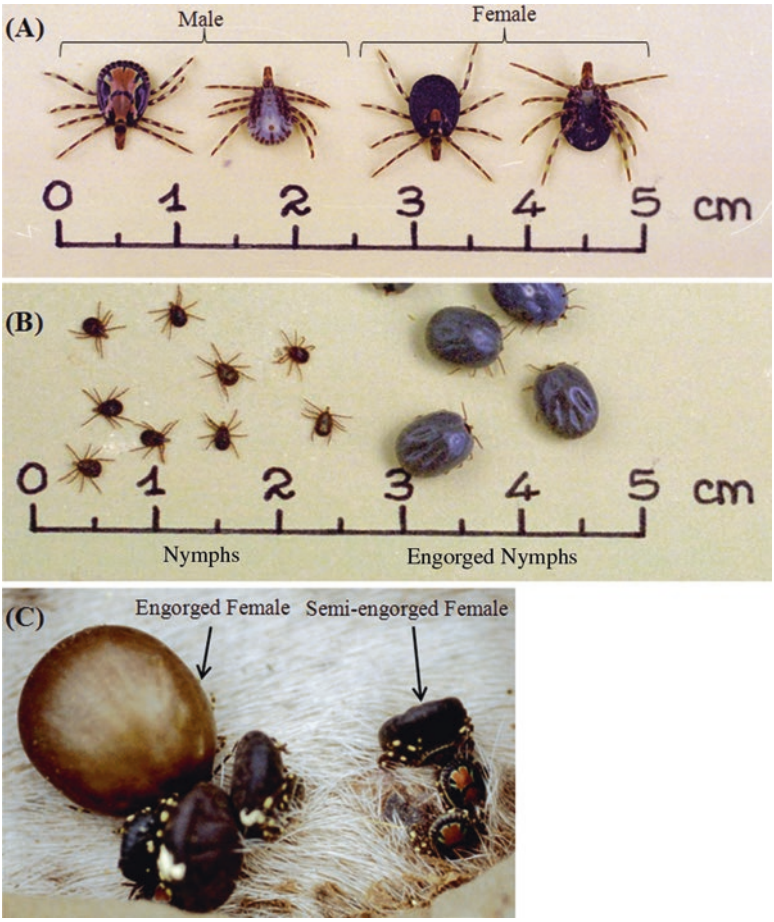

Fig. 13.4 The vector *Amblyomma variegatum*. (**A**) Male (dorsal and ventral positions) and female ticks (dorsal and ventral positions), (**B**) unengorged and engorged nymphs, (**C**) male and female on animals (CIRAD pictures: Frédéric Stachurski). The ruler aims at showing the large difference in size between *A. variegatum* nymphs and adults

the beginning of the dry season, in tropical areas, small ruminants born at other periods do not have the occasion to acquire active immune protection. They thus have no enzootic stability, which explain why local goats and sheep suffer mortalities due to heartwater, unlike local cattle breeds reared under traditional systems.

3.2 The Developmental Cycle of E. ruminantium

As above mentioned, *E. ruminantium* has a biphasic developmental cycle in host mammalian cells with two morphologically distinct forms, the extracellular infectious elementary body (EB) and the intracellular metabolically active reticulate

body (RB) (Fig. 13.2B). EBs are small (0.2–0.5 μm in diameter) and, after cell colonization, they reside within intracytoplasmic inclusions where they convert into the larger (0.75–2.5 μm) non-infectious RBs (Prozesky 1987a; Marcelino et al. 2005). The RBs multiply by binary fission, rapidly filling the inclusion (named morula), which expands in size (Prozesky 1987a). RBs re-condense back into EBs towards the end of the cycle and are then released from the host cell (Figs. 13.5 and 13.6). Microscopic observation of in vitro-cultivated *E. ruminantium* demonstrated the

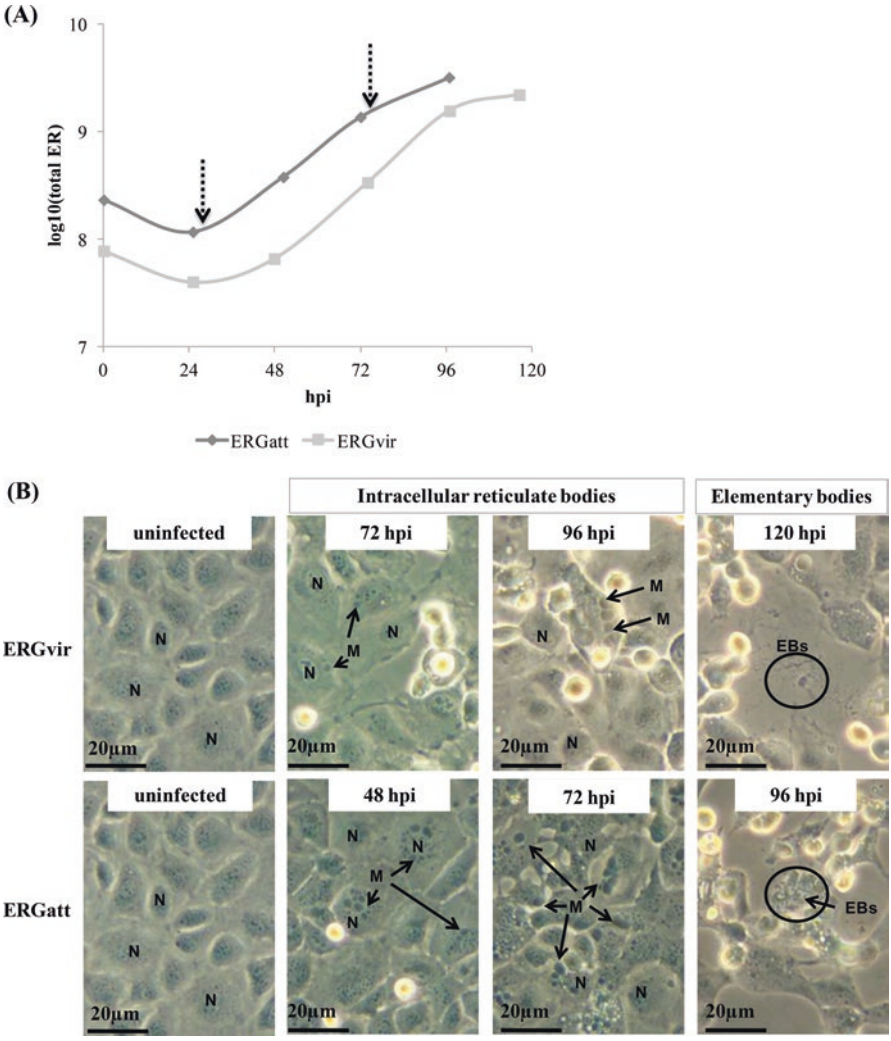

Fig. 13.5 Representative growth kinetics of *E. ruminantium* Gardel virulent (ERGvir) and attenuated (ERGatt) strains obtained by (**A**) real-time PCR targeting *map-1* gene (*dashed arrows* represent the time of total medium exchange) and (**B**) reverse phase microscopy (N stands for host cell nucleus, M for morula and EBs for elementary bodies) (Marcelino et al. 2015b)

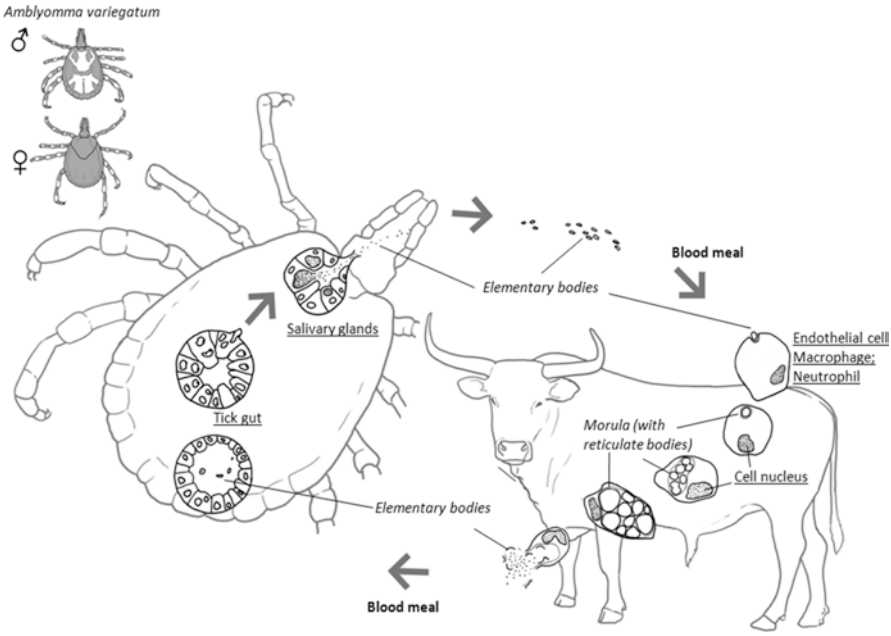

Fig. 13.6 *Ehrlichia ruminantium* life cycle (Marcelino et al., 2012b)

presence of intracellular RBs 2–4 days after infection (Marcelino et al. 2005, 2015b) (Fig. 13.5). A high number of EBs are observed after rupture of endothelial cells 5–6 days after infection (Jongejan et al. 1991; Moumene and Meyer 2015b; Marcelino et al. 2005) (Fig. 13.5). The relation between the stage of development and time post-infection depends on the strain and its adaptation to in vitro conditions. For instance, the virulent *E. ruminantium* Gardel strain (isolated in Guadeloupe, FWI) lyses 5 days post-infection whereas the attenuated Gardel phenotype lyses after 4 days (Marcelino et al. 2015b) (Fig. 13.5). In culture, EBs lose their infectivity within a few hours (Marcelino et al. 2005), but the organism, together with suitable cryoprotectants, may be viably preserved in liquid nitrogen for years (Marcelino et al. 2007; Brayton et al. 2003; Vachiery et al. 2006).

E. ruminantium developmental cycle and its infectivity within the tick are poorly understood. Transmission of the bacterium from the host to the vector occurs during an infected blood meal; *E. ruminantium,* present in the blood of the ruminant, initially develops in the gut epithelial cells of the attached tick and subsequently invades the salivary glands (Fig. 13.6). After attachment of the next tick stage to a new host, *E. ruminantium* develops and multiplies during few days in the salivary glands before being injected to the host via saliva during the blood meal. A single infected nymph or adult is able to cause the disease because of high multiplication of *E. ruminantium* in acini of salivary glands during the first phase of tick blood meal. More generally, tick saliva is thought to play a major role in the transmission of the bacterium from the vector to the vertebrate. Indeed, as other

ixodid ticks, *Amblyomma* spp. secrete a cocktail of immunomodulatory molecules in their saliva during blood-feeding that help to control the activity of host immunocompetent cells and, as a consequence, favour the establishment of *E. ruminantium* in the host target cells. One well described process is the neutralization of host cellular communication through the binding of specific saliva molecules to cytokines that have important roles in innate and adaptive immunity. A pioneering study focused on modulation of interleukin (IL)-8 that plays a critical role in inflammatory processes, and demonstrated that tick salivary gland molecules are able to bind to IL-8 preventing binding of the chemokine to its specific receptor (Kocakova et al. 2003). Further analyses demonstrated that tick saliva targets different cytokines providing a gateway for tick-borne pathogens that helps explain why ticks are such efficient and effective disease vectors. In particular, anti-IL-2, IL-4, IL-8 (CXCL8), MCP-1 (CCL2), MIP-1 (CCL3), RANTES (CCL5) and eotaxin (CCL11) activities were evidenced in *A. variegatum* salivary gland extracts (Hajnicka et al. 2005; Vancova et al. 2007, 2010a; Peterkova et al. 2008). Interestingly, the level of anticytokine activity depends on the species, developmental stage (adult or nymph), as well as on the number of days the tick has been feeding (Vancova et al. 2010b). The local immunodepression induced by tick saliva will probably indirectly help the initial multiplication of the bacteria, which probably takes place in reticulo-endothelial cells and macrophages in the lymph nodes draining the tick biting site. From here, the bacteria are disseminated via the blood stream to invade endothelial cells of blood vessels of various organs where further multiplication occurs (Du Plessis 1970).

4 Epidemiology

4.1 Heartwater Geographical Distribution

Heartwater is present in sub-Saharan Africa, the Comoros islands, including Mayotte, Madagascar, and the Mascarenes, La Réunion and Mauritius, where the major vector, *A. variegatum*, is established (Fig. 13.7). Countries like Lesotho, Somalia, southern Angola, Botswana, Namibia, and western and south-central South Africa have not been threatened by heartwater since their climate is unsuitable for *Amblyomma* ticks (Yunker 1996). *A. variegatum* is also present in the Caribbean islands, and heartwater is endemic in Guadeloupe, Marie Galante and Antigua. According to OIE, global geographical areas with reported cases of heartwater see their size decreasing since 2010 (http://www.oie.int/wahis_2/public/wahid.php/Diseaseinformation/Diseasedistributionmap) (Fig. 13.7).

As *A. variegatum* proved to be able to establish itself after accidental introduction in areas where climate is suitable and where hosts of adults, i.e. ruminants, and mainly cattle, are present, like the islands of Ocean or Atlantic oceans, there is fear that infected ticks could be introduced, by various means, into the American mainland where it could settle. Other areas, in tropical Asia or in north-east Australia for example, would also be suitable for *A. variegatum* (Barre et al. 2010).

