IN VITRO CULTIVATION OF *EHRLICHIA RUMINANTIUM* AND DEVELOPMENT OF AN ATTENUATED CULTURE-DERIVED VACCINE

In vitro kweek van *Ehrlichia ruminantium* en de ontwikkeling van een verzwakt celkweek vaccin

(met een samenvatting in het Nederlands)

PROEFSCHRIFT

Ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de Rector Magnificus, Prof. Dr. W.H. Gispen,

ingevolge het besluit van het College voor Promoties in het openbaar te verdedigen op 29 mei 2006 des middags te 14.30 uur

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In loving memory of my mum

Klara A. Zweygarth

(23.7.1921 - 28.9.2004)

Front cover: *Ehrlichia ruminantium* in DH82 cells

Back cover: *Ehrlichia ruminantium* in horse fibroblastoid cells

Printed by: Atalanta Drukwerkbemiddeling, Houten.

The research described in this thesis was supported by the Agricultural Research Council of South Africa, the Department of Arts, Culture, Science and Technology of South Africa, the South African Red Meat Board, and the European Union (Cowdriosis Network, Grant no. IC18-CT95-0008 [DG12-SNRD]; Contract No. ICA4-CT- 2000 – 30026; Grant no. EU DGVIII8ACP-TPS-040:613067). Additional support was provided by the International Consortium on Ticks and Tick-borne Diseases (ICTTD-3) financed by the INCO programme of the EU through Coordination Action Project No. 51 0561.

CONTENTS

Chapter 1

Introduction and literature review

Contents

1.1 Introduction

Heartwater, or cowdriosis, is an infectious, non-contagious, tick-borne disease, caused by the intracellular rickettsial agent previously known as *Cowdria ruminantium* but now reclassified as *Ehrlichia ruminantium* (Dumler *et al.* 2001). The disease affects cattle, sheep and goats, and also some wild ruminants, and is transmitted by ticks of the genus *Amblyomma* (Lounsbury 1900). Heartwater is usually an acute disease and may be fatal within days of the onset of clinical signs. Depending on the susceptibility of the host and the virulence of the strain, different forms of the disease may be recognised, ranging from peracute, to acute, sub-acute and even mild. The disease is characterized by pyrexia, anorexia, malaise, nervous symptoms, dyspnoea, gastro-enteritis, hydrothorax and hydropericardium. The latter, a common pathological lesion, is probably the origin of the name heartwater.

The improvement of livestock productivity in Africa is severely hampered by tick-borne parasites and the diseases they transmit (Musoke *et al.* 1997). Uilenberg (1983b) ranked heartwater second only to East Coast fever and tsetse-transmitted trypanosomosis, but no matter which source is cited, heartwater is always to be found high on the list of economically important diseases. In South Africa alone, it was estimated that 40-50% of all livestock deaths within the endemic areas were caused by heartwater (FAO 1996), not to mention the non-lethal losses. A safe and effective method to control heartwater could have a tremendous positive economic impact.

1.2 Historical background

Louis Trichardt (1838), one of the first South African pioneers, or "voortrekkers" to leave the Cape Province to move north, may be the person to whom the first written record of heartwater can be attributed. On 16th February 1838 he wrote in his diary that his small animals got many ticks, and again on 28th February he noted: "We did not move far this day because our livestock were crippled by ticks". He then reported on the 9th March that five of his sheep died of a sort of "Nintas". Nintas is probably what was later described as Nenta, Krimpsiekte or cerebro-spinal meningitis (Soga 1891; Hutcheon 1899). This is a disease characterized by paralysis of the central nervous system, symptoms also seen in heartwater, which is caused by the toxin cotyledontoxin, found in the Nenta bush (*Cotyledon wallichi Harv.*) (Watt and Breyer-Brandwijk 1932). The massive tick infestation, followed three weeks later by a disease with nervous symptoms, led Neitz (1947; 1968) to assume that what Trichardt described were cases of heartwater.

In 1877, a farmer by the name of John Webb gave evidence before the Cattle Disease Commission of the Cape of Good Hope in Grahamstown, where he reported: "I was farming on a farm without ticks, directly this tick appeared all my stock did badly, calves died of gall-sickness, boschsickness (both synonyms of cowdriosis) one man lost 2 or 3,000 sheep and goats, I believe the

tick caused it, I found water on the heart, caused by inflammation brought on by the tick". This was probably the first reference in which the disease was connected with the tick: "as this tick increases, so diseases increase". Webb also mentioned that bushbucks were "suffering from the same causes". He continued by saying that a certain Mr. W. Bowker told him that he had taken a "boute-tick" (*Amblyomma hebraeum*) from a cow from Zululand, about 40 years ago. This would have been around 1837, and was the first mention of the bont tick occurring in the Cape of Good Hope.

At that time, the term "heart water" was used for two different conditions, one was due to various intestinal parasites, such as flukes, tapeworms and small round worms (Anonymous 1888; Anonymous 1896), whereas the other heartwater was a separate disease peculiar to certain localities along the coast (Borthwick 1889). Edington (1898) described the first transmission of the disease, injecting blood from an infected ox into sheep and goats, which produced virulent heartwater, more severe than that which was produced by goat or sheep blood. Dixon (1898) showed in his experiments that heartwater was not due to anything taken into the stomach, and that if he kept the ticks off the sheep, they did not fall ill. He considered that ticks played some part in the spread of the disease, not as the direct cause, but that they conveyed the infective material, probably by inoculation. He also succeeded in transmitting the disease by subcutaneous and intravenous inoculations of infected blood into susceptible animals. The origin of the infective agent was still unknown when Hutcheon (1900) described heartwater as having all the characteristics of a special, or specific disease, that is, a disease which owes its origin to some living microorganism which grows and multiplies in the animal's body. The implication of tick involvement in heartwater was eventually demonstrated by Lounsbury (1900), who provided conclusive evidence that the bont tick, *A. hebraeum*, was involved in the transmission of the heartwater agent. The agent reponsible for heartwater was suggested to be an "ultra-visible virus" transmitted by *A. hebraeum* (Spreull 1922).

An important milestone in heartwater investigation was provided by the work of the Canadian-born rickettsiologist E.V. Cowdry, who, at a suggestion of Sir Arnold Theiler that the disease might be due to a rickettsia, successfully demonstrated gram-negative, intracellular coccus-like microorganisms in the tissues of heartwater infected animals (Cowdry 1925a). Cowdry proposed the designation *Rickettsia ruminantium* to reflect the association of microorganisms resembling *Rickettsia* with a disease of ruminants, at the time the only ruminant disease in which a *Rickettsia* had been reported. He also described the organisms in the tick vector (Cowdry 1925b).

1.3 *Ehrlichia ruminantium*

1.3.1 Classification

After the heartwater agent was named *Rickettsia ruminantium* (Cowdry 1925a), Rake *et al.* (1945) questioned the classification, suggesting that the organism was related to both *Rickettsia* and *Chlamydia* (then the lymphogranuloma-psittacosis group). Moshkovski (1947) suggested that

Rickettsia ruminantium should be assigned to a new genus, and in honour of Cowdry he renamed it *Cowdria ruminantium*. Pienaar (1970) suggested that "The taxonomic position of *C. ruminantium* should be an intermediate grouping between the genus *Rickettsia* and the psittacosis-lymphogranuloma-trachoma (PLT) agents", and subsequently numerical taxononomic studies indicated that *Cowdria* was close to the genus *Chlamydia* (Scott 1987). More evidence of a relationship with *Chlamydia* included the finding that a monoclonal antibody directed against the major outer membrane protein of *C. trachomatis* recognized *C. ruminantium* (Jongejan *et al.* 1991a), and the observation of a developmental cycle in *C. ruminantium* which appeared to resemble a cycle described for *Chlamydia* (Jongejan *et al.* 1991c). At the same time, however, serological cross-reactions between *C. ruminantium* and *E. equi* and *E. canis* indicated a relationship with *Ehrlichia* (Logan *et al.* 1986).

The taxonomic classification had, up until this point, been based on morphological and behavioural grounds. The first use of genetic analysis was when the 16S ribosomal RNA gene of *C. ruminantium* was sequenced, and the organism was found to be phylogenetically closely related to several members of what was then known as the tribe Ehrlichieae. The clustering of *C. ruminantium* with some, but not all, *Ehrlichia* species indicated that the tribe Ehrlichieae needed readjustment (van Vliet *et al.* 1992). In 2001, Dumler *et al.* proposed a new classification of the Rickettsiales based on phylogenetic analysis of the sequences of the 16S rRNA gene and the *groESL* operon. *Ehrlichia (Cowdria) ruminantium* comb. nov. together with *E. canis*, *E. chaffeensis*, *E. ewingii* and *E. muris*, were assigned to the genus *Ehrlichia*. According to this analysis *E. ruminantium* is classified as follows:

Phylum: Proteobacteria
Class: Alphaproteobacteria
Order: Rickettsiales
Family: Anaplasmataceae
Genus: *Ehrlichia*
Species: *Ehrlichia ruminantium*

1.3.2 Developmental cycle

Electron microscopic studies of *E. ruminantium* propagated in endothelial cell cultures showed that it has a *Chlamydia*-like developmental cycle (Jongejan *et al*. 1991c). *E. ruminantium* divides by binary fission within the intracytoplasmatic vacuoles forming large colonies of reticulate bodies. After three to four days in cell culture, they develop into smaller intermediate bodies characterized by an electron-dense core, which condense further into electron dense bodies shortly before disruption of the host cells. A new infectious cycle is initiated when the released elementary bodies invade other endothelial cells. Each infectious cycle lasts between five and six days. However, shorter cycles of 3 days (author's unpublished results) or 4 days (Prozesky *et al.* 1993) have been observed.

In the vertebrate host organisms are believed to initially replicate in the reticulo-endothelial cells in lymph nodes, from where they are released into the efferent lymph stream and eventually into the blood to enter endothelial cells (du Plessis 1970). The organisms, which are in contact with endothelial cell membranes, are taken up through a process resembling phagocytosis and enclosed in a vacuole. The vacuole membrane is therefore probably derived from host cell membranes. *E. ruminantium* then exists within the membrane bound vacuole in the cytoplasm of endothelial cells, developing from a single organism to form a large colony. This eventually causes the cell to rupture and to dissiminate the organisms in the form of elementary bodies into the bloodstream, thus repeating the cycle (Prozesky and du Plessis 1987). Feeding ticks probably take up *E. ruminantium* organisms during this phase of the cycle.

In the invertebrate host, after ingestion of an infected blood meal, *E. ruminantium* initially appeared to develop in the midgut epithelial cells (Cowdry 1925b). Electron microscopy studies revealed that subsequent stages of the organism invaded and developed in salivary gland acini. This suggested that *E. ruminantium* may be transferred to the vertebrate host via the salivary glands (Kocan and Bezuidenhout 1987) rather than by gut regurgitation as suggested by Cowdry (1925b). The presence of colonies of *E. ruminantium* in salivary glands of ticks, along with the demonstration of different morphological forms of the organism, led Kocan and Bezuidenhout (1987) to suggest that a developmental cycle occurs in the invertebrate host.

1.4 Epizootiology

1.4.1 Host range

All livestock belonging to the family Bovidae, sheep, goats and cattle, are susceptible to infection with the heartwater agent. Water buffalo (*Bubalus bubalis*), although only represented by a very small number in the African heartwater belt, are extremely susceptible to heartwater (Mammerickx 1961).

Several wild animal species have been implicated as hosts of *E. ruminantium*. Webb in 1877 noted that bushbucks (*Tragelaphus scriptus*) which he had shot showed symptoms similar to those of his sheep suffering from heartwater. Alexander (1931) contradicted Webb's statement and thought that heartwater was not a cause of mortality in game, but rather that game formed a source of infection for ticks. The first experimentally-confirmed infection of a blesbuck (*Damaliscus albifrons*) was carried out by Neitz (1933). He showed that the blesbuck can act as a heartwater carrier, and a subinoculation done on the 18th day of infection produced a positive result in a sheep. Further experiments also confirmed the black wildebeest (*Connochaetes gnu*) as a carrier of heartwater (Neitz 1935). It was also shown that *A. hebraeum* can transmit heartwater to a blesbuck and that the ticks can become infected by feeding on an infected blesbuck (Neitz 1937). In 1944 Neitz demonstrated the susceptibility of the springbuck (*Antidorcas marsupialis*) to heartwater, and attributed the animals' death to it. Typical clinical signs and lesions found at necropsy, and the presence of *E. ruminantium,* were observed in an

eland (*Taurotragus oryx*) (Young and Basson 1973). A summary of findings concerning heartwater in wild ruminants, non-African ruminants, wild rodents, birds, reptiles and other species has been published (Oberem and Bezuidenhout 1987). Experiments with the common guineafowl (*Numida meleagris*), the leopard tortoise (*Geochelone pardalis*) and the scrub hare (*Lepus saxitilis*) proved that these animals can act as asymptomatic carriers of *E. ruminantium*. Furthermore, larvae and nymphae of *A. hebraeum* fed on infected guinea fowl or tortoises, and larvae fed on an infected scrub hare, were able to transmit the infection in the subsequent stages of their life cycle (Bezuidenhout *et al.* 1987). Contradictory results were obtained by Peter *et al.* (2001a) whose attempts to detect *E. ruminantium* infections in ticks fed on guineafowls and leopard tortoises, which were either injected with culture-derived organisms or exposed to infected ticks, were unsuccessful. Kock *et al.* (1995) detected *E. ruminantium* in blood and bone marrow samples from clinically normal, free-ranging Zimbabwean wild ruminants. Using a polymerase chain reaction assay based on the *E. ruminantium map1* gene, positive reactions were obtained with tsessebe (*Damaliscus lunatus*), waterbuck (*Kobus ellipsiprymnus*) and impala (*Aepyceros melampus*). The host range of *E. ruminantium* in wildlife and their role in epidemiology has been reviewed recently (Peter *et al.* 2002).

1.4.2 Transmission

Heartwater is transmitted by members of the three-host tick genus *Amblyomma*. In South Africa, *A. hebraeum* is the major vector for *E. ruminantium* (Walker and Olwage 1987). *A. hebraeum* was the first tick species incriminated in the transmission of the heartwater agent (Lounsbury 1900). Infections in *A. hebraeum* ticks are transmitted transstadially, i.e. from larvae to nymphae, from nymphae to adults, and from larvae through nymphae to adults, and a single infected nymph feeding on a susceptible animal was fatal (Lounsbury 1902). Intrastadial transmission by male *A. hebraeum* ticks from a sick to a susceptible animal had no adverse effects (Lounsbury 1902). In contrast, Andrew and Norval (1989) found that intrastadial transmission by male ticks does occur. Heartwater was not transmitted transovarially (Lounsbury 1902); there is, however, a single contradictory report (Bezuidenhout and Jacobsz 1986). Among the 102 species of *Amblyomma* ticks presently known, only 10 African species transmit *E. ruminantium.* Natural and proven experimental vectors of heartwater are summarized in Table 1.

Table 1 African *Amblyomma* tick species shown to be experimental and natural vectors heartwater*

Amblyomma sp	Reported mode of transmission	Reference
A. hebraeum	I, II, III	Lounsbury (1900)
A. variegatum	I, II, III	Daubney (1930)
A. pomposum	I, II	Neitz (1947)
A. gemma	II	Lewis (1949); Ngumi et al. (1997)
A. lepidum	I, II	Karrar (1960); Karrar (1966)
A. tholloni	I, II, III	MacKenzie and Norval (1980)
A. sparsum	I, III	Norval and MacKenzie (1981)
A. astrion	I, III	Uilenberg and Niewold (1981)
A. cohaerens	I, III	Uilenberg (1983a)
A. marmoreum	I, III	Bezuidenhout et al. (1987); Peter et al. (2000b)

I from larval to nymphal stage
II from nymphal to adult stage
III from larval through nymphal to adult stage
* from Bezuidenhout (1987) with some modifications

Three New World *Amblyomma* species, *Amblyomma maculatum*, an efficient vector (Uilenberg 1982) *Amblyomma cajennense,* an inefficient vector (Uilenberg 1983a) and *Amblyomma dissimile* (Jongejan 1992) have been shown to transmit *E. ruminantium* experimentally. *Amblyomma dissimile* is a tick of reptiles, and is therefore not considered to play a significant role in the transmission of heartwater between ruminants, but it could maintain a rickettsial reservoir in the reptile population (Jongejan 1992).

Besides transmission through a vector tick, vertical transmission of *E. ruminantium* from cows to their calves has been demonstrated by xenodiagnosis and by a polymerase chain reaction assay. Furthermore, the transmission of *E. ruminantium* was shown after injection of viable colostral cells from dams living in a heartwater-endemic area into goats (Deem *et al.* 1996).

1.4.3 Distribution

In Africa, the distribution of heartwater coincides with the distribution of the *Amblyomma* vector ticks. Heartwater has been reported from almost all African countries south of the Sahara, from the Indian

Ocean islands of Madagascar (Poisson 1927), Grande Comore (du Plessis *et al.* 1989), Réunion and Mauritius (Perreau *et al.* 1980), and from the Atlantic Ocean islands of São Tomé (Uilenberg *et al.* 1982).

A. variegatum is as yet the only African vector of heartwater that has established itself outside that continent. In 1828, animals were imported onto islands in the Caribbean from Senegal and *A. variegatum* was probably introduced simultaneously. The introduction of *A. variegatum* could have been even earlier, because cattle were shipped from Africa to the Caribbean in the 18th century, possibly in 1733 (Maillard and Maillard 1998). In Guadeloupe, *A. variegatum* is therefore still called the "Senegalese" tick (Buck 1966). Although the vector tick was present in the Caribbean, heartwater was not diagnosed until much later. Neitz still stated in 1968 that although *A. variegatum* was reported to be prevalent in the West Indies, heartwater was not (Neitz 1968). Finally heartwater was diagnosed in 1980 on Guadeloupe (Perreau *et al.* 1980). Subsequently, Marie Galante (Camus *et al.* 1984) and Antigua (Birnie *et al.* 1984) were added to the list. In addition to possible dissemination through livestock trade, ticks are carried from island to island by migrating cattle egrets (*Bubulcus ibis*) (Uilenberg 1990) and thus heartwater could be introduced into the Americas, where large areas are climatically suitable for *A. variegatum*.

1.5 *In vitro* cultivation

1.5.1 Propagation of E. ruminantium *in arthropod cell cultures*

When attempts were made to cultivate *E. ruminantium* in an *Aëdes albopictus* cell line intracellular granular structures resembling *E. ruminantium* were demonstrated but their identity was not determined with certainty (du Plessis 1972, unpublished results). Sf9 cells, derived from the fall armyworm, *Spodoptera frugiperda,* could not be infected using endothelial cell-culture-derived elementary bodies of the Welgevonden stock of *E. ruminantium* (author's unpublished results, 2001).

Andreasen (1974) reported the first successful primary cultures of tick cells initiated from moulting nymphs of *A. hebraeum* and *A. variegatum* which were infected with infective blood from *E. ruminantium* infected sheep. Nine-day-old cultures were injected intravenously into two susceptible sheep, both of which died of heartwater, as diagnosed by the presence of typical *E. ruminantium* organisms in Giemsa-stained brain squash smears. Other attempts to propagate *E. ruminantium* in tick cells failed (Uilenberg 1983b, Yunker *et al.* 1988) until Bell-Sakyi *et al.* (2000) achieved the first continuous propagation of the Gardel stock of *E. ruminantium* in an *Ixodes scapularis* tick cell line for more than 500 days. Furthermore, Bekker *et al.* (2002) infected a *Rhipicephalus appendiculatus* cell line, designated RAN/CTVM3 with the Gardel stock of *E. ruminantium*. Bell-Sakyi (2004) infected continuous tick cell lines derived from *A. variegatum*, *I. scapularis* and *Ixodes ricinus* with *E. ruminantium* derived from bovine endothelial cell cultures. Subsequently this author infected another eight tick cell lines, derived from six different tick species (*A. variegatum*, *B. decoloratus,*

Boophilus microplus, I. scapularis, *I. ricinus* and *R. appendiculatus*), with *E. ruminantium* derived from the already established infected tick cell cultures. All *E. ruminantium* isolates, five in total, grew continuously in at least one tick cell line. Three of the isolates could be reestablished in bovine endothelial cell cultures following prolonged maintenance in tick cell cultures.

1.5.2 Short-term in vitro *culture of* E. ruminantium *in mammalian cells*

Initial attempts to propagate *E. ruminantium* in primary ruminant cell cultures, or cell lines from non-ruminant species, met with limited success. Cultures of BHK21 cells, inoculated with macerated brain material from an ewe which had died of heartwater, failed to become infected (Anonymous 1968). The first attempt to culture *E. ruminantium* in leukocytes of reacting animals also failed (Ramisse and Uilenberg 1971). Similarly, suspension cultures of various different types of sheep cells failed to become infected after inoculation with infectious heartwater blood. The cells used came from adult and foetal spleens, peritoneal inflammatory exudate, amniotic membrane, choroid plexus, and foetal stomach, testis and skin. None of these cultures permitted multiplication of the organism or were infective to susceptible sheep (Ramisse 1971, 1972). Jongejan *et al.* (1980) established primary kidney cell cultures from *E.ruminantium*-infected goats and 5, 12 and 13 day old cultures injected into susceptible goats induced heartwater in the recipients. Despite this, *E. ruminantium* could not be detected microscopically in the cell cultures and cultures older than 13 days were non-infective. Stewart and Howell (1981) inoculated foetal choroid plexus cells, which had been infected with *E. ruminantium* and subpassaged 3 or 8 times, into susceptible sheep but none became infected. Inclusion bodies of *E. ruminantium* were demonstrated in primary leukocyte cell cultures prepared from the blood of infected goats, sheep, and cattle (Sahu *et al.* 1983). *In vitro* cultured macrophages and buffy coat cultures also revealed the presence of inclusion bodies of *E. ruminantium* in neutrophils and macrophages (Sahu 1986). A primary neutrophil culture was devised by Logan *et al.* (1987) which was suitable for the production of *E. ruminantium* to be used in serological tests. They observed *E. ruminantium* colonies in up to 35% of neutrophils maintained *in vitro* for 18h to 5 days, and although of short duration the system did permit intracellular multiplication of the organisms. This primary neutrophil culture technique was successfully taken up by Jongejan *et al.* (1989) and Martinez *et al.* (1990).

1.5.3 Continuous in vitro *propagation of* E. ruminantium *in mammalian cell lines*

From studies using histological sections Cowdry (1926) described the multiplication of *E. ruminantium* within the endothelial cells of infected animals. It was therefore not surprising that the first successful *in vitro* propagation of *E. ruminantium* was achieved using bovine umbilical cord endothelial cells as host cells (Bezuidenhout *et al.* 1985). Almost all subsequent *in vitro* propagations of *E. ruminantium* used endothelial cells of various ruminant species, and from various anatomical sites. The following

cells, mostly endothelial cells from large blood vessels, were all used successfully: bovine aorta, bovine pulmonary artery, foetal bovine heart (Yunker *et al.* 1988); ovine pulmonary artery (Byrom *et al.* 1991); sheep brain (Brett *et al.* 1992); bovine saphenous vein (Neitz and Yunker 1996); caprine jugular vein (Totté *et al.* 1996); and bovine testicular vein (Mwangi *et al.* 1998). The ability of *E. ruminantium* to invade endothelial cells was not, however, restricted to large vessel endothelial cells, and it was shown that bovine endothelial cells isolated from brain microvasculature could also be infected with *E. ruminantium* (Martinez *et al.* 1993c; Totté *et al.* 1993a, b). Even human endothelial cells, derived from both the umbilical vein and brain microvasculature, were infected by *E. ruminantium* (Totté *et al.* 1993a). Furthermore, Smith *et al.* (1998) propagated *E. ruminantium* in endothelial cell lines obtained from three species of African wild ruminants, sable antelope (*Hippotragus niger*), buffalo (*Syncerus caffer*), and eland (*Tragelaphus oryx*), and also from a bush pig (*Potamochoerus porcus*), an omnivore. The latter two groups (Totté *et al.* 1993a; Smith *et al.* 1998) showed that growth *of E. ruminantium* in culture is not restricted to cells derived from natural hosts of the organism. Jongejan and Bekker (1999) tested several cell lines which were in use for the cultivation of other ehrlichial species (P388D1; MDH-SP; DH82) and were able to grow *E. ruminantium* in monocyte-macrophage cell lines from mice and dogs, and also in a human leukaemia cell line (HL-60). It should be noted, however, that the infection rates remained low, and no persistent infections were established.

Although most endothelial cell lines were found to be suitable for the propagation of *E. ruminantium*, Yunker *et al.* (1988) failed to infect ovine aorta or ovine pulmonary artery cells. Other cell types, such as African green monkey kidney cells (Vero cells), lamb foetal kidney cells and mouse connective tissue cells (L-cells) were refractory to infection with *E. ruminantium* (Bezuidenhout 1987), as were mouse fibroblasts (McCoy cells) (Yunker *et al.* 1988). An overview of the mammalian host cells used for the *in vitro* propagation of *E. ruminantium* is presented in Table 2.

Table 2 Mammalian host cells used for the *in vitro* propagation of *E. ruminantium*

Host cells	Designation	Reference
Calf umbilical cord endothelial	E5	Bezuidenhout *et al.* 1985
African green monkey kidney cells* Lamb foetal kidney* Mouse connective tissue cells*	Vero LFK L-cells	Bezuidenhout 1987
Bovine aorta Bovine pulmonary artery Ovine aorta* Ovine pulmonary artery* Cow pulmonary artery endothelium Foetal bovine heart Ovine choriod plexus* Mouse fibroblasts*	BA886; BA987 BPA987 CPAE; CPA-47 FBHE SCP McCoy	Yunker *et al.* 1988
Sheep brain endothelial	SBE 189	Brett *et al.* 1992
Bovine brain capillary endothelial	BBEC	Martinez *et al.* 1993c
Bovine endothelial, brain micro-vasculature Human endothelial, microvasculature Human endothelial, umbilical vein	BMC HEMEC HUVEC	Totté *et al.* 1993a
Bovine saphenous vein endothelial	BSV-793	Neitz and Yunker 1996
Caprine jugular vein endothelial	CJE	Totté *et al.* 1996
Bovine testicular vein endothelial	EC	Mwangi *et al.* 1998
Pulmonary artery or aorta endothelial of Sable antelope (*Hippotragus niger*) Buffalo (*Syncerus caffer*) Eland (*Tragelaphus oryx*) Bush pig (*Potamochchoerus porcus*)	Sable 1194 Buffalo 595 Eland 895 Bushpig 1194	Smith *et al.* 1998
Monocyte-macrophage cell lines murine canine	 MDHSP; P388D1 DH82	Jongejan and Bekker, 1999

* Cell lines were refractory to infection

Commercially available synthetic culture media are a major constituent of the media for the *in vitro* cultivation of *E. ruminantium*. The first medium used consisted of Glasgow's minimal essential medium (GMEM) (Bezuidenhout *et al.* 1985, as specified in Bezuidenhout and Brett 1992). Other media have been used: Leibovitz L-15 and Leibovitz L-15 supplemented by 0.45% glucose (Byrom

and Yunker 1990); Dulbecco's modified Eagle's medium and RPMI 1640 (Martinez *et al.* 1993c). Commonly used supplements for complete culture medium were foetal bovine serum (Byrom and Yunker 1990), newborn calf serum (Jongejan 1991) or bovine serum (Bezuidenhout *et al.* 1985). Another poorly defined supplement frequently used for complete culture medium was tryptose phosphate broth (TPB) (Bezuidenhout *et al.* 1985; Yunker *et al.* 1988; Byrom and Yunker 1990; Byrom *et al.* 1991). The addition of TPB was, however, not necessary for the successful cultivation of *E. ruminantium;* Jongejan (1991) omitted TPB without negative effects on the *E. ruminantium* cultures.

A tick-derived stabilate prepared from *A. hebraeum* nymphae was used for the first successful experiment to initiate *E. ruminantium* cultures (Bezuidenhout *et al.* 1985). Later, freshly drawn heparinized blood, infected choroid plexus, and suspensions of liver and spleen prepared from moribund infected mice, were all used to initiate cultures (Bezuidenhout 1987). A technique using plasma obtained from heparinized blood as culture inoculum was devised, demonstrating that infective *E. ruminantium* elementary bodies were present in the plasma (Byrom and Yunker 1990; Byrom *et al.* 1991). A modification of the method of Bezuidenhout (1987) was described by Jongejan *et al.* (1991c) who diluted heparinized blood with sucrose-potassium phosphate-glutamate buffer (SPG), which had been shown to improve the stability of *Rickettsia prowazekii* (Bovarnick *et al.* 1950).

Several procedures have been described to facilitate initial infection of the endothelial cell monolayer, either by retarding cell growth through irradiation or cycloheximide treatment of the endothelial cells, or by facilitating contact between the parasite and the cells (Bezuidenhout *et al.* 1985; Bezuidenhout 1987). None of these procedures, however, were found to be essential to initiate cultures (Yunker *et al.* 1988; Byrom and Yunker 1990; Byrom *et al.* 1991; Martinez *et al.* 1993c). However, the addition of cycloheximide to SBE 189 cultures infected with the Gardel stock shortened the subculture interval by one day and improved the subculture ratio in comparison to untreated cultures (author's unpublished results).

Without doubt, the continuous *in vitro* propagation of *E. ruminantium* in mammalian cell lines opened a new era in *E. ruminantium* research. *In vitro* propagation of *E. ruminantium* made available huge amounts of rickettsial material whenever needed and superseded cumbersome methods of purification of the organisms from tick tissue (Bezuidenhout 1981), brain (Viljoen *et al.* 1985) or blood fractions (Neitz *et al.* 1986a). Ultimately, the successful *in vitro* cultivation formed the basis of the sequencing of the complete genome of the Welgevonden stock of *E. ruminantium* (Collins *et al.* 2005)

1.5.4 Mass production of E. ruminantium

A pre-requisite for the large-scale production of *E. ruminantium* is an up-scaling process for the endothelial host cell cultures. Brett and Bezuidenhout (1989) used roller bottle cultures, amounting to 800 cm^2 of culture area, for the propagation of endothelial cells, which were subsequently infected with *E. ruminantium*. They estimated that the yield of one roller bottle could produce 20,000 doses of

live vaccine. A further basic step to up-scale the production system for *E. ruminantium* was reported by Totté *et al.* (1993a), who studied the adhesion properties of endothelial cells in a bioreactor using collagen microspheres. They showed that endothelial cells attached efficiently on the collagen microspheres but they did not extend their experiments further to infect these cells with *E. ruminantium*. Recently Marcelino *et al.* (in press) described optimization of a bioreaction strategy for the production of *E. ruminantium* elementary bodies under stirring culture conditions using microcarriers as anchors for the endothelial host cells. Their results indicated that the production of *E. ruminantium* in stirred tank bioreactors was possible and that the production of *E. ruminantium* increased by a factor of 6.5 when using a serum-free medium (this thesis, Chapter 4) instead of conventional serum-containing media.

1.6 Control

1.6.1 Treatment

Spreull (1922) wrote that many medicinal remedies had been tried as treatments for heartwater but none had been successful. The situation remained thus until 1939 when Neitz described the action of Uleron, a sulphonamide [4-(4'-aminobenzolsulfonamide) benzol-sulfonedimethylamide] on heartwater in sheep (Neitz 1939). He observed a recovery rate of 39 out of 41 experimentally infected sheep after treatment with Uleron, whereas 26 out of 37 untreated control sheep died (Neitz 1940). Uleron and sulphapyridine were found to be of real value in the specific treatment of the disease (Alexander *et al.* 1946). They stated that treatment should start early, because by the time a definite diagnosis could be made, i.e. when defined nervous symptoms occurred, the condition was too far advanced to respond successfully to treatment. Beside these two drugs, a wide variety of other sulphonamides had been used for the treatment of heartwater (Weiss *et al.* 1952). The sulphonamides ended the era of symptomatic treatment of heartwater by specific treatment of the heartwater agent.

The first antibiotic which was shown to have a beneficial effect against heartwater was chlortetracycline (Aureomycin^{R}). Moreover, sheep which had been cured by chlortetracycline were immune against a subsequent challenge with the same strain of *E. ruminantium* (Lewis 1951). Chlortetracycline was considered more effective than the sulphonamides (Weiss *et al.* 1952) and these authors further suggested chlortetracycline alone, or in combination with a sulphonamide, as the drug of choice for the treatment of heartwater. Other compounds of the tetracycline group, like oxytetracycline (Haig *et al.* 1954), rolitetracycline (Poole 1961) and doxycycline (Immelmann and Dreyer 1982) were also effective. Up to now, tetracyclines remain the drugs of choice for the treatment of heartwater.

1.6.2 Vaccine Developments

1.6.2.1 Artificial premunition (blood vaccine)

The first practicable method to convey protection against heartwater was applied by Neitz and Alexander (1941). It was based on the fact that young animals possess an innate resistance to heartwater, which lasts in calves up to the age of four weeks and lambs up to at least seven days. Low mortality in calves was observed after injection of virulent blood, which was even lower after treatment with a sulphonamide (Uleron). 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 (Alexander 1931). These findings were exploited by Neitz and Alexander (1945) who recommended the use of Uleron to control the reaction to the injection of infectious blood in cattle for immunization purposes. Weiss *et al.* (1952) suggested the use of aureomycin alone or in combination with a sulphonamide as the drug choice for treatment after a live "virus" immunization. These procedures of artificial premunition were referred to as the "infection and treatment method" by Uilenberg (1983b). The vaccine produced at Onderstepoort consists of blood from sheep infected with live organisms of the Ball 3 stock of *E. ruminantium*. The spectrum of protection of the Ball 3 blood vaccine stock against other *E. ruminantium* stocks is limited (Collins *et al.* 2003). However, the Ball 3 isolate was chosen because it caused a marked febrile response a few days prior to the onset of other clinical signs (Oberem and Bezuidenhout 1987) so that an effective antibiotic treatment could be initiated in time. The Welgevonden stock has been shown to stimulate protective immunity against several virulent South African stocks and would therefore be more suitable for immunization purposes than the Ball 3 stock, but its high virulence has precluded it from being used as a vaccine stock (du Plessis *et al.* 1989). The "infection and treatment" method, which is currently practised in South Africa, is far from ideal, being expensive and dangerous, but has been the only commercially available "vaccine" for more than 50 years. The sale figures of the Onderstepoort heartwater blood vaccine over the last five years are summarized in Table 3 (M.P. Combrink, personal communication 2005).

