DFID ANIMAL HEALTH PROGRAMME PROJECT R7363

FINAL TECHNICAL REPORT

Investigation of the immunogenic potential of heartwater (*Cowdria ruminantium*) grown in tick cell lines

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Executive Summary

Growth of *Cowdria* (now known as *Ehrlichia*) *ruminantium*, the causative agent of the tick-borne disease heartwater in domestic ruminants, was recently achieved in tick cell lines at CTVM. The project aimed to exploit this development by investigating the immunogenic potential of *Cowdria* in tick cells. Existing vaccines and diagnostic assays for heartwater require improvement, and it is hoped that tick cell-derived *Cowdria* will contribute to this. The research activities focussed on optimising the *in vitro* culture system, characterising *Cowdria* in tick cells at the morphological, antigenic and molecular levels, and testing its infectivity and immunogenicity in sheep.

Growth of six antigenically diverse *Cowdria* isolates from West and South Africa and the Caribbean was achieved in ten cell lines representing six species of four ixodid tick genera, including the vector *Amblyomma variegatum*. Light and electron microscopy revealed that *Cowdria* in tick cell lines resembled the developmental stages seen *in vivo* in *Amblyomma* ticks, and differed from *Cowdria* in mammalian cells, and from other ehrlichial pathogens in tick cells, both *in vivo* and *in vitro*. The immunodominant antigen expressed in tick cell stages of *Cowdria* was not the 32kD MAP1 seen in mammalian stages, but a smaller 29kD protein which shared some epitopes with MAP1. RT-PCR revealed differential stage-specific transcription of some members of the *Cowdria map1* multigene family in tick and mammalian cell stages.

Cowdria in tick cell lines was generally non-pathogenic; only 1/105 sheep inoculated with *Cowdria*-infected tick cells developed heartwater. However, certain combinations of *Cowdria* isolate and tick cell line, grown under specific conditions and inoculated intravenously as freshly harvested whole live cultures, were highly immunogenic in sheep. Of 27 sheep inoculated with the Gardel isolate in *A.variegatum* cells, 25 were fully protected (no clinical response) against a virulent homologous experimental challenge which induced severe heartwater in 11/11 naïve control sheep. A further 6/6 sheep immunised with the same material were fully protected against virulent heterologous challenge with the antigenically related Ball 3 isolate. *Cowdria* in tick cells induced an atypical and highly variable serological response in protected sheep, suggesting that this material engendered an immune response different from that produced following natural infection or immunisation with mammalian stage *Cowdria*.

Laboratory trials were carried out in sheep by collaborators in Ghana, The Gambia and Kenya, using the Gardel isolate in *A.variegatum* cells sent from CTVM and a local heterologous challenge isolate. The results indicated that travel for 6-10 days by courier adversely affected the immunogenicity of the material, although some sheep did seroconvert following inoculation. The Gardel isolate was found to offer a poor level of protection against the African challenge isolates, indicating the need to immunise with local isolates. A subsequent unsuccessful trial in Kenya, with homologous challenge, indicated a possible important role for CO_2 in the culture system.

The project demonstrated that *Cowdria* grown in tick cell lines offers an alternative and complementary source of antigens and effective immunogens which will contribute to improved immunisation and diagnosis of heartwater.

1. Background

Heartwater is a disease of domestic and wild ruminants transmitted by ticks of the genus *Amblyomma*, which occurs throughout sub-Saharan Africa and on some islands in the eastern Caribbean (Camus et al, 1996). The causative agent is the rickettsia *Cowdria ruminantium*, an obligately intracellular prokaryote which resides in membrane-lined vacuoles within cells of the reticulo-endothelial system of susceptible mammals and gut cells, haemocytes and salivary gland cells of vector ticks. During the course of the present project, *Cowdria* was reclassified, on the basis of extensive genetic analyses, as a member of the genus *Ehrlichia* (Dumler et al, 2001) in the family Anaplasmataceae; however for clarity the name *Cowdria* will be retained in this report.

C.ruminantium infection causes sudden death in a significant proportion (up to 100%, reviewed by Camus et al, 1996) of susceptible affected stock, particularly when introduced into an endemic area either by importation from other countries in or outside Africa for crossbreeding programmes, or when locally-raised animals are moved from a tick-free (e.g. urban) to a tick-infested (e.g. rural) area, as has been reported in Ghana (Anon, 1997). Intensive acaricide regimes for valuable or highly productive stock will result in fully susceptible populations which may suffer enormous losses following cessation of tick control, as seen in Zimbabwe (Norval & Lawrence, 1979; Peter et al, 1998). The expected relaxation in intensive dipping in Kenya following the introduction of a commercial vaccine against East Coast fever is likely to lead to a similar upsurge in heartwater cases in grade cattle (S. Mbogo, pers. comm. to EU/African consortium of laboratories engaged in heartwater vaccine research, 1998).

Control of heartwater in local ruminant breeds in some parts of Africa can be achieved by maintenance of endemic stability, at the expense of productivity and with the problems associated with the vector ticks (tick worry, hide damage, secondary infections, immunosuppression, dermatophilosis) (Bell-Sakyi et al, 1996). The options for keeping alive and maintaining productivity in more valuable, exotic or improved livestock remain at present intensive tick control, zero-grazing or vaccination (Uilenberg, 1983). A live blood vaccine used for many years in Southern Africa and a tick-derived vaccine (Oberem & Bezuidenhout, 1987) both suffer disadvantages of requiring trained personnel, monitoring, possible treatment of severe reactions, and lacking cross protectivity (Camus et al, 1996).