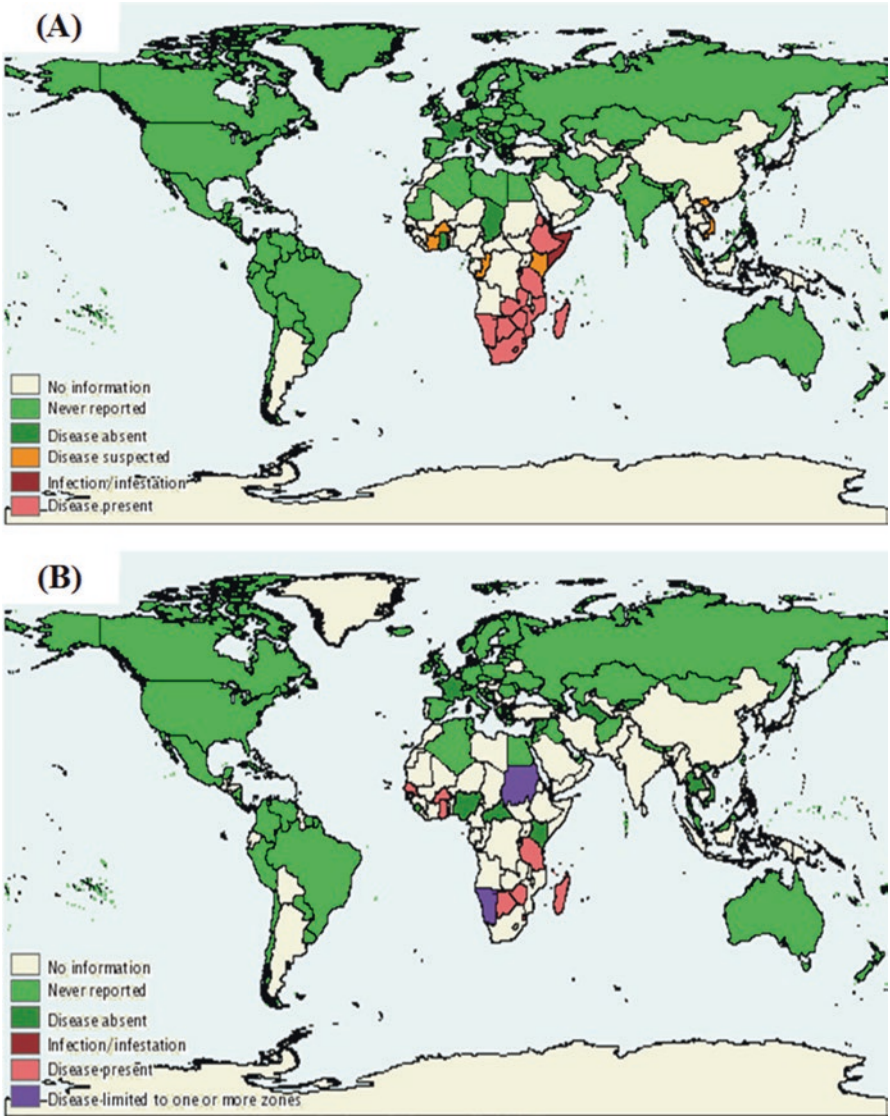

Fig. 13.7 Geographical distribution of Heartwater in the world (Source WAHID—OIE). (**a**) January to July 2010 and (**b**) January to July 2015

A. variegatum was introduced from West Africa into Guadeloupe and Martinique at the end of the eighteenth century and spread to Antigua in the nineteenth. Further spreading to other Lesser Antilles islands occurred at mid-twentieth century together with the spread of an erratic bird, the cattle egret, *Bubulcus ibis* (Barre and Uilenberg 2010). To control tick spreading in the Caribbean and reduce introduction danger to American mainland, several eradication programmes were created. From 1994 to 2008, the Caribbean *Amblyomma* programme (CAP) was implemented in the

English Lesser Antilles (Anguilla, Antigua and Barbuda, Barbados, Dominica, Montserrat, Nevis, St Kitts, St Lucia and Saint Maarten) to eradicate *Amblyomma* ticks (Ahoussou et al. 2010; Pegram et al. 2004). It allowed a decrease of tick infestation on six islands at the end of the project: Sainte Lucia, Saint Kitts, Montserrat, Anguilla, Dominica and Barbados. Currently, four islands have rare *Amblyomma* ticks (Saint Vincent and Saint Croix) or are *Amblyomma* free (Anguilla, Barbuda, Montserrat and Barbados; Fig. 13.8). In Dominica and Sainte Lucia, restricted area with high numbers of ticks (hot spots) are currently observed whereas Martinique, Saint Kitts and Nevis have low to moderate level of tick infestation. Another *A. variegatum* control programme targeting French islands was also implemented at the same time but with less success than CAP, resulting in a remaining high level of infestation in Guadeloupe and Marie Galante (Molia et al. 2008). Previous studies performed in these two islands indicated that the tick infested 35.6 % and 73.8 % of the herds in Guadeloupe and Marie Galante, respectively, with 36.7 % and 19.1 % of *A. variegatum* ticks infected with *E. ruminantium*, respectively (Molia et al. 2008; Vachiery et al. 2008a). Therefore, these islands constitute a reservoir for ticks and *E. ruminantium* in the Caribbean, threatening the American mainland through the spreading of infected *A. variegatum* nymphs by migratory birds or uncontrolled movement of animals (Kasari et al. 2010). If an accidental introduction of a tick-free *E. ruminantium* carrier animal would happen, autochthonous *A. maculatum* and *A. cajennense* (which have proven to be experimental vectors for heartwater) could promote *E. ruminantium* spreading from the North of Mexico down to the South of Brazil (except Andean region). Since 2012, heartwater has been identified within the 12 most important animal transboundary diseases for US (Vachiéry et al. 2013; Roth et al. 2013).

Although eradication programmes are now in standby in the Caribbean, heartwater control and early diagnostic are a major concern in Guadeloupe in parallel with the development of research programmes at international level to fill the gap of efficient vaccines and diagnostics. A surveillance network (RESPANG, surveillance network for nervous ruminant pathology in Guadeloupe) was also created from 2010 to 2015, in collaborations with veterinarians, French Ministry of Agriculture and the OIE reference laboratory for heartwater (CIRAD), in order to detect sick animals with heartwater suspicion, and to perform a sensitization campaign for farmers concerning acaricide treatment and recommendations.

4.2 Animal Species Affected

Small ruminants, goat and sheep, are more susceptible to heartwater than cattle. Moreover, there is also a variation between breeds: for instance, *Bos indicus* (zebu-type cows) breeds are generally more resistant than European breeds (Uilenberg 1983), not only because of enzootic stability. A wide variety of wild ruminant species may become infected with *E. ruminantium*, some showing symptoms (bleskbok, black wildebeest, giraffe, eland, etc.) and others not (buffalo, impala, greater Kudu). Other animals such as helmeted guinea fowl, leopard tortoise and scrub hare have

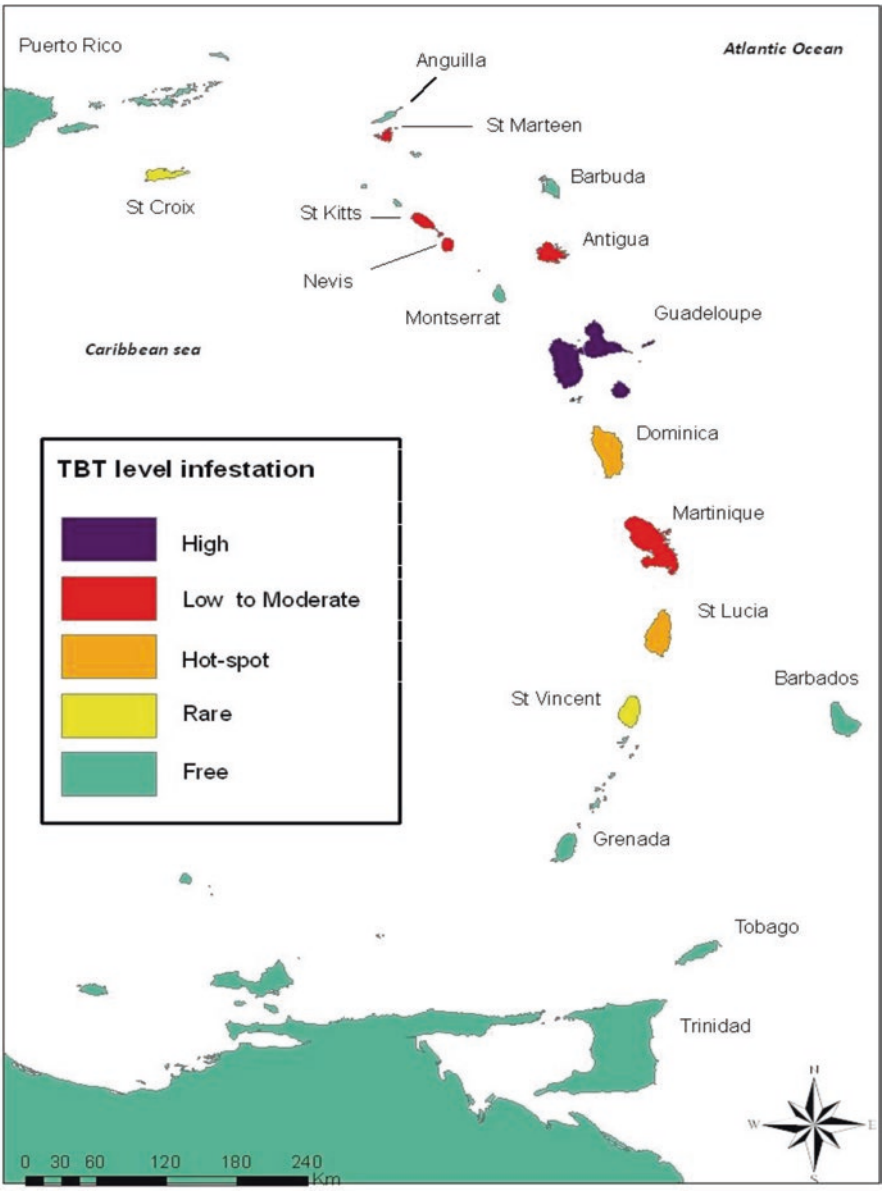

Fig. 13.8 *Amblyomma variegatum* infestation in Lesser Antilles, data from CaribVET network (2011)

been reported to develop sub-clinical heartwater (Oberem et al. 1987; Kock et al. 1995; Peter et al. 1999a). Knowledge of the susceptibility of wild ruminants to heartwater is particularly important where re-introduction of ruminant game species into heartwater endemic areas is considered. Wild ruminants also play a role as sources of infection for ticks, particularly in those areas where stringent tick control in domestic

animals is practiced (Peter et al. 1999b). Still the role of wildlife in the epidemiology of heartwater has not been elucidated (van Vuuren and Penzhorn 2015).

E. ruminantium can also infect ferrets and mice (Oberem and Bezuidenhout 1987). Although the mouse was used as an animal model for the characterization of immune responses during infection or for the development of vaccine candidates (Du Plessis et al. 1991; Nyika et al. 1998; Kock et al. 1998; Byrom et al. 2000a, b; Brayton et al. 2003; Simbi et al. 2006), very few *E. ruminantium* strains are known to infect mouse, limiting the use of this animal model for further studies.

Although there are no official records in the literature of heartwater disease in humans, three fatal cases of possible human infection by *E. ruminantium* have been reported in South Africa, revealed by genetic typing of *E. ruminantium* DNA sequences in brain tissue and serum samples associated with clinical features of heartwater such as pulmonary oedema and an oedematous, hyperaemic brain (Allsopp et al. 2005b, c). No confirmatory isolation of bacteria in culture and further genetic characterization were performed, and no new record of such infection was published since. Still heartwater is now considered as a tick-borne pathogen of potential zoonotic importance in the Southern African region (Esemu et al. 2011; Chitanga et al. 2014).

5 Diagnosis of Heartwater

Suspicion of heartwater occurs because of the recognition of clinical signs (essentially neurological damages changing the behaviour of the infected animals), presence of *Amblyomma* spp. ticks, and presence of transudates in the pericardium and thorax on post-mortem examination. The traditional method of diagnosis is the identification of the pathogen through post-mortem microscopic examination of brain smears, or bacterium isolation from infected blood or tick homogenates. Serological assays (indirect fluorescent antibody (IFA) test, enzyme-linked immunosorbent assays (ELISA) and immunoblotting (western blotting)) have been developed but suffer from poor sensitivity and specificity. Development of several *E. ruminantium* molecular diagnostics has been performed based on classical bacterial DNA amplification improved by nested and real-time PCR techniques.