Table 3 Sale figures of the Onderstepoort heartwater blood vaccine over the last five years

Year	Heartwater blood vaccine doses sold
2000	91,305
2001	75,450
2002	84,567
2003	68,481
2004	58,611

1.6.2.2 Attenuated vaccines

Early attempts to attenuate *E. ruminantium* through serial passages through sheep failed; considerable variations in virulence were observed during the first 75 generations, but no consistent attenuation was demonstrated (du Toit and Alexander 1931). Attempts to attenuate *E. ruminantium* by irradiation of the nymphal stages of the tick vector, using a ^{60}Co source, also failed and no attenuation was demonstrated (Spickett *et al.* 1981). Jongejan (1991) reported that the Senegal stock was attenuated spontaneously after only eleven passages in bovine endothelial cell cultures, and it was shown that when culture suspensions were used as vaccine, they conferred a strong protection against homologous challenge in sheep and goats. In contrast, the Welgevonden stock did not attenuate after 226 days in culture, equivalent to 17 culture passages (Jongejan 1991). The attenuated Senegal stock, however, did not provide efficient cross-protection against other virulent stocks (Jongejan *et al.* 1993b). Field trials using the attenuated stock vaccine were conducted in Senegal (Gueye *et al.* 1994). It was shown that out of a group of thirty sheep thirteen animals died, but five of these were suffering from ehrlichiosis or anaplasmosis and *E. ruminantium* was only found in two sheep, whereas in the control group twenty-one out of thirty animals died due to heartwater. The Gardel stock, originally highly virulent for domestic ruminants, was found to be attenuated after more than 200 passages in culture (Martinez 1997). The Zimbabwean Crystal Springs stock of *E. ruminantium* was not attenuated and still fatally infective in sheep after 60 and 192 passages *in vitro* (Mahan *et al.* 1995), whereas at higher passages this stock lost its virulence as demonstrated by lower fatallity rates in sheep upon challenge (S.M. Mahan, personal communication 2005).

1.6.2.3 Inactivated vaccine

With the development of methods for the *in vitro* cultivation of *E. ruminantium*, culture-derived elementary bodies became available. At the Onderstepoort Veterinary Institute (OVI), Brett (1989, unpublished results) attempted the first vaccination using culture-derived, formalin-inactivated, elementary bodies. Two sheep injected twice with the inactivated vaccine recovered from a virulent challenge, whereas none of the control animals survived. Although the results were quite promising this work was abandoned. Martinez *et al.* (1993b, 1994) used inactivated elementary bodies mixed with Freund's adjuvant to immunize goats, of which two thirds were protected and survived the challenge. Similar results were reported by Mahan *et al.* (1995) who immunised sheep against heartwater with inactivated *E. ruminantium* organisms emulsified in Freund's complete adjuvant. Cattle were also successfully immunized, and animals proved fully resistant to virulent challenge even 10 months after vaccination (Totté *et al.* 1997). In the second phase of development, Freund's complete adjuvant was replaced by more improved formulations such as Montanide ISA 50 (Martinez *et al.* 1996) or Quil A. (Mahan *et al.* 1998a). In field experiments, an inactivated vaccine used to immunise cattle, sheep and goats reduced the overall mortality among the vaccinated animals (Mahan *et al.* 2001).

1.6.2.4 DNA vaccines

A DNA vaccine containing the *map1* gene of *E. ruminantium* protected mice against a lethal dose of cell culture-derived *E. ruminantium* challenge, leading to survival rates of 23% to 88% in the various groups, whereas survival rates of 0% to 3% were recorded for the control mice (Nyika *et al.* 1998). DNA vaccination in mice with the *map1* gene followed by a protein boost augmented protection against challenge with *E. ruminantium* (Nyika *et al.* 2002). Brayton *et al.* (1998) cloned mini-libraries *of E. ruminantium* into a *Salmonella* vaccine delivery system, and used the recombinant bacteria to immunize outbred mice. However, only 14% of the mice were protected against a lethal *E. ruminantium* challenge. The genomic region encoding the polymorphic *cpg1* gene from the Welgevonden stock of *E. ruminantium* was used to test for protection against lethal *E. ruminantium* challenge in mice and sheep (Louw *et al.* 2002). Four out of five immunized sheep survived a lethal challenge whereas all immunized mice died. Another experimental vaccine, consisting of a cocktail of four *E. ruminantium* genes from a genetic locus involved in nutrient transport and cloned into a DNA vaccine vector, was tested in sheep and mice. Protection rates in mice were much lower, and very variable, but the sheep were completely protected against a subsequent lethal needle challenge, either with the homologous stock or with any one of five different virulent heterologous isolates (Collins *et al.* 2003).

1.7 Laboratory diagnosis

1.7.1 Serology

The application of serological tests for *E. ruminantium* was hampered for a long time due to the unavailability of suitable antigen preparations. Brain smears of infected animals used as antigen produced either negative or inconclusive results in the indirect fluorescent antibody test (IFAT) (du Plessis 1970; Ilemobade 1976). Considerable improvements were brought about when peritoneal cells of mice infected with the "Kümm" strain (du Plessis and Kümm 1971) were used in the IFAT (du Plessis 1981), and the test was initially considered to have a high degree of specificity (du Plessis and Malan 1987). However, sera of cattle from the Caribbean and Namibia, where heartwater had never been diagnosed, reacted positively in the IFAT (du Plessis *et al.* 1987). Furthermore, sera from domestic ruminants in regions of South Africa where *A. hebraeum* does not occur, also reacted positively in the IFAT (du Plessis *et al.* 1994), suggesting that other *Ehrlichia* species could be responsible for the high prevalence of seropositivity.

Logan *et al.* (1987) suggested the use of primary neutrophil cultures as a rapid and simple method to obtain large amounts of antigen from any *E. ruminantium* isolate. This primary neutrophil culture technique was successfully applied by Jongejan *et al.* (1989), who were able to produce antigens of the Welgevonden, Kümm and Senegal stocks in neutrophils, which were used in an IFAT. The Kwanyanga and Ball3 stocks could not be used, due to low infection rates in caprine or ovine

neutrophilic granulocytes. Martinez *et al.* (1990) used caprine leukocyte primary cultures obtained from goats infected with the Gardel, Senegal, Welgevonden and Umm-Banein stocks as antigen in the IFAT.

The IFAT was used to demonstrate *E. ruminantium* in smears from infected bovine endothelial cell cultures, in which extracellular elementary bodies fluoresced brightly (Yunker *et al.* 1988). Infected endothelial cell cultures were also used as antigen in the IFAT for titration of serum samples (Jongejan and Thielemans 1990). Bovine endothelial cells infected with three different stocks of *E. ruminantium* were used as antigen in an IFAT for the serodiagnosis of heartwater. The use of endothelial cell cultures as antigen proved to be superior to infected peritoneal macrophages from mice and to primary cultures of infected neutrophils (Martinez *et al.* 1990).

The enzyme-linked immunosorbent assay (ELISA) technique was used for the characterization of *E. ruminantium* cells isolated either by cellular affinity chromatography (Viljoen *et al.* 1985), or by Percoll density gradient centrifugation (Neitz *et al.* 1986b). A first application of the ELISA for the detection of antibodies against *E. ruminantium* during the course of a heartwater infection was described by Viljoen *et al.* (1987). Using *E. ruminantium* organisms propagated in bovine endothelial cells as antigen for an ELISA, antibodies to *E. ruminantium* were demonstrated in the serum of domestic ruminants (Martinez *et al.* 1993a). However, cross-reactivity was found between *E. ruminantium* antigen and sera raised against *Ehrlichia bovis* or *Ehrlichia ovis* but not with *Ehrlichia phagocytophila* (new name suggested by Dumler *et al.* 2001: *Anaplasma phagocytophilum*). Camus *et al.* (1993) used this ELISA to test for the presence of antibodies to *E. ruminantium* in domestic ruminants on eleven islands of the Lesser Antilles. The low percentage of positive sera, and the absence of clinical cases on some of the islands, suggested that the positive reactions were due to cross-reactions between *E. ruminantium* and other, unidentified *Ehrlichia* species.

Western blotting revealed that the immunodominant 32 kDa protein of *E. ruminantium* contained conserved antigenic determinants, which were recognized by sera against nine different stocks from Africa and the Caribbean (Jongejan and Thielemans 1989). A competitive ELISA for heartwater using monoclonal antibodies to the *E. ruminantium* 32 kDa protein was developed by Jongejan *et al.* (1991b). An immunoblotting diagnostic assay for heartwater based on the 32 kDa protein was also developed (Mahan *et al.* 1993) but this assay also detected false positives in field sera, with ruminant sera from heartwater-free areas of Zimbabwe reacting strongly with the 32 kDa protein. This too was suggested to be due to antigenic cross-reactivity between *E. ruminantium* and other agent(s), such as other *Ehrlichia* species. In a comparison of five tests for the sero-diagnosis of heartwater, including the competitive ELISA, du Plessis *et al.* (1993) found that all tests showed false positive reactions. A possible explanation for this was given by Jongejan *et al.* (1993a) who showed that the immunodominant 32 kDa protein was conserved within the genus *Ehrlichia*. These authors then suggested cloning recombinant *E. ruminantium* antigens for the development of second generation serological tests for heartwater.

A monoclonal antibody which identified an epitope on a 43-kDa protein from eight different strains was also used in a competitive ELISA (Shompole *et al.* 2000). The test detected antibodies in infected ruminants and did not react with *E. canis* antigen. The use of recombinant major antigenic protein1 (MAP1) of *E. ruminantium* for serodiagnosis was investigated and used in an indirect ELISA (van Vliet *et al.* 1995). These authors identified a region of the MAP1, designated as MAP1-B, which did not cross react with *A. bovis*, *E. ovina* or *A. phagocytophilum*, but there was still a reaction with *E. canis* and *E. chaffeensis*. For further validation they tested several serum samples which were considered to be false positives as a result of immunoblotting or indirect ELISA testing; the majority, but not all, of these serum samples did not react with the MAP-1B protein, indicating that the specificity had been increased drastically. Mahan *et al.* (1998b) evaluated the indirect MAP1-B ELISA on field sera from livestock in non-heartwater areas of Zimbabwe and demonstrated improved specificity compared to that obtained with immunoblotting. When three ELISA tests were validated, the tests using recombinant antigens showed improved specificity (Mondry *et al.* 1998). The indirect MAP1-B was recommended for epidemiological studies in regions where the distribution of heartwater is unknown, whereas the competitive ELISA, as described by Katz *et al.* (1997), should be used for studies in wildlife for which species-specific conjugates do not exist. Evaluation of the MAP1-B ELISA in South Africa revealed up to 32.9 % of presumed false positive reactions in goats from a non-*Amblyomma* area (De Waal *et al.* 2000). Using the MAP1-B ELISA to detect antibodies to *E. ruminantium* in goat sera from three communal land areas of Zimbabwe it was shown that, of 480 samples tested from a non-heartwater area, 425 were positive and comparable figures were obtained from goats in endemic areas (Kakono *et al.* 2003). Cattle sera analysed from heartwater-endemic areas in Zimbabwe produced 33% sero-prevalence by the MAP1-B ELISA (Mahan *et al.* 1998b). These findings were confirmed by Peter *et al.* (2001b). They considered the MAP1-B ELISA as an unreliable indicator of past exposure to heartwater in field-infected cattle in Zimbabwe. The low responses of cattle were explained by a down regulation of antibody responses *to E. ruminantium* in cattle (Semu *et al.* 2001).

1.7.2 Molecular techniques

Waghela *et al.* (1991) used two cloned DNA probes to identify *E. ruminantium* in *A. variegatum* ticks, the pCR9 and the pCS20, of which the pCS20 probe had greater specificity. In their experiments, *E. ruminantium* was detected in midguts of *A. variegatum* nymphs infected as larvae, and in midguts of adults infected as nymphs, but not in midguts from control ticks. By means of the pCS20 probe, *E. ruminantium* was detected in *A. hebraeum* (Yunker *et al.* 1993). The pCS20 probe, which was cloned from the DNA of the Crystal Springs strain from Zimbabwe, hybridized with four other strains from Zimbabwe, two strains from South Africa, one from Nigeria and one from Guadeloupe. The probe detected DNA from *E. ruminantium* in plasma samples from infected sheep before and during the febrile reaction (Mahan *et al.* 1992). Peter *et al.* (1995) described the development and evaluation of a PCR assay targeting the pCS20 genomic region for detecting *E. ruminantium* infection in

Amblyomma ticks. They showed that PCR amplification was necessary for the detection of low-level infections that were below the detection limit of the pCS20 DNA probe test. The test based on PCR was used successfully to determine the prevalence of *E. ruminantium* infection in *Amblyomma* ticks from heartwater–endemic areas of Zimbabwe (Peter *et al.* 1999; Peter *et al.* 2000a).

The *map*1 gene of *E. ruminantium* was cloned and found to be conserved among four isolates of *E. ruminantium* originating from Senegal, Sudan, South Africa and Zimbabwe (van Vliet *et al.* 1994). A PCR assay based on the *E. ruminantium map*1 gene detected *E. ruminantium* in blood and bone marrow samples from healthy ruminants. Furthermore, positive reactions were obtained with tsessebe (*Damaliscus lunatus*), waterbuck (*Kobus ellipsiprymnus*) and impala (*Aepyceros melampus*) (Kock *et al.* 1995). Reddy et al. (1996) found that the sequence of the MAP1 genes is variable among *E. ruminantium* isolates from different geographical areas. Allsopp *et al.* (1999) tested three probes for the detection of *E. ruminantium*, targeting the 16S gene, the *map*1 gene, and the pCS20 genomic region, and the pCS20 probe was the most sensitive. Comparison of the pCS20 PCR assay and the MAP-1B ELISA has shown that the PCR-based assay is more reliable and should be the method of choice for the evaluation of *E. ruminantium* infection (Simbi *et al.* 2003).

1.8 Aims of this thesis

The first successful *in vitro* cultivation of *E. ruminantium* in 1985 represented a major breakthrough and boosted a renaissance in heartwater research. For the first time, large quantities of *E. ruminantium* organisms became available. Although the availability of culture-derived organisms stimulated research into the biochemical, immunological and molecular aspects of the organism, the culture technique itself remained almost unchanged following its introduction. The aim of the research presented here in this thesis was therefore to further exploit and improve the culture system.

The first step in this research was to replace semi-defined media by a commercial, chemically defined hybridoma HL-1 medium kit for the *in vitro* propagation of *E. ruminantium* as presented in **Chapter 2.** Although the complete culture medium was chemically defined, the exact formulation was not entirely published. In order to further characterize the culture medium requirements we tested several synthetic media supplemented with a proprietary solution (components solution A of the HL-1 medium kit), and attempts were made to replace solution A by alternative compounds. These results are presented in **Chapter 3**. In **Chapter 4** the first protein-free, chemically defined medium for the propagation of *E. ruminantium* is presented; all semi-defined or proteinaceous components were omitted and replaced by chemically defined products. The amino acid content of cell cultures infected with *E. ruminantium* propagated in a protein-free medium were compared with uninfected cultures, and the results are presented in **Chapter 5**.

Endothelial cells are considered the main target cells for *E. ruminantium*, but neutrophils and macrophages are also infected. The latter cell types are unsuitable for the continuous cultivation of the

organism, because granulocytes and normal mature macrophages do not proliferate. In **Chapter 6** a cell culture system is described where *E. ruminantium* was grown continuously in a transformed canine monocyte-macrophage cell line. **Chapter 7** describes how the Kümm isolate of *E. ruminantium*, which previously resisted all attempts at *in vitro* isolation using conventional culture initition procedures, was isolated using non-endothelial cells. These experiments also showed that the Kümm isolate contained two distinct genotypes *of E. ruminantium*. In **Chapters 8 and 9** we describe the ability of *E. ruminantium* to infect, and grow in, several cell lines of non-endothelial and non-ruminant origin.

The development of two new experimental vaccines, a live attenuated vaccine and a nucleic acid vaccine, is described in **Chapter 10**. Virulent *E. ruminantium* was grown in a continuous canine macrophage-monocyte cell line and, upon inoculation into mice or sheep to test its virulence, it was found to have become attenuated. The nucleic acid vaccine consisted of a cocktail of four *E. ruminantium* genes, from a genetic locus involved in nutrient transport, cloned in a DNA vaccine vector. **Chapter 11** describes a study performed on the attenuated vaccine described in the previous chapter. The Welgevonden stock of *E. ruminantium*, attenuated by continuous propagation in canine DH82 cells, was re-adapted to grow in bovine endothelial cells and was then used to induce protection of sheep and goats against homologous and heterologous needle challenge with virulent organisms.

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Chapter 2

Development of defined media for the *in vitro* cultivation of *Ehrlichia ruminantium*

Basal media are complex media which consist of chemically-defined ingredients. They form the backbone of each medium so far used for the *in vitro* cultivation of. *E. ruminantium*: Glasgow minimum essential medium (GMEM) (Bezuidenhout *et al.*, 1987), Leibovitz L-15 (Byrom and Yunker, 1990) and Dulbecco's minimum essential medium (DMEM) (Martinez *et al.*, 1993) have been used by most groups working with *E. ruminantium* cultures. Growth of *E. ruminantium* was routinely achieved in a defined basal medium only when foetal bovine serum (FBS) and tryptose phosphate broth (TPB) were added. Reliable culture conditions have to prevail because the majority of heartwater research is now based on culture-produced organisms (Yunker, 1995).

Animal serum is routinely added to culture media as a source of nutrients and other ill-defined components, despite the disadvantages of its inclusion and its high cost. Disadvantages of using serum include batch-to-batch variability in composition, resulting in the need for laborious testing to identify batches of serum which are suitable for culture. Because the components are not defined it is impossible to standardise experimental protocols. Furthermore there is the risk of contamination by mycoplasma or viruses, and the high protein levels in serum complicate the identification of specific growth requirements. Both FBS and TPB should be omitted if possible. Ultimately, the use of a chemically-defined protein-free cell culture medium would offer the advantages of batch to batch consistency and reduced production costs. It would facilitate downstream processing and would improve the biosafety of the *E. ruminantium* elementary bodies thus generated. Once cells had been adapted to serum-free media, benefits would include improved control over culture conditions, the elimination of contaminant interference, improved reproducibility between cultures, consistency of media avoiding the need for batch screening; and avoidance of any serum cytotoxicity.

Furthermore, ethical considerations should make it imperative to move away from the use of FBS wherever possible, and the removal of FBS from culture media would enhance animal welfare. Foetuses are likely to be alive during blood collection, they have normal brain function and they can be expected to experience suffering until death actually occurs.

The purest and most consistent cell culture environment is a totally chemically defined medium that is entirely free of animal-derived components. Weaning cells off serum-supplement medium and onto serum-free conditions may cost some time and effort, but this investment would be repaid in terms of consistency and quality of the cultures. The objectives of the following experiments were therefore to formulate a culture medium for *E. ruminantium* which was chemically as closely defined as possible by omitting the poorly-defined constituents FBS, TPB and any other serum-derived products.

Chapter 2.1

In vitro isolation and cultivation of *Cowdria ruminantium* under serum-free culture conditions

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Research in Veterinary Science **63:** 161-164 (1997)

Abstract

Three stocks of *Cowdria ruminantium*, the causative agent of heartwater in domestic ruminants, were propagated in bovine endothelial cells in a serum-free culture medium. The Vosloo, Welgevonden and Senegal stocks were propagated for a period of more than 203, 134, and 43 days, respectively. Two of the *C. ruminantium* stocks (Vosloo and Senegal) were also successfully initiated under serum-free culture conditions. The serum-free medium consisted of a modified HL-1 medium. The Senegal stock was successfully propagated in Dulbecco's modified Eagle's medium nutrient mixture Ham F-12 containing 10% fetal bovine serum.

Heartwater is an infectious, non-contagious disease of domestic ruminants caused by the rickettsia *Cowdria ruminantium*. It is transmitted by ticks of the genus *Amblyomma* (Lounsbury, 1900). The disease prevalence coincides with the distribution of the vector ticks, and is found in sub-Saharan Africa and surrounding islands (summarized by Uilenberg, 1983), and the Caribbean (Perreau *et al.*, 1980; Birnie *et al.*, 1984).

In 1926, Cowdry described the multiplication of *Rickettsia ruminantium*, as *C. ruminantium* was then known, within the endothelial cells of infected animals. It took more than half a century until this organism was propagated *in vitro* in calf endothelial cell cultures (Bezuidenhout *et al.*, 1985). Endothelial cells of various origin were then used as host cells: endothelial cells from bovine aorta (Byrom and Yunker 1990), from ovine aorta (Byrom *et al.*, 1991), from bovine umbilical endothelial cells (Jongejan *et al.*, 1991), from bovine brain (Martinez *et al.*, 1993) from bovine microvascular, human umbilical and from microvascular endothelial cells (Totté *et al.*, 1993).

Glasgow minimum essential medium (GMEM) (Bezuidenhout *et al.*, 1987), Leibovitz L-15 (Byrom and Yunker, 1990) and Dulbecco's minimum essential medium (DMEM) (Martinez *et al.*, 1993) have been used by most groups working with *Cowdria* cultures. All of these media were supplemented with serum and, in addition, most of them contained tryptose phosphate broth (TPB). Both serum and TPB are undefined products which vary from batch to batch and it is well established that some batches of serum have toxic effects on *Cowdria* or are simply unable to support the propagation of the organisms (unpublished observations). Laborious testing is therefore necessary to identify batches of serum which are suitable for culture but there remain other serious disadvantages. Because the components are not defined it is impossible to standardise the experimental protocols, there is a risk of contamination with mycoplasma or viruses, and the high protein levels complicate the identification of specific growth requirements. We have therefore attempted to replace serum-containing medium by a HL-1-based medium, a medium which was originally formulated for serum-free cultivation of hybridoma cells.

Materials And Methods

Stocks of C. ruminantium

The Vosloo stock of *C. ruminantium* was isolated in the district of Lichtenburg (North West Province South Africa; J.L. du Plessis, personal communication) and the infectivity, pathogenicity and immunogenicity of this stock has been described in detail by Du Plessis (1993). Culture material was used to further characterize the stock. The MAP 1 gene of the Vosloo stock was identical in sequence with the Welgevonden stock, and the 16S RNA gene reacted with the Crystal Springs probe (M.T.E.P. Allsopp, personal communication). The Welgevonden stock was isolated by the injection into a mouse of a single homogenised male *Amblyomma hebraeum* which was collected on the farm Welgevonden in the Northern Transvaal, South Africa (Du Plessis, 1985). The Senegal stock was isolated in 1981 from cattle in Senegal by subinoculation into sheep (Jongejan *et al.*, 1988). The Senegal and the Welgevonden stock expressed an immunodominant and conserved 32 kD protein which is currently used for serodiagnosis of the disease (Van Vliet *et al*., 1994).

Endothelial cell lines

The E_5 calf endothelial (Bezuidenhout *et al.*, 1985) and the BA 886 (Yunker *et al.*, 1988) cell lines were used as host cells for *C. ruminantium*. Both cell lines were propagated in medium consisting of Dulbecco's modified Eagle's medium (Highveld Biological, Kelvin 2054, South Africa) with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Delta Bioproducts, Kempton Park, South Africa), buffered with 20 mM HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) (Sigma, St. Louis, MO, USA), 0.84 g/l sodium bicarbonate, and supplemented with 2 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. Cells were cultured as monolayers at 37°C in a humidified atmosphere of 5% CO_2 in air. Endothelial cell lines were used at passage 48 to 65 (BA 886) and 75 to 90 (E_5).

Medium

The medium for serum-free propagation of *C. ruminantium* consisted of HL-1 (Hycor Biomedical Inc., Portland, Maine, USA), 2 mM L-glutamine, 0.2 mM hypoxanthine, 0.04 mM thymidine, 100 IU/ml

penicillin and 100 μg/ml streptomycin, and 0.825 % (v/v) of supplement A of the HL-1 medium kit. The medium contained 15 mM HEPES and 2.2 g/l sodium bicarbonate. This medium is referred to as SFMC-1 *(***s**erum-**f**ree **m**edium for **C***owdria* No. **1**)

The serum-containing medium used in the experiments consisted of Dulbecco's modified Eagle's medium nutrient mixture Ham F-12 (DME/F-12) (Sigma, product no. D 0547), 2 mM L-glutamine, 0.2 mM hypoxanthine, 0.04 mM thymidine, 100 IU/ml penicillin and 100 μg/ml streptomycin. The medium contained 15 mM HEPES and 2.2 g/l sodium bicarbonate as well as 10% (v/v) heat-inactivated FBS. This medium is referred to as DF-12.

Infection of cell monolayers

Vosloo stock: A merino sheep was infected with *C. ruminantium* by intravenous injection of a blood stabilate. Body temperature was monitored daily and a blood sample was drawn on day 11 after infection when the body temperature rose to 41°C. Blood was collected by venipuncture into sterile Vac-u-test^{R} tubes containing heparin (lithium heparin, 14.3 USP/ml blood) as anticoagulant. Infection of the cell monolayer was carried out according to the method described by Byrom *et al.* (1991) with minor modifications. In summary; the blood was centrifuged (800 x g; 10 min; room temperature) and 3 ml of plasma were inoculated into each of two 25 cm² culture flasks containing a confluent layer of E_5 cells; the cultures were incubated at 37°C for 2 to 3 h on a rocking platform at 3 cycles/min after which the plasma was decanted and the cell monolayer was rinsed with 2 x 5 ml of serum-free medium DME/F-12 supplemented with 15 mM HEPES and 2.2 g/L sodium bicarbonate. Finally SFMC-1 was added.

Senegal stock: A Boer goat was infected with a blood stabilate of the Senegal stock. All subsequent procedures were as outlined above for the Vosloo stock. Blood was taken on 10 day after infection, when the body temperature was 41.3°C. When culture initiation was carried out with serum-free medium the cell monolayers were washed with 2 x 5 ml of DME/F-12 medium without supplements after the infective plasma had been removed.

Welgevonden stock: Elementary bodies of the Welgevonden stock were obtained at passage 56 from a continuous culture propagated at the Onderstepoort Veterinary Institute, Protozoology Division. The cell suspension material was centrifuged (800 x g; 10 min; room temperature) in order to remove most of the endothelial cells and cell debris and 3 ml of supernatant was then introduced into each culture flask containing a confluent endothelial cell monolayer. Further procedures were as described below.

Propagation of cultures

Heavily infected cultures were harvested for subcultivation by scraping off the cell monolayer into the medium using a Pasteur pipette bent at the thinner end to form a small hook. Cells were dispersed by pipetting the suspension up and down with a variable pipette (0.5 to 5 ml, Socorex, Lausanne, Switzerland) and the suspension was then transferred at a ratio of 1:2 on to new endothelial cell monolayers. When infecting an endothelial cell line other than that in which the *Cowdria* was initially grown the cell suspension material was centrifuged (800 x g; 10 min; room temperature) and 2 to 3 ml of supernatant was distributed into each culture flask containing the replacement endothelial cell line. The culture flasks were then rocked for 30 min to 2 h (Bezuidenhout and Brett, 1992) after which 2 ml of fresh medium was added. After 24 h all of the medium was discarded and replaced with 5 ml fresh medium. Before a monolayer was used for *Cowdria* cultivation it was washed at least twice with a 5 ml aliquot of DME/F-12 medium without supplements to remove serum contamination.

Microscopic examinations were carried out as follows in order to determine the presence of *C. ruminantium* in the endothelial cells. Small samples were removed from the monolayer using a sterile 21 gauge needle with a bent tip and smears were prepared which were air-dried, methanol-fixed and stained with RapiDiff (Clinical Sciences Diagnostics, Booysens, South Africa).

Results

Vosloo I

The first experimental propagation of *Cowdria* in serum-free medium was carried out using the Vosloo stock and is referred to as Vosloo I. This stock had been in culture for 33 days before the medium was changed to the serum-free SFMC-1. The Vosloo stock was subcultured at an average of 7 days, the period ranging from 2 to 23 days (23 days was the period from culture initiation to the first subculture). Culture data (days in culture, the average passage intervals and the range of passage intervals) from all experiments are summarized in Table 1.

Vosloo II

The second experiment was aimed at comparing the effectiveness with which cultures could be initiated in serum-free and serum-containing media. In both media small colonies of *Cowdria* were first detected on day 6. Serum-free propagation of Vosloo II was maintained for 63 days when the experiment was terminated. During that period 9 subcultures were carried out with passage intervals between 3 and 13 days. After initiation, the first three passage intervals were identical for both medium formulations, i.e. 10, 4 and 6 days, respectively. After passage 3, the experiment using serum-containing medium was terminated.

Welgevonden

Cultures were initiated using elementary bodies from cultures propagated at the Protozoology Division of the Onderstepoort Veterinary Institute. The first subculture was carried out 7 days after the switch to serum-free culture medium and thereafter subcultures were made at an average passage interval of 3.8 days, ranging from 3 to 6 days. Figure 1 shows a microphotograph taken of this stock on the third day after passage 18 in serum-free culture.

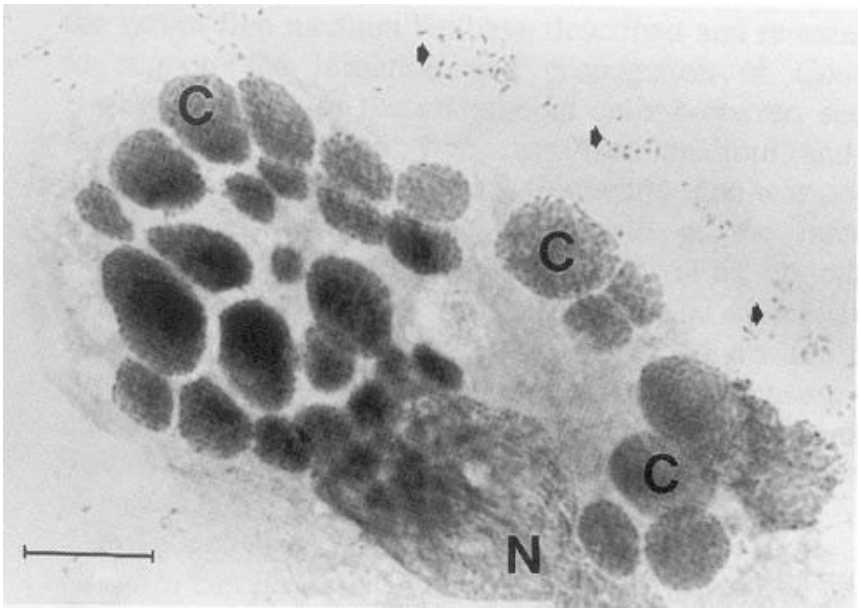

Figure 1 Microphotograph of a smear of bovine aortic endothelial cell culture heavily infected with *C. ruminantium* (Welgevonden stock). Intracellular colonies of rickettsiae and large numbers of extracellular elementary bodies are shown. Bar represents 10 μm.

Table 1 Cultivation of stocks of *Cowdria ruminantium* in bovine endothelial cells

Cowdria stock	Days in culture	Passage intervals [days]	
		Average	Range
Vosloo I[1]	203	7.0	2 - 7
Vosloo II[2]	63	7.0	3 - 13
Welgevonden	134	3.8	3 - 6
Senegal T[3,4]	120	6.7	3 - 16
Senegal S[3]	71	5.5	3 - 16
Senegal SF[3]	43	4.8	3 - 9

[1] Stock Vosloo I was propagated in serum-containing medium before SFMC-1 was applied, data presented here refer to serum-free culture conditions.

[2] Vosloo II refers to the initiation and propagation in SFMC-1 of the Vosloo stock.

[3] Senegal T refers to the overall cultivation regardless of the medium used. After passage 6 dual culture lines were established, serum-containing (S) and serum-free (SF) cell lines.

[4] The passage intervals were calculated not taking into account the period from initiation up to the first passage.

Senegal

Cultures of the Senegal stock were initiated only under serum-free culture conditions because the parallel experiments with serum-containing medium failed owing to fungal contamination of the cultures. Small colonies of *Cowdria* were first detected on day 19 after culture initiation and the stock was then propagated under serum-free culture conditions up to passage 5. At this stage a deterioration in the condition of the endothelial cells necessitated the replacement of SFMC-1 with DF-12. After passage 6, dual culture lines were established, both a serum-free and a serum-containing cell line was maintained, referred to as Senegal SF and Senegal S, respectively (Table 1). In the table Senegal T refers to the overall cultivation regardless of the medium used.

Discussion

Three different stocks of *C. ruminantium* were propagated continuously in bovine endothelial cells under serum-free culture conditions. Two of the stocks were also initiated directly from plasma of infected animals into cultures using serum-free medium. The technique included both a serum-free and a serum-containing phase of cultivation: propagation of endothelial cells was carried out with medium containing 10% FBS followed by a switch to serum-free culture conditions for the propagation of *Cowdria*.

Previously GMEM (Bezuidenhout *et al.*, 1987), Leibovitz L-15 (Byrom and Yunker, 1990) and DMEM (Martinez *et al.*, 1993) were the media commonly used to propagate *Cowdria*. All of these media were supplemented with at least 10% (v/v) serum, although Yunker *et al.* (1988) reported that *Cowdria* organisms can be isolated and propagated continuously *in vitro* in the presence of only 3% FBS. In addition most of the media used for the *in vitro* propagation of *Cowdria* also contained TPB. The present experiments were therefore undertaken in order to reduce the biological variation brought into the culture system by FBS and TPB. Serum-containing DMEM medium (currently used at the OVI for routine *Cowdria* production) was replaced by an HL-1-based medium which was originally formulated for serum-free cultivation of hybridoma cells. HL-1 medium is chemically defined, contains less than 30 μg/ml protein, and is based on a modified DME/F-12 containing HEPES buffer, insulin, transferrin, testosterone, sodium selenite, ethanolamine, saturated and unsaturated fatty acids and proprietary stabilizing proteins. The medium used for the present experiments, however, was not supplemented with insulin. Since all *Cowdria* stocks grew in the modified medium it is obvious that insulin is not an essential requirement.

The results of the serum-free cultivation of all three *Cowdria* stocks compare very favourably with results obtained by others using the same stocks. Yunker *et al.* (1988) cultured the Welgevonden stock for 133 days, during which it was passaged 10 times. Under the new conditions 10 passages were completed after 47 days and after 133 days the serum-free Welgevonden cultures had been passaged 35 times. Similar results were reported by Jongejan (1991) who found that after 226 days in culture, the Welgevonden stock has been passaged 17 times, compared with 68 days in serum-free culture for the same number of passages. Likewise the Senegal stock propagated in serum-free media for 291 days required 16 passages, whereas in serum-free medium (except for passages 5 and 6 where serum-containing medium was used) it only took 112 days to reach passage 16. Although we have used different cell lines from those employed by the other authors mentioned above it is not likely that this was the reason for the improved performance of our serum-free cultures.

Martinez *et al.* (1993) considered TPB to have a protective effect on the parasite and attribute the absence of such protective agents in the culture medium to be responsible for the failure of many attempts to initiate *C. ruminantium* cultures. However our results show that neither serum nor TPB is essential for either initiation or propagation of *C. ruminantium*.

When DF-12 and the modified HL-1 medium were used for the initiation of the Vosloo stock the first three passage intervals were identical in both media (thereafter the serum-containing culture was terminated). This might indicate that DME/F-12 represents a suitable alternative to the media used up to now for the *in vitro* propagation of *Cowdria*.

The scheme of cultivation did not take into account any specific requirement for the growth or maintenance of endothelial cells and no specific supplement was added. However, as shown in the case of initiation of the Senegal stock, endothelial cells can survive for at least 27 days in the serum-free medium we have described and remain able to support the initiation and propagation of *Cowdria*. Higher passages of the endothelial cells however, seemed to be more 'stressed' by serum-free medium and cell detachment was observed more frequently than was considered normal for the stage of growth of the infecting *Cowdria* (data not shown). When cultivating endothelial cells in a serum-free medium, Tauber *et al.* (1981) found that with late-cell passage cultures the medium had to be supplemented with fibroblast growth factor to ensure optimal growth. This was not done in the present experiments, instead the endothelial cells were used after a lower number of passages.

In conclusion the present experiments show that *Cowdria* can be initiated and propagated *in vitro* in serum-free media. Unfortunately the complete formulations of HL-1 are not provided by the manufacturer so there are still gaps in our knowledge of the *in vitro* metabolic requirements of *Cowdria* organisms. Further experiments are being carried out in order to investigate this aspect further.