Development of effective, safe and affordable vaccines is a priority area for heartwater research worldwide (Uilenberg, 1996). At the time the project started, the mid-term review of the World Bank sponsored Ghana NARP had identified tick-borne diseases (TBD) (dermatophilosis and heartwater) as a major constraint to the livestock sector and prioritised the need for further research in this area. A specific objective of proposals submitted to DFID by the Ghana Government for bilateral funding was control of TBD including heartwater. In Kenya, a KARI/DFID research priority setting panel had concluded that TBD vaccine development was of the highest priority for KARI animal health research activities. In The Gambia, ITC had reported heartwater as a serious health problem in indigenous small ruminants. Subsequently the economic importance of heartwater in Southern Africa and the need for improved control measures have been emphasised (Minjauw, 2000).

Cowdria can be cultivated *in vitro* in bovine, ovine and caprine and human endothelial cell cultures (Bezuidenhout et al, 1985; Brett et al, 1992; Martinez et al, 1990; Totte et al, 1993) as well as cells from some wild African mammals (Smith et al, 1998). *Cowdria* elementary bodies (EB) harvested from such cultures have been used as the basis for experimental inactivated or attenuated vaccines by research groups in Zimbabwe (Mahan et al, 1995), Kenya (Anon, 1999), Guadeloupe (Martinez et al, 1994) and Senegal (Gueye et al, 1994). Being derived from mammalian cell culture systems, these vaccines carry the innate risk of inducing immunity to mammalian products and the potential risk of transmitting other mammalian pathogens. At present the high level of dose required (100-600μg per animal per dose, 2-5 doses) combined with the low yield of antigen (around 2.5μg/ml of culture

supernate under CTVM culture conditions), would render such vaccines too expensive for widespread use in endemic areas, particularly in small ruminants. Field trials of inactivated EB vaccines in southern Africa have reported significant reduction in mortality but not morbidity (Mahan et al, 2001). A major drawback of such inactivated vaccines remains their ability to reduce mortality but not prevent clinical disease, and the requirement for 2-3 immunisations up to 8 weeks apart (Mahan et al, 2001) during which time the animals must presumably be kept tick-free. Since each *Cowdria* isolate cross-protects against a limited range of other antigenically related organisms, the potential for live attenuated EB vaccines is severely limited by the small number of isolates so far attenuated *in vitro*.

Recent developments in *Cowdria* serodiagnosis, of ELISA tests using crude or recombinant EB antigens (Van Vliet et al, 1995; Anon, 1997; Mboloi et al, 1999; Katz et al, 1997; Sumption et al, in preparation), have greatly improved the accuracy and range of epidemiological studies and experimental diagnosis (Jongejan & Bekker, 1999). However, the available assays still suffer from lack of specificity or sensitivity in some circumstances, particularly with field cattle in heartwater-endemic areas (Anon, 1997: Semu et al, 2001) and small ruminants exposed to other closely-related *Ehrlichia* species (Grace, 1999). Identification of additional *Cowdria*-specific antigens, which may be expressed by developmental stages other than the EB, could lead to further improvements in serodiagnosis.

Attempts to grow *Cowdria* in tick cells date back over 25 years. Andreasen (1974a & b) reported growth of *C.ruminantium* in primary cultures of nymphal *Amblyomma* cells and infectivity for sheep after 9 days *in vitro*. Subsequent attempts using tick organ cultures, primary cell cultures and established cell lines met with repeated failure (reviewed by Uilenberg, 1983; Yunker et al, 1988; L.Bell-Sakyi unpublished results, 1991-4). Recently, an *Ixodes scapularis* cell line IDE8 established by Munderloh and colleagues (Munderloh et al, 1994) was shown to be infectable with *Ehrlichia canis* (Ewing et al, 1995), *Ehrlichia equi* (Munderloh et al, 1996a), *Anaplasma marginale* (Munderloh et al, 1996b), *Ehrlichia chaffeensis* (U.Munderloh, pers. comm.) and the human granulocytic ehrlichiosis (HGE) agent (Munderloh et al, 1999). These economically important tick-borne animal and human pathogens are closely related phylogenetically to *Cowdria* (Van Vliet et al, 1992); indeed, the recent reclassification (Dumler et al, 2001) placed *Cowdria* together with *E.canis* and *E.chaffeensis*, while combining *E.equi*, HGE agent and *Ehrlichia phagocytophila* as *Anaplasma phagocytophila*, all within the family Anaplasmataceae. *A.marginale* grown in IDE8 cells has been shown to be highly immunogenic in cattle when administered live (Blouin et al, 1998) or as an inactivated preparation (Kocan et al, 2001), and when used as antigen in a c-ELISA gave superior results to the standard complement fixation test for anaplasmosis (Saliki et al, 1998).