5.1 Clinical Signs

The incubation period in natural infections is usually 2–3 weeks, but can vary from 10 days to 1 month, without any early clinical or physiological indicators, except that the rickettsemia coincides with the onset of fever. The course of the disease may range from the relatively rare peracute form (with sudden death without symptoms apart from high hyperthermia up to 42 °C) to mild, depending on age, immune status, breed and virulence of *E. ruminantium* strain (Van de Pypekamp and Prozesky

1987). The clinical signs may include a sudden fever, severe respiratory distress, hyperesthesia, lacrimation, terminal convulsions, and sudden death. Occasionally, animals also have diarrhoea. Animals with the acute form of heartwater are restless and show nervous symptoms such as rapid blinking of the eyes, hypersensitivity to touch, walking in circles, sucking movements, rigidly standing with tremors of the superficial muscles and finally, they fall to the ground, pedalling. They usually die within a week after the onset of the disease. Recovery is rare when nervous symptoms have started (Van de Pypekamp and Prozesky 1987; OIE 2005). Immune animals may have small transient hyperthermia and natural rapid recovery, or even no sign of infection.

5.2 Pathogenesis

The pathogenesis of the disease, despite still poorly understood, remains of help in confirming the suspicion of heartwater. Increased vascular permeability results in transudation of fluid into various body tissues (e.g. brain and lungs) and body cavities (e.g. pericardial and thoracic cavities) (Fig. 13.9), but the precise mechanisms responsible for the transudation are poorly understood. At necropsy, it is possible to observe hydro-pericardium (origin of the name "heartwater"), with straw-coloured to reddish pericardial fluid; this phenomenon appears to be more pronounced in sheep and goats than in cattle (van Amstel et al. 1988; Van Amstel et al. 1987; Brown et al. 1990). Brain oedema leads to nervous signs, hydropericardium contributes to cardiac dysfunction during the terminal stages of the disease, and progressive pulmonary oedema and hydrothorax result in asphyxiation (Uilenberg 1971; Owen et al. 1973). The pathogenesis of vascular permeability remains speculative as the intracytoplasmic development of the organisms (reticulate bodies) seems to have little detectable cytopathic effect upon the endothelial cells (Pienaar 1970), and there is also no apparent correlation between the number of parasitized cells in the pulmonary blood vessels and the severity of the pulmonary oedema (Prozesky and Du Plessis 1985). It has been proposed that an endotoxin (Amstel et al. 1988) and increased cerebrospinal fluid pressure (Brown and Skowronek 1990) play a role in the development of lung oedema.

5.3 "Brain Squash Smears"

In clinical cases, heartwater must be differentiated from a wide range of infectious and non-infectious diseases, especially plant poisonings, which also cause central nervous system signs. For acute clinical cases in endemic areas, clinical signs alone may suggest the aetiology, but demonstration of the organism in the cytoplasm of capillary endothelial cells is necessary for a definitive diagnosis. Typical colonies of *E. ruminantium* can be observed in "brain squash smears" made after death of the

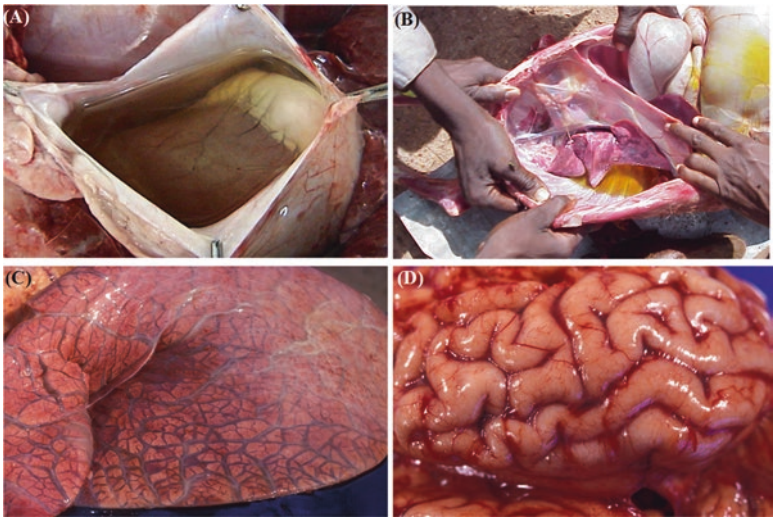

Fig. 13.9 Lesions due to *Ehrlichia ruminantium* infection in the ruminants. (**A**) Oedematous pericardium of a goat died of heartwater disease; (**B**) Severe hydrothorax in cattle dead of heartwater; (**C**) Severe lung oedema associated with heartwater (bovine); (**D**) Brain oedema associated with heartwater (bovine) (adapted from Allsopp et al. (2005a)), photos (**A**) and (**B**) are from Ken Giraud-Girard and Frédéric Stachurski (both from CIRAD)

infected animal. For this, a piece of gray matter from the cortex (~3 × 3 mm) is crushed between two microscope slides; the softened material is then spread like a blood smear with the material pushed rather than pulled along. A slight lifting of the spreader slide about every 5–10 mm creates several thick ridges across the slide, from which capillaries are arranged straight and parallel in the thin sections of the smear for easier examination. Brain smears are then air dried, fixed with methanol and stained with Eosine and methylene blue (RAL555) or Giemsa and observation of *E. ruminantium* is then possible (Fig. 13.2).

E. ruminantium occurs as clumps of reddish-purple to blue, coccoid to pleomorphic organisms in the cytoplasm of capillary endothelial cells close to the nucleus (Fig. 13.2). Colonies can be difficult to find in some samples depending on the *E. ruminantium* strain, in samples from animals treated with antibiotics or from animals with peracute disease. Colonies are still visible 2 days after death in a brain stored at room temperature (20–25 °C) and up to 34 days in a brain stored at 4 °C. Technical expertise is required to differentiate *E. ruminantium* colonies from other haemoparasites (such as *Babesia bovis*), certain blood cells (thrombocytes, granulocytes), normal subcellular structures (mitochondria, mast cell granules), or stain artefacts (stain precipitates).

To improve the histological diagnosis, an immunohistochemical staining technique was developed using a specific serum targeting the major antigenic protein-1 (MAP-1), but is now rarely used. In naturally infected cattle, sheep and goats, *E. ruminantium* morula are identified in formalin-fixed tissues as clearly defined,

brown-staining rickettsial colonies within the cytoplasm of endothelial cells, whereas no positive staining is observed in the control group (Jardine et al. 1995).

5.4 Serological Tests

Two serological diagnostics based on the detection of antibodies against *E. ruminantium* MAP-1protein are currently used: a competitive ELISA MAP1 and an indirect ELISA using a fraction of MAP1 protein, MAP1-B (van Vliet et al. 1995; Katz et al. 1997). Unfortunately, these assays display cross-reaction with other *Ehrlichia* species, specifically with *E. chaffeensis* and *E. muris*. The indirect MAP1-B ELISA is used routinely at the regional OIE reference laboratory for heartwater (CIRAD). ELISAs for heartwater diagnosis are suitable for prevalence studies at herd level but cannot be used either for specific diagnostic purposes on clinical cases or to evaluate the infectious status of imported animals. Indeed, there is a 15-day delay in seroconversion after animal infection, and the seropositivity period lasts only several weeks for bovines and less than 6 months for small ruminants, whereas animals remain immune and possibly reservoir of the pathogen for months and sometimes up to 2 years after infection. Moreover, according to the current knowledge, seropositivity to *E. ruminantium* appears to be asynchronous with the infectious kinetics as a whole, as well as with the status of immunocompetent animals. Serodiagnosis of animals previously exposed to the disease, i.e. recovered from subclinical or clinical infection, still poses problems.

5.5 Molecular Diagnosis

Molecular diagnostics are the gold standards for the diagnosis of heartwater. In the last 15 years, important improvements have resulted in the development of better molecular tools for the diagnosis as well as genetic typing of different strains of *E. ruminantium*.

5.5.1 PCR and Nested PCR

The molecular method consisting in PCR amplification of a Crystal Springs strain *pCS20* DNA fragment, specific of *E. ruminantium* (GenBank accession number X58242), followed by membrane hybridization has been first developed (Mahan et al. 1992). Low levels of infection in animals and in ticks fed on carrier animals are detected by PCR, while a hybridization reaction with the *pCS20* probe alone (without PCR first) usually remains negative (Peter et al. 1995). Experimentally, the detection limit of the conventional PCR assay was found to be between 10 and 10^2

organisms per sample, whereas it was between 1 and 10 organisms after PCR/hybridization. Thirty-seven strains from all endemic areas have been detected by PCR/hybridization with a high specificity (98 %). However, the sensitivity of the PCR assay is variable depending on the sample nature and *E. ruminantium* load (Peter et al. 2000).

Two nested PCR assays were developed to enhance the detection of low levels of rickettsemia (Semu et al. 2001; Martinez et al. 2004); both use the *pCS20* region as the target sequence. The method defined by Semu and co-workers (Semu et al. 2001) is based on two external primers U24 and L24 primers for the first amplification step followed by primers AB 128 and AB 129 as the nested primers, which amplify a 279-bp fragment from within open reading frame 2 of the 1306-bp pCS20 DNA sequence (Peter et al. 1995). The sensitivity of detection of this assay is one organism per sample. The other nested PCR assay uses a pair of external primers and comprises the AB128 sense primer together with an anti-sense primer called AB130 (Martinez et al. 2004). These amplify a 413 bp fragment used as a template in a second round PCR using also AB128 and AB129 as internal primers. The nested PCR shows an average detection limit of six organisms per sample (Martinez et al. 2004). The *pCS20* nested PCR allowed regular detection of *E. ruminantium* organisms from ticks, blood, brain and lungs from infected animals, whether the samples were processed fresh, after freezing or preservation in 70 % ethanol. Presently, the molecular diagnosis is much more sensitive and faster than histological or microbiological diagnosis. For example, brain smear observations and pCS20 nested PCR on the same brain samples demonstrated a higher percentage of heartwater positive cases that increased from 75 % based on brain smears observations to 97 % using *pCS20* nested PCR (Adakal et al. 2010).

The range of strain detection was later on increased by the use of new primers AB128' AB130' and AB129' modified from the original AB128, AB129 and AB130 by including universal nucleotides and this method is used routinely for *E. ruminantium* detection in field samples, especially in ticks (Molia et al. 2008; Adakal et al. 2009, 2010b). The detection of *E. ruminantium* by nested PCR is possible in the blood of animals 1 or 2 days before hyperthermia and during the hyperthermia period but not on asymptomatic animals. PCR-based methods appear to be more reliable in detecting infection in ticks and this could have epidemiological value in determining the *E. ruminantium* prevalence in ticks and the geographical distribution of *E. ruminantium*.

A nested PCR targeting the entire *map1* polymorphic gene has been developed in parallel in order to type the strains by restriction fragment length polymorphism (RFLP) or sequencing of the amplification fragment directly from the *pCS20* positive samples (Martinez et al. 2004). Its detection limit was evaluated at around 60 organisms per sample.

The main drawback of nested PCRs is the possibility to get cross-contamination due to second round of PCR and, technical cautions should be implemented to limit this risk particularly when manipulating the first PCR product with a high quantity of *pCS20* targeted gene. Moreover, this method is also time-consuming particularly for screening a large amount of samples.

5.5.2 Quantitative Real-Time PCR

Several quantitative real-time PCR have been developed for the detection of *E. ruminantium* targeting *map-1*, *map1-1* and *pCS20* genes (Peixoto et al. 2005; Postigo et al. 2002; Steyn et al. 2008). These methods have been described for the detection and quantitative determination of *E. ruminantium* organisms either for *E. ruminantium* kinetics in the blood of experimentally infected sheep (during the hyperthermia reaction period) (Steyn et al. 2008) or in vitro (Marcelino et al. 2005, 2006, 2007; Peixoto et al. 2005, 2007). Real-time PCR targeting *map1* and *map1-1* polymorphic genes were tested on a limited number of strains (up to six), and therefore, should not be used for diagnostics. Another real-time PCR assay targeting *pCS20* gene has a sensitivity level similar to the nested PCR, but asit was tested so far only on 15 different strains, screening on additional strains should be performed before using as diagnostic tools. Recently, Sayler and coworkers (Sayler et al. 2016) developed and validated a dual-plex Taqman QPCR assay targeting the *groEL* gene of Panola Mountain *Ehrlichia* and *E. ruminantium*. Twenty-three *E. ruminantium* strains originated from 12 countries (from Africa and Caribbean) were detected but *E. chaffeensis*, *E. ewingii* or *E. canis*, or *Anaplasma* spp. were not detected. It represents a promising method compared to nested PCR due to limitation of cross-contamination, but the strain recognition spectra need to be enlarged to validate the proof of concept before using it as a gold standard molecular diagnostic tool.