Acknowledgements

This research was supported by the South African Meat Board and the South African Agricultural Research Council. We thank Dr. F. Jongejan (Faculty of Veterinary Medicine, Utrecht, The Netherlands) for providing stabilates of the Senegal stock, and Mr. Frans Mavhungu for his valuable help in the care of the animals.

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Chapter 2.2

Serum-free Media for the *in vitro* Cultivation of *Cowdria ruminantium*

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Annals of the New York Academy of Science **849:** 307-312 (1998)

Abstract

The *in vitro* culture of *Cowdria ruminantium*, the causative agent of heartwater in domestic ruminants, was first achieved in 1985; since then, most groups working with this culture system have used media which were supplemented with serum and, in addition, most of them contained tryptose phosphate broth. These undefined products vary from batch to batch and often fail to support the growth of *C. ruminantium*. We are therefore working towards the replacement of serum-containing medium by a chemically wholey defined medium for *Cowdria* culture. We attempted the propagation of the Welgevonden stock of *C. ruminantium* in bovine endothelial cell cultures in a variety of serum-free culture media. Four synthetic media gave unsatisfactory results, these were: SFRE-199, Iscove's modified Dulbecco's medium, Dulbecco's modified Eagle's medium, and Leibovitz' L-15. These media were all supplemented with a proprietary solution A (components solution A of the HL-1 medium kit, containing transferrin, testosterone, sodium selenite, ethanolamine, saturated and unsaturated fatty acids, and stabilizing proteins). Three other serum-free media did support the growth of *C. ruminantium*: a modified HL-1 medium, Dulbecco's modified Eagle's medium nutrient mixture Ham F-12 (DME/F-12), and RPMI 1640. The chemical composition of DME/F-12 and RPMI 1640 are published, but not that of the HL-1 medium. Each of these media was supplemented with solution A. Various supplements were investigated as alternatives to the incompletely specified solution A; bovine lipoproteins and bovine transferrin were identified as essential supplements which effectively replaced solution A when added to synthetic medium DME/F-12. *Cowdria ruminantium* was propagated in the three growth-supportive media for at least 10 passages.

Heartwater is a tick-borne rickettsial disease of ruminants caused by *Cowdria ruminantium*. The disease is prevalent in sub-Saharan Africa and surrounding islands (summarized by Uilenberg, 1983), and in the Caribbean (Perreau *et al.*, 1980; Birnie *et al.*, 1984). *Amblyomma hebraeum* was first identified as a vector of the disease (Lounsbury, 1900). At present, twelve species of Amblyomma ticks are known to be capable of transmitting the disease (Walker and Olwage, 1987).

The *in vitro* culture of *C. ruminantium* was first achieved in 1985 by Bezuidenhout *et al.* who used calf endothelial cells as host cells. Glasgow minimal essential medium (GMEM) (Bezuidenhout *et al.*, 1987), Leibovitz L-15 (Byrom and Yunker, 1990) and Dulbecco's minimal essential medium (DMEM) (Martinez *et al.*, 1993) supplemented with various amounts of fetal bovine serum and tryptose phosphate broth (TPB) were used for Cowdria culture by various groups. However, culturing *C. ruminantium* in the presence of undefined components, serum and TPB, has some serious disadvantages, such as batch to batch variation, lack of standardization of experimental protocols, and the difficulty to identify specific growth requirements, to name but a few.

Recently, Zweygarth *et al.* (in press) succeeded in propagating three stocks of *C. ruminantium* in a serum-free medium, two of which were also initiated under these conditions. A commercial medium kit was used and although the complete culture medium was chemically defined, the exact formulation is not completely published. Therefore we attempted, firstly, to replace the synthetic culture medium with a medium of known composition, and secondly, to identify the compounds which may support the growth of *C. ruminantium in vitro* in order to develop a medium which takes into account the specific growth requirements of *Cowdria*.

Materials and Methods

Stock of C. ruminantium

The Welgevonden stock was originally isolated by injecting a tick homogenate into a mouse. The tick, a male *Amblyomma hebraeum*, had been collected on the farm Welgevonden in the Northern Transvaal, South Africa (Du Plessis, 1985).

Endothelial cell line

The bovine aortic endothelial cell line, BA 886 (Yunker *et al*. 1988), referred to as BA cells, was used. BA cells were propagated in Dulbecco's modified Eagle's medium (DME; Highveld Biological, Kelvin, South Africa, CN 1140A) with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Delta Bioproducts, Kempton Park, South Africa), buffered with 10 mM HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) (Sigma, St. Louis, MO, USA), 0.84 g/L sodium bicarbonate, and supplemented with 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin. Cells were cultured as monolayers at 37EC. Endothelial cells were used at passage 30 to 65.

Media

Various synthetic culture media were used: HL-1 (Hycor Biomedical Inc., Portland, Maine, USA), Dulbecco's modified Eagle's medium nutrient mixture Ham F-12 (DME/F-12)(Sigma, St. Louis, MO, USA; D 0547), SFRE 199-1 (Sigma; S 2013), Iscove's modified Dulbecco's medium (IMDM) (Sigma; I 7633), DME (Highveld Biological), Leibovitz's L-15 (Highveld Biologicals; CN 1225-1) and RPMI 1640 (Highveld Biologicals; CN 1735). The complete, serum-free media prepared from these synthetic culture media are referred to as SFMC-1 (HL-1)(Zweygarth *et al.*, in press), SFMC-3 (DME/F-12), SFMC-9 (SFRE 199-1), SFMC-10 (IMDM), SFMC-13 (Leibovitz L-15) and SFMC-14 (RPMI 1640) (serum-free medium for Cowdria No. ..). These media were supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 0.825 % (v/v) of supplement A of the HL-1 medium kit (Hycor Biomedical Inc.). Supplement A consists of transferrin, testosterone, sodium selenite, ethanolamine, saturated and unsaturated fatty acids and proprietary stabilizing proteins. Furthermore, all media but Leibovitz's L-15 contained 10 mM HEPES, and sodium bicarbonate (according to the manufacturer's recommendations). SFMC-13 was additionally supplemented with 0.45% (w/v) glucose (Byrom and Yunker, 1990).

Media based on RPMI 1640 were used to determine the influence of transferrin and bovine lipoproteins on the growth of *C. ruminantium*. SFMC-15, SFMC-16, and SFMC-17 consisted of SFMC-14 where the HL-1 supplement A was replaced by bovine transferrin (BTF; Sigma) and bovine lipoproteins (BLP; Sigma) alone, and a combination of the two, respectively. SFMC-37 consisted of RPMI 1640 only supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin.

Infection of cell monolayers

Elementary bodies of the Welgevonden stock were obtained at passage 62 from a continuous culture propagated in serum-containing DME-based medium. 2.5 ml of supernatant was distributed into each culture flask (25 cm5) containing a confluent endothelial cell monolayer. The culture flasks were then put onto a rocker platform (3 cycles/min) for 30 min (Bezuidenhout and Brett, 1992). Thereafter, the liquid content of the culture flasks was discarded, and the infected monolayer was rinsed 3 times with Dulbecco's balanced salt solution (DBSS) before the respective serum-free medium was added.

Propagation of cultures

Propagation of cultures was carried out as described previously (Zweygarth *et al.*, in press) with minor modifications. Briefly, infected cultures were harvested for subcultivation by scraping off the cell monolayer into the medium using a Pasteur pipette bent at the tip to form a small hook. Cells were dispersed by pipetting the suspension up and down a variable pipette. The contents were then distributed into two new culture flasks containing an endothelial cell monolayer. The culture flasks were put onto a rocker platform (3 cycles/min) for 30 min, after which 2.5 ml of fresh medium was added. After 24 h all of the medium was discarded and replaced with 5 ml fresh medium. Before each monolayer was used for *C. ruminantium* cultivation, it was rinsed 3 times with 5 ml of DBSS to reduce serum contamination.

Microscopic examinations were carried out to demonstrate *C. ruminantium* in the endothelial cells. Small samples from the monolayer were removed by means of a sterile 21 gauge needle with a bent tip from which smears were prepared. They were air-dried, methanol-fixed, and quick-stained (RapiDiff; Clinical Sciences Diagnostics, Booysens, South Africa).

Results

Synthetic culture media containing supplement A

SFMC-1, SFMC-3, and SFMC-14 were successfully used to propagate *C. ruminantium*. The growth characteristics obtained with SFMC-1 and SFMC-14 were identical, with an average passage interval of 4.4 days, ranging from 3 to 6 days. Both experiments were terminated after 13 passages. *C. ruminantium* propagated in SFMC-3 had the shortest average passage interval of 3.3 days, ranging from 3 to 5 days. This culture was terminated after 11 passages. The results obtain with SFMC-1, SFMC-3, and SFMC-14 are summarized in Table 1. Attempts to propagate *C. ruminantium* in SFMC-9, SFMC-10, SFMC-12, and SFMC-13 were unsatisfactory. Although the organisms survived in these cultures (data not shown), the growth was very poor compared to the above media and these cultures were terminated before passage 6.

Table 1 Cultivation of *C. ruminantium* in serum-free media

	Passage intervals [days]		
Medium designation	Average	Range	Passages at termination
SFMC-1	4.4	3 - 6	13
SFMC-3	3.3	3 - 5	11
SFMC-14	4.4	3 - 6	13

Replacement of supplement A

Various experiments were carried out to find a replacement for supplement A in the serum-free culture media (data not shown). Transferrin and bovine lipoproteins were identified to fully replace supplement A

when used together. SFMC-4 was used for 19 subcultures. The average passage interval was 4.6 days with a range of 3 to 12 days (Table 1).

Media based on RPMI 1640 were used supplemented with either a combination of BTF and BLP (SFMC-15), or with BTF (SFMC-16) or BLP (SFMC-17) alone to determine the influence of these compounds on the growth of *C. ruminantium*. SFMC-15 was the only medium suitable for continuous cultivation. The two other media, SFMC-16 and SFMC-17, gave unsatisfactory results and these cultures could not be maintained on the same high level of infection achieved with SFMC-15. RPMI 1640 supplemented only with L-glutamine, penicillin, and streptomycin (SFMC-37) was used as control. It did not support the continuous propagation and cultures became microscopically negative after several passages. The results obtained with RPMI 1640-based serum-free media are summarized in Table 2.

Table 2 RPMI 1640-based serum-free media for the *in vitro* cultivation of *C. ruminantium*

Medium designation	Supplementation			Growth
	Supplement A of HL-1[1)]	Transferrin[2)]	Bovine Lipoproteins[3)]	
SFMC-14	+	-	-	+++
SFMC-15	-	+	+	+++
SFMC-16	-	+	-	(+)
SFMC-17	-	-	+	(+)
SFMC-37	-	-	-	(+)

[1)] Supplement A was used at 0.825% (v/v)
[2)] Bovine transferrin was used at 2 mg/L
[3)] Bovine lipoproteins were used at 1% (v/v)
(+) Growth over several passages with decreasing infection rates
+++ Continuous growth with high infection rates

Discussion

The *in vitro* isolation and cultivation of *C. ruminantium* under serum-free culture conditions has been reported previously (Zweygarth *et al.*, in press). Three stocks of *C. ruminantium* were propagated in a serum-free medium based on a modified HL-1 medium, consisting of a DME/F-12-based medium and supplement A (see under Materials & Methods). Although HL-1 medium is chemically defined, the supplier did not publish the complete formulation and only limited information is made available. Therefore the first attempt was to replace the DME/F-12-based component of the modified HL-1 medium by a synthetic one with published composition. Secondly, based on the results of those experiments, we aimed at replacing the supplement A solution with single components thereof.

Several synthetic media were tested, most of which had previously been used for the successful propagation of *Cowdria* in the presence of serum, namely GMEM (Bezuidenhout *et al.*, 1987), Leibovitz L-15 (Byrom and Yunker, 1990), DMEM (Martinez *et al.*, 1993) and DME/F-12 (Zweygarth *et al.*, in press). In addition, Iscove's modified Dulbecco's medium, SFRE 199-1, and RPMI 1640 were also tested. However, only two media, DME/F-12 and RPMI 1640, convincingly supported the propagation of the Welgevonden stock of *C. ruminantium*. The remaining media were used with limited success only because the growth of *Cowdria* was retarded. Therefore experiments using these media were terminated. However, at the time of termination, there were still *Cowdria* colonies present.

To identify the single components of the HL-1 supplement A responsible for the growth-promoting action upon *Cowdria*, several compounds (data not shown), among them bovine transferrin and a source of lipids, were tested in RPMI 1640-based medium. Bovine transferrin and bovine lipoproteins were the only compounds which supported a continuous propagation comparable to media containing supplement A. Nevertheless, the Welgevonden stock of *C. ruminantium* could be propagated over several passages even without these supplements. This indicates that the intracellular pool of

nutrients may contribute to the growth of *Cowdria* so that deficiencies in the culture medium become obvious only after several passages, which hindered the present experiments considerably.

Bovine lipoproteins as a biological product, are definitely subject to batch variation. In our experience, different bovine lipoprotein preparations have been shown variations which have led to the death of *Babesia equi* in culture as a result of changing from one batch to another (Zweygarth *et al.*, unpublished results). Therefore it may be more convenient to use HL-1 medium since it is chemically defined even though the exact formulation is not made known.

In conclusion we have shown that *Cowdria* can be propagated in serum-free medium provided bovine transferrin and bovine lipoproteins were added. Further experiments are presently being carried out to ultimately reach the goal of a chemically defined medium not subject to biological variation.

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Chapter 2.3

A chemically defined medium for the growth of *Cowdria ruminantium*

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Onderstepoort Journal of Veterinary Research **68:** 37-40 (2001).

Abstract

Chemically defined media, termed SFMC-23 and SFMC-36, were devised for the *in vitro* culture of *Cowdria ruminantium*, the causative agent of heartwater in domestic ruminants. Both media were based on Dulbecco's modified Eagle's medium nutrient mixture Ham F-12 (DME/F-12) containing various supplements. Medium SFMC-23 and SFMC-36 supported the long-term growth of the Welgevonden stock of *C. ruminantium* for a total of 55 and 28 passages, respectively, with regular passage intervals of 3 days. Using SFMC-23, split ratios varied from 5 to 10, depending on which host cell line was used. Other stocks of C. *ruminantium* (Sankat, Blaauwkrantz, Senegal) were successfully propagated for a test period of 10 passages.

Introduction

Heartwater is a tick-borne rickettsial disease of ruminants caused by *Cowdria ruminantium*. The disease is prevalent in sub-Saharan Africa and surrounding islands (Uilenberg 1983), and in the Caribbean (Perreau, Morel, Barré & Durand 1980; Birnie, Burridge, Camus & Barré 1984). The first vector of the disease to be identified was *Amblyomma hebraeum* in South Africa (Lounsbury 1900). Currently, twelve species of *Amblyomma* ticks are known to be capable of transmitting heartwater (Walker & Olwage 1987).

The *in vitro* culture of *C. ruminantium* was first achieved by Bezuidenhout, Paterson & Barnard (1985). They used calf endothelial cells as host cells and Eagle's medium containing 10% bovine serum. Subsequently, Glasgow minimal essential medium (GMEM) (Bezuidenhout 1987), Leibovitz L-15 (Byrom & Yunker 1990) and Dulbecco's minimal essential medium (DMEM) (Martinez, Sheikboudou, Couraud & Bensaid 1993) supplemented with various amounts of foetal bovine serum and tryptose phosphate broth (TPB) were used for *Cowdria* culture. However, the culture of *C. ruminantium* in the presence of undefined components such as serum and TPB has some serious disadvantages. Batch-to-batch variation of components, lack of standardisation of experimental protocols, and the difficulty of identifying specific growth requirements are a few of the factors involved.

Recently, the propagation of *C. ruminantium* in serum-free media supplemented with bovine lipoproteins and transferrin was achieved (Zweygarth, Josemans & Horn 1998). In this report it is shown that *C. ruminantium* can be cultured in a protein-free, chemically defined medium, by replacing bovine lipoproteins by chemically defined lipids and transferrin by protein-free iron complexes.

Materials and Methods

Stocks of C. ruminantium

Four different stocks of *C. ruminantium* were used, the origins of which are described below. The Welgevonden stock was isolated by injecting a homogenate from a tick collected on the farm Welgevonden (Northern Transvaal, South Africa) into a mouse (Du Plessis 1985). The Senegal stock was isolated in 1981 from cattle in Senegal by subinoculation into sheep (Jongejan, Uilenberg, Franssen, Gueye & Nieuwenhuijs 1988). Both the Senegal and the Welgevonden stock express a conserved immunodominant 32 kDa protein which is currently used for serodiagnosis of the disease (Van Vliet, Jongejan, Van Kleef & Van Der Zeijst 1994). The Pokoase stock was isolated in Ghana by Dr. Bell-Sakyi, Centre for Tropical Veterinary Medicine, Edinburgh, Scotland. The Blaauwkrantz stock was originally isolated from an eland on the farm Blaauwkrantz (Eastern Cape, South Africa) in 1996 by Dr. J. L. du Plessis, formerly of the Onderstepoort Veterinary Institute.

Endothelial cell lines

The E_5 calf endothelial (Bezuidenhout *et al.* 1985) and the bovine aorta BA 886 (Yunker, Byrom & Semu 1988), cell lines were used. Uninfected cells were propagated at 37°C as monolayers in medium consisting of Dulbecco's modified Eagle's medium (Highveld Biological, Kelvin, South Africa) with 10% (v/v) heat-inactivated foetal bovine serum (FBS; Delta Bioproducts, Kempton Park, South Africa). The medium was buffered with 10 mM HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) (Sigma, St. Louis, MO, USA), and 10 mM sodium bicarbonate, and supplemented with 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin. Before being used for *C. ruminantium* cultivation, each monolayer was rinsed three times with 5 ml of phosphate-buffered saline to reduce serum contamination. Endothelial cell lines were used at passage 128 to 177 (E_5) and 33 to 65 (BA 886).

Media for the propagation of C. ruminantium

Dulbecco's modified Eagle's medium nutrient mixture F-12 Ham (Sigma) containing 15 mM HEPES and 1.2 g/L sodium bicarbonate was used. This was supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, and is referred to as serum-free medium for *Cowdria* No. 36

(SFMC-36). SFMC-23 was prepared by the addition of a mixture containing 10 µl of 40 mM ferric chloride hexahydrate (Sigma) in 10 mM HCl and 1 ml of 0.5 M glycylglycine (Sigma) to 100 ml SFMC-36 supplemented with 0.1 % (v/v) chemically defined lipids (CDL Life Technologies, Paisley, Scotland).

Propagation of cultures

Propagation of cultures was carried out as described previously (Zweygarth, Vogel, Josemans & Horn 1997) with minor modifications. Briefly, infected cultures were harvested for subcultivation by scraping off the cell monolayer into the medium using a Pasteur pipette bent at the tip to form a small hook. Cells were dispersed by pipetting the suspension up and down. The cell suspension was centrifuged (800 x g for 10 min at room temperature) and the appropriate amount (0.5 ml or 1 ml) of supernatant was distributed into each of two culture flasks containing the uninfected replacement endothelial cell line. Fresh medium was then added to 2.5 ml. The culture flasks were placed onto a rocker platform (three cycles/min) for 30 min, after which a further 2.5 ml aliquot of fresh medium was added. After 24 h all the medium was discarded and replaced with 5 ml fresh medium. Smears were prepared for microscopic examination by removal of small samples from the monolayer using a sterile 21 gauge needle with a bent tip, in order to determine the presence of *C. ruminantium* in the endothelial cells. The smears were air-dried, methanol-fixed and stained with RapiDiff (Clinical Sciences Diagnostics, Booysens, South Africa).

Results

SFMC-23 and SFMC-36 were successfully used to propagate the *C. ruminantium* (Welgevonden) stock. The growth characteristics obtained with SFMC-23 and SFMC-36 were almost identical with an average passage interval of 3 days. After transfer of the Welgevonden stock into SFMC-36, a 4-day passage interval was initially observed, followed by 3-day intervals. The split ratio used was five when BA cells were used and ten when E_5 cells were used as host cells. The cultures were terminated after 55 and 28 passages, respectively, due to a *Mycoplasma* contamination of the host cells. The Blaauwkrantz, Sankat and Senegal stocks were propagated in SFMC-23 over an observation period of ten passages with average passage intervals of 3.3 days, 4.6 days and 4.4 days, respectively. A split ratio of two for the latter three stocks was observed throughout the cultivation period. The results are summarised in Table 1.

Table 1 Cultivation of *C. ruminantium* stocks in medium SFMC-23 or SFMC-36

Stock of *C. ruminantium*	Total number of passages at termination	Number of passages in serum-free medium	Passage intervals [days]	
			Average	Range
Welgevonden	157	55	3.0	3
Welgevonden (SFMC-36)	157	28	3.0	3 - 4
Blaauwkrantz	25	10	3.3	3 - 5
Sankat	20	10	4.6	3 - 12
Senegal	26	10	4.4	3 - 6

Discussion

Two protein-free, chemically defined media, referred to as SFMC-23 and SFMC-36, one of which supports the growth of at least four different stocks of *C. ruminantium* are described in this study. The media are, however, only chemically defined to the extent of the purity of culture ingredients themselves. The *in vitro* cultivation of *C. ruminantium* under serum-free culture conditions has been reported previously (Zweygarth *et al.* 1997). Three *C. ruminantium* stocks were propagated in a serum-free medium based on a modified HL-1 medium, a DME/F-12-based medium supplemented with the HL-1 proprietary solution A, consisting of transferrin, testosteron, sodium selenite, ethanolamine, saturated and unsaturated fatty acids, and stabilising proteins. In further experiments, two components, namely bovine transferrin and bovine lipoproteins that have a growth-promoting

action on *Cowdria* were identified. These two components were able to replace solution A in the culture medium (Zweygarth *et al.* 1998). A further step towards a completely defined medium was then attempted by replacing the bovine lipoproteins and transferrin with chemically defined compounds. Instead of bovine lipoproteins, a commercially available mixture of chemically defined lipids was used. Transferrin was replaced with a complex prepared from glycylglycine and ferric chloride (Yabe, Kato, Matsuya, Yamane, Iizuka, Takayoshi & Suzuki 1987). This supplemented medium, SFMC-23, supported the propagation of the Welgevonden stock in a way never observed in our laboratory before with respect to regularity of subculture and level of infection. *Cowdria* was propagated for 55 passages at regular 3-day intervals at split ratios, which are rarely achieved by conventional, serum-containing medium. During culture propagation it also became evident that the E_5 cell line allowed a higher split ratio than the BA cell line. The reasons for this are not yet clear. Both cell lines were bovine-derived, although from different anatomical sites, the umbilical cord (E_5) and the aorta (BA). Therefore, careful selection of the host cell line is necessary for optimal *Cowdria* growth.

Surprisingly, SFMC-36, the medium which was supplemented with L-glutamine and antibiotics only, gave similar results to SFMC-23. This conflicted with previous findings, where supplements of lipids and transferrin had to be added to maintain growth of *C. ruminantium* in culture (Zweygarth *et al.* 1998). Nevertheless, DME/F-12, the basis of the serum-free media, contains ferric nitrate and ferrous sulphate as a source of iron, and linoleic acid as a source of fatty acids. The results presented here demonstrate that supplementation of the *Cowdria* culture medium with components other than L-glutamine and antibiotics is not necessary.

In addition to the Welgevonden stock, three other stocks of *C. ruminantium* were propagated in SFMC-23. However, passage intervals were slightly longer and subculture ratios usually lower than with the Welgevonden stock. This may have been due to the fact that these stocks had far fewer passages in culture than the Welgevonden stock and were therefore less adapted to growth *in vitro.*

Although the experiments are not completely conclusive as to the requirement of iron and/or lipid acids, it has been shown that *Cowdria* can be propagated in a protein-free, completely defined synthetic medium (DMEM/F-12) which is commercially available. The media described here may be of value for experiments in which serum has a negative impact on results. They can certainly contribute to the elucidation of the amino acid requirements of cultured *Cowdria*. Furthermore, these media may contribute to a more economical production of *C. ruminantium* organisms *in vitro*. Whether these media can also be used for culture initiation remains to be evaluated.

Acknowledgements

We thank Dr M. T. E. P. Allsopp for helpful comments on the manuscript. This research was supported by the Agricultural Research Council of South Africa and the European Union (Cowdriosis Network) Grant no. IC18-CT95-0008 (DG12-SNRD).

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Chapter 2.4

Amino acid content of cell cultures infected with *Cowdria ruminantium* propagated in a protein-free medium

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Annals of the New York Academy of Science **969:** 141-146 (2002).

Abstract

The *in vitro* culture of *Cowdria ruminantium*, the causative agent of heartwater in domestic animals, was first achieved in 1985. Culture media were usually supplemented with serum and tryptose phosphate broth, both undefined components, contributing to great variability . Recently, we reported about the propagation of stocks of *C. ruminantium* in a protein-free culture medium referred to as SFMC-23, which is chemically fully defined. To clarify whether the amino acid composition of SFMC-23 is adequate for the in vitro propagation of *Cowdria*, the Welgevonden stock was propagated in SFMC-23medium. After a 3-day culture period, samples were taken from uninfected and infected bovine endothelial cell cultures. They were analyzed for free amino acids by the Pico Tac reverse-phase HPLC precolumn derivatization method. Eighteen different amino acids were examined. A considerable decrease in concentration was observed with proline (29%) and glutamine (62%). Further dramatic changes were observed with amino acids which accumulated in the culture medium: aspartic acid, serine, asparagine, tryptophane, glycine, and alanine. The concentration of alanine increased by approximately 660%. The concentrations of all other amino acids analyzed remained within a 25% range, either increasing or decreasing. These results suggest that only glutamine may run short during in vitro cultivation. It seems more likely that accumulation of various amino acids may impact negatively on long-term *Cowdria* propagation.

Heartwater is a rickettsial disease of ruminants caused by *Cowdria ruminantium*. It is prevalent in sub-Saharan Africa, some islands off its eastern and western coasts (Uilenberg, 1983), and in the Caribbean (Perreau *et al.*, 1980; Birnie *et al.*, 1984). It is transmitted by ixodid ticks of the genus *Amblyomma* and is often fatal. The *in vitro* culture of *C. ruminantium* was first achieved by Bezuidenhout, Paterson & Barnard (1985). Today, the majority of heartwater research is based on culture-produced organisms (Yunker, 1995). However, the production of *C. ruminantium in vitro* in a consistent fashion remains difficult to achieve. Recent advances have been made towards culture in a completely chemically defined protein-free medium. *C. ruminantium* has been grown in serum-free media, supplemented with bovine lipoproteins and transferring (Zweygarth *et al.*, 1998). These two components were replaced with chemically defined lipids and protein-free iron complexes (Zweygarth and Josemans, 2001). The replacement of undefined components has enabled us, in this study, to determine the amino acid utilisation of *C. ruminantium*, to identify substrate deficiencies, and to gain an insight into the metabolic processes of the organism.

Materials and Methods

The Welgevonden stock of *C. ruminantium* (Du Plessis, 1985) was grown in a continuous bovine aorta endothelial cell line, BA886 (Yunker *et al.*, 1988). These cells were initially screened for the absence of mycoplasma contaminants by staining with bis-benzamide (Hoechst 33258) and examination under the fluorescence microscope. The mycoplasma-free status of the cells and the *Cowdria* cultures were confirmed by means of a mycoplasma-specific polymerase chain reaction (PCR) test (Van Kuppeveld *et al.*, 1994). Cultures were propagated as previously described (Zweygarth *et al.*, 1997) with minor modifications. Uninfected cells were propagated at 37°C as monolayers in medium consisting of Dulbecco's modified Eagles medium nutrient mixture F-12 Ham (Sigma, St. Louis, MO, USA) with additives as previously described (Zweygarth and Josemans, 2001). Infected cultures of *C. ruminantium* were propagated in a protein-free medium referred to as "serum-free medium for *Cowdria* No. 23" (SFMC-23) (Zweygarth and Josemans, 2001). The amino acid concentrations in the medium were determined before and after a 3-day cultivation period, on a Waters Automated Analyzer (Millipore Corp.) using Pico-Tag phenyl isothiocyanate (PTC) pre-derivatization methodology (Bidingmeyer *et al.*, 1984). Fresh medium (3 samples), infected culture supernatants (9 samples) and uninfected culture supernatants (3 samples) were analysed for 18 different amino acids. Amino acid concentrations in 3-day culture supernatants were expressed as percentages of concentrations in the original medium (Table 1).

Results and Discussion

The average amino acid concentrations are shown in Figure 1, while the magnitude of changes are summarized in Table 1. Glutamine was the amino acid that was most markedly depleted in both infected and uninfected cultures, by 62% and 53%, respectively. In mammalian cell cultures glutamine is not used directly for protein synthesis, but metabolised into glutamic acid, aspartic acid, asparagine and proline, and to a lesser degree into serine and alanine (Eagle, 1959). All these metabolites were increased in uninfected cultures, except for glutamic acid, which was consumed by the BA886 cells. The largest increase in uninfected cultures was found for alanine (1047 %), while a slightly lower increase for alanine (761%) was observed in *Cowdria*-infected cultures. Assuming that the host cell metabolism is not grossly disrupted until close to the time of release of the elementary bodies, amino acids from the host cells released into the medium can be used by *Cowdria* organisms. Thus, in our experiments the concentration of proline in the supernatant was 64% lower in infected than in uninfected cultures after 3 days, indicating that there must have been a large consumption of this amino acid by the *Cowdria* organisms. Similar results were found with alanine and tryptophan, which were consumed at a lower rate. In another study (Neitz and Yunker, 1996) it was found that *C. ruminantium* in serum-containing cultures consumed proline at a similar rate to that described here. Another rickettsia, *Rickettsia prowazekii*, appears to require proline for growth as maximal rickettsial growth occurred only in host cells with an intracellular proline pool of 1 mM or greater (Austin and Winkler, 1988).

Neitz and Yunker (1996) found that within 2 days arginine was almost completely depleted both in infected and uninfected cultures in a serum-containing medium. They concluded that their

medium (Glasgow's MEM) might have been deficient in arginine. In contrast, in our cultures only 4% or 6% of the initial arginine was consumed in uninfected and infected cultures respectively. A possible explanation for this discrepancy may lie in the work of Capiaumont *et al.* (1995), who suggested that if the arginine in a culture medium is suddenly completely consumed after 2 or 3 days there is probably mycoplasma contamination.

These results suggest that the concentrations for glutamine and proline may be limiting during *in vitro* cultivation. It also seems likely that the accumulation of various amino acids or metabolic by-products in the medium could have a negative impact on long term *Cowdria* propagation.

TABLE 1. Concentrations of 18 amino acids in SFMC-23 medium and after 3 days in BA866 cell cultures and *Cowdria*-infected cultures

	A	B		C	
Amino acid	**Concentra-tion in SFMC-23 MM/ml**	**Concentra-tion after 3 days in uninfected culture as % of A**	**Change vs. A**	**Concentra-tion after 3 days in infected culture as % of A**	**Change vs. B %**
Glycine	0.267	**190***	↑	**317**	**+67**
Aspartic Acid	0.056	**150**	↑	**166**	+11
Alanine	0.049	**1047**	↑	**761**	**-27**
Proline	0.098	**198**	↑	**71**	**-64**
Tryptophan	0.034	**191**	↑	**132**	**-31**
Asparagine	0.039	**182**	↑	**159**	-13
Serine	0.151	**178**	↑	**166**	-7
Threonine	0.247	114	↑	112	-1
Glutamic Acid	0.600	**55**	↓	101	**+84**
Phenylalanine	0.188	86	↓	87	+2
Tyrosine	0.222	82	↓	83	+1
Methionine	0.104	82	↓	83	+1
Lysine	0.441	79	↓	83	+5
Leucine	0.442	77	↓	79	+2
Glutamine	5.145	**47**	↓	**38**	**-**19
Arginine	0.623	96	↓	94	-2
Valine	0.445	81	↓	81	-1
Isoleucine	0.401	76	↓	75	-1

***changes in concentration > 25% in bold**

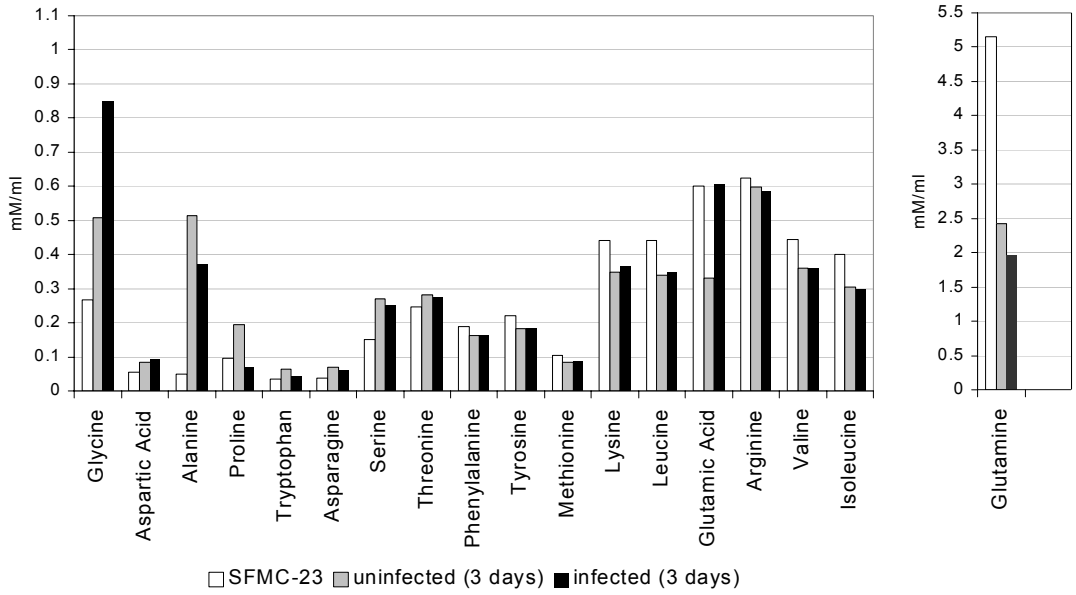

Figure 1. Average amino acid concentrations in serum-free medium, and in supernatant of *Cowdria*–infected cultures and uninfected controls

Acknowledgements

We thank Dr B.A.Allsopp for helpful comments on the manuscript. This research was supported by the Agricultural Research Council of South Africa and the European Union (Cowdriosis Network) Grant no. IC18-CT95-0008 (DG12-SNRD)

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Chapter 2.5

Discussion – Defined Media

The culture media originally used for the *in vitro* propagation of *E. ruminantium* consisted of a chemically defined, complex, basal medium with additional undefined components, usually tryptose phosphate broth (TPB) and a source of mammalian serum. The complete medium was thus a semi-defined medium, and examples include Glasgow's MEM (GMEM) (Bezuidenhout 1987), Leibovitz L-15 (Byrom and Yunker 1990) and DMEM (Martinez *et al.* 1993). All of these were supplemented with 10% (v/v) serum and 10% (v/v) TPB. The experiments presented here were undertaken in order to develop defined culture media which would not suffer from the biological variation entailed by the inclusion of ingredients such as foetal bovine serum (FBS) and TPB. The approach was a stepwise replacement of the undefined components with more defined ingredients, until finally a chemically fully-defined medium was formulated which supported the continuous cultivation of *E. ruminantium*. The availability of a serum-free, chemically defined medium is an important tool for carrying out cell cultures under controlled conditions.