Work ongoing at CTVM at the start of the project had resulted for the first time in the successful infection with *Cowdria* of two tick cell lines, IDE8 and an *A.variegatum* larva-derived cell line AVL/CTVM13 (Anon, 1999; Bell-Sakyi et al, 2000a & b). Initial studies indicated establishment times of 6 and 10 weeks for the Gardel isolate of *Cowdria* in the IDE8 and AVL/CTVM13 lines respectively. Once established however, rickettsial growth in the IDE8 line kept up with cell growth, such that an infected culture could survive and be propagated for over a year. There was little evidence of significant cytopathic effect in infected cells of either line, and actively dividing cells containing *Cowdria* inclusions could be seen in Giemsa-stained preparations. The presence of *Cowdria* in the tick cells was confirmed by PCR and transmission EM, and karyotyping of infected IDE8 cells confirmed their tick origin and absence of bovine cells. Light microscopy revealed differences in morphology between *Cowdria* in bovine and tick cells *in vitro*; in particular, the spherical EB stage was not seen in infected tick cell cultures (Bell-Sakyi et al, 2000a & b). *Cowdria* in IDE8 cells was infective *in vitro* for bovine endothelial cells but, at the time the project started, sheep inoculated with *Cowdria* in IDE8 cells (4 animals) or AVL/CTVM13 cells (2 animals) had not become detectably infected. These observations indicated significant differences between mammalian and tick stages of the *Cowdria* life cycle, which might be reflected in exploitable antigenic differences.

Concurrent with the work of this project, advances in molecular biological methods enabled researchers to begin to investigate stage-specific gene transcription and protein expression in tick-borne pathogens at the molecular level. In particular, reverse-transcriptase PCR (RT-PCR) allowed identification of the genes transcribed at particular development stages and improved understanding of the role of these genes, and the proteins for which they code, in the host-vector-pathogen relationship. Multigene families, coding for slightly different versions of major surface proteins, have been identified as being stage-specifically transcriptionally active in *A.marginale* (Rurangirwa et al, 1999, 2000; Barbet et al, 2001) and *E.canis* (Unver et al, 2001). The immunodominant surface protein in *Cowdria*, the 32kD MAP1, is known to be encoded by one of such a multigene family (Sulsona et al, 1999). The tick-borne spirochaete *Borrelia burgdorferi* has been shown to modulate expression of a family of antigenic surface proteins depending on its environment in the mammalian host or tick vector, and in response to temperature changes (Ryan et al, 1998; Obonyo et al, 1999). Similarly, temperature affects protein expression in the tick-borne human pathogen *Rickettsia rickettsii* propagated in tick cell lines (Policastro et al, 1997).

At the start of the project, *Cowdria* grown in tick cells was seen as offering a potentially safer source of material for immunisation compared with mammalian cell culture systems, minimising the risk of accidental transfer of other pathogens such as viruses and prions. Mammalian cell/*Cowdria* systems generally involve monolayer cultures, with resultant surface area limitations, but tick cells do not exhibit contact inhibition *in vitro*, offering the possibility of much higher yields of cells, and consequently *Cowdria* antigen, per ml of culture either as monolayers or as suspension cultures. The *Cowdria*/tick cell culture system was also seen as an alternative or complementary source of immunogenic material for improving diagnostic tests and development of recombinant vaccines. It was expected that characterisation of *Cowdria* grown in tick cells would help to elucidate the relationship between *Cowdria* and its tick vector, and how the organism makes the transition between the physiologically very diverse environments of mammalian host and vector tick.

2. Project Purpose

The project purpose agreed at the outset was to develop improved methods of diagnosis and control of tickborne disease, specifically, a simple and cheap *in vitro* cultivation system for *Cowdria* using tick cells instead of mammalian cells, which should result in immunogenic material for vaccination against heartwater and disease diagnosis free of mammalian-derived contaminants. The project was originally funded for two years; during this period certain combinations of *Cowdria* isolate and tick cell line were found to be highly immunogenic in sheep. Accordingly, the project was granted a year's extension with the aim of confirming this finding in laboratory trials with larger groups of animals.

3. Research Activities

The research was carried out mainly at CTVM, by Lesley Bell-Sakyi and, in years 1-2, Edith Paxton. *In vivo* sheep experiments were carried out with technical assistance from Paul Wright, Maureen Jordan and Kim Davaies. Some aspects of the antigenic and molecular characterisation of *Cowdria* in tick cells were done in collaboration with Keith Sumption, Stuart Smith and Milagros Postigo of CTVM, as part of the EU-funded Cowdriosis network. Throughout the project, Ulrike Munderloh of the University of Minnesota provided advice on propagation of ehrlichial pathogens in tick cells. Overseas laboratory trials were carried out in collaboration with the Ghana Government Veterinary Services Department (E.B.M.Koney, Otilia Dogbey), International Trypanotolerance Centre, The Gambia (Raffaele Mattioli, Joseph Faye), NVRC/KARI, Kenya (Sam Mbogo, F.D.Wesonga, Peter Gitau), and ILRI, Kenya (Subhash Morzaria, Duncan Mwangi, Paul Spooner and others). Links were also established with other groups in the EU Cowdriosis network, in particular with Utrecht University (Cornelis

Bekker, Frans Jongejan) and CIRAD-EMVT (Dominique Martinez, Philippe Totte). At CTVM, administrative support was provided by Pauline McManus, Lesley More and Sophie Carver, and technical backup by Ann Morrison.

The project activities can be divided into three main sections: 1) *In vitro* cultivation of *Cowdria* in tick and bovine endothelial cell lines, 2) Characterisation of *Cowdria* grown in tick cell lines, and 3) *In vivo* laboratory trials of the immunogenicity for sheep of *Cowdria* grown in tick cell lines.

3.1. *IN VITRO* CULTIVATION OF *COWDRIA* IN TICK AND BOVINE ENDOTHELIAL CELL LINES

The aim of this activity was to optimise the methods required to cultivate *Cowdria* in tick cells *in vitro*, and to establish as many *C.ruminantium* isolates in as many tick cell lines as possible.