5.5.3 Typing Methods

The genetic characterization and structure of *E. ruminantium* population at regional scale is essential in order to select potential vaccine strains. The genetic typing of strains was previously done using RFLP on the polymorphic gene *map-1* after PCR amplification (Faburay et al. 2007; Adakal et al. 2010). Based on the genome analysis of two different strains, Gardel and Welgevonden, truncated and unique coding sequences specific of strains have been identified. This analysis allowed the development of a differential strain-specific diagnosis using nested PCRs targeting six unique and four truncated CDS (Vachiery et al. 2008b). New multi-locus methods adapted to *E. ruminantium* have been validated such as multi-locus sequence typing (MLST) (Adakal et al. 2009, Nakao et al. 2011, Cangi et al. 2016) and multi-locus variable number of tandem repeated sequence analysis (MLVA) (Pilet et al. 2012). Two studies on restricted areas in Burkina Faso demonstrated the presence of several different clusters and identified one strain population in stasis and another strain population in clonal expansion. Cangi and co-workers recently used MLST to analyse the genetic diversity and population structure of 194 *E. ruminantium* strains circulating worldwide. This study highlighted the importance of recombination events in the generation of *E. ruminantium* diversity and evolution and revealed that the strains were clustered into two major genetic groups: a West African cluster, and a worldwide cluster which includes West Africa, East Africa, Southern Africa, Indian Ocean, and Caribbean (ref: Cangi, N., Gordon, J. L., Bournez, L., Pinarello,

V., Aprelon, R., Huber, K., … Vachiéry, N. (2016). Recombination Is a Major Driving Force of Genetic Diversity in the Anaplasmataceae Ehrlichia ruminantium. Frontiers in Cellular and Infection Microbiology, 6, 111. http://doi.org/10.3389/fcimb.2016.00111)

With the important progress in the performances of sequencers (Illumina or PGM Ion torrent) since 2010, sequencing of whole genome of *E. ruminantium* strains is now possible in a very short time allowing getting information on all genes. However, this method is restricted to samples produced in cell culture as field samples (ticks of blood of infected animals) do not contain enough *E. ruminantium* organisms for the analyses.

5.6 *Identification of Suitable Diagnostic for Heartwater Diagnosis*

A comparison of the different molecular diagnostic methods for heartwater is presented in Table 13.1. The nested PCR *pCS20* presents several advantages and it is the gold standard assay for diagnostic of clinical heartwater specimens. Nevertheless, the real-time PCR *pCS20* is faster and less prone to cross-contamination. It is thus essential to do further validation and compare the detection efficiency between real-time and nested PCR *pCS20* in order to validate the real-time PCR assay for heartwater diagnosis.

A molecular multi-pathogen method, the reverse line blot (RLB), is also available, targeting *Ehrlichia* and *Anaplasma* sp. including *E. ruminantium*, *A. margi-*

Table 13.1 Comparative analysis of the available molecular tools for heartwater diagnosis (Vachiery et al. 2013)

Critical criteria	Nested PCR *pCS20*	qPCR *map1*	qPCR *map1-1*	qPCR *pCS20*	Research needs
Rapidity	5	8	8	8	Development of quick tests: Multi-pathogen detection by microarrays or qPCR
Sensitivity	8	6	5	8	
Handling and caution to avoid cross-contamination	5	8	8	8	
Detection of different ER strains	10	2	2	3	Comparison of detection capacity between *pCS20* nested PCR and qPCR
Adapted to field samples	10	NT	5	5	
Total score	38	24	29	32	

NT not tested
Scoring from 0 to 10, where 0 = the worst and 10 = the best

nale, A. centrale, A. ovis and *A. phagocytophilum* (Bekker et al. 2002). Nevertheless, it is less sensitive than the nested PCR and it has not been validated at large scale and during epidemiological studies. There is therefore a strong interest and usefulness in developing improved rapid multi-pathogen detection assay, i.e. using microarray technology or multiplex qPCR as done by Sayler and coworkers (Sayler et al. 2016) and that could also include other tick-borne pathogens such as *Babesia* and *Theileria* spp.

6 Treatment, Control and Prevention

As mentioned above, in enzootic areas, indigenous cattle have developed resistance to heartwater due to long-term interaction with the bacteria. Although natural enzootic stability should be considered as the desirable situation where no control measures would need to be implemented, this stability can be easily disrupted by variations in climate, host and pathogen phenotypes, and management strategies (Florin-Christensen et al. 2014). As enzootic stability can be sometimes difficult to achieve, several strategies are developed to control heartwater. These include chemotherapy and chemoprophylaxis, vector chemical control and vaccination.

6.1 Chemotherapy and Chemoprophylaxis

Treatment with antibiotics (tetracyclines) of infected animals during the early febrile stages is very efficient and confers long-lasting immunity. The main problem is the timing of treatment of field cases: in general, animals display visible (nervous) symptoms and are presented for treatment when it is too late to treat.

In goats, it was proposed to use short-acting tetracyclines administered at a dosage rate of 3 mg/kg body weight on 10, 20, 30, 45 and 60 days after introduction in an endemic area, during the nymph infestation periods, to allow acquisition of protective immunity. In that case, animals should not be dipped during all the immunization procedure (Infection and treatment method, see below) (Gruss 1981). Similarly, injections of long-acting tetracycline formulations (10–20 mg/kg body weight) given on days 7, 14 and 21, or even on only two occasions (days 7 and 14) in cattle are sufficient to protect them from contracting heartwater, while at the same time allowing them to develop a natural immunity, when introduced in heartwater endemic regions during peak infestation level by nymph or adult ticks (Purnell 1987). The success of this regime is of course dependent on all the animals becoming naturally infected with heartwater during the time that they are protected by the drug: time of introduction and treatment has to be determined according to seasonal variations of ticks.

6.2 Vector Chemical Control

Heartwater is usually introduced into free areas by infected animals, including subclinical carriers, or by infected ticks. Sustained and intensive tick control measures may thus succeed in preventing outbreaks of heartwater in tick-free areas, and even, under certain conditions, in enzootic areas. The disease can however only be controlled successfully if all the animals on the farm are treated regularly with acaricides throughout the year and if there are no, or an absolute minimum, of game animals and birds on which ticks can survive. Because of the high infection rate of the vector ticks, it is nevertheless very difficult and expensive to prevent heartwater by vector control in enzootic areas. Moreover, the use of acaricides also has a negative impact on environment and human health. Although tick populations resistant to acaricide have only been very rarely found in *Amblyomma* spp. ticks, it is moreover considered that high frequency treatment programmes may lead, sooner or later, to the development of tick resistance most probably on other tick species such as *Rhipicephalus microplus* to the used compound. Such intensive programmes can also lead to disappearance of enzootic stability, even in local cattle breeds.

6.3 Vaccination

Four different vaccine strategies against heartwater have been developed: the "infection and treatment" method using live bacteria, infection with in vitro attenuated bacteria, immunization with inactivated in vitro grown bacteria and recombinant or subunit (using DNA or recombinant proteins, respectively) (Table 13.2). For all these vaccines, the main problem is the presence of numerous strains in the field with high genetic and/or phenotypic diversity (as above mentioned) and, sometimes, the reduced level of cross-protection between them. The choice of the vaccine strain(s) is therefore crucial and depends on the region, as it was previously demonstrated by Adakal and co-workers (Adakal et al. 2010).

6.3.1 The "Infection and Treatment" Method

Field observations and experiments under laboratory conditions have shown that cattle, sheep and goats are capable of developing a protective immunity against heartwater after surviving a virulent infection. In South Africa, this led to the development of an "infection and treatment" type of immunization where animals are injected with fully virulent *E. ruminantium* organisms of the Ball 3 strain and are subsequently treated at onset of hyperthermia with tetracyclines to prevent disease, which requires daily monitoring of body temperature (du Plessis and Bezuidenhout 1979). Despite the low cross-protection of the Ball 3 strain against some other *E. ruminantium* strains and the fact that this is an expensive and dangerous

Table 13.2 Examples of vaccination strategies tested for *Ehrlichia ruminantium* infection

Type of vaccine	Host	Vaccine isolate	Challenge isolate[b]	Survival ratio (%)	References
Live	Cattle	Ball 3	Ball 3	100	du Plessis and Bezuidenhout (1979)
Attenuated	Sheep	Senegal	Senegal, Welgevonden, Umpala, Lutale, Ball 3, Gardel, Um Banein	0–100	Jongejan (1991), Jongejan et al. (1993)
	Sheep	Welgevonden	Wegevonden, Ball 3, Gardel, Mara 87/7, Blaauwkrans	100	Zweygarth et al. (2005), Collins et al. (2003a)
	Goat	Welgevonden	Welgevonden	80	
Inactivated cell cultured organism (+ adjuvant)	Goats	Gardel	Gardel	65 70–100	Martinez et al. (1994, 1996), Vachiery et al. (2006), Marcelino et al. (2015a, b), Marcelino et al. (2007)
	Sheep	Crystal Spring	Crystal Spring	100	Mahan et al. (1995, 1998a, b)
			Crystal Spring (ticks) Beatrice (ticks)		Mahan et al. (1998a)
		Mbizi	Mbizi, Beatrice, Isiolo, Welgevonden	60–100	
		Mbizi	Beatrice (ticks)	70	
	Cattle	Gardel	Gardel	100	Totte et al. (1997)
	Goats/Sheep/Cattle	Mbizi, Sunnyside, Lutale, Bathurst	Field tick challenge	26–80	Mahan et al. (2001)

(continued)

Table 13.2 (continued)

Type of vaccine	Host	Vaccine isolate	Challenge isolate[b]	Survival ratio (%)	References
Recombinant (DNA and/or recombinant protein)	Mice	*map-1* DNA vaccine (Crystal Spring)	Crystal Spring	25–90	Nyika et al. (1998); Nyika et al. (2002).
	Sheep	Cocktail of genes (Welgevonden)	Welgevonden	20–100	Collins et al. (2003a, b), Pretorius et al. (2002, 2007, 2008, 2010)
	Mice	*cpg1* gene (Welg)	Welgevonden	0	Louw et al. (2002)
	Sheep			80	
	Mice	*E. coli* lysates expressing 5 different genes	Highway	Highly variable	Barbet et al. (2001)

methodology, it has been the only commercially available vaccine strategy for more than 50 years (Du Plessis et al. 1989, Onderstepoort Biological Products SOC Ltd)). Whenever large numbers of commercial ruminants of high value are introduced to heartwater endemic regions, the block method of vaccination is also used: after vaccine administration, the animals are treated simultaneously and indiscriminately with antibiotics whether a febrile reaction occurs or not (Du Plessis and Malan 1987). This method has many drawbacks such as the requirements of a cold chain, a short shelf-life and the potential for the transmission of other pathogens and could not be used widely (Shkap et al. 2007).

6.3.2 Live Attenuated Vaccine

In the early 1990s, an attenuated strain of *E. ruminantium* (Senegal) was prepared as a live vaccine by serial passage in vitro in endothelial cells (Jongejan 1991). This attenuated strain, while providing immunity to homologous challenge, was nevertheless not fully effective to provide cross-protection against other virulent strains (Jongejan et al. 1993) (Table 13.2). Another strain from Guadeloupe (Gardel) can also be attenuated after 200 passages in endothelial cells (Marcelino et al. 2015b, Martinez 1997). This strain provides a good protection against heterologous challenge with other strains (Martinez 1997). Zweygarth and co-workers have successfully attenuated the virulent Welgevonden strain of *E. ruminantium* by 50 continuous passages in a canine macrophage-monocyte cell line (Zweygarth and Josemans 2001). The use of such live attenuated vaccines is nevertheless limited since cross-protection against different isolates is not complete. In comparison with other immunization methods, the main disadvantage of attenuated vaccines is the possible reversion to virulence and the need to storage in liquid nitrogen until used.

6.3.3 Inactivated Vaccine

The inactivated vaccine is based on purified *E. ruminantium* organism (produced in bovine endothelial cells) that are chemically inactivated or lysed (Martinez et al. 1994, 1996; Mahan et al. 1998a). In 2006, Marcelino and co-workers developed a fully scalable process for the large-scale production of the antigen using bioreactors and microcarriers; this bioprocess also allows to decrease the price of a vaccine dose (Marcelino et al. 2006). The development of a large-scale purification process using membrane (Peixoto et al. 2007) and theoptimization of theantigen buffer also improved the level of purity of the vaccine and its storage conditions (Marcelino et al. 2007). Field tests proved however that a strain isolated in a region is less effective when used elsewhere, and that local strains should be added to improve vaccine effectiveness (Adakal et al. 2010).

In 2015, Marcelino and co-workers developed a ready-to-use inactivated vaccine that could be easily used in the field and even withstand up to 3–4 days at 37 °C before injection (Marcelino et al. 2015a). As soon as regional isolates would be

available in culture after isolation, it could be possible to produce an inactivated vaccine including a cocktail of regional strains. The main difficulty is to choose the strains which could protect against other circulating strains. The choice will depend on genetic characteristics and markers, which are not yet precisely defined. The main inconvenient of the inactivated vaccines is the observation of animal morbidity during the infectious challenge.

6.3.4 Recombinant Vaccine

Besides the increased safety and reduced price, the use of recombinant vaccines could permit the correct presentation of the antigen after endogenous processing leading to a long-lasting immunity. To develop such a vaccine, it is nonetheless necessary to identify *E. ruminantium* antigens that would induce a protective immune response. The *map1* gene was cloned and tested as a naked-DNA vaccine in a mouse model system (Nyika et al. 1998, 2002). Others genes such as groE operon (*groES* and *groEL)* (Lally et al. 1995) and *cpg 1* (Louw et al. 2002) have also been cloned and tested as a recombinant DNA vaccine to protect animals against lethal *E. ruminantium* infection (van Vliet et al. 1993, 1994, 1995, 1996; Reddy et al. 1996; Mahan et al. 1994; Nyika et al. 1998, 2002) (Table 13.2). Subunit vaccines using denatured *E. ruminantium* have also been tested, although no protection was achieved (van Vliet et al. 1993). Since 2007, Pretorius and co-workers evaluated the ability of several *E. ruminantium* ORFs as an efficient component of a recombinant vaccine against heartwater (Pretorius et al. 2007, 2008, 2010). When the authors immunized the animals either with a recombinant DNA cocktail of four 1H12 pCMViUBs_ORFs followed by a r1H12 protein or with 1H12 plasmid rDNA, a survival ratio of 100 % against a virulent *E. ruminantium* Welgevonden needle challenge was obtained (Pretorius et al. 2007). When a similar strategy was used but using a tick challenge, only 20 % of protection was obtained (Pretorius et al. 2008). A prime/boost vaccination trial using the polymorphic *cpg1* gene and the recombinant protein also resulted in complete protection of vaccinated animals after homologous challenge; no trials with heterologous strains have yet been performed (Pretorius et al. 2010). Due to the polymorphic property of *cpg1*, a cocktail of representative *CpG1* from different strains should be included in the vaccine before any field trial. In the same year, Sebatjane and co-workers also tested five *E. ruminantium* ORFs (Erum7340, Erum7350, Erum7360, Erum7380, and Erum4360) coding for membrane proteins of low molecular weights as potential antigen against heartwater (Sebatjane et al. 2010). Unfortunately, the vaccination experiment in sheep using a DNA/protein prime/boost resulted in a low survival ratio (20 %).