Serum-supplemented Dulbecco's MEM medium, which was routinely used at the Onderstepoort Veterinary Institute (OVI) for *E. ruminantium* production, was replaced by an HL-1-based medium which was originally formulated for serum-free cultivation of hybridoma cells (Chapter 2). HL-1 medium is chemically defined, contains less than 30 μg/ml protein, and is based on a modified DME/F-12 medium containing HEPES buffer. It is further supplemented with transferrin, testosterone, sodium selenite, ethanolamine, saturated and unsaturated fatty acids and proprietary stabilizing proteins (supplement A of the HL-1 medium kit) and insulin (supplement B of the HL-1 medium kit). All *E. ruminantium* stocks grew in the modified medium when insulin was omitted, indicating that the latter was not an essential requirement.

Three different stocks of *E. ruminantium* were propagated continuously in bovine endothelial cells under serum-free conditions in a medium based on HL-1, and the results compared very favourably with those reported by other researchers using the same stocks in but semi-defined media. For example, Yunker *et al.* (1988) had propagated the Welgevonden stock for 133 days in GMEM with TPB and serum, during which it was passaged 10 times. Under the serum-free conditions reported here, 10 passages were completed after only 47 days, and after 133 days the serum-free Welgevonden cultures had been passaged 35 times. Similarly Jongejan (1991) reported that after 226 days in culture the Welgevonden stock had been passaged 17 times, compared with 68 days in serum-free culture for the same number of passages in the present study. Likewise, the Senegal stock propagated in serum-

supplemented medium required 291 days for 16 passages, whereas in serum-free medium it only took 112 days to reach passage level 16.

E. ruminantium is an obligatory intracellular organism and the *in vitro* culture system must therefore contain host cells. The host cells, in this case bovine endothelial cells, have different nutrient requirements from *E. ruminantium,* and this was not taken into account when formulating the serum-free medium. Nevertheless, endothelial cells could survive for at least 27 days in the serum-free medium, as has been shown during the initiation phase of the Senegal stock. Although the serum-free medium based on the HL-1 medium kit represented a step forward in the propagation of *E. ruminantium*, the complete formulation of HL-1 was not published by the manufacturers. It was therefore still not possible at this stage to formulate a fully-defined medium for the propagation of *E. ruminantium in vitro*.

In a second series of experiments, attempts were made to replace the DME/F-12-based component of the modified HL-1 medium by a synthetic one with a published composition (Chapter 3). Several synthetic media were tested, some of which had previously been used for the successful propagation of *E. ruminantium* in the presence of serum and/or TPB: they were Glasgow's MEM (Bezuidenhout 1987), Leibovitz L-15 (Byrom and Yunker, 1990), Dulbecco's MEM (Martinez *et al.* 1993) and DME/F-12 in the presence of serum only (Chapter 2). In addition, Iscove's modified Dulbecco's medium, SFRE 199-1, and RPMI 1640 were tested. Only two of these media, DME/F-12 and RPMI 1640, convincingly supported propagation of the Welgevonden stock, while the growth of *E. ruminantium* was retarded in the other media. These results clearly showed that the common synthetic media used for the *in vitro* cultivation of *E. ruminantium* in the past were inferior to a DME/F-12-based medium.

Next, it was necessary to identify the individual components of the HL-1 supplement A which were responsible for its growth-promoting action upon *E. ruminantium*. Several compounds were tested in an RPMI 1640-based medium (data not shown) which, because it did not contain sources of iron or lipids, allowed controlled supplementation with these components. Bovine transferrin and bovine lipoproteins were found to be the only compounds which supported continuous propagation comparable to that in media containing supplement A of the HL-1 medium kit. Nevertheless, the Welgevonden stock of *E. ruminantium* could be propagated over several passages even without these supplements. This suggests that the intracellular pool of nutrients provided by the endothelial host cells may have contributed to the growth of *E. ruminantium* so that deficiencies in the culture medium became obvious only after several passages, a factor which considerably hindered the experiments.

After first identifying a suitable synthetic medium, and then successfully replacing serum with transferrin and bovine lipoproteins, the third series of experiments was aimed at replacing the transferrin and the bovine lipoproteins by chemically defined components. Two protein-free, chemically defined media, referred to as SFMC-23 and SFMC-36, one of which supported the growth of at least four different stocks of *E. ruminantium*, were described (Chapter 4). These media were,

however, only chemically defined to the extent of the purity of the culture ingredients themselves. Medium SFMC-23 was supplemented with a commercially-available mixture of chemically defined lipids, and transferrin was replaced with a complex prepared from glycylglycine and ferric chloride (Yabe *et al.* 1987). SFMC-23 supported the propagation of the Welgevonden stock more effectively than any culture system previously used in our laboratory. *E. ruminantium* was propagated in this medium for 55 passages, subcultured at regular 3-day intervals with split ratios of between 1 in 5 and 1 in 10. This regularity of subculture and level of infection was only rarely achieved in conventional serum-containing medium at that time.

Surprisingly, SFMC-36, where the iron and lipid supplementation was omitted, gave similar results to SFMC-23. This conflicted with previous findings, where supplements of bovine lipoproteins and transferrin had to be added to maintain growth of *E. ruminantium* in culture (Chapter 3). DME/F-12, the basis of the serum-free media, contained ferric nitrate and ferrous sulphate as sources of iron, and linoleic acid as a source of fatty acids, so that an extra supplementation with a non-proteinaceous iron complex and the chemically defined mixture of lipids may have been superfluous.

Although the experiments were not completely conclusive as to the requirement of iron and/or lipids, it has been shown that *E. ruminantium* could be propagated in a protein-free, synthetic medium (DMEM/F-12) which is commercially available, and of fully published composition. An added advantage of using a chemically defined protein-free medium lies in the cost saving derived from omitting serum, the most expensive component of semi-defined media. Moreover, an increase in productivity has been reported by Marcelino *et al.* (in press) who demonstrated the superiority of SFMC-23 over Glasgow's MEM, supplemented with serum and TPB. They observed a 6.5-fold increase in *E. ruminantium* yield when serum-free medium was used during and after infection.

The protein-free medium SFMC-23 has made it possible to determine the amino acid utilization of *E. ruminantium*, has allowed identification of possible substrate deficiencies, and has yielded insight into the metabolic processes of the organism (Chapter 5), without interference from serum proteins or from proteins provided by TPB. Glutamine was the amino acid that was most markedly depleted in both infected and uninfected cultures, by 62% and 53%, respectively. In mammalian cell cultures glutamine is not used directly for protein synthesis, but is metabolised into glutamic acid, aspartic acid, asparagine and proline, and to a lesser degree into serine and alanine (Eagle 1959). All these metabolites were increased in uninfected cultures, except for glutamic acid, which was consumed by the endothelial cells. The largest increase in amino acid concentration found in uninfected cultures was 1047 % for alanine, while a slightly lower increase for alanine (761%) was observed in *E. ruminantium*-infected cultures. Assuming that the host cell metabolism is not grossly disrupted until near to the time of release of the elementary bodies, amino acids from the host cells, which would otherwise be released into the medium, can be used by *E. ruminantium* organisms. Thus, the concentration of proline in the supernatant was 64% lower in infected than in uninfected cultures, indicative of high levels of consumption of this amino acid by *E. ruminantium*. In a similar study, Neitz

and Yunker (1996) found that *E. ruminantium* in serum-supplemented cultures consumed proline at a similar rate to that described here. Another rickettsia, *Rickettsia prowazekii*, has been shown to require proline for growth (Austin and Winkler 1988), with maximal rickettsial growth only occurring in host cells with an intracellular proline pool of 1 mM or greater. Only two of the culture media described for the *in vitro* propagation of *E. ruminantium*, namely RPMI 1640 and DME/F-12, contain proline, whereas Glasgow's MEM, Leibovitz L-15 and DME do not. In the latter medium preparations, proline had to be provided in the form of FCS and TPB. *E. ruminantium* genes for the biosynthesis of proline have been identified (Collins *et al.* 2005) but the level of synthesis may have been too low to adequately support the high level of reproduction observed in protein-free cultures. Therefore, the high proline depletion of the culture medium underlines the necessity of sufficient levels of proline in the culture medium. In retrospect it seems quite obvious that only RPMI 1640 and DME/F-12-based media were suitable for the serum-free cultivation of *E. ruminantium.*

In the present serum-free culture system 4% and 6% of the initial arginine was consumed in uninfected and infected cultures, respectively. In contrast, Neitz and Yunker (1996) reported an almost complete depletion of this amino acid both in infected and uninfected cultures in a serum-supplemented medium. They concluded that their medium (Glasgow's MEM) might have been deficient in arginine. Another reason for the observed depletion of the medium of arginine might have been due to arginase, an arginine-degrading enzyme which can be present at high levels in some foetal calf serum batches (Mellman and Cristofalo 1972). Furthermore, Capiaumont *et al.* (1995) suggested that if the arginine in a culture medium is completely consumed within 2 to 3 days there is probably mycoplasma contamination, although Neitz and Yunker (1996) did not report any evidence of mycoplasma contamination in their cultures.

Overall the results suggest that concentrations of glutamine and proline could become a limiting factor during the *in vitro* cultivation of *E. ruminantium*, and that accumulation of various amino acids or metabolic by-products in the medium could have a negative impact on the organisms.

Chapter 3

Mammalian host cell repertoire

Ehrlichia ruminantium was originally reported to occur within the vascular endothelial cells of infected animals (Cowdry 1926) and to use this cell type for the first *in vitro* propagation seemed appropriate (Bezuidenhout *et al.* 1985). Almost all the continuous cultures of *E. ruminantium* which have been achieved since then have used endothelial cells of various animal species and from various anatomical sites, and several stocks have been isolated, propagated and expanded *in vitro* using these culture systems (Byrom and Yunker 1990; Byrom *et al.* 1991; Jongejan *et al.*1991; Martinez *et al.* 1993; Totté *et al.* 1993). The first propagation of *E. ruminantium* in non-ruminant endothelial cells was carried out by Totté *et al.* (1993) using human umbilical and human microvascular endothelial cells. Smith *et al.* (1998) propagated *E. ruminantium* in endothelial cell lines derived from three species of wild African ruminants and from an omnivore.

It has been shown that other cells could be infected *in vitro*, including leukocytes (Logan et al. 1987) and monocytes (Sahu 1986), but not fibroblasts (Bezuidenhout 1987). Although a primary neutrophil culture system has been devised (Logan et al. 1987), this system was unsuitable for continuous *in vitro* propagation of the organism, because the supply of host cells was too cumbersome. Macrophages are also infected by *E. ruminantium* (Du Plessis 1975) but these cells are unsuitable for the continuous cultivation of the organism, because normal mature macrophages do not proliferate. However, Jongejan & Bekker (1999) tested several cell lines, which were in use for the cultivation of other ehrlichial species (P388D1; MDH-SP; DH82), and were able to grow *E. ruminantium* in monocyte-macrophage cell lines from mice and dogs, however, persistent infections were not established.

Besides the cell types mentioned above, Da Graça (1966) reported finding *E. ruminantium* in fibroblasts of the interstitial spaces and alveolar septa, and Ilemobade (1976) described the presence of *E. ruminantium* in the epithelium of a renal tubule of one animal. The reports of *E. ruminantium* being found in cell types other than endothelial cells, however, have been questioned (Uilenberg, 1983). Clear evidence of whether a certain cell type is potentially susceptible to *E. ruminantium* infection can only be provided by cell culture methods.

The aim of these experiments was, firstly, to evaluate whether a malignantly transformed cell line of the monocyte/macrophage lineage can be used for the continuous propagation of *E. ruminantium*, and secondly, to determine whether *E. ruminantium* can infect and multiply in various cells of non-endothelial cell origin others than monocytes and leukocytes.

Chapter 3.1

Continuous *in vitro* propagation of *Cowdria ruminantium* (Welgevonden stock) in a canine macrophage-monocyte cell line

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Onderstepoort Journal of Veterinary Research **68:** 155-157 (2001)

Abstract

The Welgevonden stock of *Cowdria ruminantium*, aetiologic agent of heartwater, was continuously propagated in DH82 cells, a continuous canine macrophage-monocyte cell line. Cultures of DH82 cells were readily infected provided that the culture medium was supplemented with cycloheximide. Cultures were split at regular 3-day intervals and infection rates ranged between 60 % and 95 %. Cultures were continuously propagated through more than 125 passages over a period of more than one year.

Introduction

Heartwater is an infectious, non-contagious disease of domestic ruminants caused by the rickettsia *Cowdria ruminantium*. It is transmitted by ticks of the genus *Amblyomma* (Lounsbury 1900). The prevalence of the disease throughout sub-Saharan Africa, the islands of Madagascar, La Réunion, Mauritius and São Tomé coincides with the distribution of the vector ticks (Uilenberg 1983). The disease also occurs on three Caribbean islands (Perreau, Morel, Barré & Durand 1980; Birnie, Burridge, Camus & Barré 1984).

Cowdry (1926) was the first to describe, from a histological study, the multiplication of *C. ruminantium* (then known as *Rickettsia ruminantium*) within the endothelial cells of infected animals. It is therefore not surprising that the first successful *in vitro* propagation of *Cowdria* was achieved using bovine umbilical cord endothelial cells (Bezuidenhout, Paterson & Barnard 1985), although other cell types have also been used. Logan, Whyard, Quintero & Mebus (1987) devised a primary neutrophil culture system suitable for the production of *Cowdria* to be used in serological tests. This system was, however, of short duration and unsuitable for continuous *in vitro* propagation of the organism. Macrophages are also infected by *Cowdria* (Du Plessis 1975) but these cells are unsuitable for the continuous cultivation of the organism, because normal mature macrophages do not proliferate. However, Jongejan & Bekker (1999) tested several cell lines, which were in use for the cultivation of other ehrlichial species (P388D1; MDH-SP; DH82) and were able to grow *Cowdria* in monocyte-macrophage cell lines from mice and dogs. Infection rates remained low, however, and no persistent infections were established.

We report here the first continuous propagation of the Welgevonden stock of *C. ruminantium* in DH82 cells through more than 125 passages over a period of more than one year.

Materials and Methods

Stock of C. ruminantium

The Welgevonden stock was isolated by injecting into a mouse a tick homogenate prepared from a single tick. The tick, a male *Amblyomma hebraeum*, was collected on the farm Welgevonden in the Northern Province of South Africa (Du Plessis 1985).

Cell cultures

DH82 cells originally derived from a dog suffering from malignant histiocytosis (Wellman, Krakowka, Jacobs & Kociba 1988) were grown in Dulbecco's modified Eagle's medium nutrient mixture Ham F-12 (DME/F-12)(Sigma, St. Louis, MO, USA; D 0547) containing 15 mM HEPES and 1.2 g/*l* sodium bicarbonate. It was further supplemented with 10 % (v/v) heat-inactivated foetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin. This medium was used for infected and uninfected cell cultures.

Infection of DH82 cells

The propagation of *C. ruminantium* in endothelial cells was carried out as described in detail elsewhere (Zweygarth, Vogel, Josemans & Horn 1997). In brief, endothelial cell cultures heavily infected with *C. ruminantium* were harvested by scraping off the cell monolayer into the medium. The cell suspension was centrifuged (800 x g for 10 min at room temperature) and 2.5 ml of the supernatant, containing mainly elementary bodies, was distributed into culture flasks containing DH82 cells. Attempts were made to initiate two different cultures, one using the medium described above and one using the same medium supplemented with 1 µg/ml cycloheximide. Cycloheximide is a specific inhibitor of protein synthesis in eucaryotic cells (Ennis & Lubin 1964) and has been used to promote growth of chlamydial organisms in cell cultures (Hobson, Johnson & Byng 1977). The cultures were incubated at 37 °C. In each case the initial medium was removed after 24 h and replaced with 5 ml of the same medium.

Monitoring of cultures

C. ruminantium infections were detected by light microscopy. Small samples of adherent cells were removed from the cultures, using a sterile 21 gauge needle with a bent tip, and smears were prepared. These were air-dried, methanol-fixed, and quick-stained (RapiDiff; Clinical Sciences Diagnostics, Booysens, South Africa). Occasionally cytospin preparations of infected cells floating in the culture supernatant were also monitored.

Results

Infection rates with *C. ruminantium* in DH82 cell cultures in the absence of cycloheximide were 3.6 % in adherent cells, and less than 1% in cells floating in the supernatant, and because of these low infection rates the cultures were terminated after two passages. The experiments were repeated twice with similar results (data not shown).

In contrast, the cultures of DH82 cells which contained cycloheximide were readily infected by the Welgevonden stock of *C. ruminantium*, with approximately 35 % of adherent cells and 70 % of detached cells showing infection before the first subculture. Cultures were split at regular intervals of 3 days at a ratio of 2. Occasionally many of the adherent cells were found to have detached from the surface of the flask and were subsequently lost when the medium was changed 24 h after passage. In such cases the cultures were passaged onto fresh host cells without being split. Infection rates were high, i.e. usually between 60 % and 95 %.

Continuous cultures of *Cowdria* could be maintained only when cycloheximide was added to the medium, and this continued to be true even after 116 passages. If cycloheximide was omitted from cultures having an infection rate close to 100 % the rate began to decline after 3 days and was close to zero percent after only four passages.

Discussion

Cowdria ruminantium is particularly closely related to those *Ehrlichia* species in the phylogenetic clade known as Genogroup III (Allsopp, Visser, Du Plessis, Vogel & Allsopp 1997) with somewhat more distant relatives in Genogroups II and I. Several of these species have been successfully propagated in DH82 cells: from Genogroup III *Ehrlichia canis* (Dawson, Rikihisa, Ewing & Fishbein 1991) and *Ehrlichia chaffensis* (Dawson, Anderson, Fishbein, Sanchez, Goldsmith, Wilson & Duntley 1991) and from Genogropup I *Ehrlichia risticii* (Van Heeckeren, Rikihisa, Park & Fertel 1993) and *Neorickettsia helminthoeca* (Rikihisa, Stills & Zimmerman 1991). The results of our study demonstrate that *C. ruminantium* too can readily be propagated in DH82 cells, a permanent canine macrophage-monocyte cell line, but only if cycloheximide as present in the culture medium. Split ratios of two and subculture intervals of 3 days were regularly observed, although in rare instances a split ratio of one had to be used.

Jongejan & Bekker (1999) failed to establish persistent infections of *Cowdria* in the DH82 cell line, and we speculate that this was because they did not incorporate cycloheximide in the medium. It should be noted that Bezuidenhout (1987) and Yunker, Byrom & Semu (1988) found that the presence of cycloheximide in the culture did not improve the propagation of *Cowdria* in bovine endothelial cells. This might have been due to the fact bovine endothelial cells usually show contact inhibition after confluency, with a concomitant reduction in their rate of metabolism. DH82 cells do not show this characteristic, and we propose that the crucial factor in our experiments was the suppression of protein synthesis in the DH82 cells by cycloheximide (Ennis & Lubin 1964) thus reducing their high metabolic turnover. It has been shown that *Chlamydia psittaci* and L cells compete for the amino acid isoleucine (Hatch 1975) and it is likely that competition between *Cowdria* and DH82 cells for certain amino acids would have been suppressed by cycloheximide. Such competition could have been the factor limiting the propagation of *Cowdria* in the absence of cycloheximide.

In conclusion, it has been shown that the Welgevonden stock of *C. ruminantium* can be propagated in DH82 cells, provided suitable culture conditions prevail.

Acknowledgements

We thank Dr B.A. Allsopp for helpful comments on the manuscript. This research was supported by the Agricultural Research Council of South Africa and the European Union (Cowdriosis Network) Grant no. IC18-CT95-0008 (DG12-SNRD).

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Chapter 3.2

The Kümm isolate of *Ehrlichia ruminantium*: *In vitro* isolation, propagation, and characterization

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Onderstepoort Journal of Veterinary Research **69:** 147-153 (2002)

Abstract

An effective culture system for *Ehrlichia* (*Cowdria*) *ruminantium* comb. nov. was first established in 1985 and many stocks were subsequently isolated and propagated *in vitro*. A notable exception, however, was the Kümm isolate which resisted all attempts at *in vitro* culture until the successful experiment described here. In one experiment white blood cells were harvested from heparinized blood derived from a sheep infected with the Kümm isolate. The cells were added to DH 82 cells and incubated at 37 °C. The high metabolic activity of the DH 82 cells necessitated that cell growth be retarded by the addition of cycloheximide. Colonies were first detected 19 days after culture initiation and, once the cultures were established, they could be passaged every 3 days. Bovine and sheep endothelial cells were readily infected with culture supernatant obtained from the infected DH 82 cells. In a further experiment another sheep was infected, using a higher dose of the same batch of Kümm stabilate, and we attempted to infect several different cell lines: these were DH 82 cells, bovine aorta (BA 886) cells, sheep brain endothelial (SBE 189) cells and sheep fibroblastoid cells (E_2). Ten days after culture initiation only the E_2 cells had become positive for *E. ruminantium*. Culture supernatant from the first cultured isolate (Kümm-1) was less virulent for mice than that of the second cultured isolate (Kümm-2) which killed all mice. Upon molecular characterization with *E. ruminantium* 16S probes we found that Kümm-1 hybridized with a Senegal 16S genotype probe, whereas Kümm-2 hybridized only with an Omatjenne 16S genotype probe. The original stabilate used to infect the sheep hybridized with both probes. These results clearly indicate that two different stocks had been isolated in culture.

Introduction

Heartwater is a tick-borne rickettsial disease of ruminants caused by *Ehrlichia* (*Cowdria*) *ruminantium* comb. nov. (Dumler, Barbet, Bekker, Dasch, Palmer, Ray, Rikihisa, & Rurangirwa 2001). The disease is prevalent in sub-Saharan Africa (Uilenberg, 1983), and in the Caribbean (Perreau, Morel, Barré & Durand 1980; Birnie, Burridge, Camus & Barré 1984). The *in vitro* culture of *E. ruminantium* was first achieved using bovine umbilical cord endothelial cells as host cells (Bezuidenhout, Paterson & Barnard 1985). Almost all the continuous cultures of *E. ruminantium* which have been achieved have used endothelial cells of various animal species and from various anatomical sites (Byrom and Yunker 1990; Byrom, Yunker, Donavan & Smith 1991; Jongejan, Zandbergen, Van de Wiel, De Groot & Uilenberg 1991; Martinez, Sheikboudou, Couraud & Bensaid 1993; Totté, Blankaert, Zilimwabagabo & Wérenne 1993), and several stocks have been isolated, propagated and expanded *in vitro* using these culture systems. However, the Kümm isolate (Du Plessis & Kümm 1971) has yielded constantly negative culture results (Bezuidenhout, Brett, Erasmus & Rossouw 1988; Bezuidenhout & Brett 1992). Here we report on the first successful attempt at the isolation and *in vitro* propagation of the Kümm isolate of *E. ruminantium* and its subsequent characterization.

Materials and Methods

Infective agent

The Kümm isolate of *E. ruminantium* was originally obtained from a naturally infected goat in Rust de Winter, Northern Province, South Africa (Du Plessis, 1982). The animal had a disease which was described at the time as heartwater (Du Plessis & Kümm 1971) and a lymph node suspension from the animal was inoculated intravenously into a sheep. The sheep reacted and a saline suspension of its mesenteric lymph nodes was inoculated intraperitoneally (i.p.) into mice, in which it was found to be fatal. Lung and spleen homogenate from infected mice was preserved in liquid nitrogen (Ramisse & Uilenberg, 1970) and the isolate has subsequently been passaged more than 100 times in our laboratory, mainly in mice but also in sheep. This isolate has also been used to prepare antigen slides for heartwater serology (Du Plessis 1982). The number of animal passages at which the present experiments were conducted is unknown.

Host cells

Four different mammalian cell lines were used as potential host cells. The DH 82 cells were from a canine macrophage-monocyte cell line (Wellman, Krakowka, Jacobs & Kociba 1988), the BA 886 cell line was established from a bovine aorta (Yunker, Byrom & Semu 1988), the SBE 189 cell line was from sheep brain endothelium (Brett, Bezuidenhout & De Waal 1992) and the E_2 cells were obtained by cultivation of blood mononuclear cells from a sheep. In order to characterize the E_2 cells we used acetylated low density lipoprotein labelled with the fluorescent probe 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate (DiI-Ac-LDL) (Molecular Probes, Eugene, OR, USA). DiI-Ac-LDL has been shown to label endothelial cells metabolically, thus allowing their identification by fluorescence microscopy (Voyta, Via, Butterfield & Zetter 1984). The E_2 cells were labelled, and fluorescence microscopy was carried out, as described in detail by Aherne, Davis & Sordillo (1995). As no fluorescence was observed we believe that the E_2 cells are not of endothelial origin.

Culture medium

Infected and uninfected cell cultures were grown in Dulbecco's modified Eagle's medium nutrient mixture Ham F-12 (DME/F-12)(Sigma, St. Louis, MO, USA; D 0547) containing 15 mM HEPES and 1.2 g/*l* sodium bicarbonate. The medium was further supplemented with 10 % (v/v) heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin.

Infection of host cells

DNA samples from the Merino sheep used in these experiments were initially examined by PCR (Mahan Waghela, McGuire, Rurangirwa, Wassink & Barbet 1992), and probed with the *E. ruminantium*-specific pCS20 probe (Waghela, Rurangirwa, Mahan, Yunker, Crawford, Barbet, Burridge & McGuire 1991) to ascertain that they were negative for *E. ruminantium.*

A Merino sheep was infected with the Kümm isolate by intravenous injection of 5 ml of a blood stabilate prepared in 1994. Body temperature was monitored daily and a blood sample was drawn on day 18 after infection when the body temperature had risen to 41 °C. Blood was collected by venipuncture into sterile Vac-u-test^{R} tubes containing heparin (lithium heparin, 14.3 USP ml^{-1} blood) as anticoagulant and put on ice. The cooled blood was centrifuged (800 x g; 10 min; 4 °C) and the buffy coat was collected and washed with cold phosphate buffered saline solution (PBS). The red blood cells were lysed in 5 ml sterile distilled water followed by the addition of an equal volume of 1.8 % (w/v) NaCl solution. The white blood cells were washed twice with PBS solution and then resuspended in complete culture medium containing 1 µg ml^{-1} cycloheximide (Ennis & Lubin 1964). The cell suspension was then inoculated into each of two 25 cm² culture flasks containing DH 82 cells and the cultures were incubated at 37 °C. The stock isolated in this experiment is referred to as Kümm-1.

In order to repeat the isolation experiment another Merino sheep was infected with a higher dose (9 ml) of the same batch of blood stabilate, using a procedure which was otherwise as described above. In this experiment we attempted to infect all four of the cell lines mentioned earlier, and two flasks of each cell line were initiated using heparinized blood. The stock isolated in this instance is referred to as Kümm-2.

Propagation of cultures

Infected cultures were harvested for subcultivation by scraping the cell monolayer into the medium and the cells were dispersed by pipetting the suspension up and down. The suspension was distributed into two new culture flasks containing uninfected host cells, and 2.5 ml of fresh medium were added. After 24 h all the medium was discarded and replaced with 5 ml fresh medium.

Infectivity testing

The infectivity of the organisms derived from Kümm-1 and Kümm-2 cultures was tested by injecting culture suspensions, either i.p. or intravenously (i.v.) into mice (0.2 ml) or i.v. into sheep (2 ml).

Molecular characterization

DNA was extracted from Kümm-1 and Kümm-2 infected cell cultures, as well as from the 1974 stabilate of the Kümm isolate, using either the QIAmp (Qiagen, Germany) tissue kit or the FastDNA® Kit (Bio 101 Inc., California, USA). PCR was performed using primers AB 128 and AB 129 for pCS20 amplification (Mahan *et al*. 1992), and primers 930 and BAA5, which amplify the V1 loop of the 16S rRNA genes of all Rickettsiales species, as previously described (Allsopp, Visser, Du Plessis, Vogel & Allsopp 1997). The pCS20 amplicons were slot-blotted onto nylon membranes (Hybond N+, Amersham International), and probed with the pCS20 probe and the 16S amplicons were slot blotted and probed for five different genotypes of *E. ruminantium* (Ball 3, Senegal, Omatjenne, Mara 87/7 and Welgevonden) using procedures as described by Allsopp, Hattingh, Vogel & Allsopp (1999).

The full length 16S rDNA genes of Kümm-1 and Kümm-2 were amplified using primers fD1 and rD1 (Weisburg, Barns, Pelletier & Lane 1991) as previously described (Allsopp *et al.*, 1997) and cloned into pMOS*Blue* (Amersham International). Clones were sequenced on both strands using T7, M13(-48) and internal primers (Allsopp *et al.* 1997) on an ABI 377 automatic DNA sequencer (BigDye terminator cycle sequencing kit, Perkin Elmer Applied Biosystems) and the data were assembled and analyzed using the Staden package (Bonfield, Smith & Staden 1995).

Results

In vitro *cultures*

Kümm-1

Nineteen days after culture initiation colonies were found in two culture flasks. Twenty-four days post initiation, cultures were subcultured into BA 886 cells and DH 82 cells. From then on, cultures were subcultured every 3 d for another 14 passages until they were cryopreserved in liquid nitrogen. Treatment of the DH 82 cells with cycloheximide during the culture period was a prerequisite for continuous propagation.

Kümm-2

All flasks containing DH 82, BA 886 and SBE 189 cells remained negative throughout the observation period (30 days). One of the two flasks containing E_2 cells became positive 10 d after culture initiation. This culture had been initiated with whole blood. This flask was incubated for 3 h before the blood was removed and replaced with medium. Regular subculture intervals could not be achieved because of the slow growth of the organisms and their low infection rate for the host cells. Kümm-2 cultures were passaged ten times with an average splitting interval of 10.5 d (range 8 – 21 d). Once the number of infected cells increased, attempts were made to infect DH 82, BA 886 and SBE 189 cells. The SBE 189 cells became infected and were subcultured after 15 d but failed to grow subsequently. Neither the DH 82 nor the BA 886 cells became infected by the Kümm-2 stock. The major differences observed between Kümm-1 and Kümm-2 are summarized in Table 1.

Table 1 Differences observed between *Ehrlichia* stocks Kümm-1 and Kümm-2

	Stock Kümm-1	Stock Kümm-2
Growth in BA886 or DH82 cells	+	-
Growth in E_2 cells	+	+
Regular subculture intervals	+	-
Virulence in mice	+/-	+
16S genotype	Senegal	Omatjenne

Animal infectivity

Culture supernatant harvested from Kümm-1 cultures was injected i.p. and i.v. into mice. None of the mice died nor did they develop signs of disease. However, in a second experiment, culture material was injected into a group of 20 mice, 13 of which died. A Merino sheep which was injected with 2 ml of culture supernatant became sick and its body temperature rose to 42 °C six days after infection. The sheep was cured after being treated with oxytetracycline.

Culture supernatant obtained from Kümm-2 cultures was highly virulent for mice, all of which died 9 - 11 d after infection regardless of whether the inoculum was injected i.p. or i.v. Seven days after injecting a Merino sheep with 2 ml of culture supernatant its body temperature rose to 42 °C and was elevated for 3 d. The sheep was treated on the third day of elevated body temperature with oxytetracycline.

Molecular characterization

The pCS20 probe hybridized with amplicons of Kümm-1 and Kümm-2, indicating that both stocks were *E. ruminantium*. The 16S V1 loop PCR amplification and probing results were as follows. The *E. ruminantium* (Omatjenne) probe hybridized with amplicons from the 1974 Kümm stabilate and the Kümm-2 culture material, but not with the Kümm-1 culture material. The *E. ruminantium* (Senegal) probe gave hybridization signals with amplicons from the 1974 Kümm stabilate and the Kümm-1 culture material, but not with the Kümm-2 culture material (Table 1). The 16S rDNA gene sequence of Kümm-1 was identical to that of the Senegal stock of *E. ruminantium* (Senegal, Genbank accession #U03775) while that of Kümm-2 was identical to that of the Omatjenne isolate of *E. ruminantium* (Omatjenne, Genbank accession #U03776).

Discussion

In vitro cultivation allowed the discrimination and separation of two different organisms, here referred to as Kümm-1 and Kümm-2, within the stock known as the Kümm isolate of *E. ruminantium*. Many previous attempts to isolate this organism using the conventional method, by inoculation of blood onto a layer of endothelial cells, have failed. It was previously reported that the Kümm stock infects mouse peritoneal macrophages (Du Plessis 1982) so a range of monocyte cell lines was used in initial experiments which are not described here. The DH 82 cell line was the most promising candidate and all subsequent experiments were carried out using this cell line.

In the first experiment described here colonies were first detected 19 d after culture initiation. Shortly thereafter the cultures could be subcultured and, surprisingly, bovine endothelial cells (BA 886) were easily infected. These Kümm-1 organisms proliferated in a manner typical of other *E. ruminantium* stocks, with a period from infection to subculture within 3 d. The reason why the Kümm-1 stock has not been previously isolated in attempts using BA 886 cells remains unclear, although a similar phenomenon has been observed using tick cell cultures for *E. ruminantium* isolation (Dr. Lesley Bell-Sakyi, personal communication). In that work, *E. ruminantium* organisms grew if they were derived from infected endothelial cell cultures, but not if they were derived from the blood of an infected animal.

The purpose of the second series of experiments was to prove that the Kümm stock only infects DH 82 cells and all the other cell lines which were available in our laboratory were used as controls. Interestingly, only one culture of E_2 cells became infected, with a few colonies being detected. This culture, Kümm-2, showed different behaviour to that of the first isolation. Kümm-2 could be propagated only at irregular intervals and cell infection rates remained rather low. BA 886 and DH 82 cells could not be infected at all, SBE 189 cells were infected, but infection was lost on subculturing. In contrast, Kümm-1 was subcultured regularly at 3 d intervals and infection rates were high.

The E_2 cell line, obtained by cultivation of blood mononuclear cells from a sheep, did not accumulate Dil-Ac-LDL, from which it was assumed that they were not of endothelial origin. It is generally accepted that *E. ruminantium* infects only endothelial cells, neutrophils and monocytes, and although there are scattered reports of *E. ruminantium* being found in other cell types, their accuracy has been questioned (Uilenberg 1983), and no real cultural evidence has been presented. Da Graça (1966) reported finding *E. ruminantium* in fibroblasts of the interstitial spaces and alveolar septa, while Ilemobade (1976) described the presence of *E. ruminantium* in the epithelium of a renal tubule of one animal. If E_2 cells are in fact fibroblastoid cells then this is, to the best of our knowledge, the first report of *E. ruminantium* cultures initiated in mammalian cells other than endothelial cells, apart from the short-term cultures of *E. ruminantium* in neutrophils (Logan, Whyard, Quintero & Mebus 1987). Yunker *et al.* (1988) have reported that a cell line changed from an endothelioid cell morphology, susceptible to *E. ruminantium* infection, to a fibroblastic morphology, thus losing its susceptibility to infection. Furthermore, they claimed that E5 cells, which were of fibroblastic morphology and negative for Factor VIII, were susceptible to infection. In our hands E5 cells, although of questionable morphology, stained positively with Dil-Ac-LDL (data not shown). Bezuidenhout (1987) found that Vero cells and lamb foetal kidney cells did not support the growth of the Ball 3 isolate of *E. ruminantium*, and that mouse L-cells did not become infected with the Welgevonden isolate. These conflicting data suggest that further experiments need to be carried out to investigate the infectivity of *E. ruminantium* to fibroblastoid cells.