3.1.1 Tick cell lines: tick cell lines were maintained at CTVM by standard procedures, with weekly medium changes (removal and replacement of 1/2-3/5 volume), subculture at 2-8 week intervals, and cryopreservation with 10% DMSO as required (Bell-Sakyi 1991; Munderloh et al 1994; Bell-Sakyi et al 2000a & b). Tick cells were grown in 5ml medium in sealed 25cm^2 flasks (Nunc) or 2ml medium in sealed flat-sided culture tubes (Nunc) in dry incubators (28, 31, 37°C); some cultures were also held at 37°C in a humidified incubator gassed with 5% CO_2 in air. Tick cell lines tested for ability to support growth of *Cowdria* are summarised in Table 1.

3.1.2 Bovine endothelial cell line: the cell line designated BPC, established at CTVM from bovine pulmonary artery endothelium, was grown at 37°C in Glasgow MEM supplemented with 20% FCS, 2mM L-glutamine, penicillin/streptomycin/amphotericin B (Mutunga et al, 1998). Cells were maintained in 25 or 75cm^2 flasks (Nunc) either with filter caps in a humidified incubator gassed with 5% CO_2 in air (years 1-2) or sealed in a dry incubator (year 3), and were subcultured at 4-11 day intervals following dissociation with trypsin/EDTA.

3.1.3. Growth of *Cowdria* in BPC: Six *C.ruminantium* isolates (Table 2) were maintained in BPC at 37°C in Glasgow MEM supplemented with 10% TPB, 10% newborn calf serum, L-glutamine and antibiotics as above (GMEM/TPB/NCS) (Mutunga et al, 1998). Growth of *Cowdria* was monitored visually and in Giemsa-stained cytospin smears. An experiment was carried out to investigate the decline in infectivity with time of *Cowdria* EB harvested from BPC cultures; the method and results are given in Appendix 4.

Table 1. Origins and culture conditions of tick cell lines maintained at CTVM and tested for ability to support growth of *C.ruminantium*.

Cell line	Tick species	Instar	Culture medium[1]	Incubation temp.	Reference
AVL/CTVM13	*Amblyomma variegatum*	Larva	L-15/L-15B	37°C	Bell-Sakyi et al, 2000b
AVL/CTVM17	*Amblyomma variegatum*	Larva	L-15/L-15B, L-15/H-Lac/L-15B	37°C	Unpublished
BDE/CTVM16	*Boophilus decoloratus*	Embryo	L-15	28-31°C	Unpublished
BME/CTVM2	*Boophilus microplus*	Embryo	L-15	28-31°C	Unpublished
BME/CTVM6	*Boophilus microplus*	Embryo	L-15	28-31°C	Unpublished

HAE/CTVM9	*Hyalomma a.anatolicum*	Embryo	L-15/MEM	31°C	Bell-Sakyi, 1991
HAE/CTVM10	*Hyalomma a.anatolicum*	Embryo	L-15/H-Lac	31°C	Bell-Sakyi, 1991
IDE8	*Ixodes scapularis*	Embryo	L-15B	31°C	Munderloh et al, 1994
IRE/CTVM18	*Ixodes ricinus*	Embryo	L-15/H-Lac	28°C	Unpublished[2]
RAE/CTVM1	*Rhipicephalus appendiculatus*	Embryo	L-15	31°C	Bell, 1983
RAN/CTVM3	*Rhipicephalus appendiculatus*	Nymph	H-Lac	28-37°C	Bekker et al, 2002
RAE25	*Rhipicephalus appendiculatus*	Embryo	L-15	31°C	Kurtti et al, 1982

[1] Culture media - (all supplemented with 2mM L-glutamine, penicillin/streptomycin) - L-15: L-15 Leibovitz medium with 10% tryptose phosphate broth (TPB), 20% foetal calf serum (FCS); H-Lac: Hanks BSS with 0.5% lactalbumin hydrolysate, 20% FCS; L-15/H-Lac: equal parts L-15 and H-Lac; L-15/MEM: equal parts L-15 Leibovitz medium and MEM(with Hanks salts) with 10% TPB, 20% FCS; L-15B: as L-15 but supplemented according to Munderloh & Kurtti (1989) and with 5% FCS; L-15/H-Lac/L-15B: equal quantities of the three complete media.

[2] This cell line was established during the lifetime of the project from embryonic *I.ricinus* (originating from Dorset, UK) provided by Dr Ernest Gould, NERC-CEH Oxford; method based on that of Bell-Sakyi (1991).

Table 2. Origins of *C.ruminantium* isolates tested for ability to infect and grow in tick cell lines.