From the above mentioned, recombinant vaccines look promising under experimental conditions, but results during field trials have been less successful. Moreover, simple intramuscular immunization is not sufficient to induce protection, and the use of a gene gun necessary for prime DNA injection is not suitable for large field vaccination campaign.

6.3.5 Identification of theBest Vaccine Currently Available

A comparison between the different vaccines is presented in Table 13.3. This analysis is based on critical criteria: efficacy against homologous/heterologous strains, safety, induction of low morbidity, availability of industrial process, stability, supply, easiness of administration and ability to elaborate a regional vaccine. At the moment, the inactivated vaccine seems to be the most appropriate for worldwide vaccination strategy against heartwater since: (1) a bioprocess is already optimized, (2) it includes killed bacteria, (3) storage condition is compatible with field use and (4) it is suitable for regional cocktail of strains to improve vaccine efficacy. .

The diversity of *E. ruminantium* strains remains the main problem for all candidate vaccine mentioned above. For instance, only one Caribbean strain is currently available in culture; it is thus necessary to isolate and cultivate new Caribbean strains to prepare a vaccine cocktail that will be suitable for all the regional area. This will

Table 13.3 Comparative analysis of different vaccines currently available for heartwater (adapted from Vachiery et al. 2013)

Critical criteria	Infection and treatment	Recombinant vaccine	Attenuated vaccine	Inactivated vaccine	Research needs
Efficacy/ homologous challenge	10	10	10	10	
Efficacy/ heterologous challenge	5	**2**	5	5	Genetic and phenotypic characterization
Safety	**0**	8	**3**	8	Knowledge of virulence mechanisms[a]
Low morbidity	**0**	8	8	3	
Time to availability	7	7	4	6	
One dose	10	**2**	8	4	
Industrial process	**0**	**0**	**0**	10	Development of bioprocess[b]
Stability/shelf life	8	NT	8	7	
Storage Distribution Supply	**0**	**3**	**0**	8	
Administration	**3**	**2**	**3**	8	
Regional vaccine	**0**	6	**0**	6	Development of regional vaccine: isolation of live Caribbean strains
Total score	43	48	49	**75**	

NT not tested
[a]Only for attenuated vaccine
[b]Graded on 1–10 scale, where 0 = the worst and 10 = the best

also be essential at the African continent level, to characterize and study genetic and antigenic features and compare them, in order to define efficient cocktails for vaccination against heartwater and their corresponding geographic areas of use.To understand the structure of the rickettsia population,the genetic characterization of some of the strains circulating in the field in Africa and Caribbean areas has been performed as mentioned above(Adakal et al. 2010; Pilet et al. 2012) Still, further improvements and experiments are required. Indeed,Cross-protection studies were performed at the OIE reference laboratory for heartwater (CIRAD) in attempt to link genotyping to cross-protection, but the correlation has proven to be somewhat difficult to establish (unpublished results). Still, these epidemiological molecular studies could help to choose for optimal vaccine strains as previously shown (Adakal et al. 2010).

Despite the efforts to develop an effective recombinant vaccine, few genes and/or recombinant proteins have been tested so far (Table 13.2). This might be due to the difficulty of selecting the best genes as until now little knowledge on virulence mechanisms is available. To overcome this issue, virulent and attenuated *E. ruminantium* strains from geographical distinct regions are being compared using high-throughput "Omics approaches" such as genomics, transcriptomics and proteomics(discussed in more detail in the following sections).

7 Immune Response to *Ehrlichia ruminantium* and Against Heartwater

The knowledge of protective immunity to *E. ruminantium* is still fragmentary. However, significant advances have been made towards the understanding of the mechanisms underlying antibody and cell-mediated immune response to this pathogen.

High antibody titres are normally detected in infected animals at the height of the febrile reaction, and this has led to the initial hypothesis that a humoral response might be involved in protection against heartwater (Semu et al. 1992). However, transfer of immune serum or gamma globulins from immunized to naïve animals have failed to protect animals or even modify the course of the disease (Du Plessis et al. 1984; Prozesky 1987b; Alexander 1931; Du Plessis 1993). Although these results show the limitations of using immune sera to induce protection, the possible existence of protective antibodies should not be fully excluded. Indeed, antibodies may be crucial in opsonization, complement-mediated killing and antibody-dependent cell-mediated toxicity and therefore deserve further investigations.

The apparent lack of an effective humoral response together with the report of a T-cell-mediated response in experimentally infected mice (Du Plessis 1982) have then led to the belief that immunity against heartwater is likely to be mediated by cellular responses directed against infected cells (Stewart 1987). *E. ruminantium* has a profound effect on endothelial cells. In vitro, it elicits the synthesis of IL-1β, IL-6 and IL-8 mRNA in infected host cells, and this effect appears to be potentiated by IFN-γ (Bourdoulous et al. 1995). IL-1 and IL-6 can act as co-stimulatory signals for T- and B-cells activation, while IL-8 might participate in the recruitment of neu-

trophils towards brain endothelial cells with potentially deleterious effect. Additionally, infection of endothelial cells with *E. ruminantium* strongly affect the expression of IFN-γ-induced MHC I and MHC II molecules at the surface of the host cells (Vachiery et al. 1998). Therefore, endothelial cells may have a pivotal role in the development of a protective immune response against heartwater.

Further experiments have shown that an array of molecular and cellular effectors is involved and that different responses can be obtained according to the immunization strategy (infection and treatment method, inactivated vaccine and recombinant vaccines). To understand which cell subsets and antigens are involved in the immune response against heartwater, in vitro lymphocyte proliferation tests were performed. When PBMCs obtained from live vaccines immunized cattle were stimulated with the two immunodominant recombinant antigens of *E. ruminantium* (MAP1 or MAP2), T-cell responses specific to MAP1 and MAP2 were generated. Proliferation of PBMCs was also elicited in vitro by infected, autologous endothelial cells, and *E. ruminantium*-primed monocytes, but not by killed organisms (Mwangi et al. 1998a). These proliferative responses were characterized by a mixture of $CD4^+$, $CD8^+$ and γδ T cells and strong expression of IFN-γ, tumour necrosis factors alpha and beta (TNF α/β), and interleukin-2 (IL-2), all which are strong indicators of a Th1-driven immune response. When PBMCs from animals immunized with inactivated antigens, $CD4^+$ T cells and IFN-γ were generated in response to *E. ruminantium* lysates and to *E. ruminantium*-primed autologous monocytes (Totte et al. 1997). These cell lines were also able to proliferate in vitro when stimulated with soluble *E. ruminantium* proteins between 20 and 32 kDa, previously fractionated by fast-performance liquid chromatography (FPLC) (Totte et al. 1998b) but they did not respond to the two immunodominant recombinant antigens of *E. ruminantium* (MAP1 or MAP2) (Totte et al. 1998a). Thus, during infection with live virulent *E. ruminantium*, T cell responses may be preferentially directed against certain epitopes expressed by infected cells but absent from the elementary body, the free extracellular stage of the organism (Fig. 13.5) (Totte et al. 1999). Flow cytometric analysis of PBMCs also showed that during vaccination experiments with inactivated vaccines, no significant change in the immune cell population occurred. However, after the challenge with virulent *E. ruminantium*, significant alterations were observed. After an initial progressive depletion of $CD4^+$, $CD8^+$ and T-cell subsets, an impressive rise in $CD8^+$ cells was observed (Martinez 1997; Mwangi et al. 1998b). This last finding is in accordance with the previous studies which led the authors to suggest that $CD8^+$ T cells might play a major role in immunity to heartwater described by Du Plessis with a murine model (Du Plessis 1982; Du Plessis et al. 1991, 1992).

In another approach, a naked-DNA vaccine containing the *map1* gene of *E. ruminantium* was used (Nyika et al. 1998). Immunized DBA/2 mice produced antibodies against MAP1 antigen and elicited a Th1 response, characterized by the production of IFN-γ and IL-2 in supernatant of splenocyte cultures stimulated with *E. ruminantium* lysates or recombinant MAP1 antigen. In 2010, Sebatjane and co-workers performed a DNA prime-protein boost immunization in sheep based on low molecular weight (LMW, <20 kDa) proteins of *E. ruminantium*, and confirmed the importance of sustained IFN-gamma production in conferring a protective immunity against heartwater (Sebatjane et al. 2010)

8 "Omics" Approaches for Improved Understanding of *E. ruminantium* Infection and Pathophysiology

Global "Omics" approaches (genomics, proteomics, transcriptomics, and metabolomics) in a systems biology context are becoming key tools to increase knowledge on the biology of infectious diseases, specially to improve knowledge of the complex host–vector–pathogen interactions (Marcelino et al. 2012b) (Fig. 13.10). These interactions consist of dynamic processes involving genetic traits of hosts, pathogens, and ticks that mediate their development and survival (Popara et al. 2015). .Nowadays, three complete *E. ruminantium* genomes (Frutos et al. 2006a, b; Collins et al. 2005) are available and the sequencing of three *new E.ruminantium* strains is being performed Nakao et al. (2016). This overall genomic information paves the way of using "Omics" approaches for this pathogen.

In 2009, Emboulé and co-workers optimized the Selective Capture of Transcribed Sequences (SCOTS) methodology to successfully capture *E. ruminantium* mRNAs, avoiding the contaminants of host cell origin and eliminating rRNA which accounts for 80 % of total RNA encountered (Emboule et al. 2009). This method is essential to perform transcriptomic studies on the intracellular form of the bacterium (reticulate body, RB) avoiding host cell contaminants. In 2011, Pruneau and co-workers (Pruneau et al., 2011) determined the genome-wide transcriptional profile of *E. ruminantium* replicating inside bovine aortic endothelial cells (BAECs) using cDNA microarrays. Interestingly, over 50 genes were found to have differential expression levels between RBs and EBs. A high number of genes involved in metabolism, nutrient exchange and defence mechanisms, including those involved in resistance to oxidative stress, were significantly induced in RBs, indicating an active metabolism of *E. ruminantium* inside host cells (for bacterial growth inside vacuoles) and the need to protect themselves against host cell defence mechanisms. Finally, the authors demonstrate that the transcription factor *dksA,* known to induce virulence in other microorganisms, is overexpressed in the infectious form of *E. ruminantium*.

In a complementary way, proteomics provides information on the protein content of cells and tissues that may differ from results at the transcriptomics level and may be relevant either for basic biological studies or for vaccine antigen discovery (Popara et al. 2015). Marcelino and co-workers used bidimensional electrophoresis (2DE) coupled to mass spectrometry (MS) analysis to establish the first 2DE proteome map of *E. ruminantium* cultivated in endothelial cells (Marcelino et al. 2012a). In 2015, the authors combined gel-based and gel-free approaches to identify proteins and/or mechanisms involved in *E. ruminantium* virulence, by performing an exhaustive comparative proteomic analysis between a virulent strain (ERGvir) and its high-passaged attenuated strain (ERGatt). Despite their different behaviours in vivo and in vitro, the results from 1DE-nanoLC-MS/MS showed that the two strains share 80 % of their proteins; this core proteome includes chaperones, proteins involved in metabolism, protein–DNA–RNA biosynthesis and processing, and bacterial effectors. Conventional 2DE revealed that 85 % of the identified proteins are proteoforms, suggesting that post-translational modifications (namely glycosyl-