The original Kümm isolate was the first isolate of *E. ruminantium* described to be pathogenic for mice by i.p. injection. Mice infected with spleen and liver suspension via the i.p. route consistently died from 10 to 14 d after injection. Sheep inoculated with these organisms developed severe febrile reactions and some of the animals died (Du Plessis & Kümm 1971). In contrast, in our first experiment described here, mice injected with Kümm-1 did not develop any clinical signs of disease, nor did they die. However, when the experiment was repeated 65 % of the infected mice succumbed to the infection. Despite these conflicting results Kümm-1 was consistently less virulent for mice than Kümm-2, which was highly virulent in this host, even after i.p. injection. The Kümm-2 stock, therefore, bears the closest resemblance to the description of the original Kümm isolate (Du Plessis & Kümm 1971). The fact that the Kümm stabilate (original) and the Kümm-2 stock cause more than 90 % mortality in mice when injected i.p. is a characteristic not shared by other isolates (MacKenzie & McHardy 1987).

The striking differences in growth patterns, host cell repertoire and subculture intervals clearly indicated that two different organisms were isolated. The molecular characterization further confirmed that the Kümm-1 and Kümm-2 stocks were genetically distinct organisms, with the Kümm-1 stock being of the Senegal 16S genotype while the Kümm-2 stock was of the Omatjenne 16S genotype. It is interesting to note that the Kümm-1 stock's 16S gene sequence is an exact match with the original Senegal stock isolated in 1988 by Jongejan, Uilenberg & Franssen. The Kümm-2 stock was of the Omatjenne *E. ruminantium* 16S genotype, and this isolate was originally made by infecting a mouse with homogenate prepared from a single *Hyalomma truncatum* tick collected in a heartwater- and *Amblyomma*-free area of Namibia (Du Plessis 1990). Previous attempts to molecularly characterize the Kümm isolate gave conflicting results. Examination of a long-stored Kümm blood stabilate revealed the

presence of Omatjenne type V1 loop sequences and also of sequences of the *E. ruminantium* Crystal Springs 16S genotype (Allsopp *et al.* 1997). Subsequently, ticks experimentally infected with the Kümm isolate were positive only with the Omatjenne 16S probe (Allsopp *et al.* 1999).

The presence of the two different genotypes poses the question as to whether they were already present in the original isolate of 1971. As described above, the characteristics of Kümm-2 appear to be typical of the original description, so it is possible that the Kümm-1 component was introduced during passages, which were carried out over many years. This is not impossible, given the difficulty of detecting *E. ruminantium*-carrier animals in the pre-PCR era (Andrew & Norval, 1989; Camus, 1992). The question could be resolved by examining the original stabilate of the Kümm isolate, but unfortunately, the earliest stabilate still available stored in liquid nitrogen originates from 1974. The presence of both genotypes was demonstrated in this material, so we can be sure that, if inadvertent contamination occurred, it was at an early stage in the stock's history. Unfortunately the question as to whether both genotypes were present in the original isolate in 1971 cannot now be answered.

Acknowledgements

This research was supported by the Agricultural Research Council of South Africa and the European Union (Cowdriosis Network) Grant no. IC18-CT95-0008 (DG12-SNRD).

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Chapter 3.3

In vitro infection by *Ehrlichia ruminantium* of Baby Hamster Kidney (BHK), Chinese Hamster Ovary (CHO-K1) and Madin Darby Bovine Kidney (MDBK) cells

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Onderstepoort Journal of Veterinary Research **70:** 165-168 (2003)

Abstract

The Welgevonden stock of *Ehrlichia ruminantium,* aetiological agent of heartwater, was propagated in Baby Hamster Kidney (BHK) cells, Chinese Hamster Ovary (CHO-K1) cells and Madin Darby Bovine Kidney (MDBK) cells. The cultures required supplementation of the medium with cycloheximide for reliable growth of *E. ruminantium*. Growth of the Welgevonden stock in BHK and CHO-K1 cells could lead to the development of suspension cultures suitable for the mass production of *E. ruminantium* for an inactivated elementary body vaccine.

Introduction

Heartwater is a tick-borne rickettsial disease of ruminants caused by *Ehrlichia ruminantium*, the multiplication of which was originally reported to occur within the endothelial cells of infected animals (Cowdry 1926). As a logical consequence of this observation endothelial cells were used for the first successful *in vitro* propagation of *E. ruminantium* (Bezuidenhout, Paterson & Barnard 1985). Subsequent attempts to propagate *E. ruminantium* continuously were therefore carried out almost exclusively in cultures of endothelial cells derived from various animal species and humans. It has been shown that other cells could be infected *in vitro*, including leukocytes (Logan, Whyard, Quintero & Mebus 1987) and monocytes (Sahu 1986), but not including fibroblasts (Bezuidenhout 1987). Nevertheless, Da Graça (1966) described *E. ruminantium* in fibroblasts of the interstitial spaces and alveolar septa of the lung of a ewe, and Ilemobade (1976) described the presence of *E. ruminantium* in the epithelium of a renal tubule of one experimentally infected ox. It was recently shown that the Kümm isolate (Du Plessis & Kümm 1971) consisted of two distinct genotypes, both of which were isolated and propagated in cells of non-endothelial origin (Zweygarth, Josemans, Van Strijp, Van Heerden, Allsopp & Allsopp 2002). These observations prompted us to further investigate the possibility that *E. ruminantium* could enter, survive and grow in cells of non-endothelial origin. We used Baby Hamster Kidney (BHK) cells, morphologically described as being fibroblast-like, and Chinese Hamster Ovary (CHO-K1) and Madin Darby Bovine Kidney (MDBK) cells, morphologically described as being epithelial-like.

Materials and Methods

Stock of E. ruminantium

The Welgevonden stock of *E. ruminantium* was used for the experiments (Du Plessis 1985).

Cells and cell lines

Four different cell lines were used, one of which was endothelial. A bovine aorta cell line (BA 886) (Yunker, Byrom & Semu 1988) was used for the initial propagation of the Welgevonden stock of *E. ruminantium*. BHK cells and MDBK cells were obtained from the Division's cryobank. CHO-K1 were purchased from Highveld Biologicals, Kelvin, South Africa. The passage history of these cell lines is unknown.

Infected and uninfected cells were propagated at 37 °C in a medium consisting of Dulbecco's modified Eagle's medium nutrient mixture F-12 Ham (Sigma, St. Louis, MO, USA) with 10 % (v/v) heat-inactivated foetal bovine serum (FBS; Delta Bioproducts, Kempton Park, South Africa). The medium was buffered with 15 mM HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) (Sigma) and 1.2 g/*l* sodium bicarbonate, and supplemented with 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin.

Propagation of cultures

Endothelial cell cultures heavily infected with *E. ruminantium* were harvested by scraping the cell monolayer off the walls of the culture flask into the medium. The cell suspension was centrifuged (800 x g for 10 min at room temperature) after which the supernatant contained predominantly elementary bodies. Varying amounts of supernatant, up to 2.5 ml, were distributed into culture flasks containing the host cells. Attempts were made to initiate two different types of culture, one using the medium described above and the other in which the same medium was supplemented with 0.5 µg ml^{-1} or 1 µg ml^{-1} cycloheximide (CyX). The cultures were incubated at 37 °C and the medium was removed after 24 h and replaced with 5 ml of the respective medium. When cellular infections had been initiated subsequent passages were carried out using infected cell suspensions which were not centrifuged beforehand.

Microscopic examinations were carried out to demonstrate the presence of *E. ruminantium* organisms in the host cells. Small cell samples were removed by means of a sterile 21-gauge needle with a bent tip, and smears were prepared. They were air-dried, methanol-fixed, and quick-stained (RapiDiff; Clinical Sciences Diagnostics, Booysens, South Africa).

Results

Three days after inoculation with elementary bodies obtained from bovine endothelial cell cultures it was observed that all cell lines were infected and contained colonies. The first culture passage was carried out on Day 3. The addition of CyX to the culture medium was necessary for continuous cultivation and to achieve regular passage intervals. Infected BHK cultures were kept for 33 passages, infected CHO-K1 cultures for 26 passages, whereas infected MDBK cultures were terminated after 11 passages. These results are summarized in Table 1.

Table 1 Propagation of the Welgevonden stock of *E. ruminantium* in the BHK, CHO-K1 and MDBK cell lines

Host cells	Number of passages	Passage intervals [days]
Baby Hamster Kidney cells (BHK)	33	3
Chinese Hamster Ovary cells (CHO-K1)	26	3
Madin Darby Bovine Kidney cells (MDBK)	11	3

Discussion

Continuous *in vitro* propagation of *E. ruminantium* has been restricted to endothelial cell cultures until recently. Early unsuccessful attempts to propagate *E. ruminantium* in fibroblastoid cell lines include the inability of Vero cells and lamb foetal kidney cells to support the growth of the Ball 3 isolate, and of mouse L-cells to become infected with the Welgevonden stock (Bezuidenhout 1987). The situation changed, however, when DH82 cells were infected with the Welgevonden stock of *E. ruminantium* and propagated continuously for more than 100 passages (Zweygarth & Josemans 2001a). It has also been reported that the Kümm isolate of *E. ruminantium* was propagated in a cell line of non-endothelial origin (Zweygarth *et al.* 2002)

In the present experiments we demonstrated that *E. ruminantium* can enter, survive and grow in cells of non-endothelial origin, morphologically described as being fibroblast-like (BHK) or epithelial-like (CHO-K1, MDBK). However, all infected cell lines required the presence of CyX for a regular growth pattern with regular subculture periods and consistent subculture ratios. Cycloheximide is a specific inhibitor of protein synthesis in eucaryotic cells (Ennis & Lubin 1964) which has been used to promote the growth of chlamydial organisms in cell cultures (Hobson, Johnson & Byng 1977), and also of *E. ruminantium* in DH82 cells (Zweygarth & Josemans 2001a). The propagation of the cultures without CyX was abandoned because of irregular growth patterns (data not shown) although it cannot be excluded that they could have been potentially viable.

The culture of *E. ruminantium* in non-endothelial cells, as described here, could have a practical implication for the bulk production of *E. ruminantium* elementary bodies for use in an inactivated elementary body vaccine (Martinez, Maillard, Coisne, Sheikboudou & Bensaid 1994; Mahan, Andrew, Tebele, Burridge & Barbet 1995). A three-step protocol for adapting an anchorage-dependant, serum-dependant, cell lineage of recombinant CHO cells to a serum-free suspension culture has been described (Sinacore, Drapeau & Adamson 2000), and media for the serum-free propagation of *E. ruminantium* have also been developed (Zweygarth, Vogel, Josemans & Horn 1997; Zweygarth, Josemans & Horn 1998; Zweygarth & Josemans 2001b). Our present results therefore suggest that a large-scale production system for *E. ruminantium* could be feasible, using either CHO-K1 or BHK suspension cultures as the host cells. Such a system would be more convenient than the present one, in which collagen microspheres as carriers for endothelial cells are used (Totté, Blankaert, Marique, Kirkpatrick, Van Vooren & Wérenne 1993). While the transition from *E. ruminantium*-infected stationary cultures to suspension cultures is likely to present some difficulties, there is a real need for a large scale, economical production system for this organism.

In conclusion, it has been shown that the Welgevonden stock of *E. ruminantium* was able to grow in non-endothelial cells from different animal species, both ruminant and non-ruminant. Whether *E. ruminantium* infects non-endothelial cells other than leucocytes and monocytes/macrophages *in vivo*, however, remains to be elucidated.

Acknowledgements

We are grateful to Dr B.A. Allsopp for helpful comments on the manuscript. This research was supported by the Agricultural Research Council of South Africa and the European Union (Cowdriosis Network) Grant no. IC18-CT95-0008 (DG12-SNRD).

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Chapter 3.4

In vitro infection of non-endothelial cells by *Ehrlichia ruminantium*

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Annals of the New York Academy of Science **990:** 573-580 (2003)

Abstract

The Welgevonden stock of *Ehrlichia ruminantium* was propagated in eight non-endothelial cell cultures derived from different animal species, both ruminants and non-ruminants. The origins of the cells were: bovine foetal testis (BFT), cat ovary (COC), donkey fibroblasts (DFC), sheep fibroblasts (E_2), horse testis (HTC), lamb foetal testis (LFT), mouse connective tissue (L), and African green monkey kidney (Vero). Four cell culture types (BFT, E_2, LFT and Vero) required supplementation of the medium with cycloheximide for suitable growth of *E. ruminantium*, whereas the other four (COC, DFC, HTC and L) did not. Three other stocks of *E. ruminantium*, Senegal, Ball 3 and Gardel, were also propagated, either in LFT cultures only or in both E_2 and LFT cell cultures. The Welgevonden stock was successfully initiated using E_2 and LFT cell cultures.

In a histological study the multiplication of *Rickettsia ruminantium* (as *Ehrlichia ruminantium* was then known) was described within the endothelial cells of infected animals (Cowdry, 1995), so it is not surprising that the first successful *in vitro* propagation of *E. ruminantium* was achieved using bovine umbilical cord endothelial cells as host cells (Bezuidenhout *et al*., 1985). Endothelial cells of various species and from various anatomical sites were used for almost all subsequent propagations of *E. ruminantium,* and a few examples of those of ruminant origin are: bovine aorta (Byrom and Yunker, 1990), ovine aorta (Byrom *et al*., 1991), bovine umbilical cord (Jongejan *et al*., 1991), bovine brain (Martinez *et al*., 1993), bovine brain microvasculature (Totté *et al*., 1993), caprine jugular vein (Totté *et al*., 1996), and bovine testicular vein (Mwangi *et al*., 1998). The first propagation of *E. ruminantium* in non-ruminant endothelial cells was carried out by Totté *et al.* (1993) using human umbilical and human microvascular endothelial cells. Smith *et al.* (1998) propagated *E. ruminantium* in endothelial cell lines derived from three species of wild African ruminants (sable antelope [*Hippotragus niger*], buffalo [*Syncerus caffer*], eland antelope [*Tragelaphus oryx*]) and from a bush pig (*Potamochoerus porcus*), an omnivore.

Logan *et al.* (1987) devised a primary neutrophil culture system suitable for the production of *E. ruminantium* to be used in serological tests. This system was, however, of short duration and unsuitable for continuous *in vitro* propagation of the organism. Macrophages constitute another cell type infected by *E. ruminantium* (du Plessis, 1975), but these cells are also unsuitable for continuous cultivation of the organisms because normal mature macrophages do not proliferate. Despite this limitation, Jongejan and Bekker (1999) were able to grow *E. ruminantium* in transformed monocyte-macrophage cell lines from mice and dogs (P388D1; MDH-SP; DH82), although no persistent infections were established. Continuous propagation of *E. ruminantium* in DH82 cells was only achieved recently by supplementing the culture medium with cycloheximide (Zweygarth and Josemans, 2001). Cycloheximide is a specific inhibitor of protein synthesis in eucaryotic cells (Ennis and Lubin, 1964) and has been used to promote growth of chlamydial organisms in cell cultures (Hobson *et al*., 1977).

Besides the cell types mentioned above, Da Graça (1966) reported finding *E. ruminantium* in fibroblasts of the interstitial spaces and alveolar septa, and Ilemobade (1976) described the presence of *E. ruminantium* in the epithelium of a renal tubule of one animal. The reports of *E. ruminantium* being found in other cell types, however, have been questioned (Uilenberg, 1983), because no clear evidence has been presented so far. Here we report on the successful propagation of *E. ruminantium* in several non-endothelial cell cultures and cell lines derived from ruminants and non-ruminants.

Materials and Methods

Stocks of E. ruminantium

Four stocks of *E. ruminantium* were used for these experiments: Welgevonden (du Plessis, 1985) and Ball 3 (Haig, 1952), both from South Africa; Senegal from Senegal (Jongejan *et al*., 1988); and Gardel, which was isolated in Guadeloupe (Uilenberg *et al*., 1985). The Gardel stock was initially propagated in sheep brain endothelial cells (SBE 189) (Brett *et al*., 1992), and a bovine aorta cell line (BA 886) (Yunker *et al*., 1988) was used for the initial propagation of the other three stocks. The passage history for each stock is unknown.

Cells and cell lines

A total of 8 different non-endothelial cell lines was used. Donkey fibroblastoid cells (DFC) were obtained by propagating mononuclear cells which adhered to the culture flask after incubation of heparinized donkey blood. E_2 cells were obtained by cultivation of blood mononuclear cells from a sheep (Zweygarth *et al*., 2002). Horse testis (HTC) and cat ovary (COC) cell cultures were initiated from surgical material obtained from adult animals, using trypsin to disaggregate the tissues (Freshney, 1987). Bovine foetal testis (BFT) and lamb foetal testis (LFT) cell cultures were kindly provided by D. Wallace (Onderstepoort Veterinary Institute). All the above-mentioned cells were used at passage levels below 40. Vero cells were used between passage levels of 137 to 147, whereas the passage history for the mouse L-cells used is unknown. The latter two cell lines were obtained from the Division's cryobank.

Infected and uninfected cells were propagated at 37°C in medium consisting of Dulbecco's modified Eagle's medium nutrient mixture F-12 Ham (Sigma, St. Louis, MO, USA) with 10% (v/v) heat-inactivated foetal bovine serum (FBS; Delta Bioproducts, Kempton Park, South Africa). The medium was buffered with 15 mM HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) (Sigma) and 1.2 g/L sodium bicarbonate, and supplemented with 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin.

Propagation of cultures

The propagation of *E. ruminantium* in endothelial cells was carried out as described in detail elsewhere (Zweygarth *et al*., 1997). In brief, endothelial cell cultures heavily infected with *E. ruminantium* were harvested by scraping the cell monolayer off the walls of the culture flask into the medium. The cell suspension was centrifuged (800 x g for 10 min at room temperature) and varying amounts (up to 2.5 ml) of the supernatant, which contained elementary bodies, was distributed into culture flasks containing the respective host cells. Attempts were made to initiate two different cultures, one using the medium described above and another in which the same medium was supplemented with 0.5 µg ml^{-1} or 1 µg ml^{-1} cycloheximide (CyX). The cultures were incubated at 37°C. The medium was removed after 24 h and replaced with 5 ml of the respective medium.

Microscopic examinations were carried out to demonstrate the presence of *E. ruminantium* organisms in the host cells. Small cell samples were removed by means of a sterile 21-gauge needle with a bent tip, and smears were prepared. They were air-dried, methanol-fixed, and quick-stained (RapiDiff; Clinical Sciences Diagnostics, Booysens, South Africa).

Initiation of cultures

The inocula for culture initiation consisted of suspensions of spleen prepared from moribund mice infected with the Welgevonden stock of *E. ruminantium*. One ml of suspension, diluted with 4 ml of culture medium, was inoculated into 25 cm^2 culture flasks containing either E_2 cells, or LFT cells or DFC cells. The flasks were incubated at 37° C for 3 h before the spleen cell suspension was rinsed off and replaced by fresh medium. Cultures were observed for 30 d.

Results

Growth of* E. ruminantium *(Welgevonden stock) in E_2, LFT, BFT and Vero cells

When using E_2, LFT, BFT and Vero cells, the presence of CyX in the medium was required in order to achieve regular passage intervals of 3 d. When using LFT cells in the absence of CyX, the passage intervals were irregular, with an average of 5.4 d and a maximum of 22 d. Infecting LFT cells with elementary bodies resulted in an average infection rate of approximately 80% before the first passage. Attempts to use BFT and Vero cells without CyX in the medium were discontinued, usually after the second or third passage, owing to unsatisfactory growth results, comprising either low infection rates or too few free elementary bodies. Results are summarized in Table 1.

Growth of* E. ruminantium *(Welgevonden stock) in HTC, COC, DFC and L cells

Three days after inoculation with elementary bodies, HTC, COC, DFC and L cells were observed to contain *E. ruminantium* colonies, which ruptured when smeared onto glass slides, releasing masses of elementary bodies. The addition of CyX to the culture medium was not necessary for continuous cultivation or for regular passage intervals with these four cell types. The passage interval was 3 d, which was the same as when the stock was propagated in BA 886 cells. Cultures were kept for at least 10 passages. Results are summarized in Table 1.

Initiation of* E. ruminantium *(Welgevonden stock) cultures

Suspensions of spleen cells prepared from moribund mice infected with the Welgevonden stock were used to initiate cultures using LFT and E_2 cells, which became positive after 25 d and 16 d respectively. In control experiments using BA 886 cells for initiation it took just 8 d before the first passage. *E. ruminantium* cultures could not be established in DFC owing to early detachment of the cells from the culture flask.

Growth of **E. ruminantium** *(Senegal stock) in LFT cells*

In order to see whether stocks other than the Welgevonden stock could be propagated in fibroblastoid cells, the Senegal stock was propagated in LFT cells and CyX-containing medium. The cultures were grown over 5 passages, with an average interval of 3 d, before the experiment was terminated.

Growth of **E. ruminantium** *(Ball 3 stock) in E_2 and LFT cells*

The Ball 3 stock was propagated in E_2 cells over 5 passages at 4 d intervals before the cultures were terminated. Passage intervals were the same as for the Ball 3 stock when propagated in BA 886 cells. The Ball 3 stock was

used to infect LFT cells in a culture medium containing 0.5 $\mu g \cdot ml^{-1}$ CyX, but an infection rate of less than 1% before the first passage decided us to make no further propagation attempts with this cell/stock combination.

Growth of **E. ruminantium** *(Gardel stock) in E_2 and LFT cells*

The Gardel stock was propagated in E_2 cells over 10 passages at 4 d intervals before the cultures were terminated. At the last passage a comparison was made between flasks containing medium with or without CyX, and CyX clearly improved the infection rate of the cultures. Passage intervals were 4 d, which was the same as for the Gardel stock when propagated in SBE 189 cells. The Gardel stock was propagated in LFT cells, in culture medium containing 0.5 $\mu g \cdot ml^{-1}$ CyX, over 8 passages at 4 d intervals before the cultures were terminated.

Table 1 Propagation of the Welgevonden stock of *E. ruminantium* in non-endothelial cell lines

Host cell	Number of passages	Passage intervals [days]	Cycloheximide supplementation
Bovine foetal testis cells (BFT)	10	3	+
Cat ovary cells (COC)	10	3	–
Donkey fibroblastoid cells (DFC)	25	3	–
E_2 cells	10	3	+
Horse testis cells (HTC)	10 10	3 3	+ –
Lamb foetal testis cells (LFT)	32 16	3 5.4 (3-22)	+ –
Mouse connective tissue cells (L cells)	20 20	3 3	+ –
African Green monkey kidney cells (Vero cells)	8	3	+

Discussion

Ehrlichia ruminantium is most often found *in vivo* in endothelial cells, neutrophils and macrophages, whereas *in vitro* it has been propagated in cell lines derived nearly exclusively from endothelial cells. This has resulted in it being described as an obligatory parasite of endothelial cells (Martinez *et al*., 1993). Endothelial cells of ruminants were the first cells shown to be infected by *E. ruminantium* (Cowdry, 1926), but in culture, endothelial cells of ruminants and non-ruminants can be infected. Totté *et al.* (1993) showed that human umbilical and microvascular endothelial cells were readily infected and were suitable for the continuous propagation of *E. ruminantium*. Furthermore, Smith *et al.* (1998) propagated *E. ruminantium* in an endothelial cell line derived from a bushpig (*Potamochoerus porcus*), which is an omnivorous animal. These results clearly show that, under experimental culture conditions, infections can be produced in endothelial cells of non-ruminant hosts not normally susceptible to *E. ruminantium* infection, hence suitable attachment sites must be present on non-ruminant endothelial cells. In addition it has been reported that *E. ruminantium* can be propagated in a cell line of non-endothelial origin

(Zweygarth *et al*., 2002). These conflicting data prompted us to investigate the infectivity of *E. ruminantium* to fibroblastoid cells and we have found that, under culture conditions, the organism is capable of entering, surviving and growing in fibroblastoid cells from several different animal species.

Early attempts to propagate the Ball 3 isolate of *E. ruminantium* in fibroblastoid cell lines failed when it was found that Vero cells and lamb foetal kidney cells did not support the growth of the organism (Bezuidenhout, 1987). In our experiments the Ball 3 stock could also not be established satisfactorily in LFT cells, although it was successfully propagated in E_2 cells as long as CyX was present. Furthermore, Bezuidenhout (1987) did not succeed in infecting mouse L-cells with the Welgevonden isolate, whereas in our experiments mouse L-cells were easily infected with this isolate, and cultures were propagated with and without CyX over 20 passages before the cultures were terminated. The reasons for this experimental discrepancy are unclear. One reason could be that the Welgevonden stock which we have used is culture-adapted, and propagates better in culture than that used by Bezuidenhout more than 15 years ago. The high concentrations of CyX used by Bezuidenhout may also have had a negative impact, his media contained 0.1 - 0.5 % of CyX compared to 0.5 – 1 $\mu g \cdot ml^{-1}$ in the present experiments.

The present results indicate that stocks differ in their ability to infect and grow in fibroblastoid cells, since the Welgevonden stock produced an infection rate in LFT cells of approximately 80% while the Ball 3 stock infected less than 1% of the host cells. The conventional method of isolation, using endothelial cell cultures, was superior in terms of time, at least as far as the Welgevonden stock was concerned, as was shown when we attempted to initiate cultures in E_2 or LFT cells. There are, however, reasons for attempting to isolate uncharacterised genotypes of *E. ruminantium* in systems other than the conventional one. It has been shown recently that cultures of two different genotypes of *E. ruminantium* could be initiated from a sheep infected with the Kümm isolate, by using either DH 82 cell cultures or the E_2 cell line, but not by using endothelial cells (Zweygarth *et al*., 2002). It is evident, therefore, that organisms are only isolated from field cases if they are susceptible to growth in the particular culture system used. Using a broader spectrum of cell culture types should therefore increase the chance of isolating organisms which would otherwise remain uncharacterised.

E. ruminantium has been demonstrated in lymph node reticulo-endothelial cells of infected sheep several days before typical colonies could be demonstrated in brain endothelial cells (du Plessis, 1970). In the event of natural tick infection, cells of the regional lymph nodes are likely to be the first to be infected, hence fibroblastoid cells in the lymph node tissue could become infected as well. It has been shown that persisting parasites were associated with fibroblasts in the draining lymph nodes of mice that had healed a cutaneous infection with *Leishmania major*, although *Leishmania* usually infect macrophages, granulocytes and dendritic cells (Bogdan *et al*., 2000). Although we do not have any proof that *E. ruminantium* infects fibroblasts *in vivo*, it could be speculated that, in a similar manner to *L. major*, *E. ruminantium* may survive and even grow in fibroblasts. Carriers of *E. ruminantium* have been demonstrated in domestic ruminants and in African buffalo. Kock *et al.* (1995) demonstrated *E. ruminantium* DNA in the bone marrow of carrier tsessebe (*Damaliscus lunatus*), impala (*Aepyceros melampus*) and sheep, and assumed that this anatomical site is a possible reservoir for *E. ruminantium*. In our experiments using LFT cell cultures without CyX, growth of the organisms was greatly reduced, indicating that the organisms were maintained at a lower state of activity and did not divide as rapidly as they would normally do in endothelial cells. Fibroblasts are widely distributed in various tissues, and these cells could serve as an important reservoir in animals carrying low levels of *E. ruminantium* infection.

In conclusion, it has been shown that stocks of *E. ruminantium* isolated from different geographical regions were all able to grow in fibroblastoid cells from a number of different animal species, both ruminants and non-ruminants. Whether *E. ruminantium* infects fibroblasts *in vivo*, however, remains to be elucidated.

Acknowledgements

We are grateful to D. Wallace for the provision of the lamb and bovine foetal testis cell cultures and Dr B.A. Allsopp for helpful comments on the manuscript.

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Chapter 3.5

Discussion - Host cell repertoire

E. ruminantium was at one time considered to be an obligatory parasite of endothelial cells (Martinez *et al.* 1993), although other cell types have been found to be infected. These include neutrophils (Logan *et al.* 1987) and macrophages/monocytes (Du Plessis 1975; Sahu 1986), while du Plessis (1982) reported that the Kümm stock infected mouse peritoneal macrophages. Many attempts to culture the Kümm isolate, using the conventional method of inoculation of blood onto a monolayer of endothelial cells, had failed, and therefore we decided to investigate unconventional approaches. In a series of experiments we tried to establish whether the Welgevonden stock, derived from endothelial cell cultures, could be propagated in a range of monocyte-derived, transformed cell lines (J 774A, P 388D1, DH82). The DH82 line, a continuous canine macrophage-monocyte cell line, was the most promising candidate and all subsequent experiments were carried out using this cell line (Chapter 6). *E. ruminantium* was readily propagated in DH82 cells, but only if cycloheximide was present in the culture medium. A possible action of cycloheximide in mammalian cells is the suppression of protein synthesis (Ennis and Lubin 1964) thus reducing their high metabolic turnover. In contrast, the presence of cycloheximide in the culture medium did not improve the propagation of *E. ruminantium* in bovine endothelial cells (Bezuidenhout 1987, Yunker *et al.* 1988), probably because with these adherent, monolayer-forming cells, contact inhibition occurs after confluency, with a concomitant reduction in the rate of metabolism. DH82 cells do not exhibit these characteristics, and therefore the addition of cycloheximide may have been a crucial factor. Hatch (1975) showed that *Chlamydia psittaci* and L cells compete for the amino acid isoleucine. A similar competition between *E. ruminantium* and DH82 cells for certain amino acids could have been suppressed by cycloheximide, and reduction of metabolic turnover and/or competition for certain amino acids could have been the limiting factor for the propagation of *E. ruminantium* in the absence of cycloheximide

After the successful *in vitro* propagation of the Welgevonden stock in DH82 cells, attempts were made to isolate the Kümm stock in culture using DH82 cells (Chapter 7). All previous attempts to isolate the Kümm organism by conventional culture methods, i.e. using endothelial cells as host cells, had failed. The rationale for choosing DH82 cells was threefold: they could be continuously propagated, they were easily available, and they were of monocyte/macrophage origin, which was promising since it was reported that the Kümm isolate infects peritoneal macrophages of mice (Du Plessis 1982). Indeed, colonies were detected in DH82 cells 19 days after initial infection with the Kümm isolate. The Kümm organisms could easily be subcultured into DH82 cells, but also from DH82 cells into bovine endothelial cells (BA 886). From their behaviour *in vitro*, these organisms, referred to as

Kümm-1, could not be differentiated from other *E. ruminantium* stocks propagated in culture. The reason why the Kümm-1 stock had not been previously isolated in BA 886 cells remained unclear.

In order to prove that the Kümm organisms isolated in the first experiment only infected DH82 cells, a second series of experiments was carried out. All the cell lines which were available in our laboratory at the time were used, including the DH82 cells. This time, however, only one culture of uncharacterised ovine cells (E_2) became infected. The Kümm organisms, now referred to as Kümm-2, showed behaviour quite different from the Kümm-1 stock which was obtained during the first isolation. The most prominent characteristics were their irregular subculture intervals and low infection rates. BA 886 and DH82 cells could not be infected at all, and SBE 189 cells were infectable from the E_2 cells but the infection was inexplicably lost on subculturing. In contrast, the Kümm-1 stock was subcultured regularly in BA 886 and SBE 189 cells at 3-day intervals with high infection rates.

Assuming that *E. ruminantium* only infects endothelial cells, the E_2 cell line was initially considered as being of endothelial origin. Cultivation of sheep blood mononuclear cells, which gave rise to the E_2 cell line, favours the establishment of fibroblastoid rather than of endothelial cells. Therefore the E_2 cell line was further characterised using acetylated low-density lipoprotein labelled with the fluorescent probe Dil-Ac-LDL (1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate), a marker for endothelial cells (Aherne *et al.* 1995). However, the E_2 cells did not accumulate the probe, whereas the known endothelial cell lines, BA 886 and SBE 189, readily accumulated the probe. Therefore it was concluded that the E_2 cell line was not of endothelial origin. There have been scattered reports of *E. ruminantium* being found in cell types other than endothelial cells, however, their accuracy has been questioned (Uilenberg 1983), and no real evidence from *in vitro* cultures has been presented up to now. Bezuidenhout (1987) found that Vero cells (African green monkey kidney cells) and lamb foetal kidney cells did not support the growth of the Ball 3 isolate of *E. ruminantium*, and that mouse L-cells (mouse connective tissue cells) did not become infected with the Welgevonden isolate. These conflicting data suggested that further experiments were necessary to investigate the possibility that *E. ruminantium* could infect fibroblastoid cells under the culture conditions currently in use.

The question whether *E. ruminantium* could infect other non-endothelial cells in addition to the E_2 line (Chapter 7) was examined in Chapter 8. It was shown that the Welgevonden stock of *E. ruminantium* could be propagated in Baby Hamster Kidney (BHK) cells and Chinese Hamster Ovary (CHO-K1) cells. These cell lines were chosen as candidate host cells because they are widely used in bioreactors and would as such be suitable for the mass production of *E. ruminantium* elementary bodies. These could then be used in the production of an inactivated elementary body vaccine (Martinez *et al.* 1994; Mahan *et al.* 1995). A cell line of genetically modified CHO cells, anchorage- and serum-independent and suitable for suspension culture, has been described (Sinacore *et al.* 2000). Serum-free propagation of *E. ruminantium* has been developed (Chapter 2) and the use of an anchorage-independent host cell line such as CHO-K1 or BHK suspension cultures, could be feasible. The present results therefore suggested that such a large-scale production system for *E. ruminantium* could replace the

current system in which collagen microspheres are used as carriers for endothelial cells in large scale cultures (Totté *et al.* 1993).

Once the Welgevonden stock was shown to infect BHK, CHO-K1 and MDBK cells, the question arose of whether other stocks of *E. ruminantium* could infect fibroblastoid cells, which were isolated not only from natural *E. ruminantium* hosts but also from animal species refractory to *E. ruminantium* infection (Chapter 8). Earlier attempts to propagate the Ball 3 stock of *E. ruminantium* in Vero cells and lamb foetal kidney cells had failed (Bezuidenhout 1987). In our experiments the Ball 3 stock could not be established satisfactorily in LFT cells, although it was successfully propagated in E_2 cells as long as cycloheximide was present. Similarly, in our experiments mouse L-cells were easily infected with the Welgevonden stock, and cultures were propagated with and without cycloheximide over 20 passages before the cultures were terminated, whereas Bezuidenhout (1987) did not succeed in infecting mouse L-cells with the the same stock. The reasons for this experimental discrepancy are unclear. One reason could be that by the time we used the Welgevonden stock it had become culture-adapted, and grew better *in vitro* than when it was used by Bezuidenhout more than 15 years before. The high concentrations of cycloheximide used by Bezuidenhout may also have had a negative impact.

The present results further indicate that stocks differ in their ability to infect and grow in fibroblastoid cells, since the Welgevonden stock produced an infection rate in LFT cells of approximately 80% while the Ball 3 stock infected less than 1% of the host cells. The conventional method of isolation, using endothelial cell cultures, was superior in terms of time, at least as far as the Welgevonden stock was concerned, since it took longer to initiate cultures in E_2 or LFT cells. The importance of these experiments has been demonstrated earlier (Chapter 7), namely that cultures of two different genotypes of the Kümm isolate of *E. ruminantium* were only initiated using both DH82 cell cultures and the E_2 cell line, but not by using endothelial cells. The use of a single culture system for epidemiological studies can therefore be misleading because not all organisms are equally likely to grow in that particular system. Using a broader spectrum of cell culture types should therefore increase the chance of isolating organisms which would otherwise remain uncharacterised, or even undetected.