***C.ruminantium* isolate**	**Geographical origin**	**Reference**
Gardel	Guadeloupe, West Indies	Uilenberg et al, 1985
Attenuated Gardel*	Guadeloupe, West Indies	Martinez 1997
Attenuated Senegal*	Senegal, West Africa	Jongejan, 1991
Sankat 430	Ghana, West Africa	Bell-Sakyi et al, 1997
Pokoase 417	Ghana, West Africa	Bell-Sakyi et al, 1997
Ball 3	South Africa	Haig, 1952
Welgevonden	South Africa	Du Plessis, 1985

*Kindly provided by Dominique Martinez, CIRAD-EMVT

3.1.4. Preparation of *Cowdria* EB stabilates: Stabilates for *in vivo* challenge of sheep were prepared from four *Cowdria* isolates: Gardel at passage 15 (STAB1), Sankat 430 at passage 10 (STAB2), Ball 3 at passage 8 (STAB3) and Welgevonden at passage 8 (STAB4). For each isolate, supernate from heavily infected cultures (with most of the monolayer destroyed) was centrifuged at 400 × g for 5 min to remove the majority of intact endothelial cells, and the resultant EB suspension was chilled on ice for stabilate preparation as follows. Dimethyl sulphoxide (DMSO) was added dropwise with swirling to give a final DMSO concentration of 10%, the suspension was gently mixed and dispensed immediately in at least 20 × 1ml aliquots into ice cold cryotubes (Nunc), held on dry ice, and transferred within 15 minutes to the vapour phase of a liquid nitrogen refrigerator. Pre- and post-freezing viability and *in vitro* infectivity of all stabilates were assayed; all except Welgevonden were also characterised post-freezing *in vivo* in sheep.

3.1.5. Infection of tick cell lines with *Cowdria*: The method described in Bell-Sakyi et al (2000a & b; Appendix 12) was followed. Supernate containing EB from *C.ruminantium*-infected BPC cultures was added to tick cell cultures in volumes of 0.5-1ml per tube, 1-2.5ml per flask. Tick cells were then incubated at the appropriate temperature, sometimes preceded by 24hr at 37°C, and maintained with weekly medium changes for up to 20 weeks. Giemsa-stained cytospin smears of whole resuspended cultures were prepared at regular intervals and examined for presence of spherical *Cowdria* EB from the inoculum both extra- and intracellular, intracellular *Cowdria* colonies, and extracellular pleomorphic *Cowdria* organisms. If there was no evidence of *Cowdria* infection or growth in the tick cells after 10-20 weeks,

the cultures were discarded. If however infection was detected, the cultures were maintained further and subculture onto cells of the same or a different tick cell line was attempted. In the latter case, if transfer was between cell lines capable of growth at the same temperature, the supernate was centrifuged for 5-10min at 1500 × g to remove intact tick cells prior to inoculation. This was not done when the donor cells were known to be incapable of growth at the recipient culture's incubation temperature.

3.1.6. Maintenance of *Cowdria*-infected tick cell lines: *Cowdria*-infected cultures were maintained with weekly medium changes and subcultures, as described in Bell-Sakyi et al (2000a), carried out as required. Infected cultures were cryopreserved as follows: cells were resuspended by pipetting, centrifuged at 200 × g for 5 min, resuspended in 1-2ml of original supernate and held on ice. An equal volume of ice-cold culture medium with 20% DMSO was added dropwise with swirling, mixed gently and dispensed immediately into 2-4 ice-cold cryotubes. The tubes were held on dry ice and transferred within 1 hour to the vapour phase of a liquid nitrogen refrigerator. Infected cultures were resuscitated by thawing rapidly, and the contents of 1 cryotube were added to a fresh 25cm^2 flask of the appropriate cell line. Medium was changed after 24hr, and weekly thereafter.

3.2. CHARACTERISATION OF *COWDRIA* GROWN IN TICK CELL LINES

This activity involved comparison of *Cowdria* grown in tick and bovine endothelial cell lines at the morphological, antigenic and molecular levels.

3.2.1. Light microscopy: Growth and development of *Cowdria* in tick cells was followed in cytospin smears prepared from whole resuspended cultures or from cell-free supernate prepared by centrifugation at 1,500 × g for 5 min. Smears were air-dried, fixed for 2min in methanol, stained with Giemsa with added Azur II diluted in tap water (10% for 20min or 5% for 40min), and examined at ×500 and ×1,000 magnification under oil immersion using a Leitz Laborlux microscope.

3.2.2. Electron microscopy: Samples from uninfected and *C.ruminantium*-infected IDE8 and AVL/CTVM13 cultures were prepared for transmission electron microscopy as follows. Adherent cells were removed by gentle pipetting and the resultant 5ml cell suspension was centrifuged at 400 x g for 10 min at room temperature. The supernatent medium was removed and discarded, and the cell pellet resuspended in cold 3% glutaraldehyde in 0.1M sodium cacodylate buffer. The samples were post-fixed in 1% osmium tetroxide in 0.1M sodium cacodylate buffer, dehydrated through a graded acetone series and embedded in araldite; 60nm sections were cut on a diamond knife using an ultramicrotome (Reichert OMU4 Ultracut), mounted on 200 mesh copper grids, stained with uranyl acetate and lead citrate (LKB Ultrostainer), and viewed and photographed using a Philips 400 transmission electron microscope.