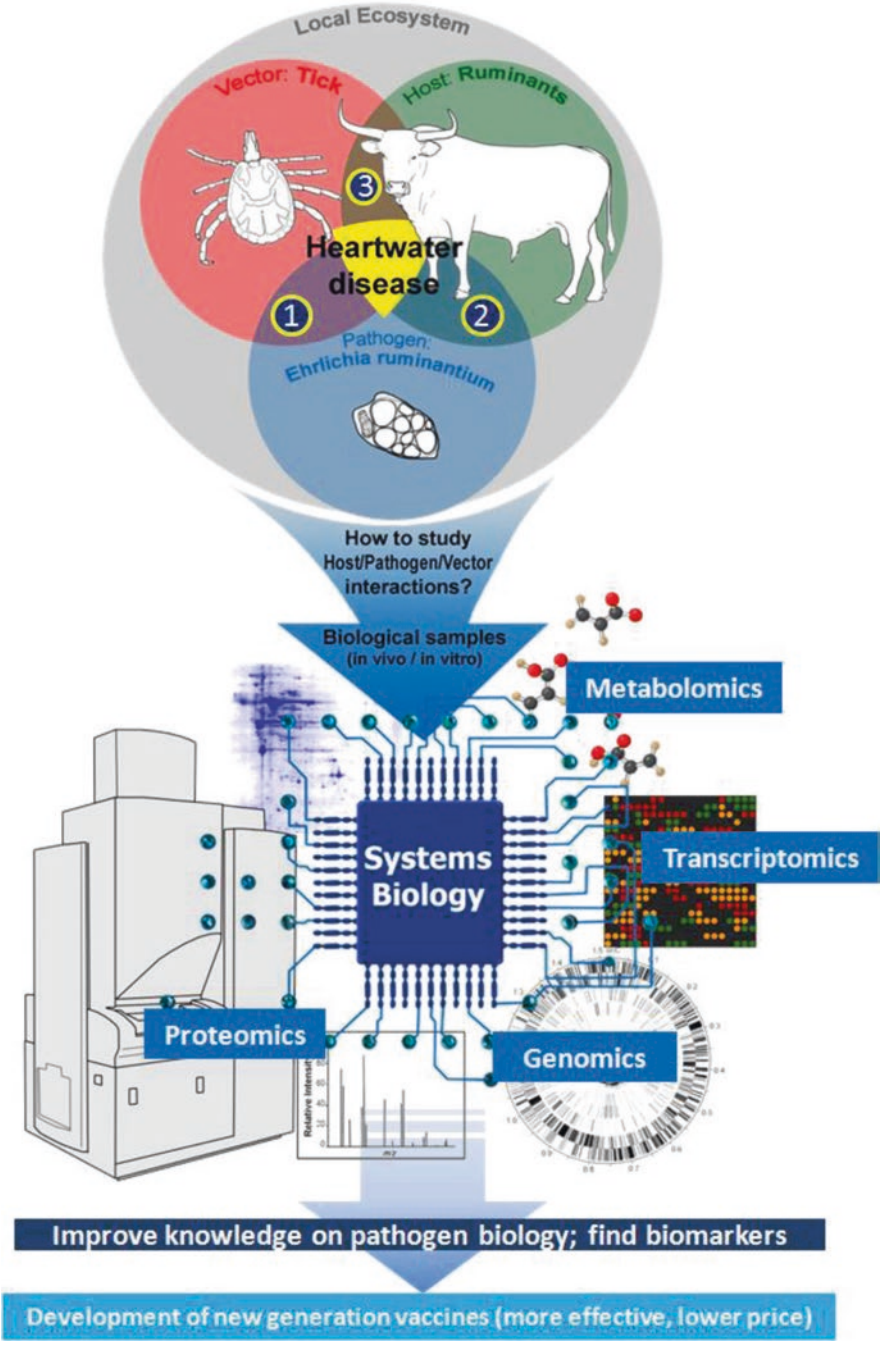

Fig. 13.10 The triangle of interactions between tick-borne pathogen, their vector and vertebrate host. The development of new vaccines against tick-borne diseases such as heartwater requires the profound knowledge of the intimate relations between (1) pathogen–tick, (2) pathogen–host and (3) tick–host (Marcelino et al. 2012b)

ation) are important in *E. ruminantium* biology. Strain-specific proteins were also identified: while ERGatt has an increased number and overexpression of proteins involved in cell division, metabolism, transport and protein processing, ERGvir shows an overexpression of proteins and proteoforms (DIGE experiments) involved in pathogenesis such as Lpd, AnkA, VirB9 and B10, providing molecular evidence for its increased virulence in vivo and in vitro. Overall, this work revealed that ERGvir and ERGatt proteomes are streamlined to fulfil their biological function (maximum virulence for ERGvir and replicative capacity for ERGatt), and the authors provide both pioneering data and novel insights into the pathogenesis of this obligate intracellular bacterium (Marcelino et al. 2015b). This work also suggests that virulence or attenuation phenomena might not be only a question of presence or lack of a specific protein but can also result from differential levels of expression of common proteins. Bioinformatics tools can also be very useful to identify virulence factors, in particular, those secreted by the Type Four Secretion System (T4SS). In 2013, Meyer and co-workers developed an algorithm to search for putative Type Four Effectors (T4Es) in the whole genome of *E. ruminantium*. This tool called S4TE (searching algorithm for type-IV secretion system effectors) predicts and ranks T4E candidates by using a combination of 13 sequence characteristics, including homology to known effectors, homology to eukaryotic domains, presence of subcellular localization signals or secretion signals, etc. Recently, Tago and Meyer (2016) used game theory tools to provide a theoretical basis to the process of generating attenuated strains of obligate intracellular bacterial pathogens. Interestingly, the authors hypothesize that *E.ruminantium* virulence might not just be a reflect of the bacterium's characteristics but would be the outcome of the interaction between the bacterium and the host defense system.

"Omics" approaches have been also very useful to study tick saliva. This fluid contains a cocktail of, potent anti-haemostatic, anti-inflammatory and immunomodulatory molecules, and it became in the last 10 years an attractive target to control tick-borne diseases. Sialome (or saliva transcriptome/proteome) studies benefit from recent advances in molecular biology, protein chemistry and computational biology, and highlighted newly isolated genes that code for proteins with homologies to known proteins allowing identification or prediction of their function. However, most of these genes code for proteins with unknown functions therefore opening new ways to functional genomic approaches to identify their biological activities and roles both in blood feeding and pathogen transmission (Valenzuela 2004). A recent proteomics study on *Amblyomma* spp., revealed that host and tick proteins involved in blood digestion, heme detoxification, development and innate immunity were differentially represented between adults and nymphs, whereas proteins involved in tick attachment, feeding, heat shock response, protease inhibition and heme detoxification were differentially represented between *Amblyomma* spp., suggesting adaptation processes to biotic and abiotic factors (Villar et al. 2014).

9 Conclusions and Future Perspectives

The vaccination strategies developed so far have proven not to be fully effective due to genetic and antigenic diversity of *E. ruminantium*. At the moment, the experimental inactivated vaccine is the most suitable for large-scale application, because of the availability of an optimized industrial process and the ability to include several strains within the vaccine to design an appropriate regional vaccine. To improve the vaccine efficacy, it will be necessary to isolate in vitro several strains from each enzootic geographic region to study their ability of protection; genotyping of protective strains will be also crucial to identify genetic markers linked to clusters of protection. More globally, it is essential to perform molecular epidemiology studies to evaluate the variability of strains in order to design regional vaccines.

On the other hand, further studies are required to better understand *E. ruminantium* pathogenesis in order to identify protective antigens and elaborate next generation vaccines. New breakthroughs in vaccine research are increasingly reliant on novel "Omics" approaches such as genomics, proteomics, transcriptomics, and other less known "Omics" such as metabolomics, immunomics, and vaccinomics (Bagnoli et al. 2011). These "Omics" approaches will deepen our understanding on: (1) *E. ruminantium* pathogenesis and attenuation mechanisms, (2) *E. ruminantium* host subversion mechanisms (including those driven by tick saliva), and (3) the key biological processes leading to protective immunity. These high-throughput technologies will also significantly contribute to overcome knowledge gaps on the role of key parasite molecules involved in cell invasion, adhesion, tick transmission and, surely revolutionize the capacity for discovering potential candidate vaccines, such as proteins involved in protective immune response, tick feeding or parasite development. These studies will contribute to the development of new treatments or next-generation vaccines.

References

Adakal H, Stachurski F, Konkobo M, Zoungrana S, Meyer DF, et al. (2010) Efficiency of inactivated vaccines against heartwater in Burkina Faso: impact of *Ehrlichia ruminantium* genetic diversity. Vaccine 28: 4573–4580.

Ahoussou S, Lancelot R, Sanford B, Porphyre T, Bartlette-Powell P, et al. (2010) Analysis of *Amblyomma* surveillance data in the Caribbean: lessons for future control programmes. Vet Parasitol 167: 327–335.

Alexander RA (1931) In Director of Veterinary Services and Animal IndustryUnion of South Africa, South Africa, 89–150.

Allsopp BA (2015) Heartwater–*Ehrlichia ruminantium* infection. Rev Sci Tech 34: 557–568.

Allsopp BA, Bezuidenhout JD, Prozesky L (2005a) In Infectious diseases of livestock(Ed, Tustin RC) Oxford University Press Southern Africa, Cape Town.

Allsopp MT, Allsopp BA (2001) Novel Ehrlichia genotype detected in dogs in South Africa. J Clin Microbiol 39: 4204–4207.

Allsopp MT, Allsopp BA (2007) Extensive genetic recombination occurs in the field between different genotypes of *Ehrlichia ruminantium*. Vet Microbiol.

Allsopp MT, Louw M, Meyer EC (2005b) *Ehrlichia ruminantium*: An Emerging Human Pathogen? Ann N Y Acad Sci 1063: 358–360.

Amstel Sv, Oberem P, Didomenico M, Kirkpatrick R, Mathee J (1988) The presence of endotoxin activity in cases of experimentally-induced heartwater in sheep. Onderstepoort Journal of Veterinary Research 55: 217–220.

Bagnoli F, Baudner B, Mishra RP, Bartolini E, Fiaschi L, et al. (2011) Designing the next generation of vaccines for global public health. Omics 15: 545–566.

Barbet AF, Byrom B, Mahan SM (2009) Diversity of *Ehrlichia ruminantium* major antigenic protein 1-2 in field isolates and infected sheep. Infect Immun 77: 2304–2310.

Barre N, Garris G, Camus E (1995) Propagation of the tick *Amblyomma variegatum* in the Caribbean. Rev Sci Tech 14: 841–855.

Barre N, Uilenberg G (2010) Spread of parasites transported with their hosts: case study of two species of cattle tick. Rev Sci Tech 29: 149–160, 135–147.

Bekker CP, de Vos S, Taoufik A, Sparagano OA, Jongejan F (2002) Simultaneous detection of *Anaplasma* and *Ehrlichia* species in ruminants and detection of *Ehrlichia ruminantium* in *Amblyomma variegatum* ticks by reverse line blot hybridization. Vet Microbiol 89: 223–238.

Bell-Sakyi L (2004) *Ehrlichia ruminantium* grows in cell lines from four ixodid tick genera. J Comp Pathol 130: 285–293.

Bell-Sakyi L, Paxton EA, Munderloh UG, Sumption KJ (2000) Growth of *Cowdria ruminantium*, the causative agent of heartwater, in a tick cell line. J Clin Microbiol 38: 1238–1240.

Bezuidenhout J (1988) University of Pretoria.thesis title: Certain aspects of the transmission of heartwater, the occurrence of the organism in ticks and in vitro culture.

Bezuidenhout J, Bigalke R (1987) The control of heartwater by means of tick control. Onderstepoort Journal of Veterinary Research.

Bezuidenhout JD, Paterson CL, Barnard BJ (1985) In vitro cultivation of *Cowdria ruminantium*. Onderstepoort J Vet Res 52: 113–120.

Bourdoulous S, Bensaid A, Martinez D, Sheikboudou C, Trap I, et al. (1995) Infection of bovine brain microvessel endothelial cells with *Cowdria ruminantium* elicits IL-1 beta, -6, and -8 mRNA production and expression of an unusual MHC class II DQ alpha transcript. J Immunol 154: 4032–4038.

Brayton KA, Collins NE, van Strijp F, Allsopp BA (2003) Preparation of *Ehrlichia ruminantium* challenge material for quantifiable and reproducible challenge in mice and sheep. Vet Parasitol 112: 63–73.

Brown CC, Skowronek AJ (1990) Histologic and immunochemical study of the pathogenesis of heartwater (*Cowdria ruminantium* infection) in goats and mice. Am J Vet Res 51: 1476–1480.

Byrom B, Barbet AF, Obwolo M, Mahan SM (2000a) CD8(+) T cell knockout mice are less susceptible to *Cowdria ruminantium* infection than athymic, CD4(+) T cell knockout, and normal C57BL/6 mice. Vet Parasitol 93: 159–172.

Byrom B, Obwolo M, Barbet AF, Mahan SM (2000b) A polarized Th1 type immune response to *Cowdria ruminantium* infection is detected in immune DBA/2 mice. J Parasitol 86: 983–992.

Chitanga S, Gaff H, Mukaratirwa S (2014) Tick-borne pathogens of potential zoonotic importance in the southern African Region. J S Afr Vet Assoc 85: 1084.

Collins NE, Liebenberg J, de Villiers EP, Brayton KA, Louw E, et al. (2005) The genome of the heartwater agent *Ehrlichia ruminantium* contains multiple tandem repeats of actively variable copy number. Proc Natl Acad Sci U S A 102: 838–843.

Cowdry EV (1925) Studies on the Etiology of Heartwater : Ii. *Rickettsia Ruminantium* (N. Sp.) in the Tissues of Ticks Transmitting the Disease. J Exp Med 42: 253–274.

Cowdry EV (1926) Studies on the Etiology of Heartwater : Iii. The Multiplication of *Rickettsia Ruminantium* within the Endothelial Cells of Infected Animals and Their Discharge into the Circulation. J Exp Med 44: 803–814.

Deem SL, Norval RA, Donachie PL, Mahan SM (1996) Demonstration of vertical transmission of *Cowdria ruminantium*, the causative agent of heartwater, from cows to their calves. Vet Parasitol 61: 119–132.