It has been shown that stocks of *E. ruminantium* originating from different geographical regions were all able to grow in fibroblastoid cells from a number of different animal species, both ruminants and non-ruminants. Whether or not *E. ruminantium* infects fibroblasts *in vivo*, however, remains to be elucidated.

Chapter 4

Application of *in vitro* culture techniques for the attenuation of *Ehrlichia ruminantium*

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 (Alexander 1931). After the discovery of an effective treatment against the heartwater agent, an "infection and treatment" method was developed to immunize animals. Fully virulent *E. ruminantium* organisms of the Ball 3 stock were injected and the animals were subsequently treated with tetracyclines to prevent serious disease (Bezuidenhout 1989). This procedure has been the only commercially available heartwater "vaccine" for more than 50 years. The spectrum of protection of the Ball 3 blood vaccine stock against other *E. ruminantium* stocks is limited, whereas the Welgevonden stock has been shown to stimulate protective immunity against several virulent South African stocks and should therefore be more suitable for immunization purposes than the Ball 3 stock (Collins *et al.* 2003). However, its high virulence has precluded it from being used for immunization (Du Plessis *et al.* 1989).

Attenuation is usually achieved by passage of an infective agent in a foreign host such as embryonated eggs or tissue culture cells. From among the many mutants that exist in a population, some will be selected that have a better ability to grow in the foreign host resulting in a higher virulence. However, these organisms tend to be less virulent for the original host. The basis of attenuation is not known since attenuation was mostly achieved empirically. Advantages of attenuated vaccines are that they activate all phases of the host immune system and raise immune responses to all protective antigens, thus inducing a durable immunity. The Senegal stock of *E. ruminantium* became attenuated after a limited number of passages *in vitro* (Jongejan 1991). This attenuated stock provided immunity to homologous challenge (Jongejan 1991), but did not provide efficient cross-protection against other virulent stocks (Jongejan *et al.* 1993). The Gardel stock from Guadeloupe has also been attenuated *in vitro*, although more than 200 passages were required (Martinez 1997).

In the following chapters a Welgevonden stock which was attenuated by continuous passage in a canine macrophage-monocyte cell line (Chapter 3.1) was used to vaccinate sheep and goats under controlled experimental conditions and its potential as a possible new vaccine against heartwater was evaluated.

Chapter 4.1

Development of improved attenuated and nucleic acid vaccines for heartwater

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Developmental Biology (Basel) **114:** 121-36 (2003)

Abstract

Heartwater, an economically important tickborne disease of wild and domestic ruminants, is caused by the intracellular rickettsia *Ehrlichia* (formerly *Cowdria*) *ruminantium*. The only commercially available immunization procedure is more than 50 years old and employs an infection and treatment regimen using a preparation of virulent organisms in cryopreserved sheep blood. Much research has been conducted into the development of attenuated, inactivated, and nucleic acid vaccines over the last half-century, with relatively little success until recently. We describe here the development of two new experimental vaccines, a live attenuated vaccine and a nucleic acid vaccine. The attenuation of virulent *E. ruminantium* was achieved by growing the organisms in a continuous canine macrophage-monocyte cell line. After more than 125 passages the cultures produced no disease when inoculated into mice or sheep, and the animals were completely protected against a subsequent lethal homologous needle challenge. The nucleic acid vaccine consists of a cocktail of four *E. ruminantium* genes, from a genetic locus involved in nutrient transport, cloned in a DNA vaccine vector. Sheep immunized with this cocktail were completely protected against a subsequent lethal needle challenge, either with the homologous isolate or with any one of five different virulent heterologous isolates. Protection against a field challenge in a heartwater endemic area, however, was relatively poor. Genetic characterization of the *E. ruminantium* genotypes in the challenge area did not identify any having major differences from those used in the heterologous needle challenge experiments, so lack of cross-immunity between the vaccine genotype and those in the field was unlikely to be the main reason for the lack of protection. We believe that a needle challenge is far less acute than a tick challenge, and that the immunity engendered by the DNA vaccine alone was not sufficient to protect against the natural route of infection. Boosting with live organisms after DNA vaccination results in much higher levels of protection against tick challenge than DNA vaccination alone, suggesting that improved methods of boosting could lead to more effective immunization.

Introduction

Heartwater, or cowdriosis, is a tick-borne disease of cattle, sheep, goats and some wild ruminants, caused by an intracellular rickettsia previously known as *Cowdria ruminantium* but recently reclassified as *Ehrlichia ruminantium* (Dumler *et al.* 2001). The disease distribution coincides with that of the tick vectors, the most important of which are *A. variegatum* and *A. hebraeum*, and the endemic area encompasses most of sub-Saharan Africa except the dry south west (Provost and Bezuidenhout 1987). Heartwater also occurs in the French Antilles (Camus and Barre 1995), to which infected *A. variegatum* ticks were introduced, probably with cattle from Africa during the eighteenth century (Maillard and Maillard 1998). The possible spread of infected *A. variegatum* ticks on to the American mainland (Deem 1998), where a suitable tick vector is already present (Mahan *et al.* 2000), will remain a permanent threat to the American livestock industry until a safe and effective vaccine becomes available.

Animals which recover from heartwater are subsequently immune to homologous challenge, and this observation led, more than 50 years ago, to the development of an infection and treatment immunization procedure (van der Merwe 1987). Animals are infected with cryopreserved sheep blood containing virulent *E. ruminantium* organisms of the Ball 3 genotype (Bezuidenhout 1989) and antibiotic treatment is administered when a rise in temperature occurs. This procedure, however, does not fully protect against all the genotypes which circulate in the field. Limited cross-protection between genotypes has been shown by several workers (du Plessis *et al.* 1998; Jongejan *et al.* 1988; Jongejan *et al.* 1991) but it must be remembered that these experiments were performed before reliable genetic characterization methods became available. At least one of the stocks used is now known to be genetically heterogeneous (Zweygarth *et al.* 2002).

The Senegal isolate of *E. ruminantium* has been attenuated in cell culture and the attenuated organisms have been shown to protect animals against subsequent lethal homologous challenge (Jongejan 1991). Protection against other isolates is poor, however (Gueye *et al.* 1994), and most other isolates have not proved susceptible to *in vitro* attenuation (Jongejan *et al.* 1991). A nucleic acid (naked DNA) vaccine, expressing the *map*1 gene of *E. ruminantium*, protects mice against a lethal homologous needle challenge, both when administered alone (Nyika *et al.* 1998), and when a MAP1 protein boost was given (Nyika *et al.* 2001). Other *E. ruminantium* genetic vaccines have also shown some protection in the mouse model (Barbet *et al.* 2001) but in all these mouse studies the levels of protection were very variable.

Immunity to the Welgevonden isolate of *E. ruminantium* has previously been shown to confer immunity to a number of other virulent stocks (du Plessis *et al.* 1989). We have confirmed this observation using several genetically characterised isolates in culture and this isolate, which is the type specimen of *E. ruminantium* comb. nov., was therefore chosen for our vaccine development work. We have used two different approaches: classical attenuation, and genetic immunization, which consists of the presentation of protection-stimulating genes in a DNA vaccine vector. Both of these methods of immunization have been shown, in other systems, to be capable of stimulating a cellular immune response, which is a particularly important consideration for protection against heartwater (du Plessis *et al.* 1991; du Plessis *et al.* 1992; Martinez 1997; Mwangi *et al.* 1998; Totté *et al.* 1997).

Materials and Methods

E. ruminantium *stocks, culture, and preparation of genomic DNA*

Six *E. ruminantium* stocks or isolates were used for cross immunity trials, the full details of these are given in Table 1. For the preparation of genomic DNA *E. ruminantium* (Welgevonden) was cultured in a serum-free medium (Zweygarth *et al.* 1998) and elementary bodies were purified and DNA prepared as described elsewhere (de Villiers *et al.* 2000).

Table 1 *E. ruminantium* stocks used for cross immunity experiments

Isolate	Remarks	Origin	Reference
Ball 3	S. African blood "vaccine" isolate	Natural field infection, mammalian host not specified Northern Province, S. Africa	Haig 1952
Blaauwkrans	Highly pathogenic to goats in Eastern Cape, S. Africa.	*A. hebraeum* tick from an eland, Eastern Cape, S. Africa	-
Gardel	Common Carribean genotype	Guadeloupe, French West Indies	Uilenberg et al 1985
Kwanyanga	Not highly pathogenic to sheep	Naturally infected sheep in the Eastern Cape, S. Africa	Mackenzie and Van Rooyen 1981
Mara 87/7	Widespread and virulent S. African genotype	*A. hebraeum* tick, Northern Province, S. Africa	Du Plessis et al 1989
Welgevonden	Type specimen of *E. ruminantium* comb. nov	Northern Transvaal, S. Africa, close to area of the original *Rickettsia ruminantium* isolation (Cowdry 1925)	Du Plessis 1985

***Needle challenge of animals with stocks of virulent* E. ruminantium**

All needle challenges were with 10 LD_{50} of titrated single-genotype stabilate prepared in the target animal species (Brayton *et al.* 2003). Genetically immunized animals were challenged five weeks after the final immunization, and animals in coss immunity trials were challenged as described below. In the case of small ruminants the body temperatures were monitored daily before and after challenge. Other symptoms were also observed daily after challenge and a reaction index (Table 2) was totalled daily to provide a measure of the severity of the reaction.

Cross immunity trials in sheep

Animals were age-matched as far as possible and screened using PCR and pCS20 probing (Allsopp *et al.* 1999) to ensure that they were free from any *E. ruminantium* infection. Groups of 20 animals were infected with an *E. ruminantium* stock and treated when they became febrile. Homologous challenge was carried out, a minimum of four weeks later, to check their immune status. After a minimum of a further four weeks groups of four animals were subjected to heterologous challenge. One naive control animal was included with each group and, in conformity with animal ethics considerations, tetracycline was administered to any animal if death appeared likely.

Table 2 Reaction index estimate for small ruminants challenged with *E. ruminatium*. Body temperatures and symptoms were monitored for the duration of the experiment. After challenge, animals were scored daily according to their temperature reaction, symptoms displayed and treatment received. Scores were totalled throughout the duration of the experiment.

	Indicator		Score
1.	**Before challenge:**		
	Record body temperature for each animal for 10 days prior to challengeDetermine average temperature for each animal		
2.	**After challenge:**		
	Temperature:	for every 1°C above the average temp for every 1°C below the average temp	+1 -1
	Symptoms:	Loss of appetite, heavy breathing, hanging head, stiff gait, depression, exaggerated blinking and chewing movements with anorexia	+5
		Hyperesthesia, lacrimation and convulsions	+10
	Treatment:	i/m treatment i/v treatment	+10 +20
	Death / euthanasia:		+20

In vitro *attenuation of* E. ruminantium *(Welgevonden)*

The organism was continuously propagated in a continuous canine macrophage-monocyte cell line (DH82) as described elsewhere (Zweygarth and Josemans 2001) through more than 125 passages over a period of more than one year.

Immunization of animals with attenuated* E. ruminantium *culture material

Three-day cultures of *E. ruminantium* in DH82 cells were used. The cells were resuspended in the culture medium and inoculated intravenously into six to eight week old Balb/C mice (0.2 ml per mouse at a range of dilutions) or six to eight month old Merino sheep (2.0 ml per sheep without any dilution). The body temperatures of the sheep were monitored daily after inoculation.

The indirect fluorescent antibody (IFA) test for anti-*E. ruminantium *antibody

The IFA test was performed on sheep which had received immunizing inoculations of attenuated *E. ruminantium* (Welgevonden) culture material, using virulent *E. ruminantium* (Welgevonden) endothelial cell culture material as antigen (du Plessis *et al.* 1993).

Cloning of selected* E. ruminantium *genes

Clone 1H12, a stable clone isolated from an *E. ruminantium* (Welgevonden) cosmid library (Brayton *et al.* 1997b), was subcloned and sequenced (Collins *et al.* 1998). Open reading frames (ORFs) were identified and amplified from genomic *E. ruminantium* DNA by PCR using primers designed to amplify the complete sequence, including predicted start and stop codons. The amplicons were cloned into the genetic immunization vector plasmid pCMViUBs (Sykes and Johnston 1999) and propagated in *E. coli* bacterial cells under ampicillin resistance. ORFs in this vector are expressed in mammalian cells, under the control of the CMV major immediate-early promoter, as fusion products with ubiquitin, which is designed to enhance cytotoxic lymphocyte responses. The sequences of all constructs were checked and plasmid DNA prepared for immunizations using Nucleobond[7] ion exchange cartridges (Machery-Nagel).

Genetic immunization of animals with selected* E. ruminantium *genes cloned in plasmid pCMViUBs

Each animal was inoculated with plasmid DNA by two routes, intramuscular injection and intradermal administration. The latter method employed tungsten or gold microbeads, coated with DNA, which were delivered into the skin of the ear by means of a gene gun (the OpgunJ) (Brayton *et al.* 1997a). Six to eight week old C57BL/6J mice were given a total of 25 µg of plasmid DNA i.m. and 1µg of plasmid DNA by gene gun. Six to eight month old Merino sheep, tested for freedom from *E. ruminantium* infection by PCR as described above, were given 200µg of plasmid DNA by i.m. inoculation and 20 µg of plasmid DNA by gene gun delivery. All animals received three immunizations given at three week intervals.

Lymphocyte proliferation assays

Peripheral blood mononuclear cell (PBMC) lymphocyte proliferation assays were performed on genetically immunized sheep, once before immunization and then weekly after the third inoculation until the time of challenge (Brown *et al.* 1995). Assays were carried out in triplicate in 96-well plates and each well contained a single cell suspension of 2 x 10^5 PBMCs in a total volume of 100 µl. The cells were incubated with positive antigen (*E. ruminantium* infected bovine endothelial cells, 1 µg/well) or negative antigen (uninfected bovine endothelial cells, 1 µg/well). In addition negative control wells contained PBMC without antigen and positive control wells contained PBMCs stimulated with Concanavalin A (2 units/well, Sigma). The cultures were incubated for 72, 96 or 120 h and pulsed with 1 µCi/well of [^{3}H]thymidine (Amersham) for the last 6h of the incubation period. Cells were harvested and the [^{3}H]thymidine uptake was estimated. Results are expressed as Stimulation Index (SI) (counts per minute (cpm) of positive antigen divided by cpm of negative antigen) averaged from triplicate wells± the standard deviation. A SI greater than 2 was considered significant.

Natural tick challenge of animals with E. ruminantium

Sheep were transported to the farm Springbokfontein, in the Northern Province, in the heartwater-endemic area of South Africa. Here they received a natural challenge from *Amblyomma hebraeum* ticks.

Results

Cross immunity trials in sheep

The Welgevonden stock of *E. ruminantium* provided complete cross-protection in sheep against challenge with each of the other stocks used in the experiment. Only two other stocks provided solid protection against any heterologous challenge, Ball 3 against Kwanyanga, and Mara 87/7 against Ball 3. Inverse protection was not observed in these two instances, Kwanyanga and Ball 3 provided only partial protection against Ball 3 and Mara 87/7, respectively. The results are summarised in Table 3.

Table 3 Results of cross immunity experiments in sheep using six virulent isolates of *E. ruminantium*. Animals were immunized by infection and treatment and challenged with 10 LD_{50} of virulent *E. ruminantium* (Welgevonden).

Stock to which immune	**Challenge stock**					
	Welgevonden	Ball3	Gardel	Mara 87/7	Kwanyanga	Blaauwkrans
Welgevonden	+	+	+	+	+	+
Ball3	-	+	1/4	3/4	+	3/4
Gardel	-	3/4	+	-	2/4	2/4
Mara 87/7	-	+	1/4	+	3/4	2/4
Kwanyanga	-	2/4	3/4	-	+	1/4
Blaauwkrans	-	2/4	1/4	3/4	1/4	+

\+ complete cross protection = no animals treated or died
\- no cross protection = all animals treated or died
x/y partial cross protection = x of y animals survived without treatment

In vitro *attenuation of* E. ruminantium *(Welgevonden)*

Cultures of DH82 cells were readily infected provided that the culture medium was supplemented with cycloheximide (Zweygarth and Josemans 2001). Cultures were split at regular three-day intervals and infection rates ranged between 60% and 95%. Cultures were continuously propagated through more than 125 passages over a period of more than one year and 0.2 ml aliquots of undiluted culture suspension were tested in mice for virulence at 25, 50 and 100 passages (Table 4). The culture had apparently lost all virulence for mice between passages 25 and 50.

Table 4 Reaction of mice to inoculation with of *E. ruminantium* (Welgevonden) at various stages during continuous passage in DH82 cell culture.

Passages	**Survival after challenge**
25	0/5
50	5/5
100	5/5

Immunizing ability of attenuated E. ruminantium *(Welgevonden) in mice*

Mice were inoculated with 0.2 ml of a range of dilutions of attenuated culture material at passage 56 and were needle challenged after 30 days with 10 LD_{50} of virulent Welgevonden. Animals receiving dilutions of 10^{-4} or less were completely immune to challenge (Table 5).

Table 5 Reaction of mice immunized with attenuated *E. ruminantium* (Welgevonden) to challenge with $10LD_{50}$ of virulent *E. ruminantium* (Welgevonden).

Dilution	Survival after challenge
10^{-3}	10/10
10^{-4}	10/10
10^{-5}	5/10
Unimmunized	0/10

Immunizing ability of attenuated* E. ruminantium *(Welgevonden) in sheep

Six sheep were inoculated with 2 ml of attenuated culture material, three at passage 56 and three at passage 111, and anti-*E. ruminantium* antibodies were estimated weekly. Needle challenge with 10 LD_{50} of virulent Welgevonden was given 30 days after immunization, and temperatures were monitored daily throughout the experiment. All the sheep showed a temperature reaction after immunization and mounted an antibody response. Three of the six animals also showed a temperature reaction after challenge, but they continued to eat and react normally, and all six recovered without intervention (Table 6).

Table 6 Reaction of sheep to immunisation with attenuated *E. ruminantium* (Welgevonden) and survival after challenge with $10LD_{50}$ of virulent *E. ruminantium* (Welgevonden).

Culture passage	Animal	Days to temperature	Pre-challenge IFA antibody titre	Challenge reaction
56	5	5 (42.0°C)	1/1280	41.3°C
56	14	7 (41.9°C)	1/640	Nil
56	35	7 (41.3°C)	1/160	Nil
111	77	2 (41.1°C)	1/1280	40.5°C
111	211	2 (42.0°C)	1/2560	Nil
111	277	5 (42.0°C)	1/1280	40.9°C

Sequence analysis of cosmid clone 1H12

The sequence of *E. ruminantium* cosmid clone 1H12 was 20276 bp in length and it contained 11 ORFs of predicted lengths greater than 90 amino acids. There were also four regions containing long repeats, ranging from 165-220 bp (Fig. 1).

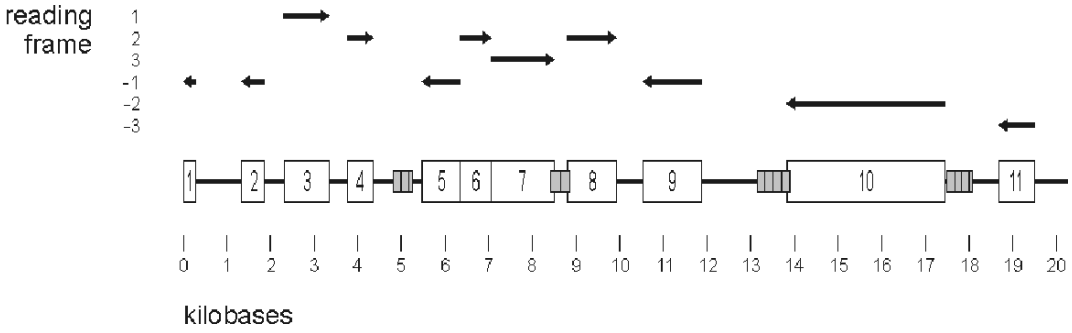

Figure 1 Schematic representation of the open reading frames (ORFs) coding for more than 90 amino acids identified in the sequence of the cosmid clone, 1H12. Open boxes represent ORFs; the arrow above each ORF indicates its orientation. Long tandem repeats are represented by grey boxes.

Screening of 1H12 ORFs for protection in mice

Eleven 1H12 ORFs cloned into the genetic immunization plasmid pCMViUBs were tested in C57BL/6J mice for their ability to induce protective immunity to lethal *E. ruminantium* (Welgevonden) challenge (Table 7). In an initial trial those groups which received ORFs 3 and 6 showed significantly

improved (P<0.1) survival rates as compared to the controls. The next highest survival rates occurred in the ORFs 2 and 5 groups, but these were not significant (P>0.3). The small sample size, however, made it difficult to be certain that the true significance of any protective effect was being seen, hence a second trial was conducted in larger groups of mice with ORFs 2, 3, 5, and 6. In this case the survival rates of mice inoculated with ORFs 3 and 6 were lower than in the first experiment, but survival in all four groups was significantly higher (P<0.1) than that of the control group.

Table 7 Survival of C57BL/6J mice inoculated with 1H12 ORFs cloned in the DNA vaccine vector pCMViUBs and needle challenged with virulent *E. ruminantium* (Welgevonden).

Plasmid/clone used for immunization	Survival after challenge	% survival	Significance (*P* value)[1]
Experiment 1			
pCMViUB/ORF 1	3/8	37.5	0.5000
pCMViUB/ORF 2	4/8	50.0	0.3042
pCMViUB/ORF 3	6/8	75.0	0.0660
pCMViUB/ORF 4	3/8	37.5	0.5000
pCMViUB/ORF 5	4/8	50.0	0.3042
pCMViUB/ORF 6	7/8	87.5	0.0203
pCMViUB/ORF 7	2/8	25.0	0.7154
pCMViUB/ORF 8	3/8	37.5	0.5000
pCMViUB/ORF 9	2/8	25.0	0.7154
pCMViUB/ORF 10	0/10	0.0	0.2333
pCMViUB/ORF 11	0/8	0.0	0.2333
pCMViUB	2/8	25.0	control
Experiment 2			
pCMViUB/ORF 2	8/19	42.1	0.0015
pCMViUB/ORF 3	4/19	21.1	0.0525
pCMViUB/ORF 5	10/19	52.6	0.0002
pCMViUB/ORF 6	4/19	21.1	0.0525
pCMViUB	0/19	0.0	control

[1] Significance by Fisher's one tailed exact probability test, as compared to control group.

Protection of sheep immunized with selected 1H12 ORFs against homologous challenge

An equimolar cocktail of the four genetic immunization plasmids containing ORFs 2, 3, 5 and 6 was used to immunize five sheep. The experiment also included five sheep immunized with empty vector (negative controls) and five infected and treated sheep (positive controls). Lymphocyte proliferation assays became positive for 4/5 test sheep and 4/5 positive controls, and remained negative for 5/5 sheep immunised with empty vector. Animals were challenged five weeks after the final immunization with 10 LD_{50} of virulent *E. ruminantium* (Welgevonden). All the positive control animals survived without any reaction, while all the negative control sheep developed severe symptoms of heartwater and were treated *in extremis*, despite which one died. All five sheep inoculated with the ORF cocktail developed elevated temperatures, and while their reaction indices were higher than those of the positive controls they were lower than those of the negative controls. All the genetically immunized animals continued to eat and react normally, and all recovered without intervention. The results are summarised in Table 8.

Table 8 Survival of sheep inoculated with a cocktail of four 1H12 ORFs cloned into the genetic vaccine vector, pCMViUBs, after challenge with 10LD_{50} of *E. ruminantium* (Welgevonden) Sheep which developed severe symptoms of heartwater were treated and were considered not to have survived challenge.

Group	Method of inoculation	Sheep number	Days to temperature above 40°C	Highest temperature reached	Reaction Index score	Survival (S) or treatment on day(s) shown
Positive control	Infection and treatment	67	10	40.5	4.9	S
		119	12	40.3	1.6	S
		161	11	40.3	3.0	S
		239	9	41.1	20.9	S
		251	10	40.6	8.7	S
Exper-imental group	DNA vaccination with 1H12 cocktail	131	9	41.6	50.0	S
		185	9	41.6	38.4	S
		194	9	41.3	39.1	S
		217	10	41.6	45.5	S
		263	9	41.7	40.8	S
Negative control	DNA vaccination with empty vector	203	11	42.0	63.9	13
		216	9	42.0	97.1	11, 13
		242	8	42.0	139.3	12, 13, 14
		255	9	42.0	125.3	11, 13, 14
		282	10	41.6	131.4	12, 13, 14, died day 14

Protection of sheep immunized with selected 1H12 ORFs against homologous and heterologous challenge

A further 30 sheep were immunized with the same cocktail of four ORFs, and this experiment included 25 negative controls and five positive controls, as described above, plus five naive (uninoculated) controls. The positive control sheep, five of the sheep immunized with the cocktail, and five of the negative control sheep, were challenged with 10 LD_{50} of the Welgevonden isolate. The remaining 20 negative control sheep and 20 immunized sheep were each challenged in groups of four with 10 LD_{50} of the five *E. ruminantium* isolates used in the initial cross immunity experiment: Blaauwkrans, Ball 3, Gardel, Kwanyanga and Mara 87/7. All sheep in the positive control group survived challenge without any reaction. All sheep immunized with the 1H12 cocktail survived challenge, irrespective of the challenge isolate, while all negative control sheep succumbed to infection, again irrespective of the challenge isolate (Table 9). Some of the immunized sheep reacted to the challenge and developed elevated temperatures, and others did not, but no immunized animal became severely ill.

The remaining five genetically immunized sheep, four naive controls, and all 25 genetically immunized sheep which had survived needle challenge, were exposed to natural heartwater challenge by *Amblyomma hebraeum* ticks on Springbokfontein farm. All the naive sheep died. Four of the five genetically immunized sheep which had not previously been needle challenged also died (Table 9). Among the DNA immunized and needle challenged sheep it was observed that reaction to the previous challenge, as shown by the reaction index, had a major effect on survival during the field challenge. All 10 of the sheep which died had not reacted to the needle challenge, while 9/9 which had reacted to the previous need challenge survived. Only 6/16 sheep which had not reacted to the needle challenge survived the field challenge (Figure 2). Post-mortem examinations confirmed that all deaths were due to heartwater.

Table 9 Survival of sheep inoculated with 1H12 ORFs cloned in the DNA vaccine vector pCMViUB and exposed to lethal homologous and heterologous heartwater challenges.

Method of inoculation	**Needle challenge 10 LD_{50} *E. ruminantium***			**Field challenge**
	Challenge stock	**Reaction**	**Survival**	**Survival**
Infected and treated	Welgevonden	0/5	5/5	nd
1H12 cocktail	Welgevonden	4/5	5/5	4/5
Empty vector	Welgevonden	5/5	0/5	-
1H12 cocktail	Ball3	2/4	4/4	3/4
Empty vector	Ball3	4/4	0/4	-
1H12 cocktail	Blaauwkrans	1/4	4/4	1/4
Empty vector	Blaauwkrans	4/4	0/4	-
1H12 cocktail	Gardel	0/4	4/4	2/4
Empty vector	Gardel	4/4	0/4	-
1H12 cocktail	Kwanyanga	2/4	4/4	3/4
Empty vector	Kwanyanga	4/4	0/4	-
1H12 cocktail	Mara 87/7	0/4	4/4	2/4
Empty vector	Mara 87/7	4/4	0/4	-
1H12 cocktail	nd	-	-	1/5
None (naive)	nd	-	-	0/4

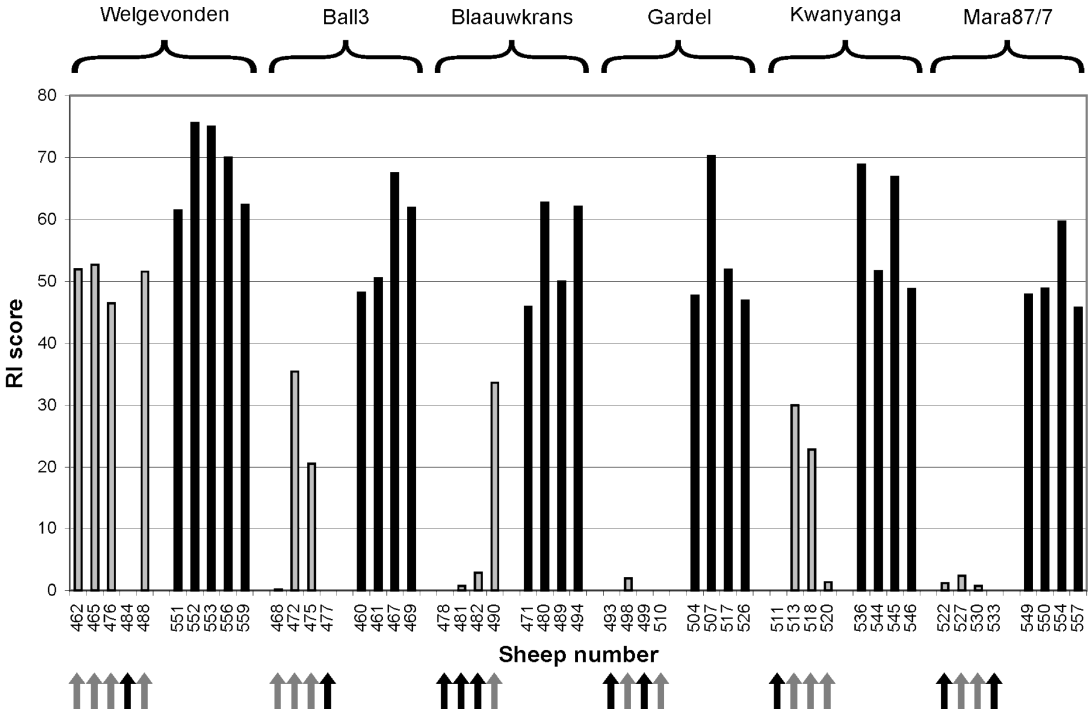

Figure 2 Reaction indices of sheep inoculated with the 1H12 ORF cocktail (grey) and negative control sheep (black). Sheep were needle challenged with 10 LD_{50} of various *E. ruminantium* isolates (indicated above the chart). Survivors (grey arrows) were subjected to a natural tick challenge on Springbokfontein farm. Those which died are indicated by black arrows.

Discussion

The only commercially available immunization procedure for protection against heartwater is in use in southern Africa. It involves infecting animals with virulent *E. ruminantium* organisms of the Ball 3 isolate, cryopreserved in sheep blood (Bezuidenhout 1989), after which the animals' body temperatures are monitored and antibiotic treatment administered at the proper time. Both production and use are expensive, and the frozen blood must be stored and transported at below –40°C, and which makes it particularly inappropriate for use in rural areas, especially in Africa. The duration of immunity is uncertain, and because live organisms are involved the procedure cannot be used in non-endemic areas. The Ball 3 stock does not protect against all isolates which circulate in the field (du Plessis *et al.* 1989; Jongejan *et al.* 1988) but it is used because it produces a temperature rise several days before serious clinical disease occurs, making it relatively easy to decide when to treat. More virulent isolates may provide better cross-protection against other stocks, but can cause death very shortly after a rapid temperature rise and cannot be used for infection and treatment. The search for an improved vaccine has addressed attenuated, inactivated and nucleic acid vaccines, but success has so far been limited.

All of the older information in the literature which describes cross protection between *E. ruminantium* isolates which had not been genetically characterised and challenge material which had not been quantitated (du Plessis *et al.* 1989; Jongejan *et al.* 1988; Jongejan *et al.* 1991). One of the stocks previously used has been demonstrated recently to be genetically heterogeneous (Zweygarth *et al.* 2002) and we felt the need to perform some new cross immunity trials using genetically characterized isolates and a known titred challenge (Brayton *et al.* 2003). The stocks we chose had each been shown to have a unique sequence for the single copy (Brayton *et al.* 1997b) *map*1 gene (Allsopp *et al.* 2001) and the only isolate which provided complete protection against subsequent challenge with all the others was the Welgevonden isolate, the type specimen of *E. ruminantium* comb. nov. We now use this isolate for all our vaccine development work.

The attenuated Senegal isolate of *E. ruminantium* provides immunity against homologous challenge (Jongejan 1991), but not to heterologous challenge (Gueye *et al.* 1994), and other *E. ruminantium* stocks, including Welgevonden, have not previously been successfully attenuated (Jongejan 1991). The present attenuation of this virulent isolate by continuous culture in DH82 cells represents a significant advance, especially as it appears to protect both mice and sheep against homologous challenge after a single immunization. Much more work needs to be done before we could advocate the use of this attenuated stock for immunization. Its spectrum of cross-protection has to be examined, and it must be induced to grow in a cell line other then the cancerous line in which it has been attenuated. If these issues were successfully resolved it would still remain a live vaccine, with all the disadvantages which that implies. It would, however, undoubtedly be cheaper to produce than the present infective blood vaccine and could well be used as an alternative in the markets where that vaccine is currently sold.

Inactivated *E. ruminantium* organisms have been shown to induce immunity against homologous challenge (Martinez *et al.* 1994; Mahan *et al*. 1995), which suggests that the development of a subunit vaccine is feasible. Until now all heartwater recombinant vaccine development work has been performed using the mouse model, and although protection has been demonstrated (Nyika *et al*. 1998; Nyika *et al*. 2001; Barbet *et al*. 2001) the results have been very variable. When we found that four 1H12 ORFs, cloned in the DNA vaccine vector pCMViUBs, provided some protection in the mouse model system we too observed that it was difficult to obtain reproducible results. We already knew that one origin of variability was the difficulty of obtaining an accurate challenge dose, although the use of quantitated challenge material went a long way towards reducing this source of error (Brayton *et al*. 2003).

We therefore decided to use a cocktail of all four potentially protective ORFs as a genetic vaccine in sheep, to see whether the protection was real. All of the immunized sheep survived a lethal needle challenge with *E. ruminantium* (Welgevonden), a result which we subsequently were able to reproduce in three further experiments. The same DNA vaccine cocktail also provided protection in sheep against a heterologous challenge with each of the isolates we had used in the cross immunity trials. The reproducibility and the level of the protection we obtained against needle challenge was highly encouraging and in marked contrast to the unpredictable results previously obtained in mice.

Since we had never tried the cocktail in mice we subsequently immunized 10 mice with the cocktail and only 3 survived (results not shown). This was good evidence that results obtained in the mouse model were unlikely to correlate with those obtained in at least one ruminant species which would be a target for the vaccine. This observation, coupled with the lack of reproducibility we had observed between experiments, has led us to abandon the use of the mouse model for evaluating recombinant vaccines for heartwater.