3.2.3. Western blotting: Extracts of whole, PBS-washed uninfected and *C.ruminantium*-infected tick cells, uninfected BPC and BPC-derived *C.ruminantium* EB were prepared by washing twice in PBS and suspending the resultant pellets in PBS containing 0.5% (v/v) Nonidet P40, 0.5% (v/v) sodium deoxycholate and 5× concentrated protease inhibitor ("General use protease inhibitor cocktail", Sigma). The suspension was mixed for 30min at 4°C, centrifuged at 15,000 × g for 20min at 4°C, and the supernate containing soluble proteins was stored at -20°C. The proteins were separated by SDS-PAGE according to Laemmli (1970) on 12% acrylamide gels; biotinylated molecular weight markers (Sigma) were included on every gel. Protein transfer was performed onto PVDF membrane (Millipore) using a semi-dry blotter (Bio-Rad) according to the manufacturer's instructions. The resultant blots were temporarily stained with 0.1% Ponceau S in 5% acetic acid to confirm protein transfer and to allow detachment of the molecular weight marker tracks prior to probing test tracks with specific antibody. All blots were rinsed repeatedly in wash buffer containing 0.5M

NaCl, 50mM Tris, 0.1% Tween 20 at pH 7.4 (TNT) to remove the dye, then blocked overnight in 5% skimmed milk (Marvel) in TNT (MTNT). Test blots were washed for 3 × 10min in TNT.
For reaction with monoclonal antibodies (mab) raised against bovine endothelial cell culture-derived *C.ruminantium* (Senegal) EB (Jongejan et al, 1991), the blots were incubated overnight with mab 4F10B4 diluted 1/100 in TNT or one of mabs 1E5H8, 3D8H11 and 1E3H10 diluted 1/5 in TNT. Test and molecular weight marker blots were then rinsed in TNT as before and incubated for 1h with gentle rocking in, respectively, horseradish peroxidase anti mouse IgG conjugate (Scottish Antibody Production Unit) diluted 1/500 in MTNT, or streptavidin-horseradish peroxidase conjugate (Scottish Antibody Production Unit) diluted 1/2000 in MTNT. After rinsing as before in TNT, all blots were rinsed once in deionised H_2O and developed in DAB/H_2O_2 (Sigma).
For reaction with pre-inoculation, pre-challenge and day 28 post-challenge sera from selected test sheep, test blots were processed as described above, except that the first incubation was carried out for 2h in sheep sera diluted 1/50 in MTNT, and the second incubation was for 1h in horseradish peroxidase anti sheep IgG (Scottish Antibody Production Unit) diluted 1/500 in MTNT.

3.2.4. Polymerase chain reaction (PCR): The PCR was carried out following the method of Ngumi (1997) on DNA extracted using a commercial kit (Qiagen) from uninfected and *Cowdria*-infected cultures. The combination of primers used (HE1, the highly variable region in the 16s rRNA gene which is specific for *C.ruminantium*, and HE3, a short complimentary sequence identified from *C.ruminantium* [Crystal Springs] which is conserved between *Ehrlichia* spp, *C.ruminantium* and *Anaplasma* spp.) produces a 388 base pair sequence in PCR. A master mix of the reaction constituents was made such that each 50µl PCR reaction comprised 10mM Tris HCl pH 8.3, 50mM KCl, 0.001% w/v gelatin, 200µM nucleotides (dNTPs, Pharmacia Biotech), 0.2µM of HE1 and HE3 primers (Cruachem), and 2 units of Ultrataq DNA polymerase (Thermometric Ltd). Aliquots of 45µl master mix were put into 0.5ml reaction tubes and overlayed with 50µl mineral oil. Test and control DNA samples were added as 5µl aliquots through the oil. The PCR conditions were a first denaturation at 95°C for 5min followed by 39 cycles of 1min at 94°C (denaturation), 1min at 55°C (annealing) and 2min at 72°C (extension), with a final extension of 10min at 72°C. The PCR products were run on 1% agarose gels with ethidium bromide in TBE buffer (Ngumi, 1997).

3.2.5. Reverse-transcriptase PCR (RT-PCR): This was carried out as described in Bekker et al (2002; Appendix 12). Total RNA was isolated from *C. ruminantium*-infected BPC and tick cells using a RNeasy mini kit (Qiagen). Total RNA was treated before elution with RNase-free DNase I (10U). Following elution, the RNA was quantified in a spectrophotometer at A_{260}, while purity was confirmed by a spectrophotometric A_{260}/A_{280} ratio of over 1.8.The RNA (1 µg) was used to generate cDNA using a first strand cDNA synthesis system (SUPERSCRIPT™, Life Technologies) and random hexamer primers, according to the manufacturer's instructions. The primer pairs (0.4 µM), one general (g-for) and one specific for each of three *map1* genes (Bekker et al, 2002) were used to amplify each *map1* gene in a PCR reaction using the same cDNA batch as template. PCR parameters were as follows: amplification for 30 cycles at 94°C for 30 sec, 50°C for 1 min, and 72°C for 1 min; and a final elongation step at 72°C for 5 minutes. A negative control that included all reagents except the reverse transcriptase was included in every test to confirm that genomic DNA was not present in the RNA preparations. The PCR products were run on 1% agarose gels with ethidium bromide in TBE buffer (Ngumi, 1997).

3.3. *IN VIVO* LABORATORY TRIALS OF THE IMMUNOGENICITY FOR SHEEP OF *COWDRIA* GROWN IN TICK CELL LINES

The aims of this activity were to determine the infectivity and/or immunogenicity for sheep of *Cowdria* grown in tick cell lines and investigate its potential use for improved vaccine development.

3.3.1. Laboratory trials carried out at CTVM: All experiments were carried out following a standard protocol, under Home Office and SAPO regulations.

3.3.1.1. Sheep: Suffolk or Suffolk cross lambs aged between 5 and 14 months and never previously exposed to ticks, were purchased from local suppliers, dewormed, and in some cases their feet were bathed in formalin to control footrot. They were maintained in the MAFF isolation facility at CTVM, and fed on hay and water *ad lib*, with concentrates twice daily. Their necks were shaved monthly to allow easy access to the jugular vein.