Du Plessis JL (1970) Pathogenesis of heartwater. I. *Cowdria ruminantium* in the lymph nodes of domestic ruminants. Onderstepoort J Vet Res 37: 89–95.
Du Plessis JL (1982) University of Pretoria, Pretoriathesis title: Mice infected with a Cowdria ruminantium-like agent as a model in the study of heartwater..
Du Plessis JL (1993) An in vitro test to demonstrate the inhibitory effect of homologous immune serum on the infectivity of *Cowdria ruminantium*. Onderstepoort J Vet Res 60: 69–73.
Du Plessis JL, Berche P, Van Gas L (1991) T cell-mediated immunity to *Cowdria ruminantium* in mice: the protective role of Lyt-2+ T cells. Onderstepoort J Vet Res 58: 171–179.
Du Plessis JL, Bezuidenhout JD (1979) Investigations on the natural and acquired resistance of cattle to artificial infection with *Cowdria ruminantium*. J S Afr Vet Assoc 50: 334–338.
Du Plessis JL, Bezuidenhout JD, Ludemann CJ (1984) The immunization of calves against heartwater: subsequent immunity both in the absence and presence of natural tick challenge. Onderstepoort J Vet Res 51: 193–196.
Du Plessis JL, Gray C, Van Strijp MF (1992) Flow cytometric analysis of T cell response in mice infected with *Cowdria ruminantium*. Onderstepoort J Vet Res 59: 337–338.
Du Plessis JL, Malan L (1987) The block method of vaccination against heartwater. Onderstepoort J Vet Res 54: 493–495.
Du Plessis JL, Van Gas L, Olivier JA, Bezuidenhout JD (1989) The heterogenicity of *Cowdria ruminantium* stocks: cross-immunity and serology in sheep and pathogenicity to mice. Onderstepoort J Vet Res 56: 195–201.
Dumler J, Barbet A, Bekker C, Dasch G, Palmer G, et al. (2001) 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*, descriptions of six new species combinations and designation of *Ehrlichia equi* and 'HGE agent' as subjective synonyms of *Ehrlichia phagocytophila*. Int J Syst Evol Microbiol 51: 2145–2165.
Dunning Hotopp JC, Lin M, Madupu R, Crabtree J, Angiuoli SV, et al. (2006) Comparative Genomics of Emerging Human Ehrlichiosis Agents. PLoS Genet 2: e21.
Emboule L, Daigle F, Meyer DF, Mari B, Pinarello V, et al. (2009) Innovative approach for transcriptomic analysis of obligate intracellular pathogen: selective capture of transcribed sequences of *Ehrlichia ruminantium*. BMC Mol Biol 10: 111.
Esemu SN, Ndip LM, Ndip RN (2011) Ehrlichia species, probable emerging human pathogens in sub-Saharan Africa: environmental exacerbation. Rev Environ Health 26: 269–279.
Faburay B, Geysen D, Ceesay A, Marcelino I, Alves PM, et al. (2007) Immunisation of sheep against heartwater in The Gambia using inactivated and attenuated *Ehrlichia ruminantium* vaccines. Vaccine 25: 7939–7947.
Ferla MP, Thrash JC, Giovannoni SJ, Patrick WM (2013) In PLoS One, Vol. 8 United States, e83383.New rRNA gene-based phylogenies of the Alphaproteobacteria provide perspective on major groups, mitochondrial ancestry and phylogenetic instability.
Florin-Christensen M, Suarez CE, Rodriguez AE, Flores DA, Schnittger L (2014) Vaccines against bovine babesiosis: where we are now and possible roads ahead. Parasitology 1–30.
Frutos R, Viari A, Ferraz C, Bensaid A, Morgat A, et al. (2006a) Comparative Genomics of Three Strains of *Ehrlichia ruminantium*: A Review. Ann N Y Acad Sci 1081: 417–433.
Frutos R, Viari A, Ferraz C, Morgat A, Eychenie S, et al. (2006b) Comparative Genomic Analysis of Three Strains of *Ehrlichia ruminantium* Reveals an Active Process of Genome Size Plasticity. J Bacteriol 188: 2533–2542.
Gruss B (1981) In Tick Biology and Control Grahamstown, South Africa, 135–136.
Hajnicka V, Vancova I, Kocakova P, Slovak M, Gasperik J, et al. (2005) Manipulation of host cytokine network by ticks: a potential gateway for pathogen transmission. Parasitology 130: 333–342.
Jardine JE, Vogel SW, van Kleef M, van der Lugt JJ (1995) Immunohistochemical identification of *Cowdria ruminantium* in formalin-fixed tissue sections from mice, sheep, cattle and goats. Onderstepoort J Vet Res 62: 277–280.

Jongejan F (1991) Protective immunity to heartwater (*Cowdria ruminantium* infection) is acquired after vaccination with in vitro-attenuated rickettsiae. Infect Immun 59: 729–731.

Jongejan F, Vogel SW, Gueye A, Uilenberg G (1993) Vaccination against heartwater using in vitro attenuated *Cowdria ruminantium* organisms. Rev Elev Med Vet Pays Trop 46: 223–227.

Jongejan F, Zandbergen TA, van de Wiel PA, de Groot M, Uilenberg G (1991) The tick-borne rickettsia *Cowdria ruminantium* has a Chlamydia-like developmental cycle. Onderstepoort J Vet Res 58: 227–237.

Kasari TR, Miller RS, James AM, Freier JE (2010) Recognition of the threat of *Ehrlichia ruminantium* infection in domestic and wild ruminants in the continental United States. J Am Vet Med Assoc 237: 520–530.

Katz JB, DeWald R, Dawson JE, Camus E, Martinez D, et al. (1997) Development and evaluation of a recombinant antigen, monoclonal antibody-based competitive ELISA for heartwater serodiagnosis. J Vet Diagn Invest 9: 130–135.

Kocakova P, Slavikova M, Hajnicka V, Slovak M, Gasperik J, et al. (2003) Effect of fast protein liquid chromatography fractionated salivary gland extracts from different ixodid tick species on interleukin-8 binding to its cell receptors. Folia Parasitol (Praha) 50: 79–84.

Kock ND, Moore PF, Jongejan F, Houghton J, Hubert J, et al. (1998) Suppression of interleukin-5 prevents mortality in BALB/C mice infected with Cowdria ruminantium. Ann N Y Acad Sci 849: 378–380.

Kock ND, van Vliet AH, Charlton K, Jongejan F (1995) Detection of *Cowdria ruminantium* in blood and bone marrow samples from clinically normal, free-ranging Zimbabwean wild ungulates. J Clin Microbiol 33: 2501–2504.

Lally NC, Nicoll S, Paxton EA, Cary CM, Sumption KJ (1995) The *Cowdria ruminantium* groE operon. Microbiology 141 (Pt 9): 2091–2100.

Louw E, Brayton KA, Collins NE, Pretorius A, Van Strijp F, et al. (2002) Sequencing of a 15-kb *Ehrlichia ruminantium* clone and evaluation of the cpg1 open reading frame for protection against heartwater. Ann N Y Acad Sci 969: 147–150.

Maass M, Dalhoff K (1995) Transport and storage conditions for cultural recovery of *Chlamydia pneumoniae*. J Clin Microbiol 33: 1793–1796.

Mahan SM, Kumbula D, Burridge MJ, Barbet AF (1998a) The inactivated *Cowdria ruminantium* vaccine for heartwater protects against heterologous strains and against laboratory and field tick challenge. Vaccine 16: 1203–1211.

Mahan SM, McGuire TC, Semu SM, Bowie MV, Jongejan F, et al. (1994) Molecular cloning of a gene encoding the immunogenic 21 kDa protein of *Cowdria ruminantium*. Microbiology 140 (Pt 8): 2135–2142.

Mahan SM, Peter TF, Simbi BH, Burridge MJ (1998b) PCR detection of *Cowdria ruminantium* infection in ticks and animals from heartwater-endemic regions of Zimbabwe. Ann N Y Acad Sci 849: 85–87.

Marcelino I, de Almeida AM, Brito C, Meyer DF, Barreto M, et al. (2012a) Proteomic analyses of *Ehrlichia ruminantium* highlight differential expression of MAP1-family proteins. Vet Microbiol 156: 305–314.

Marcelino I, de Almeida AM, Ventosa M, Pruneau L, Meyer DF, et al. (2012b) Tick-borne diseases in cattle: Applications of proteomics to develop new generation vaccines. J Proteomics. 75(14):4232–50.

Marcelino I, Lefrancois T, Martinez D, Giraud-Girard K, Aprelon R, et al. (2015a) A user-friendly and scalable process to prepare a ready-to-use inactivated vaccine: The example of heartwater in ruminants under tropical conditions. Vaccine 33: 678–685.

Marcelino I, Sousa MF, Verissimo C, Cunha AE, Carrondo MJ, et al. (2006) Process development for the mass production of *Ehrlichia ruminantium*. Vaccine 24: 1716–1725.

Marcelino I, Vachiery N, Amaral AI, Roldao A, Lefrancois T, et al. (2007) Effect of the purification process and the storage conditions on the efficacy of an inactivated vaccine against heartwater. Vaccine 25: 4903–4913.

Marcelino I, Ventosa M, Pires E, Muller M, Lisacek F, et al. (2015b) Comparative Proteomic Profiling of Ehrlichia ruminantium Pathogenic Strain and Its High-Passaged Attenuated Strain

Reveals Virulence and Attenuation-Associated Proteins. PLoS ONE 10(12): e0145328. doi:10.1371/journal.pone.0145328In PLoS One, Vol. 10 United States, e0145328.

Marcelino I, Verissimo C, Sousa MF, Carrondo MJ, Alves PM (2005) Characterization of *Ehrlichia ruminantium* replication and release kinetics in endothelial cell cultures. Vet Microbiol 110: 87–96.

Martinez D (1997) In Department of Parasitology and Tropical Veterinary MedicineUtrecht University, Utrecht.Thesis title: Analysis of the immune response of ruminants to Cowdria ruminantium infection

Martinez D, Maillard JC, Coisne S, Sheikboudou C, Bensaid A (1994) Protection of goats against heartwater acquired by immunisation with inactivated elementary bodies of *Cowdria ruminantium*. Vet Immunol Immunopathol 41: 153–163.

Martinez D, Perez JM, Sheikboudou C, Debus A, Bensaid A (1996) Comparative efficacy of Freund's and Montanide ISA50 adjuvants for the immunisation of goats against heartwater with inactivated *Cowdria ruminantium*. Vet Parasitol 67: 175–184.

Minjauw B, McLeod A (2003) Centre for Tropical Veterinary Medicine (CTVM), University of Edinburgh, Edinburgh, 116 pp.

Molia S, Frebling M, Vachiery N, Pinarello V, Petitclerc M, et al. (2008) *Amblyomma variegatum* in cattle in Marie Galante, French Antilles: prevalence, control measures, and infection by *Ehrlichia ruminantium*. Vet Parasitol 153: 338–346.

Moumene A, Marcelino I, Ventosa M, Gros O, Lefrancois T, et al. (2015a) Proteomic Profiling of the Outer Membrane Fraction of the Obligate Intracellular Bacterial Pathogen *Ehrlichia ruminantium*.In PLoS One, Vol. 10 United States, e0116758.

Moumene A, Meyer DF (2015b) *Ehrlichia*'s molecular tricks to manipulate their host cells. Microbes Infect.

Mwangi DM, Mahan SM, Nyanjui JK, Taracha EL, McKeever DJ (1998a) Immunization of cattle by infection with *Cowdria ruminantium* elicits T lymphocytes that recognize autologous, infected endothelial cells and monocytes. Infect Immun 66: 1855–1860.

Mwangi DM, McKeever DJ, Mahan SM (1998b) Cellular immune responses of cattle to *Cowdria ruminantium*. Dev Biol Stand 92: 309–315.

Nyika A, Barbet AF, Burridge MJ, Mahan SM (2002) DNA vaccination with *map1* gene followed by protein boost augments protection against challenge with *Cowdria ruminantium*, the agent of heartwater. Vaccine 20: 1215–1225.

Nyika A, Mahan SM, Burridge MJ, McGuire TC, Rurangirwa F, et al. (1998) A DNA vaccine protects mice against the rickettsial agent *Cowdria ruminantium*. Parasite Immunol 20: 111–119.

Oberem PT, Bezuidenhout JD (1987) Heartwater in hosts other than domestic ruminants. Onderstepoort J Vet Res 54: 271–275.

OIE (2005) Institute for International Cooperation in Animal Biologies (OIE collaborating center, College of Veterinary Medicine (Iowa State University).

Owen NC, Littlejohn A, Kruger JM, Erasmus BJ (1973) Physiopathological features of heartwater in sheep. J S Afr Vet Assoc 44: 397–403.

Pedregal A, de Sousa DR, Nguyen HN, das Neves EA, Lowagie S, et al. (2008) Toward prevention of cowdriosis. Ann N Y Acad Sci 1149: 286–291.

Peixoto C, Marcelino I, Amaral AI, Carrondo MJT, Alves PM (2007) Purification by membrane technology of an intracellular *Ehrlichia ruminantium* candidate vaccine against heartwater. Process Biochemistry 42: 1084–1089.