Despite the success of the experimental DNA vaccine against both homologous and heterologous needle challenge it was not, on its own, sufficient to protect sheep against a virulent heterologous tick challenge. It is possible that the Welgevonden derived vaccine is not cross protective against the stock(s) present in Springbokfontein, but we do not feel that this is likely. *E. ruminantium* isolates from Springbokfontein have been shown to be genetically very similar to Welgevonden itself, and to Mara 87/7, against which Welgevonden gives 100% cross protection (H. van Heerden, unpublished results). It is more likely that needle challenge with infected blood is fundamentally different from natural tick challenge. The saliva of *Ixodes scapularis* and *Rhipicephalus sanguineus* ticks has been shown to contain molecules that can modulate host immune responses (Wikel 1999; Ferreira and Silva 1998; Zeidner *et al*. 1997), and although tick immunomodulation has not been demonstrated for *Amblyomma* ticks it is highly likely that it occurs. There may also be phenotypic differences between *E. ruminantium* organisms released into mammalian blood and *E. ruminantium* organisms as they are extruded from tick salivary gland cells. It would indeed be surprising if the latter were not >primed= for mammalian infection, perhaps by way of expressing a particular surface receptor, or by having by up regulation of a specific metabolic pathway.

Those sheep which had been immunized with the DNA vaccine, and which subsequently had a temperature reaction upon needle challenge, survived the tick challenge. It has long been known, from experience with the infection and treatment immunization procedure, that animals which do not react do not become immune (du Plessis and Malan 1987), and effectively what we had done was to boost the immunity engendered by the DNA vaccine. DNA immunisation, followed by a recombinant live virus boost, has been effective for inducing protective levels of $CD8^+$ T cells active against the malaria parasite (Schneider *et al*. 1999; Hill *et al*. 2000). Since immunity to *E. ruminantium* is also T cell mediated (Martinez 1997; Mwangi *et al*. 1998) we intend to explore this strategy to enhance the levels of protection generated by our nucleic acid cocktail vaccine.

In summary, we have made considerable progress in the development of new vaccines for heartwater, both by attenuating a stock having wide cross protection abilities, and by demonstrating for the first time that ruminants can be protected by a cocktail of *E. ruminantium* genes presented as a genetic vaccine.

Acknowledgements

Parts of this research were sponsored by the Agricultural Research Council of South Africa, by the Department of Arts, Culture, Science and Technology of South Africa, and by the European Union under Contract No. ICA4-CT- 2000 - 30026.

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Chapter 4.2

An attenuated *Ehrlichia ruminantium* (Welgevonden stock) vaccine protects small ruminants against virulent heartwater challenge

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Vaccine **23:** 1695-1702 (2005)

Abstract

Heartwater is a tick-borne disease of ruminants caused by the intracellular rickettsia *Ehrlichia ruminantium*. The only commercially available immunization procedure involves infecting animals with cryopreserved sheep blood containing virulent *E. ruminantium* organisms, followed by treatment with tetracyclines when fever develops. The virulent Welgevonden stock of *E. ruminantium* was attenuated by continuous propagation of the organisms in a canine macrophage-monocyte cell line (DH82), followed by re-adaptation to grow in a bovine endothelial cell line (BA 886). The material used for the present experiments consisted of the attenuated stock between passages 43 and 64 after re-adaptation. When inoculated into sheep or goats the attenuated organisms did not produce disease, and the only symptom observed was a rise in body temperature in most, but not all, animals. All sheep injected with 2 ml of culture suspension were subsequently found to be fully protected against a lethal needle challenge with the virulent homologous stock or with one of four different heterologous stocks (Ball 3, Gardel, Mara 87/7, Blaauwkrans). Titrations of elementary body suspensions showed that 2 ml of a 1:10,000 dilution of culture suspension injected into sheep or goats was still sufficient to trigger an immune response which resisted a lethal needle challenge with the virulent Welgevonden stock. Adult *Amblyomma hebraeum* ticks, fed as nymphs on sheep immunized with DH82-derived organisms of passage 111, were able to transmit the attenuated stock to a naïve sheep which was found to be protected against a subsequent lethal homologous needle challenge.

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1. Introduction

Heartwater is an infectious, non-contagious, tick-borne disease of both wild and domestic ruminants caused by the rickettsia *Ehrlichia ruminantium*. The disease is prevalent in sub-Saharan Africa (Uilenberg 1983) and the Caribbean (Perreau *et al.* 1980, Birnie *et al.* 1984), from where it poses a threat to livestock in North and South America (Barré *et al.* 1987).

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 (Alexander 1931). 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 stock and are subsequently treated with tetracyclines to prevent serious disease (Bezuidenhout 1989). Although this procedure is expensive and dangerous it has been the only commercially available "vaccine" for more than 50 years. Unfortunately, the spectrum of protection of the Ball 3 blood vaccine stock against other *E. ruminantium* stocks is limited. In contrast, the Welgevonden stock has been shown to stimulate protective immunity against several virulent South African stocks and should therefore be more suitable for immunization purposes than the Ball 3 stock (Collins *et al.* 2003). However, its high virulence has precluded it from being used for immunization (Du Plessis *et al.* 1989). Attempts to develop an attenuated vaccine were first reported using a Senegalese stock of *E. ruminantium*, which became attenuated after a limited number of passages *in vitro* (Jongejan 1991). This attenuated stock, while providing immunity to homologous challenge (Jongejan 1991), did not provide efficient cross-protection against other virulent stocks (Jongejan *et al.* 1993). Another stock, from Guadeloupe, has also been attenuated, although more than 200 passages were required (Martinez 1997), but efforts to attenuate other stocks have proved unsuccessful until recently. We successfully attenuated the virulent Welgevonden stock of *E. ruminantium* by continuous passage in a canine macrophage-monocyte cell line (Zweygarth and Josemans 2001). After more than 50 passages the cultured organisms did not produce disease when inoculated into mice or sheep, and the animals were immune to subsequent lethal homologous needle challenge (Collins *et al.* 2003). Here we report the successful use of the attenuated Welgevonden stock to vaccinate sheep and goats under controlled experimental conditions.

2. Materials and methods

2.1. Stocks of E. ruminantium

The Welgevonden stock was isolated from a male *Amblyomma hebraeum* tick which was collected on the farm Welgevonden in the Northern Transvaal, South Africa (Du Plessis 1985). Another four stocks of *E. ruminantium* were used for cross protection experiments in sheep. Three of these stocks were from South Africa: the Ball 3 stock (Haig 1952), the Mara 87/7 stock (Du Plessis *et al.* 1989) and the Blaauwkrans stock isolated from an eland near Port Elizabeth in 1996. In addition we used the Gardel stock, isolated in Guadeloupe (Uilenberg *et al.* 1985).

2.2 Propagation of E. ruminantium

The medium used for all cultures, infected and uninfected, consisted of Dulbecco's modified Eagle's medium nutrient mixture Ham F-12 (DME/F-12) (Sigma, St. Louis, MO, USA; catalogue no. D 0547) containing 15 mM HEPES and 1.2 g l^{-1} sodium bicarbonate. It was further supplemented with 10 % (v/v) heat-inactivated foetal bovine serum, 2 mM L-glutamine, 100 IU ml^{-1} penicillin and 100 µg ml^{-1} streptomycin.

Propagation of the Welgevonden stock in DH82 cells, which were derived from a dog suffering from malignant histiocytosis (Wellman *et al.* 1988), has been described in more detail elsewhere (Zweygarth and Josemans 2001). Briefly, infected DH82 cell cultures were harvested by resuspending the cells into the medium, which contained 1 µg ml^{-1} cycloheximide (Sigma). The cell cultures were subcultured at a ratio of 1:2 into new culture flasks containing uninfected DH82 cells. After 24 h all of the medium was discarded and replaced with 5 ml of fresh medium containing 1 µg ml^{-1} cycloheximide. The cultures were incubated at 37 °C.

2.3. Re-infection of BA 886 cells by DH82-derived Welgevonden elementary bodies

The attenuated Welgevonden stock was propagated in DH82 cells through 61 passages and infected cell cultures were harvested by resuspending the cells into the medium. The cell suspension was centrifuged (800 x g; 10 min; room temperature) and 2.5 ml of supernatant containing elementary bodies was distributed into a culture flask containing bovine endothelial cells (BA 886) (Yunker *et al.* 1988) and 2.5 ml of fresh medium. Newly infected BA 886 cultures were harvested for subcultivation by scraping off the cell monolayer into the medium. Cells were dispersed by pipetting the suspension up and down and the suspension was then transferred at various ratios (between 1:2 and 1:5) on to new endothelial cell monolayers. After 24 h all of the medium was discarded and replaced with 5 ml of fresh medium. Subcultures were performed every 3 days. The culture medium used contained 0.25 µg ml^{-1} cycloheximide.

2.4. Experimental animals

We used a total of 63 six to eight month old Merino sheep and 25 Boer goats. The animals were purchased from the farm where they were born and raised, in Warden in the Free State (South Africa), a heartwater- and *Amblyomma*-free area (Du Plessis *et al.* 1994). DNA samples obtained from the blood of all animals used in these experiments were initially examined by PCR (Mahan *et al.* 1992) and probed with the *E. ruminantium*-specific pCS20 probe (Waghela *et al.* 1991), which confirmed that they were all negative for *E. ruminantium.* This test is the method of choice for the detection of *E. ruminantium* infection (Simbi *et al.* 2003). All animals were inspected daily for clinical symptoms of heartwater, and the rectal temperature was recorded daily. Rectal temperatures of 40°C in goats and 40.5°C in sheep were considered to constitute fever. Control animals were included in each experiment, and in compliance with the institute's animal ethics regulations, oxytetracycline was administered to any animal showing severe clinical symptoms, such as depression, laboured respiration, anorexia, recumbency or nervous signs (in-coordination).

2.5. Antibody analysis by an indirect fluorescent antibody test (IFAT)

Serum samples of all animals were subjected to a slightly modified IFAT which, as described elsewhere (Semu *et al.* 1992) detects anti-*Ehrlichia* antibodies. Serum was collected on Day 0, before immunization, and on the respective day before challenge (Tables 1-3). Two-fold dilutions of the test sera were applied to the wells of antigen slides and were incubated at 37°C for 30 min. The second antibody was rabbit anti sheep IgG or anti goat IgG, labelled with fluorescein isothiocyanate (Sigma), and diluted 1:80 in 0.1% Evans blue solution. Slides were mounted in 50% glycerol in phosphate buffered saline and the fluorescence was evaluated under a Leitz Orthoplan fluorescence microscope. Positive and negative control sera were included in each test. Titers higher than 1:40 were considered *Ehrlichia*-positive. Although all animals were free of heartwater, as determined by the pCS20 test, some were serologically positive by the IFAT owing to cross-reactions with other unknown *Ehrlichia* species (Mahan *et al.* 1993, Mahan *et al.* 1998, Kakono *et al.* 2003).

2.6. Cross protection trials in sheep

The attenuated Welgevonden stock was passaged 43 times in BA 886 cells before it was used for cross-protection trials in sheep. Groups of 5 sheep were injected intravenously (i.v.) with 2 ml of a suspension of culture-derived elementary bodies of to determine the immunogenicity of the stock and its ability to protect the animals against a virulent heartwater challenge. The immunizing dose consisted of elementary bodies obtained from heavily infected BA 886 cells with a more than 90% infection rate. All vaccinated animals were challenged on day 29 post injection with a dose of $10xLD_{50}$ of a homologous or heterologous *E. ruminantium* stock (Welgevonden, Ball 3, Mara 87/7, Blaauwkrans or Gardel). The virulent challenge material, which was used in all experiments, was prepared as described by Brayton *et al.* (2003). Briefly, aliquots of infective blood had been cryopreserved previously. After thawing test aliquots were serially diluted in sucrose-potassium-glutamate (SPG) (Bovarnick *et al.* 1950) and inoculated i.v. into susceptible sheep. From the proportion of the sheep dying in each group the LD_{50} of the cryopreserved material was determined by the method of Reed and Muench (1938).

2.7. Titration of the immunizing doses in sheep

A titration of immunizing doses of the attenuated Welgevonden stock after 60 passages in BA 886 cells was performed in sheep. A suspension of elementary bodies was serially diluted (10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}) and groups of five sheep were injected with 2 ml of each dilution. Aliquots of each immunizing dose were quantitated as follows: 5 ml of each dilution was used to infect a cell layer of BA 886 cells in a 25 cm^2 cell culture flask. After two days of incubation at 37°C the cell culture medium was removed, and the BA 886 cells were allowed to dry before being fixed with methanol and stained with eosin-methylene blue (Kyro-Quick stain; Kyron Laboratories, Benrose, South Africa). Infected and uninfected cells were counted and the percentage of infected cells was calculated. The estimated number of infective particles in the immunizing dose was then calculated from the percentage of infected cells and the average number of BA 886 cells in uninfected control cultures ($3x10^6$ cells/$25cm^2$ flask) according to the following formula: A = B x C x 2 (ml) / 100 x 5 (ml), where A equals the number of infective particles in an immunizing dose (2 ml), B the percentage of infected cells and C the average number of BA 886 cells in a culture flask. After 37 days the sheep were challenged with a dose of $10xLD_{50}$ of the virulent Welgevonden stock as described under *2.6* above. Five uninfected sheep were used as challenge controls.

2.8. Titration of the immunizing doses in goats

A titration of immunizing doses of the attenuated Welgevonden stock after 64 passages in BA 886 cells was performed in goats. A suspension of elementary bodies was serially diluted (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}) and groups of five Boer goats were injected with 2 ml of each dilution. An aliquot of each dose was evaluated as described above. After 30 days the goats were challenged with a dose of $10xLD_{50}$ of the virulent Welgevonden stock as described above. Five uninfected goats were used as challenge controls.

2.9. Transmissibility by ticks

Amblyomma hebraeum ticks, originally obtained from Pienaarsrivier (Northern Province, South Africa), were kept at the Institute's acaridarium and reared free from infection of *E. ruminantium*. Larvae were fed on rabbits, while nymphs and adults were fed on uninfected sheep, and engorged ticks were allowed to moult at 27^0C and 80% relative humidity.

Two sheep (numbers 211 and 277) were injected with 2 ml of a culture suspension of the attenuated Welgevonden stock grown in DH82 cells at passage 111. Eight days later, while the fever was declining, 100 *A. hebraeum* nymphs were allowed to feed on each sheep. Engorged nymphs were collected 6 days later and allowed to moult to adults under the maintenance conditions described above. Two months later 5 male ticks were selected and applied to an uninfected sheep (number 82) to induce the production of tick attraction, aggregation and attachment pheromones (Norval *et al.* 1992). This facilitated the attachment of female ticks, 10 of which were applied two days later. The animal was challenged after 30 days with $10xLD_{50}$ of the virulent Welgevonden stock. The IFAT for anti-*Ehrlichia* antibodies was performed on sheep 82 on day 0, before ticks were put applied, and on day 30, before challenge.

3. Results

3.1. Cross protection experiments in sheep

The attenuated Welgevonden stock at passage 43 in BA 886 cells was used to inoculate 25 sheep. All the animals developed fever between 2 and 7 days after immunization, with a duration of 1 to 6 days (Table 1). In three animals an initial fever period was interrupted by a fever-free period, followed by a second increase in body temperature. All the vaccinated sheep became positive by the IFAT for anti-*Ehrlichia* antibodies, with titers ranging from 1:640 to at least 1:2560 (highest dilution indicated in Table 1). After challenge with virulent Welgevonden, Gardel, Mara 87/7 or Blaauwkrans stocks no temperature reactions were observed. In contrast, 2 out of 5 animals challenged with the Ball 3 stock reacted with one-day temperature rises to 40.5°C and 41.3°C respectively. All challenged control animals developed severe clinical symptoms, as outlined above, and required treatment with oxytetracycline. The results are summarized in Table 1.

3.2. Titration of the immunizing doses in sheep

The immunizing material which was titrated in cell culture revealed that the suspension of the highest dilution (10^{-4}) infected 1.5% of the BA 886 cells, equivalent to $1.8x10^4$ infective particles per 2 ml dose. Dilutions of 10^{-3}, 10^{-2} and 10^{-1} infected 7%, 68% and 99% of the cells, corresponding to $8.4x10^4$, $8.2x10^5$ and $1.2x10^6$ infective particles per dose, respectively.

Elementary body suspensions, serially diluted from 10^0 to 10^{-4}, were each used to immunize groups of 5 sheep (Table 2). In the groups immunized with 10^0 and 10^{-1} dilutions all the animals reacted after 5 to 9 days with a fever for 1 to 3 days. In contrast, in the groups where higher dilutions (10^{-2}, 10^{-3} or 10^{-4}) were used, fewer animals (1, 3 and 1) reacted with an elevated temperature. After immunization all sheep developed IFAT titers ranging from 1:320 to 1:2560. After challenge, only 2 animals reacted with elevated temperatures for 2 or 3 days. In contrast, all challenged control animals developed severe clinical symptoms and required treatment with oxytetracycline. Details are summarized in Table 2.

3.3. Titration of the immunizing doses in goats

The immunizing material which was titrated in cell culture revealed that the suspension of the highest dilution (10^{-4}) was able to infect 0.5% of the BA 886 cells, equivalent to $6.0x10^3$ infective particles per 2 ml dose. Dilutions of 10^{-3}, 10^{-2} and 10^{-1} infected 2%, 20.5% and 82.5% of the cells and were equivalent to $2.4x10^4$, $2.5x10^5$ and $9.9x10^5$ infective particles per dose, respectively.

Elementary body suspensions diluted from 10^{-1} to 10^{-4} were used to immunize groups of 5 Boer goats. Incubation periods varied between 8 and 15 days, with a fever persisting for up to 3 days. Seroconversion was observed in all goats, and the titers ranged from 1:320 to 1:2560. After challenge, in those groups which had received the higher immunization doses (dilutions of 10^{-1} and 10^{-2}), 3 animals reacted with fever for one day only, and one animal for 2 days. In the groups which received the lower immunizing doses (dilutions of 10^{-3} and 10^{-4}), 2 animals of each group reacted with fever for one day after the challenge. Animal 154 died on day 4 after inoculation as a result of a fibrino-purulent peritonitis, unrelated to the vaccination. All the other animals survived the experiment, showing no clinical signs other than a transient rise in body temperature, whereas all the challenged control animals developed severe clinical symptoms and were treated with oxytetracycline. The results are summarized in Table 3.

3.4. Transmissibility by ticks

Adult *A. hebraeum* ticks, fed as nymphs on a sheep injected with the attenuated Welgevonden stock, were allowed to feed on animal 82, which was serologically negative at the time. Seventeen days after attachment of the ticks, the animal showed a temperature reaction for 5 days and a maximum temperature of 41.6°C was recorded. On day 30 after tick attachment the animal had an IFAT titer of 1:1260 and was challenged with a dose of $10xLD_{50}$ of the virulent Welgevonden stock. The animal survived the virulent challenge without showing any clinical reaction.

4. Discussion

Attenuation of virulent organisms can occur spontaneously, but not frequently. To increase the likelihood of attenuation organisms can be exposed to mutagenic substances, they can be subjected to "unnatural conditions" such as an elevated temperature, or they can be passaged in cells not derived from the natural host, as in the present experiments. The virulent Welgevonden stock was serially passaged in dog-derived DH82 cells (Zweygarth and Josemans 2001), and it lost its virulence for mice between passages 25 and 50. Sheep inoculated with attenuated culture material at passages 56 and 111 survived a needle challenge with $10xLD_{50}$ of the virulent Welgevonden stock (Collins *et al.* 2003). The previous experiments were conducted with organisms derived from the transformed cell line DH82, which may be unsuitable for the production of a commercial vaccine. The attenuated Welgevonden stock was therefore re-adapted to the bovine endothelial cell culture line, BA 886, and used for the experiments described here from passage 43 up to passage 60. In the first experiment the only symptom observed in animals injected with 2 ml of BA 886-derived culture material was an increase in body temperature, indicating that virulence had not been re-established. Immunization with the attenuated Welgevonden stock

provided full protection against a homologous virulent needle challenge, showing that immunogenicity remained as high as with DH82-derived organisms.

It has been shown previously that infection and treatment with the virulent Welgevonden stock conferred immunity against a number of other *E. ruminantium* genotypes, such as Ball 3 and Mara 87/7 (Du Plessis *et al.* 1989) or Blaauwkrans and Gardel (Collins *et al.* 2003). After using the attenuated Welgevonden stock for immunization, and subsequently challenging the animals with one of these heterologous stocks, we found that all animals were protected. These results indicated that the same pattern of cross-protection is induced by the virulent and attenuated Welgevonden stocks. In contrast, all challenge control animals which received a $10xLD_{50}$ dose of any virulent stock developed severe disease symptoms and had to be treated with oxytetracycline.

Jongejan (1991) reported that the Senegal stock was attenuated after only 11 passages, or 229 days, in bovine endothelial cell cultures, whereas the Welgevonden stock did not attenuate after 17 passages, or 226 days, in culture. The Zimbabwean Crystal Springs stock of *E. ruminantium* did not attenuate, and was still fatal for sheep, after 60 and 192 passages *in vitro* (Mahan *et al.* 1995) and the authors concluded that the attenuation of *E. ruminantium* appeared to be strain-specific, and possibly limited to the Senegalese stock. Here, however, we have shown that, when suitable conditions are used (Zweygarth and Josemans 2001), one of the most virulent stocks of *E. ruminantium* in South Africa can be effectively attenuated. While 2 ml of a 10^{-4} dilution of the virulent Welgevonden stock produced potentially fatal disease in all animals, the same dose of the attenuated stock did not cause any symptoms which necessitated treatment (data not shown). The Gardel stock became attenuated after more than 200 passages in endothelial cell cultures (Martinez 1997). In contrast, the Welgevonden stock, which is in continuous culture in bovine endothelial cells in our laboratory, remained virulent at passage levels up to 231 with an average passage interval of 3 days (data not shown).

Titration of the immunizing doses in sheep showed that all animals, no matter which dilution was used, developed a solid immunity against a $10xLD_{50}$ needle challenge with the virulent Welgevonden stock. It is noteworthy that with higher dilutions of the immunizing material, ranging from 10^{-2} to 10^{-4}, only some of the animals reacted to the immunization with an elevated body temperature (Table 2), nevertheless all the animals survived the subsequent challenge. This is in contrast to observations made during infection and treatment experiments by Du Plessis and Malan (1987), in which animals which did not react did not become immune. These authors concluded that immunity elicited by an infective inoculum is dependent on the severity of the reaction that it causes, a finding which did not hold with our attenuated organisms, as was shown by two animals which did not react to the immunization. After challenge they developed elevated temperatures for 2 or 3 days, but no other symptoms were observed and treatment was not necessary.

The experiments in Boer goats revealed that all the animals reacted to the immunizing dose with an increase in body temperature, and all the animals resisted a subsequent needle challenge. In comparison to the results obtained with sheep, more of the goats reacted to the challenge dose with an increase in body temperature for one or two days. Despite this, no treatment was necessary after inoculation of the attenuated Welgevonden stock, and all the animals were subsequently protected from a virulent challenge. Angora goats are more highly susceptible to *E. ruminantium* than Boer goats and immunization against heartwater is difficult and hazardous. Du Plessis *et al.* (1983) found that few Angora goats survived when treated on the 2nd or 3rd day of the febrile reaction following an i.v. inoculation of virulent organisms. On the other hand, if the goats were treated on the first day of the reaction the survival rate was high, but the immunity to subsequent challenge was then poor. Immunization of Angora goats with attenuated Welgevonden organisms could offer an improved method of protection for these animals, although it remains to be elucidated how they would react to this vaccine.

In the present experiment it was shown that the attenuated Welgevonden stock can be transmitted through *A. hebraeum* ticks which were fed on an immunized animal. In contrast, the attenuated Senegal and Gardel stocks were not transmitted by *Amblyomma* ticks (Martinez 1997). Furthermore, it was shown that the sheep on which the infected ticks were feeding seroconverted and were protected against a $10xLD_{50}$ challenge dose with the virulent Welgevonden stock, indicating that the sheep had been immunized as a result of the tick transmission of the attenuated organisms. Although one sheep is not a basis for statistical analysis, two valid observations can be made from this experiment:

firstly, a single tick-transmission did not cause the organisms to revert to the virulent stage, and secondly, ticks which transmit the attenuated organisms can immunize naïve animals. Ideally, a culture-derived attenuated vaccine should not be tick-transmissible. However, in areas where the virulent Welgevonden stock of *E. ruminantium* is present, tick transmissibility need not be a disadvantage, since ticks infected with the attenuated organisms could subsequently immunize naïve animals. Even the "worst case" scenario, where the attenuated Welgevonden stock reverted to virulence, would not aggravate the situation in the field where virulent stocks already exist. For safety reasons the attenuated Welgevonden stock should obviously not be used in areas where it does not protect against the field strains, nor in non-endemic *Amblyomma* infested areas.

The attenuated vaccine shares with the present blood vaccine marketed in South Africa the disadvantage that both have to be distributed frozen, due to the extreme lability of *E. ruminantium* (Oberem and Bezuidenhout 1987). The attenuated vaccine, however, offers several major advantages over the blood vaccine: it would be cheaper to produce, a single 25cm^2 culture flask could produce between 2,500 and 25,000 doses of live vaccine, a blocking treatment with tetracyclines appears to be unnecessary, and the expected pattern of cross-protection is wider than that provided by the Ball 3 blood vaccine. Furthermore, the attenuated vaccine is produced in a closed *in vitro* culture system, which minimises the risk of introducing extraneous biological contaminants, as can occur with the blood vaccine.

We have shown that readapting the attenuated Welgevonden stock to bovine endothelial cells (BA886), which are derived from a natural host species of *E. ruminantium*, and propagating the organisms for 64 passages, led neither to a reacquisition of virulence nor to a loss of immunogenicity. Furthermore, it was shown that a single immunization was sufficient to induce a solid immunity in sheep and goats, and that organisms propagated *in vitro* remained stable as far as the loss of virulence was concerned.

Acknowledgements

This research was supported by the Agricultural Research Council of South Africa and the European Union (Cowdriosis Network) Grant no. EU DGVIII8ACP-TPS-040:613067.

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Table 1 Reactions of sheep to vaccination with attenuated *E. ruminantium* (Welgevonden stock) and challenge with homologous and heterologous *E. ruminantium* stocks

Sheep number	rIFA titer [a] Day 0	Immunization			rIFA titer [a] Day 29	Challenge			
		Incubation period (days)	t_{max} (°C)	Duration of fever [b] (days)		Challenge stock	Incubation period (days)	t_{max} (°C)	Duration of fever (days)
605	20	5	41.0	4	≥2560	Welgevonden	-	-	-
607	20	6	42.0	5	≥2560	Welgevonden	-	-	-
611	neg	5	42.0	4	≥2560	Welgevonden	-	-	-
621	neg	5	41.8	3	≥2560	Welgevonden	-	-	-
629	20	7	41.3	2(1)1	2560	Welgevonden	-	-	-
601	neg	3	42.0	6	≥2560	Ball 3	14	40.5	1
602	20	5	41.7	3	≥2560	Ball 3	-	-	-
603	20	5	41.4	4	≥2560	Ball 3	-	-	-
608	neg	5	42.0	4	1280	Ball 3	7	41.3	1
612	20	5	42.0	4	1280	Ball 3	-	-	-
613	20	5	41.5	4	≥2560	Gardel	-	-	-
615	20	5	42.0	4	1280	Gardel	-	-	-
616	20	5	41.0	3	≥2560	Gardel	-	-	-
619	20	5	42.0	4(2)1	≥2560	Gardel	-	-	-
620	20	5	42.0	4	≥2560	Gardel	-	-	-
622	neg	5	41.8	3(2)1	640	Mara 87/7	-	-	-
623	20	2	41.6	5	640	Mara 87/7	-	-	-
624	20	7	41.2	1	≥2560	Mara 87/7	-	-	-
625	20	5	41.5	3	≥2560	Mara 87/7	-	-	-
628	20	5	41.6	4	2560	Mara 87/7	-	-	-
632	20	6	41.6	4	≥2560	Blaauwkrans	-	-	-
634	neg	5	41.8	5	1280	Blaauwkrans	-	-	-
635	neg	5	41.8	4	640	Blaauwkrans	-	-	-
639	20	4	42.0	5	640	Blaauwkrans	-	-	-
640	20	5	42.0	6	640	Blaauwkrans	-	-	-
610	40	-	-	-	ND	Welgevonden	10	42.0	5
614	neg	-	-	-	ND	Ball3	14	41.8	4
630	160	-	-	-	ND	Gardel	8	42.0	5
631	640	-	-	-	ND	Mara 87/7	13	42.0	4
638	neg	-	-	-	ND	Blaauwkrans	12	42.0	5

[a] Reciprocal indirect fluorescent antibody titer
neg : negative
[b] Numbers in brackets indicate fever-free periods
ND: not done

Table 2 Reactions of sheep to vaccination with different doses of the attenuated *E. ruminantium* (Welgevonden stock) and challenge with the virulent Welgevonden stock

Sheep number	Dose (dilution)	rIFA titer [a] Day 0	Immunization			rIFA titer [a] Day 37	Challenge		
			Incubation period (days)	t_{max} (°C)	Duration of fever [b] (days)		Incubation period (days)	t_{max} (°C)	Duration of fever [b] (days)
662	10^{-4}	40	-	-	-	640	-	-	-
668	10^{-4}	40	-	-	-	640	-	-	-
676	10^{-4}	40	12	40.6	1	640	-	-	-
686	10^{-4}	40	-	-	-	640	-	-	-
687	10^{-4}	40	-	-	-	1280	-	-	-
618	10^{-3}	40	11	41.5	2	640	-	-	-
651	10^{-3}	40	10	40.8	2	640	-	-	-
656	10^{-3}	40	-	-	-	640	15	41.0	3
661	10^{-3}	20	10	40.1	1	640	-	-	-
669	10^{-3}	20	-	-	-	640	-	-	-
627	10^{-2}	40	-	-	-	320	-	-	-
636	10^{-2}	20	-	-	-	640	23	41.1	2
666	10^{-2}	40	-	-	-	640	-	-	-
674	10^{-2}	20	11	40.5	1	1280	-	-	-
675	10^{-2}	20	-	-	-	2560	-	-	-
617	10^{-1}	40	8	40.8	1	640	-	-	-
664	10^{-1}	neg	7	41.1	1	640	-	-	-
671	10^{-1}	20	9	40.8	1	1280	-	-	-
680	10^{-1}	40	7	40.8	3	2560	-	-	-
684	10^{-1}	20	7	41.3	2	1280	-	-	-
650	10^{0}	40	7	41.8	1	640	-	-	-
659	10^{0}	40	7	40.8	1	2560	-	-	-
665	10^{0}	40	7	41.0	1	1280	-	-	-
677	10^{0}	40	5	41.5	1(1)1(1)1	1280	-	-	-
681	10^{0}	40	7	40.6	3	2560	-	-	-
604	-	320	-	-	-	5120	16	41.9	5
653	-	neg	-	-	-	2560	15	41.8	4
654	-	40	-	-	-	5120	14	42.0	5
655	-	neg	-	-	-	5120	12	41.9	1(1)5
658	-	neg	-	-	-	5120	15	42.0	4

[a] Reciprocal fluorescent antibody titer neg : negative

[b] Numbers in brackets indicate fever-free periods

Table 3 Reactions of goats to vaccination with different doses of the attenuated *E. ruminantium* (Welgevonden stock) and challenge with the virulent Welgevonden stock

Goat number	Dose (dilution)	rIFA titer [a] Day 0	Immunization			rIFA titer [a] Day 30	Challenge		
			Incubation period (days)	t_{max} (°C)	Duration of fever [b] (days)		Incubation period (days)	t_{max} (°C)	Duration of fever [b] (days)
325	10^{-4}	20	15	40.7	2	640	-	-	-
327	10^{-4}	80	15	40.1	2	640	14	41.0	1
331	10^{-4}	20	15	40.1	2	640	-	-	-
340	10^{-4}	40	14	40.6	3	640	14	40.8	1
351	10^{-4}	40	13	40.7	2	1280	-	-	-
227	10^{-3}	80	13	40.4	1	1280	15	41.0	1
281	10^{-3}	40	14	40.1	2	1280	-	-	-
283	10^{-3}	20	13	40.3	2(1)1	2560	14	41.0	1
303	10^{-3}	40	15	40.6	1	320	-	-	-
310	10^{-3}	40	15	40.0	1	640	-	-	-
93	10^{-2}	80	13	40.9	3	1280	13	41.0	1
99	10^{-2}	20	13	40.6	1(1)2	2560	13	40.8	1
106	10^{-2}	20	14	40.2	2	1280	-	-	-
152	10^{-2}	160	13	40.4	1	640	15	41.1	1
154[c]	10^{-2}	-	-	-	-	-	-	-	-
32	10^{-1}	40	8	40.8	3	1280	-	-	-
35	10^{-1}	80	10	40.0	1	1280	14	40.6	2
55	10^{-1}	40	8	40.8	2	320	-	-	-
57	10^{-1}	20	9	40.3	2	320	15	40.5	1
87	10^{-1}	80	10	40.5	1(2)1	320	14	41.0	1
365	-	40	-	-	-	320	12	41.8	4
368	-	320	-	-	-	1280	12	41.7	5
377	-	80	-	-	-	640	14	41.5	4
380	-	160	-	-	-	640	12	41.8	3(1)1
382	-	80	-	-	-	1280	11	41.4	4

[a] Reciprocal fluorescent antibody titer

[b] Numbers in brackets indicate fever-free periods

[c] Animal died from peritonitis on day 4

Chapter 4.3

Discussion - Attenuated vaccine

The Onderstepoort blood vaccine is the only commercially available vaccine against heartwater, and its use is limited to southern Africa. Animals are inoculated intravenously with cryopreserved blood from sheep infected with the virulent Ball 3 stock of *E. ruminantium* (Bezuidenhout 1989), their body temperature is monitored and antibiotic treatment has to be administered at the appropriate time. In senso stricto, this procedure is not a vaccination but an infection-and-treatment regime (Uilenberg 1983). Moreover, the vaccine must be stored and transported frozen on dry ice, which makes it inconvenient for use in rural areas. Furthermore, the vaccination procedure is labour-intensive, as the animals have to be closely monitored for a temperature reaction. It is also expensive, because additional costs arise through the need for tetracyclines to block infection. And last but not least, the Ball 3 stock is mainly used because it produces a temperature rise several days before serious clinical disease occurs, making it relatively easy to decide when to treat, but it does not protect against all isolates present in the field (du Plessis *et al.* 1989; Jongejan *et al*. 1988). More virulent isolates may provide better cross-protection against other stocks, but can cause death very shortly after a rapid temperature rise, and therefore cannot be used safely for the infection-and-treatment method of vaccination.

Attenuation is a decline in virulence imposed on a pathogen by chemical or physical interference, during *in vitro* cultivation. Mutagenic substances, or "unnatural conditions" such as an elevated culture temperature, can increase the likelihood of attenuation, although attenuation can also occur spontaneously. In our experiments, the virulent Welgevonden stock, serially passaged through canine DH82 cells, cells not derived from the natural host, lost its virulence for mice when tested between passages 25 and 50.

The attenuated organisms used initially were derived from DH82 cells. They produced no disease when inoculated into mice or sheep, and the animals were completely protected against a subsequent lethal homologous needle challenge. However, DH82 cells are cancerous cells and unsuitable for the production of a commercial vaccine. Therefore the attenuated Welgevonden stock was re-adapted to the bovine endothelial cell culture line BA 886. This cell line has been used for more than 8 years for the routine propagation of the virulent Welgevonden stock. For the experiments described here the attenuated organisms were serially passaged from passage 43 up to passage 60 in BA 886 cells. Animals injected with 2 ml of BA 886-derived culture material showed an increase in body temperature, but no other symptoms. This indicated that virulence had not been re-established by changing the host

cells. Furthermore, immunisation with the attenuated Welgevonden stock provided full protection against a homologous virulent needle challenge, showing that immunogenicity remained as high as with DH82-derived organisms.