3.3.1.2. Inoculum: Following pre-infection observations (see below), test sheep were inoculated intravenously in the jugular vein with 1-3ml of whole *Cowdria*-infected tick cell culture containing *Cowdria*, tick cells and culture medium, within 30min of harvest. In one experiment, an aliquot of inoculum was treated to simulated travel to Africa by courier prior to inoculation.

3.3.1.3. Monitoring: Rectal temperature was taken pre-infection and daily thereafter; blood for serum and PCR was collected by jugular puncture pre-infection, weekly thereafter, and pre-euthanasia (if applicable). Animals were monitored daily for clinical response (increased respiration rate, dullness, inappetance, diarrhoea, nervous signs etc). Any sheep showing severe clinical signs (respiratory distress/nervous signs) and/or temperature >40.8°C for more than 2 consecutive days was euthanased by intravenous inoculation of 20ml euthatal, and a post mortem carried out to determine volume of hydrothorax and hydropericardium, presence of ascites, and *Cowdria* infection rate in brain capillary endothelial cells.

3.3.1.4. Challenge: All surviving test sheep were challenged 28-42 days after initial inoculation, along with naïve control sheep, with 1ml of a 1:10 dilution of *C.ruminantium* EB stabilate. The vial of stabilate was thawed rapidly by immersion in warm water, the contents immediately mixed gently but thoroughly with 10ml GMEM/TPB/NCS, and 1ml inoculated i/v into the jugular vein of each test sheep, followed by the control sheep. In experiments involving more than 10 sheep, 2 or 3 vials of stabilate were thawed simultaneously, pooled into the appropriate volume of diluting medium to maintain the specified dilution rate, and inoculated as above. In all cases, challenge of all sheep was completed within 13min of thawing. In one experiment, two sheep were challenged with 2.4ml fresh *C.ruminantium* (Senegal) EB suspension, administered within 9min of harvest; the virulent Senegal culture was kindly provided by Cornelis Bekker, Utrecht University. Monitoring was carried out as above to day 28 of challenge, when surviving sheep were euthanased.

3.3.1.5. Serology: Serum was separated from clotted jugular blood by centrifugation and stored at -20°C. All sera were tested for antibodies to *C.ruminantium* by c-ELISA (Anon, 1997; Sumption et al, in preparation) and MAP1-B ELISA (Van Vliet et al, 1995, Mboloi et al, 1999) at the end of the experiment.

3.3.2. Laboratory trials carried out by collaborators in laboratories in Ghana, The Gambia and Kenya using infected tick cells produced at CTVM: The protocols followed were based on those used at CTVM, and are described in Appendix 9 for the experiments carried out in Ghana, The Gambia and Kenya (NVRC/KARI). The protocol followed for the experiment carried out at ILRI is described in Appendix 10. Mr Joseph Faye, a senior laboratory technician at ITC, The Gambia, underwent a month's attachment at CTVM in March 2000 during which he received training in the specific techniques required to carry out the laboratory trial at ITC.

3.4. MODIFICATIONS TO PROPOSED RESEARCH ACTIVITIES

Additions to the proposed research activities included the RT-PCR studies carried out with Utrecht University, and the establishment of the first all-British tick cell line IRE/CTVM18 from

ticks provided by CEH Oxford. Maintenance by the project of a panel of tick cell lines enabled Lesley Bell-Sakyi to supply various research institutes with tick cells and training in their cultivation (see Appendix 11). Through the link established with CIRAD-EMVT, uninfected and *Cowdria*-infected tick cell lines were transferred to Guadeloupe to enable researchers there to carry out immunisation trials and cell-mediated immunity studies. Uninfected and *Cowdria*-infected tick cells were also transferred with appropriate training on request to the Ghana Government Veterinary Services Department for locally funded immunisation trials in sheep using Ghanaian *Cowdria* isolates.

Proposed activities which were not carried out included the following: Groups of sheep immunised with inactivated EB were not included in trials at CTVM, as the current Home Office Project Licence did not permit this. Although correspondence was maintained with the Zimbabwe/Florida/USAID heartwater research group, they did not make any Zimbabwean *Cowdria* isolates available for cultivation in tick cells. Anti-MAP1 rabbit serum was not provided by Utrecht University. Although there were plans during the third year for at least two laboratory trials at ILRI, and a training visit to CTVM for an ILRI technician, only one trial was carried out. Apart from the above, all planned inputs were achieved.

4 Outputs

The original outputs specified for the project were:
1. System for growth of *Cowdria* in cell lines from one or more tick species
2. Increased knowledge of *Cowdria* development in tick cells
3. Production of useful quantities of immunogenic *Cowdria* material in tick cell cultures free from harmful contaminants.

As outlined in the Project Completion Summary Sheet above, all anticipated outputs were achieved, with the proviso that it was not possible within the lifetime of the project to attempt to purify the immunogenic *Cowdrial* tick cell material by removal of the mammalian-derived foetal calf serum present in the tick cell culture medium.

4.1. *IN VITRO* CULTIVATION OF *COWDRIA* IN TICK AND BOVINE ENDOTHELIAL CELL LINES

At the start of the project, growth of a single isolate of *C.ruminantium*, Gardel, had been successfully achieved in two tick cell lines, IDE8 and AVL/CTVM13. Throughout the lifetime of the project, attempts were made to establish all of the six available *Cowdria* isolates (Table 2) in as many of the available tick cell lines (Table 1) as possible. Tick cell cultures were inoculated with supernate containing *Cowdria* either from BPC cultures, or from other tick cell lines. Growth of *Cowdria* in tick cells was continuous (maintained for over 200 days, with regular subculture) or finite (less than 200 days, subculture to same cell line impossible or limited to one or two passages). The results are presented in tables 3 - 5.