Peixoto CC, Marcelino I, Vachiery N, Bensaid A, Martinez D, et al. (2005) Quantification of *Ehrlichia ruminantium* by real time PCR. Vet Microbiol 107: 273–278.

Peter TF, Anderson EC, Burridge MJ, Perry BD, Mahan SM (1999a) Susceptibility and carrier status of impala, sable, and tsessebe for *Cowdria ruminantium* infection (heartwater). J Parasitol 85: 468–472.

Peter TF, Bryson NR, Perry BD, O'Callaghan CJ, Medley GF, et al. (1999b) *Cowdria ruminantium* infection in ticks in the Kruger National Park. Vet Rec 145: 304–307.

Peter TF, Deem SL, Barbet AF, Norval RA, Simbi BH, et al. (1995) Development and evaluation of PCR assay for detection of low levels of *Cowdria ruminantium* infection in *Amblyomma* ticks not detected by DNA probe. J Clin Microbiol 33: 166–172.

Peterkova K, Vancova I, Hajnicka V, Slovak M, Simo L, et al. (2008) Immunomodulatory arsenal of nymphal ticks. In Med Vet Entomol, Vol. 22 England, 167–171.

Pegram R, Indar L, Eddi C, George J et al. (2014) The Caribbean Amblyomma Program: some ecologic factors affecting its success. Ann N Y Acad Sci. 2004 Oct;1026:302–11.

Pienaar JG (1970) Electron microscopy of *Cowdria* (*Rickettsia*) *ruminantium* (Cowdry, 1926) in the endothelial cells of the vertebrate host. Onderstepoort J Vet Res 37: 67–78.

Pilet H, Vachiery N, Berrich M, Bouchouicha R, Durand B, et al. (2012) A new typing technique for the *Rickettsiales Ehrlichia ruminantium*: multiple-locus variable number tandem repeat analysis. J Microbiol Methods 88: 205–211.

Popara M, Villar M, de la Fuente J (2015) Proteomics characterization of tick-host-pathogen interactions. Methods Mol Biol 1247: 513–527.

Postigo M, Bell-Sakyi L, Paxton E, Sumption K (2002) Kinetics of experimental infection of sheep with *Ehrlichia ruminantium* cultivated in tick and mammalian cell lines. Exp Appl Acarol 28: 187–193.

Pretorius A, Collins NE, Steyn HC, van Strijp F, van Kleef M, et al. (2007) Protection against heartwater by DNA immunisation with four *Ehrlichia ruminantium* open reading frames. Vaccine 25: 2316–2324.

Pretorius A, Liebenberg J, Louw E, Collins NE, Allsopp BA (2010) Studies of a polymorphic *Ehrlichia ruminantium* gene for use as a component of a recombinant vaccine against heartwater. Vaccine 28: 3531–3539.

Pretorius A, van Kleef M, Collins NE, Tshikudo N, Louw E, et al. (2008) A heterologous prime/boost immunisation strategy protects against virulent *E. ruminantium* Welgevonden needle challenge but not against tick challenge. Vaccine 26: 4363–4371.

Pretorius A, Van Strijp F, Brayton KA, Collins NE, Allsopp BA (2002) Genetic immunization with *Ehrlichia ruminantium* GroEL and GroES homologues. Ann N Y Acad Sci 969: 151–154.

Prozesky L (1987a) Heartwater. The morphology of *Cowdria ruminantium* and its staining characteristics in the vertebrate host and in vitro. Onderstepoort J Vet Res 54: 173–176.

Prozesky L (1987b) The pathology of heartwater. III. A review. Onderstepoort J Vet Res 54: 281–286.

Prozesky L, Du Plessis JL (1985) The pathology of heartwater. II. A study of the lung lesions in sheep and goats infected with the Ball3 strain of *Cowdria ruminantium*. Onderstepoort J Vet Res 52: 81–85.

Pruneau L, Emboule L, Gely P, Marcelino I, Mari B, et al. (2011) Global gene expression profiling of *Ehrlichia ruminantium* at different stages of development. FEMS Immunol Med Microbiol.

Reddy GR, Sulsona CR, Harrison RH, Mahan SM, Burridge MJ, et al. (1996) Sequence heterogeneity of the major antigenic protein 1 genes from *Cowdria ruminantium* isolates from different geographical areas. Clin Diagn Lab Immunol 3: 417–422.

Rikihisa Y (2010) *Anaplasma phagocytophilum* and *Ehrlichia chaffeensis*: subversive manipulators of host cells. In Nat Rev Microbiol, Vol. 8 England, 328–339.

Roth JA, Richt JA, Morozov IA (2013) Vaccines and Diagnostics for Transboundary Animal Diseases., Basel.

Sayler KA, Loftis AD, Mahan SM, Barbet AF (2016) Development of a Quantitative PCR Assay for Differentiating the Agent of Heartwater Disease, *Ehrlichia ruminantium*, from the Panola Mountain *Ehrlichia*. Transbound Emerg Dis.63(6):e260-e269.

Sebatjane SI, Pretorius A, Liebenberg J, Steyn H, Van Kleef M (2010) In vitro and in vivo evaluation of five low molecular weight proteins of *Ehrlichia ruminantium* as potential vaccine components. Vet Immunol Immunopathol 137: 217–225.

Semu SM, Mahan SM, Yunker CE, Burridge MJ (1992) Development and persistence of *Cowdria ruminantium* specific antibodies following experimental infection of cattle, as detected by the indirect fluorescent antibody test. Vet Immunol Immunopathol 33: 339–352.

Shkap V, de Vos AJ, Zweygarth E, Jongejan F (2007) Attenuated vaccines for tropical theileriosis, babesiosis and heartwater: the continuing necessity. Trends Parasitol 23: 420–426.

Simbi BH, Bowie MV, McGuire TC, Barbet AF, Mahan SM (2006) Evaluation of *E. ruminantium* genes in DBA/2 mice as potential DNA vaccine candidates for control of heartwater. Ann N Y Acad Sci 1078: 424–437.

Smith GE, Anderson EC, Burridge MJ, Peter TF, Mahan SM (1998) Growth of *Cowdria ruminantium* in tissue culture endothelial cell lines from wild African mammals. J Wildl Dis 34: 297–304.

Stewart CG (1987) Specific immunity in farm animals to heartwater. Onderstepoort J Vet Res 54: 341–342.

Steyn HC, Pretorius A, McCrindle CM, Steinmann CM, Van Kleef M (2008) A quantitative real-time PCR assay for *Ehrlichia ruminantium* using pCS20. Vet Microbiol 131: 258–265.

Totte P, Bensaid A, Mahan SM, Martinez D, McKeever DJ (1999) Immune responses to *Cowdria ruminantium* infections. Parasitol Today 15: 286–290.

Totte P, Blankaert D, Marique T, Kirkpatrick C, Van Vooren JP, et al. (1993) Bovine and human endothelial cell growth on collagen microspheres and their infection with the *rickettsia Cowdria ruminantium*: prospects for cells and vaccine production. Rev Elev Med Vet Pays Trop 46: 153–156.

Totte P, McKeever D, Jongejan F, Barbet A, Mahan SM, et al. (1998a) Analysis of cellular responses to native and recombinant proteins of *Cowdria ruminantium*. Ann N Y Acad Sci 849: 155–160.

Totte P, McKeever D, Martinez D, Bensaid A (1997) Analysis of T-cell responses in cattle immunized against heartwater by vaccination with killed elementary bodies of *Cowdria ruminantium*. Infect Immun 65: 236–241.

Totte P, Nyanjui J, Bensaid A, McKeever D (1998b) The use of CD4+ T-cell lines to screen for immunogenic proteins of *Cowdria ruminantium*. Ann N Y Acad Sci 849: 375–377.

Uilenberg G (1971) [Studies on cowdriosis in Madagascar. I]. Rev Elev Med Vet Pays Trop 24: 239–249.

Uilenberg G (1983) Heartwater (*Cowdria ruminantium* infection): current status. Adv Vet Sci Comp Med 27: 427–480.

Vachiery N, Jeffery H, Pegram R, Aprelon R, Pinarello V, et al. (2008a) *Amblyomma variegatum* ticks and heartwater on three Caribbean Islands. Ann N Y Acad Sci 1149: 191–195.

Vachiery N, Lefrancois T, Esteves I, Molia S, Sheikboudou C, et al. (2006) Optimisation of the inactivated vaccine dose against heartwater and in vitro quantification of *Ehrlichia ruminantium* challenge material. Vaccine 24: 4747–4756.

Vachiery N, Maganga G, Lefrancois T, Kandassamy Y, Stachurski F, et al. (2008b) Differential strain-specific diagnosis of the heartwater agent: *Ehrlichia ruminantium*. Infect Genet Evol 8: 459–466.

Vachiery N, Marcelino I, Martinez D, Lefrancois T (2013) In Dev Biol (Basel), Vol. 135 Switzerland, 191–200.

Vachiéry N, Marcelino I, Martinez D, Lefrancois T (2014) In Manual of Security Sensitive Microbes and Toxins(Ed, Liu D) CRC Press, Boca Raton, 749–759.

Vachiery N, Trap I, Totte P, Martinez D, Bensaid A (1998) Inhibitory effect of *Cowdria ruminantium* on the expression of MHC class I and class II molecules on bovine endothelial cells. Ann N Y Acad Sci 849: 181–187.

Valenzuela J (2004) Exploring tick saliva: from biochemistry to 'sialomes' and functional genomics. Parasitology Suppl:S83–94.

van Amstel SR, Guthrie AJ, Oberem PT, Killeen VM, Matthee O (1988) The clinical pathology of heartwater. II. Studies on cardiac and pulmonary function in 4 calves with experimentally-induced heartwater. Onderstepoort J Vet Res 55: 109–116.

van Amstel SR, Guthrie AJ, Reyers F, Bertschinger H, Oberem PT, et al. (1987) The clinical pathology and pathophysiology of heartwater: a review. Onderstepoort J Vet Res 54: 287–290.

van de Pypekamp HE, Prozesky L (1987) Heartwater. An overview of the clinical signs, susceptibility and differential diagnoses of the disease in domestic ruminants. Onderstepoort J Vet Res 54: 263–266.

van Vliet AH, Jongejan F, van Kleef M, van der Zeijst BA (1993) Cloning and partial characterization of the Cr32 gene of *Cowdria ruminantium*. Rev Elev Med Vet Pays Trop 46: 167–170.

van Vliet AH, Jongejan F, van Kleef M, van der Zeijst BA (1994) Molecular cloning, sequence analysis, and expression of the gene encoding the immunodominant 32-kilodalton protein of *Cowdria ruminantium*. Infect Immun 62: 1451–1456.

van Vliet AH, van der Zeijst BA, Camus E, Mahan SM, Martinez D, et al. (1995) Use of a specific immunogenic region on the *Cowdria ruminantium* MAP1 protein in a serological assay. J Clin Microbiol 33: 2405–2410.

van Vliet AH, van der Zeijst BA, Camus E, Mahan SM, Martinez D, et al. (1996) Recombinant expression and use in serology of a specific fragment from the *Cowdria ruminantium* MAP1 protein. Ann N Y Acad Sci 791: 35–45.

van Vuuren M, Penzhorn BL (2015) Geographic range of vector-borne infections and their vectors: the role of African wildlife. Rev Sci Tech 34: 139–149.

Vancova I, Hajnicka V, Slovak M, Kocakova P, Paesen GC, et al. (2010a) Evasin-3-like anti-chemokine activity in salivary gland extracts of ixodid ticks during blood-feeding: a new target for tick control. In Parasite Immunol, Vol. 32 England, 460–463.

Vancova I, Hajnicka V, Slovak M, Nuttall PA (2010b) Anti-chemokine activities of ixodid ticks depend on tick species, developmental stage, and duration of feeding. In Vet Parasitol, Vol. 167 Netherlands, 274–278.

Vancova I, Slovak M, Hajnicka V, Labuda M, Simo L, et al. (2007) Differential anti-chemokine activity of Amblyomma variegatum adult ticks during blood-feeding. In Parasite Immunol, Vol. 29 England, 169–177.

Villar M, Popara M, Mangold AJ, de la Fuente J (2014) Comparative proteomics for the characterization of the most relevant *Amblyomma* tick species as vectors of zoonotic pathogens worldwide. J Proteomics 105: 204–216.

Yunker CE (1995) Current status of in vitro cultivation of *Cowdria ruminantium*. Vet Parasitol 57: 205–211.

Yunker CE (1996) Heartwater in sheep and goats: a review. Onderstepoort J Vet Res 63: 159–170.

Zweygarth E, Josemans AI (2001) Continuous in vitro propagation of *Cowdria ruminantium* (Welgevonden stock) in a canine macrophage-monocyte cell line. Onderstepoort J Vet Res 68: 155–157.

Zweygarth E, Josemans AI (2003) In vitro infection by *Ehrlichia ruminantium* of baby hamster kidney (BHK), Chinese hamster ovary (CHO-K1) and Madin Darby bovine kidney (MDBK) cells. Onderstepoort J Vet Res 70: 165–168.

Zweygarth E, Josemans AI, Van Strijp MF, Lopez-Rebollar L, Van Kleef M, et al. (2005) An attenuated *Ehrlichia ruminantium* (Welgevonden stock) vaccine protects small ruminants against virulent heartwater challenge. Vaccine 23: 1695–1702.