The virulent Welgevonden stock conferred immunity after infection-and-treatment against a number of other *E. ruminantium* genotypes, such as Ball3, Mara 87/7 (du Plessis *et al.* 1989; Collins *et al.* 2003), Blaauwkrans and Gardel (Collins *et al.* 2003). When the attenuated Welgevonden stock was used for immunisation, and the animals were subsequently challenged with one of the heterologous stocks mentioned above, all the animals were fully protected. These results indicated that the same cross-protection pattern was induced by both virulent and attenuated Welgevonden stocks. In contrast, all control animals which received a $10xLD_{50}$ dose of any virulent stock developed severe disease symptoms and had to be treated with oxytetracycline.

The Senegal stock was found to be spontaneously attenuated after eleven passages or 229 days in bovine endothelial cell cultures, whereas the Welgevonden stock did not attenuate after 17 passages over 226 days in culture (Jongejan 1991). Similarly, the Zimbabwean Crystal Springs stock of *E. ruminantium* did not attenuate and was fatal for sheep after 60 and 192 passages *in vitro* (Mahan *et al.* 1995). The authors concluded that the attenuation of *E. ruminantium* appeared to be strain-specific and that its application was limited to the Senegal stock. However, several years later and after more passages *in vitro*, the Crystal Springs stock too became attenuated (Mahan 2005). Likewise, the Gardel stock became attenuated after more than 200 passages in endothelial cell cultures (Martinez 1997). Although no data were formally published for the latter two attenuated stocks, continuous propagation very probably led to their attenuation. In contrast, the Welgevonden stock, which is in continuous culture in bovine endothelial cells in our laboratory, remained virulent at passage levels up to 231 with an average passage interval of 3 days. It has been shown, nevertheless, that when suitable culture conditions prevail (Chapter 6) the Welgevonden stock, which is one of the most virulent stocks of *E. ruminantium* in South Africa, can be effectively attenuated at relatively low passage levels and within a short period of time.

Titration of the immunising dose in sheep showed that not more than 18,000 infective particles per dose were necessary to induce a solid immunity against a $10xLD_{50}$ needle challenge with the virulent Welgevonden stock. It is noteworthy that with higher dilutions of the immunising material, ranging from 10^{-2} to 10^{-4}, only some of the animals reacted to the immunisation with an elevated body temperature, nevertheless all animals survived the subsequent challenge. This is in contrast to observations made by Du Plessis and Malan (1987) during infection and treatment experiments, in which animals which did not react did not become immune. Their conclusion that immunity elicited by an infective inoculum was dependent on the severity of the reaction that it caused, does not appear to apply to the attenuated Welgevonden organisms.

Our experiments in Boer goats revealed that all animals reacted to the immunising dose with an increase in body temperature, and all animals resisted a subsequent needle challenge. As few as 6,000 infective particles per dose were sufficient to induce a solid immunity. In contrast to Boer goats, Angora goats are highly susceptible to *E. ruminantium*, and immunisation against heartwater is difficult and

hazardous (du Plessis *et al*. 1983). These authors found that few Angora goats survived when they were treated on the 2nd or 3rd day of the febrile reaction following the i.v. inoculation of virulent organisms. On the other hand, if they were treated on the first day of the reaction, the survival rate was high, but the immunity of the goats to subsequent challenge was poor. Because of these problems, Angora goats in South Africa are not vaccinated with the Onderstepoort blood vaccine. Instead they are prophylactically treated with tetracyclines every fortnight. Immunisation of Angora goats with the attenuated Welgevonden organisms could offer an improved method of protection for these animals, although it remains to be seen how they would react to this vaccine.

In contrast to the attenuated Senegal and Gardel stocks which were not transmitted by *Amblyomma* ticks (Martinez 1997), the attenuated Welgevonden stock was transmitted through *A. hebraeum* ticks which were fed as nymphs on an immunised animal during the febrile reaction phase. Sheep on which the resultant adult ticks were feeding seroconverted and withstood a lethal challenge with the virulent Welgevonden stock, indicating that they were in fact immunised by the tick transmission of the attenuated organisms. Tick transmissibility is not necessarily a disadvantage in areas where the virulent Welgevonden stock of *E. ruminantium* is present, because ticks infected with the attenuated organisms could subsequently immunise naïve animals. Even if the attenuated Welgevonden stock should revert to virulence, it would not aggravate the situation in the field where virulent stocks already exist. Further studies need to be conducted to show whether immunised animals become carriers of the organisms and if so, whether or not ticks will pick up infection from carrier animals.

At present, the attenuated vaccine is the only alternative to the blood vaccine marketed in South Africa. Both vaccines share the disadvantage of having to be distributed frozen, due to the extreme lability of *E. ruminantium* (Oberem and Bezuidenhout 1987). The attenuated vaccine, however, offers several major advantages: no animals are required for production, it would be cheaper to produce - a single 25cm^2 culture flask could produce between 2,500 and 25,000 doses of live vaccine, a blocking treatment with tetracyclines is unlikely to be necessary, and the expected spectrum of cross-protection is wider than that of the Ball3 blood vaccine. Furthermore, the attenuated vaccine is produced in a closed *in vitro* culture system, which minimises the risk of introducing extraneous biological contaminants, as can occur with the blood vaccine. In contrast to the blood vaccine where the vaccine doses were elaborated empirically, the immunising dose of the attenuated vaccine can be determined by *in vitro* culture techniques, and the dose adjusted accordingly. Determination of the number of infective particles as described here (Chapter??) is the only valid quantification for the vaccine dose, since neither the number of viable elementary bodies nor the amount of protein or DNA present in the vaccine, correlate with infectivity.

Future directions

The most convenient way to isolate *E. ruminantium* from an infected animal is from blood or plasma, where extracellular organisms are in abundance during the febrile reaction, and endothelial cell cultures can be easily infected. This is probably true for most *E. ruminantium* isolates, but not for all, hence modified techniques need to be developed for the isolation of as yet uncharacterised *Ehrlichia*. The importance of such alternative approaches has been documented in Chapter 7, where an unconventional approach to *in vitro* isolation and further propagation was used. This allowed the separation, from the Kümm stock of *E. ruminantium*, of two organisms which differed in their culture characteristics and which hybridised with two different *E. ruminantium* 16S genotype probes. Each stock was isolated in a different cell type of non-endothelial origin, namely a transformed canine macrophage/monocyte cell line and a sheep fibroblastoid cell culture.

It has been shown previously that *E. ruminantium* derived from endothelial cell cultures can be propagated in various tick cell lines, derived from the natural tick vector but also from non-vector ticks (Bell-Sakyi 2004). However, several attempts to isolate *E. ruminantium* directly from an infected animal using tick cell cultures failed (Bell-Sakyi 2004). In contrast, other (former) *Ehrlichia* species, *E. equi* (Munderloh et al. 1996), the human granulocytic ehrlichiosis (HGE) agent, (Munderloh et al. 1999) and *E. phagocytophila* (Woldehiwet *et al.* 2002) (now included in *Anaplasma* [*Ehrlichia*] *phagocytophilum* comb. nov. [Dumler *et al.* 2001]) have been successfully isolated from infected blood using tick cell cultures. There are no known reasons why *E. ruminantium* should not be isolated directly from a febrile animal into tick cell cultures provided favourable culture conditions prevail. Preliminary experimental evidence indicated that the IDE8 tick cell culture system could be used to isolate the Ball3, Blaauwkrans, Nonile and Welgevonden stocks of *E. ruminantium* directly from the blood of infected sheep (author's unpublished results). Therefore, non-endothelial cell cultures, as described above, or tick cell cultures could be used in future to increase the chances of culture isolation of organisms which would normally not grow in endothelial cell cultures.

There is an urgent need for a reliable serological test for animals originating from heartwater-free areas in southern Africa. Experimental animals to be used in heartwater experiments at OVI were usually purchased from farms where they were born and raised in a heartwater- and *Amblyomma*-free area of South Africa. DNA samples obtained from the blood of these animals were examined by PCR (Mahan *et al*. 1992) and probed with the *E. ruminantium*-specific pCS20 probe (Waghela *et al.* 1991) which indicated that they were negative for *E. ruminantium.* However, serological results using the IFAT were contradictory, and false positives with titres up to 1:640 were detected (author's unpublished results). Even when using the MAP1-B ELISA, which is supposed to be a more specific test (van Vliet *et al.*

1995), these samples showed many false positive reactions. Evaluation of the MAP1-B ELISA in southern Africa revealed a high number of false positive reactions in goats from non-*Amblyomma* areas (Mahan *et al* 1998; de Waal *et al.* 2000). Using the MAP1-B ELISA to detect antibodies to *E. ruminantium* in goat sera from three communal land areas of Zimbabwe, Kakono *et al.* (2003) showed that nearly 90% of goats from a non-heartwater area gave false positive reactions. The agent causing the cross-reactions has not been isolated or characterised, although from its immunological similarity to *E. ruminantium* it is assumed to be a species of *Ehrlichia*. It is of foremost importance to apply the above-mentioned approaches for culture isolation, propagation, identification, and characterisation to these uncharacterised agents.

The only heartwater control method currently used commercially is an immunisation procedure which is more than 50 years old, whereby animals are infected with cryopreserved sheep blood containing virulent *E. ruminantium* organisms of the Ball 3 genotype (Bezuidenhout 1989) and antibiotic treatment is administered when a rise in temperature occurs. This situation is far from ideal. The blood vaccine harbours the risk of introducing extraneous biological contaminants, and large numbers of experimental animals are required to produce the material. Therefore, a live culture-derived vaccine could offer an interim solution until better alternative vaccines can be introduced such as DNA vaccines. There are major arguments in favour of an attenuated, culture-derived vaccine: it is cheaper to produce than the traditional blood vaccine, a blocking treatment with tetracyclines is unlikely to be necessary, and the expected range of cross-protection, when using the attenuated Welgevonden stock, is better than that of the Ball3 blood vaccine. The Welgevonden stock has been found to provide cross protection against most South African isolates (du Plessis *et al.* 1989; Collins *et al.* 2003). Consequently, local stocks with a similar broad spectrum of cross protection need to be identified in other heartwater areas in Africa, where the attenuated Welgevonden stock may not provide protection against field strains and should not be introduced because of the risk of a possible reversion to virulence. Once isolated in culture, these stocks could be attenuated using suitable culture procedures (Jongejan 1991; Bell-Sakyi *et al.* 2002; Martinez 1997; Chapter 6). For the development of attenuated vaccines, however, only regional solutions are likely to be possible. Thus the Senegal isolate of *E. ruminantium,* attenuated in cell culture, protected animals against subsequent lethal homologous challenge (Jongejan 1991), but its protective effect against isolates from other geographical regions was poor (Jongejan *et al.* 1993). The antigenic diversity of *E. ruminantium* hampers the development of vaccines against heartwater, and cross protection studies in animals are at present the only means to identify cross protective isolates. In order to minimize the use of experimental animals, *in vitro* tests should be developed to enable prediction of possible cross protective capacity between different isolates of *E. ruminantium*. With the availability of the genome sequence of the Welgevonden (Collins *et al.* 2005) and Gardel (Martinez 2004) stocks of *E. ruminantium*, and with other stocks likely to follow, it may be possible to identify genomic similarities between isolates which induce cross protective immunity. This would facilitate the design of molecular probes to identify such similarities in field isolates. Cumbersome and expensive animal cross protection trials to identify "master strains" from the respective heartwater regions to be used in an attenuated vaccine could then be omitted.

The attenuated vaccine shares with the present blood vaccine marketed in South Africa the disadvantage of requiring to be distributed frozen, owing to the extreme lability of *E. ruminantium* (Oberem and Bezuidenhout 1987). The necessity of keeping the current heartwater vaccine deep-frozen until it is inoculated, and the rapidity with which the vaccine loses its infectivity and thereby its immunogenicity, are major constraints shared by live vaccines in general. To increase acceptance of an attenuated, live vaccine by the end user, a process to avoid the need for maintaining the cold chain and to give the product a reasonable shelf live under less rigid storage temperatures would have to be developed. Early experiments using lyophilized mouse tissues and sheep blood showed that the organisms remained infective to mice and sheep after storage at 4°C for 90 days (du Plessis *et al.* 1990). A successful freeze-drying process seems feasible because of the sheer number of heartwater organisms in such a vaccine which is much higher than that in infective organs or blood. Freeze-drying would greatly improve the acceptance of an attenuated vaccine. Infectivity, a pre-requisite for an attenuated vaccine to immunise animals successfully, could then easily be tested in an *in vitro* culture system.

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Summary

Ehrlichia ruminantium, an intracellular gram-negative rickettsia, is the causative agent of heartwater, a tick-borne disease of domestic and wild ruminants. It is a major constraint to livestock production throughout sub-Saharan Africa and the islands of Madagascar, La Réunion, Mauritius and São Tomé. The disease is also present in the Caribbean, on the French Antillean islands of Guadeloupe and Antigua, to which infected *Amblyomma variegatum* ticks were introduced, possibly as early as the eighteenth century.

Research into heartwater was hampered for many years by the impossibility of obtaining viable *E. ruminantium* organisms of a defined genotype and free from contamination with mammalian cellular material. A major turning point was reached when a suitable culture system became available. However, the first *in vitro* culture system was exceptionally fragile, and unexplained failures used to occur frequently, causing frustrating delays in heartwater research.

This thesis examines three aspects of the *in vitro* propagation of *E. ruminantium* in mammalian cell culture systems. Firstly, a chemically defined serum-free culture medium was formulated by omitting poorly-defined constituents and serum-derived products. Secondly the potential of *E. ruminantium* to infect, and to grow in, several cell lines of non-endothelial and non-ruminant origin was investigated. These cells were different from those considered to be the organism's natural host cells, which are endothelial cells, neutrophils and macrophages. Thirdly, the infectivity and immunogenicity for mice, sheep and goats of a culture variant of an *E. ruminantium* stock was examined for its potential as an attenuated culture-derived vaccine.

Development of defined media for the *in vitro* cultivation of *Ehrlichia ruminantium*

Media commonly used for the *in vitro* cultivation of *E. ruminantium* consisted of a chemically defined portion, a source of serum and, in some cases, tryptose phosphate broth (TPB). Both serum and TPB are chemically undefined products which vary in composition from batch to batch. Sometimes batches of serum have toxic effects on *E. ruminantium,* or are simply unable to support the propagation of the organisms. Therefore attempts were made to replace serum-containing medium by a HL-1-based medium, a commercially available medium originally formulated for the serum-free cultivation of hybridoma cells (Chapter 2.1). Three stocks of *E. ruminantium* were successfully propagated in bovine endothelial cells in this HL-1-based medium, namely the Vosloo, Welgevonden and Senegal stocks which were propagated for periods of more than 203, 134, and 43 days, respectively. Cultures of the Vosloo and Senegal stocks of *E. ruminantium* were also successfully initiated under serum-free culture conditions. These experiments showed for the first time that *E. ruminantium* could be initiated and propagated *in vitro* under

serum-free culture conditions. Unfortunately the complete formulation of HL-1 was not provided by the manufacturer, so that it was still not possible to prepare a fully-defined medium.

To further characterize the composition of a serum-free medium for the *in vitro* cultivation of *E. ruminantium,* seven different synthetic media of published compositions were used to replace part of the HL-1 medium kit (Chapter 2.2). Four of these synthetic media gave unsatisfactory results: these were SFRE-199 medium, Iscove's modified Dulbecco's medium, Dulbecco's modified Eagle's medium, and Leibovitz' L-15 medium. Three other serum-free media did, however, support the growth of *E. ruminantium*: these were a modified HL-1 medium, DME/F-12 medium, and RPMI 1640 medium. Each of these media was supplemented with "Solution A", obtained from the HL-1 medium kit, which contained unpublished quantities of transferrin, testosterone, sodium selenite, ethanolamine, saturated and unsaturated fatty acids, and stabilizing proteins. Based on these details, various supplements were investigated as alternatives to the incompletely specified Solution A. Bovine lipoproteins and bovine transferrin were subsequently identified as essential supplements which effectively replaced Solution A when added to the synthetic medium DME/F-12. *E. ruminantium* was propagated in the three growth-supportive media for at least 10 passages. Protein-free and chemically completely defined media for the growth of *E. ruminantium* were thus finally formulated (Chapter 2.3).

In an attempt to further define the media, bovine lipoproteins and transferrin (Chapter 2.2) were replaced with chemically defined lipids and a protein-free iron complex. A medium designated SFMC-23 was formulated, based on DME/F-12 supplemented with L-glutamine, antibiotics, chemically defined lipids and an iron complex, consisting of a mixture of ferric chloride and glycylglycine. In addition medium SFMC-36, also based on DME/F-12 medium and supplemented with L-glutamine and antibiotics only, was formulated. Both of these media supported the long-term growth of the Welgevonden stock of *E. ruminantium* for a total of 55 and 28 passages respectively, with regular passage intervals of 3 days. Using SFMC-23, split ratios varied from 5 to 10, depending on which host cell line was used. Other stocks of *E. ruminantium* (Sankat 430, Blaauwkrans, Senegal) were also successfully propagated in these media for a test period of 10 passages.

The Welgevonden stock was propagated in the chemically defined SFMC-23 medium in order to establish the amino acid requirements of *E. ruminantium* during *in vitro* propagation. After a 3-day culture period, samples of supernate were taken from infected and uninfected bovine endothelial cell cultures and the amino acid concentrations were determined (Chapter 2.4). The samples were analyzed for eighteen free amino acids by the Pico Tac reverse-phase HPLC precolumn derivatization method. The concentrations of proline (29%) and glutamine (62%) were the most reduced, while aspartic acid, serine, asparagine, tryptophan, and glycine concentrations increased by more than 25%, and that of alanine increased by approximately 660%. The concentrations of all other amino acids analyzed remained within a narrow range, increasing or decreasing by not more than 25%. These results indicated that glutamine and proline are the only amino acids depleted during *in vitro* cultivation. The accumulation of various other amino acids in the culture medium may impact negatively on long-term *E. ruminantium* propagation, and this phenomenon should be investigated.

Host cell repertoire

Multiplication of *E. ruminantium* in infected animals occurs preferentially within endothelial cells, although other cell types, leukocytes and macrophages, have also been found to be infected. The Welgevonden stock of *E. ruminantium* was continuously propagated in DH82 cells, a continuous canine macrophage-monocyte cell line. Cultures of DH82 cells were readily infected provided that the culture medium was supplemented with cycloheximide. Cultures were split at regular 3-day intervals and infection rates ranged between 60 % and 95 %. Cultures were continuously propagated through more than 125 passages over a period of more than one year (Chapter 3.1).

The *in vitro* isolation, propagation, and characterization of the Kümm isolate of *E. ruminantium* are described in Chapter 3.2. Prior to the successful experiments described here, the Kümm isolate had resisted all attempts at *in vitro* cultivation. In one experiment white blood cells were harvested from a sheep infected with the Kümm isolate, and were added to DH82 cells. Rickettsial colonies were first detected 19 days after culture initiation. Bovine and sheep endothelial cells were readily infected with culture supernatant obtained from the infected DH82 cells. In a further experiment another sheep was infected, using a higher dose of the same batch of the Kümm stabilate. For isolation, several different cell lines were used: DH82 cells, bovine aorta (BA886) cells, sheep brain endothelial (SBE189) cells and sheep fibroblastoid cells (E_2). Ten days after culture initiation only the E_2 cells had become positive for *E. ruminantium*. Upon molecular characterization with *E. ruminantium* 16S probes, the Kümm organisms from the first experiment (Kümm-1) hybridized with a Senegal 16S genotype probe, whereas the Kümm organisms from the second experiment (Kümm-2) hybridized only with an Omatjenne 16S genotype probe. The original stabilate used to infect the sheep hybridized with both probes, clearly indicating that it contained both genotypes, and that these had been separated by cultivation in the different cell types.

The Welgevonden stock of *E. ruminantium* was also propagated in baby hamster kidney (BHK) cells, Chinese hamster ovary (CHO-K1) cells and Madin Darby bovine kidney (MDBK) cells (Chapter 3.3). The cultures required supplementation of the medium with cycloheximide for reliable growth of *E. ruminantium*. Growth of the Welgevonden stock in BHK and CHO-K1 cells could lead to the development of suspension cultures suitable for the mass production of *E. ruminantium* for an inactivated elementary body vaccine.

Further experiments were carried out to investigate the ability of *E.ruminantium* to grow in a range of different cell types (Chapter 3.4). The Welgevonden stock was propagated in eight non-endothelial cell cultures derived from different animal species, both ruminants and non-ruminants. The origins of the cells were: bovine foetal testis (BFT), cat ovary (COC), donkey fibroblasts (DFC), sheep fibroblasts (E_2), horse testis (HTC), lamb foetal testis (LFT), mouse connective tissue (L), and African green monkey kidney (Vero). Four cell types (BFT, E_2, LFT and Vero) required supplementation of the medium with cycloheximide to achieve reliable growth of *E. ruminantium*, whereas the other four (COC, DFC, HTC and L) did not need cycloheximide. Three other stocks of *E. ruminantium*, Senegal,

Ball 3 and Gardel, were also propagated, either in LFT cultures only or in both E_2 and LFT cell cultures. In addition, cultures of the Welgevonden stock were successfully initiated in E_2 and LFT cell cultures.

Application of *in vitro* culture techniques for the attenuation of *Ehrlichia ruminantium*

The attenuation of the virulent *E. ruminantium* Welgevonden stock was achieved by growing the organisms in the continuous canine macrophage-monocyte cell line DH82 (Chapter 3.1). After 50 or more passages, the culture-derived organisms did not produce any disease symptoms when inoculated into mice or sheep, and all the animals were completely protected against a subsequent lethal homologous needle challenge (Chapter 4.1). The attenuated vaccine was developed further by readapting the attenuated organisms to continuous propagation in a bovine endothelial cell line (BA 886) (Chapter 4.2). When inoculated into sheep or goats the attenuated organisms did not produce disease, and the only symptom observed was a rise in body temperature in most animals. Following injection of culture material, all sheep were fully protected against a subsequent lethal homologous (virulent Welgevonden) or heterologous (Ball 3, Gardel, Mara 87/7, Blaauwkrans) needle challenge. Titrations of elementary body suspensions showed that a 1:10,000 dilution injected into sheep or goats was still sufficient to trigger an immune response which resisted a lethal needle challenge with the virulent Welgevonden stock. Adult *Amblyomma hebraeum* ticks, fed as nymphs on sheep immunized with attenuated organisms, were able to transmit the attenuated stock to a naïve sheep, which was found to be protected against a subsequent lethal homologous needle challenge.

In conclusion, the work described in this thesis has provided an important contribution to improved heartwater vaccine development. A chemically completely defined medium for the continuous *in vitro* propagation of *E. ruminantium* has been described. Additional *in vitro* studies revealed that *E. ruminantium* has a broader repertoire of host cells than previously assumed. Propagation in an unusual host cell line led to the emergence of an attenuated version of the Welgevonden stock of *E. ruminantium*, an important basis for the development of a culture-derived heartwater vaccine. Preliminary studies on the efficacy of this vaccine indicated that sheep were fully protected against a lethal challenge with the virulent homologous stock or with any of four different heterologous stocks. Future studies aimed at development of a freeze-dried formulation would greatly improve the acceptance of a live, attenuated vaccine by the end user. Effective, reliable control of heartwater using this vaccine to prevent serious disease outbreaks could have a tremendous positive economic impact for small-scale as well as commercial farmers in South Africa.

Samenvatting

Ehrlichia ruminantium, een intracellulair gram-negatieve bacterie, is de oorzaak van heartwater, een ziekte bij herkauwers die door *Amblyomma* teken wordt overgedragen. Heartwater vormt een belangrijke belemmering voor de ontwikkeling van de dierlijke productie in grote delen van Afrika ten zuiden van de Sahara.

Onderzoek aan heartwater is jarenlang belemmerd doordat *E. ruminantium* niet *in vitro* kon worden gekweekt en de eerste succesvolle *in vitro* kweek systemen moeilijk bleken te reproduceren. In dit proefschrift zijn drie specifieke aspecten van de *in vitro* kweek van *E. ruminantium* bestudeerd. Ten eerste is een chemisch gedefinieerd serum-vrij kweek medium ontwikkeld, waardoor de reproduceerbaarheid aanmerkelijk kon toenemen. Ten tweede is het gastheercel repertoir voor *E. ruminantium* onderzocht, waarbij is gekeken naar een breed scala van potentiële gastheer cellen. Ten derde is de infectiviteit van een *in vitro* geattenueerd isolaat van *E.ruminantium* voor kleine herkauwers getest met het doel om te komen tot een celkweek vaccin tegen heartwater.

Ontwikkeling van media voor de *in vitro* kweek van *Ehrlichia ruminantium*

De meeste kweek media die in eerste instantie voor de *in vitro* kweek van *E. ruminantium* werden ontwikkeld, bestonden uit een chemisch gedefinieerde component, plus serum en tryptose fosfaat broth. Zowel serum als tryptose fosfaat broth bleken van batch tot batch te verschillen en bovendien waren sommige batches serum ongeschikt voor de *in vitro* kweek.

In dit proefschrift is beschreven dat het mogelijk is om *E.ruminantium* in het serum-vrij medium HL-1 te kweken (**hoofdstuk 2.1)**. Drie isolaten van *E. ruminantium* (Welgevonden, Vosloo, en Senegal) werden met succes in celkweken van runder endotheelcellen aangehouden. Omdat de exacte samenstelling van het HL-1 medium niet beschikbaar was, zijn vervolgens een aantal synthetische media getest, waarvan de samenstelling wel bekend was (**hoofdstuk 2.2**). Vier media, SFRE-199, Iscove's modified Dulbecco's, Dulbecco's modified Eagle's en Leibovitz' L-15 medium, bleken ongeschikt. Daarentegen bleek RPMI 1640 medium, DME/F-12 en een gemodificeerd HL-1 medium wel geschikt voor het propageren van het organisme. Het nadeel was echter dat er ook een onvoldoende gedefinieerde "Solution A" moest worden toegevoegd aan deze media. Nadat het duidelijk was geworden dat lipoproteinen en transferrinen als alternatief voor "Solution A" konden worden gebruikt, is vervolgens getracht om zowel de lipoproteinen als transferrinen te vervangen door chemisch gedefinieerde lipiden en een eiwitvrij ijzer complex. Als resultaat is een synthetisch medium ontwikkeld (SFMC-23) gebaseerd op DME/F-12 aangevuld met L-glutamine, antibiotica, lipiden en een complex bestaande uit ijzerchloride en glycylglycine. In dit medium kon *E. ruminantium*

(Welgevonden) langdurig worden aangehouden tot tenminste 55 passages met een gemiddeld interval van drie dagen. Drie andere stammen van *E. ruminantium* (Sankat 430, Blaauwkrans en Senegal) werden eveneens met succes in dit volledig synthetische SFMC-23 medium gekweekt.

Vervolgens is de aminozuurbehoefte bepaald van *E.ruminantium* (Welgevonden) in SFMC-23 medium, waaruit na 3 dagen incubatie monsters werden genomen van het supernatant van geïnfecteerde en ongeïnfecteerde endotheel celkweken (**hoofdstuk 2.4)**. Uit reverse-phase HPLC bleek dat de concentratie proline (29 %) en glutamine (62 %) waren verlaagd, terwijl alanine sterk was toegenomen tot 660 %. De concentratie van de overige animozuren bleef relatief constant. Het kon niet worden bepaald wat het mogelijk effect is van de sterke verhoging van alanine op de lange termijn kweek van *E. ruminantium*.

Gastheercel repertoire

De ontwikkeling van *E. ruminantium* in geïnfecteerde dieren vindt bij voorkeur plaats in endotheel cellen, alhoewel andere typen cellen, zoals leukocyten en macrofagen, ook kunnen worden geïnfecteerd. In **hoofdstuk 3.1** is beschreven dat *E. ruminantium* (Welgevonden) met succes kon worden gekweekt in DH82 cellen, een cellijn van canine macrofagen/monocyten, mits dergelijke cultures behandeld werden met cycloheximide. Onder deze omstandigheden kon een infectiegraad van 60 % tot 95 % worden bereikt in DH82 cellen die 125 maal gepasseerd konden worden over een periode van één jaar (**Hoofdstuk 3.1**).

In hoofdstuk 3.2 is de kweek en karakterisering van de Kümm stam van *E. ruminantium* beschreven, een isolaat dat nooit eerder met succes in kweek werd gebracht. In een eerste serie experimenten werd gewerkt met een leukocyten fraktie van een schaap geïnfecteerd met *E. ruminantium* (Kümm), dat na 19 dagen zichtbare *Ehrlichia* kolonies te zien gaf in DH82 cellen. Vervolgens lukte het om endotheel cellen te infecteren met supernatant afkomstig van de geïnfecteerde DH82 kweek. In een tweede serie experimenten werd een schaap geïnfecteerd met een hogere dosis van hetzelfde Kümm isolaat. Ditmaal werden verschillende cellijnen gebruikt, waaronder DH82, runder aorta endotheel (BA886), endotheel uit de hersenen van schapen (SBE189) en een schapen fibroblasten cellijn (E_2). Na tien dagen incubatie bleken alleen de E_2 cellen positief voor *E. ruminantium*. Nader moleculaire karakterisering met specifieke 16S probes wees uit dat *E. ruminantium* (Kümm) uit de eerste serie experimenten (Kümm-1) hybridiseerden met genotype “Senegal”, terwijl *E. ruminantium* (Kümm) uit de tweede serie experimenten (Kümm-2) alleen positief waren voor het genotype “Omatjenne”. Omdat het originele materiaal hybridiseerde met beide 16S probes, bleek dat beide genotypes aanwezig waren in het oorspronkelijke materiaal en blijkbaar van elkaar gescheiden konden worden door de *in vitro* kweek in twee verschillende gastheercellen.

Nadat was gebleken dat *E. ruminantium* (Welgevonden) ook kon worden gekweekt in embryonale hamster cellen (BHK), ovarium cellen van chinese hamsters (CHO-K1) en in runder niercellen (MDBK) die met cycloheximide werden behandeld (**hoofdstuk 3.3**), werd besloten om het

gastheercel repertoir aan een nader onderzoek te onderwerpen **(hoofdstuk 3.4**). Het Welgevonden isolaat bleek zich in acht verschillende cellijnen te ontwikkelen, waarbij het in de helft van de gevallen nodig was om cycloheximide aan het medium toe te voegen om de ontwikkeling van *E. ruminantium* te waarborgen. Cycloheximide was bij de overige cellijnen niet nodig. Tenslotte werden nog drie andere isolaten van *E. ruminantium* (Senegal, Ball 3 en Gardel) met succes in verschillende cellijnen *in vitro* aangehouden.

In vitro* attenuering van *Ehrlichia ruminantium

Passage van *E. ruminantium* (Welgevonden) in de canine macrofaag/monocyten cellijn DH82 bleek volledig verlies van de virulentie tot gevolg te hebben. Indien de infectiviteit van passage 50 of hoger werd getest in muizen of schapen, bleven alle klinische reacties volledig achterwege. Bovendien bleken de ingespoten dieren beschermd te zijn tegen een lethale homologe challenge (**hoofdstuk 4.1**). De in DH82 cellen geattenueerde *E. ruminantium* stam kon verder worden aangehouden in celkweken van runder aorta endotheel (BA 886) (**hoofdstuk 4.2**). Indien vervolgens schapen of geiten met dit geattenueerde materiaal werden ingespoten, werd alleen een verhoging van de lichaamstemperatuur gezien zonder verdere klinische verschijnselen. Opnieuw bleek dat alle dieren beschermd waren tegen een lethale homologe challenge met de virulente Welgevonden oorspronkelijke stam, maar ook tegen een heterologe challenge met de Ball 3 stam, Gardel, Mara 87/7, of de Blaauwkrans stam. Bovendien was een verdunning van 1 op 10.000 van het gekweekte materiaal al voldoende om een adequate immuun response op te wekken in schapen, die daarbij een volledige bescherming kon bieden. Interessant was dat volwassen *Amblyomma hebraeum* teken, die zich als nimfen op geïmmuniseerde schapen hadden gevoed, in staat waren om de geattenueerde stam over te dragen op een naïef schaap, dat vervolgens ook beschermd bleek te zijn tegen heartwater.

Het werk dat in dit proefschrift is beschreven heeft bijgedragen aan de ontwikkeling van een geattenueerd celkweek vaccin tegen heartwater. Indien een gevriesdroogde formulering mogelijk zou zijn, dan is er met name in Zuid Afrika een aanzienlijke markt voor dit vaccin dat ingezet zou kunnen worden ter bescherming van herkauwers tegen heartwater, één van de belangrijkste door teken overgedragen infectieziekten.

Curriculum vitae

Erich Peter Zweygarth was born on March 3, 1951 in Heilbronn, Germany. He graduated in veterinary medicine at the Free University of Berlin in 1976. In 1979 he obtained the degree of Doctor in Veterinary Medicine (Doctor medicinae veterinariae) from the Free University in Berlin, Germany. Between 1977 and 1982, he worked at the Institute of Veterinary Parasitology and Tropical Veterinary Medicine of the Free University as an assistant lecturer. In 1981 he obtained a post-graduate degree as a Veterinary Parasitologist from the Veterinary Council, Berlin. Between 1983 and 1993 he worked at the Veterinary Laboratories in Kabete, Kenya, on animal trypanosomiases. In 1990 he received an award from the "Fondation Internationale pour la Substitution de l'Expérimentation Animale (FISEA), Luxembourg, in recognition of scientific work serving animals by reducing the use of experimental animals. In 1991 he received the "Felix-Wankel-Tierschutz-Forschungspreis" of the Ludwig-Maximilians-Universität, Munich, Germany, an award for research on *in vitro* tests for the determination of drug resistance in pathogenic trypanosomes. Since 1993 he has been employed at the Onderstepoort Veterinary Institute in South Africa. In September 1996 he started working in the field of cowdriosis. In March 1998 he was promoted to specialist scientist.

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Acknowledgements

I would like to express my gratitude to my promoters Profs. F. Jongejan and J.P.M. van Putten who provided me with the opportunity to obtain a Ph.D. at the Utrecht University.

Special thanks go to Prof. B.A. Allsopp, colleague, co-promoter and my programme manager from 1996 up to 2003, for his support, assistance and for "de-germanising" my written English.

Many thanks to Dr. L. Bell-Sakyi who set the ball rolling getting me interested in compiling this thesis, as well as for her moral support, for the reading of the manuscript and for her helpful comments.

I would like to extend my appreciation to Dr. S.W. Vogel, Dr. J.D. Bezuidenhout and Mrs. S. Brett who built up a heartwater literature collection at the Onderstepoort Veterinary Institute which made my life much easier when looking for all the references.

I am obliged to all my colleagues from the Onderstepoort Veterinary Institute, especially Mrs. A.I. Josemans, who collaborated and contributed in one way or the other to the work presented in this thesis, many thanks to you all.

Furthermore, I am thankful to Prof. J.S. Ahmed, who was my mentor during my first years in parasitological research, influencing my career considerably.

Last not least I would like to thank my dear wife Monika for her support and patience while I was writing up this thesis.

"When you know a thing, to hold that you know it; and when you do not know a thing, to allow that you do not know it – this is knowledge".

Kung Fu Tse
(551 -479 BC)