Tick cell lines from three species were susceptible to infection with mammalian stage *Cowdria* (EB), the vector *A.variegatum*, and two non-vector species *I.scapularis* and *I.ricinus* (Table 3). Of these, the two *Ixodes* species were the most easily infected, with success rates of 15% (14/96) for IDE8 and 43% (3/7) for IRE/CTVM18 over all the *Cowdria* isolates. *A.variegatum* cultures were only infected in 3/76 attempts, and were not considered suitable for routine establishment of *Cowdria in vitro*.

Table 3. Results of inoculation of tick cell lines with supernate from BPC cultures containing *Cowdria* EB. + = infection achieved, - = tried and failed

Tick cell	***Cowdria* isolate**

line	Gardel	Attenuated Gardel	Attenuated Senegal	Sankat 430	Pokoase 417	Ball 3	Welgevonden
AVL/CTVM13	+	-	-	-	-	-	-
AVL/CTVM17	-	-	-	+	-	-	-
BDE/CTVM16	-	-	-	-	-	-	-
BME/CTVM2	-	-	-	-	-	-	-
BME/CTVM6	-	-	-	-		-	-
HAE/CTVM9	-	-	-	-	-	-	-
HAE/CTVM10	-			-	-	-	-
IDE8	+	+	+	-	+	+	+
IRE/CTVM18	+	+	+				-
RAE/CTVM1	-			-		-	-
RAN/CTVM3	-	-	-	-	-	-	-

Cell lines from all tick species tested except *H.a.anatolicum* were susceptible to infection using *Cowdria* transferred from other already-infected tick cell lines (Table 4), suggesting that the switch from mammalian stage to tick stage was more difficult for the *Cowdria* to achieve than the change from one tick host species to another. Once established, *Cowdria* was able to grow in cells from tick species known to transmit heartwater (*A.variegatum*), species previously shown to be incapable of transmitting heartwater (*B.decoloratus*, *R. appendiculatus*; Camus et al, 1996), and species whose vector status has not been tested (*B.microplus*, *I.scapularis*, *I.ricinus*). Cell lines from *R. appendiculatus* proved to be the most versatile host cell *in vitro*, since all six *Cowdria* isolates grew in all three cell lines from this species; in addition, the RAN/CTVM3 cell line can be grown at any temperature between 28 and 37°C.

Table 4. Results of inoculation of tick cell lines with supernate from other already-infected tick cell lines (AVL/CTVM17, IDE8, IRE/CTVM18 or RAN/CTVM3). + = infection achieved, - = tried and failed

Tick cell line	***Cowdria* isolate**						
	Gardel	Attenuated Gardel	Attenuated Senegal	Sankat 430	Pokoase 417	Ball 3	Welgevonden
AVL/CTVM13	+	+	+	+	+	+	+
AVL/CTVM17	+	+	-		-	+	-
BDE/CTVM16	+	+	+	+	+	+	-
BME/CTVM2	+	+	+	-	-	+	-
BME/CTVM6	+	+	+	-	-	+	-
HAE/CTVM9	-	-	-	-	-	-	-
HAE/CTVM10	-	-	-	-	-	-	-
IDE8				+			
RAE/CTVM1	+	+	+	+	+	+	+
RAN/CTVM3	+	+	+	+	+	+	+
RAE25	+	+	+	+	+	+	+

Continuous growth of at least one *Cowdria* isolate was achieved in ten cell lines from six tick species (Table 5). However the affinity of particular isolates for cells of particular host species was variable; for example, all isolates could infect AVL/CTVM13, but only Sankat 430 grew continuously in either *A.variegatum* cell line. In contrast, all isolates grew continuously in 2/3 *R. appendiculatus* cell lines.

Table 5. Growth of *Cowdria* in different tick cell lines. C = continuous growth (>200 days, subculture possible), F = finite growth (dies out before 200 days), ? = infection >200 days old at time of writing

Tick cell	***Cowdria* isolate**

line	Gardel	Attenuated Gardel	Attenuated Senegal	Sankat 430	Pokoase 417	Ball 3	Welgevonden
AVL/CTVM13	F	F	F	C	F	F	F
AVL/CTVM17	F	F		C		F	
BDE/CTVM16	C	F	F	F	F	F	
BME/CTVM2	?	C	?			C	
BME/CTVM6	?	C	?			C	
IDE8	C	C	F	F	F	C	C
IRE/CTVM18	C	C	C				
RAE/CTVM1	?	C	?	?	?	C	C
RAN/CTVM3	C	C	C	C	C	C	C
RAE25	?	C	?	?	?	C	C

Infectivity for BPC was tested on one or more occasions for some combinations of *Cowdria* isolate and tick cell line, with variable results (Table 6); it was not possible to draw conclusions as to the culture conditions required to achieve successful infection, which was detected in the BPC between 6 and 57 days after inoculation.

Table 6. Results of attempts to infect BPC with *Cowdria* from tick cell lines. + = infection achieved, - = tried and failed

Tick cell line	***Cowdria* isolate**						
	Gardel	Attenuated Gardel	Attenuated Senegal	Sankat 430	Pokoase 417	Ball 3	Welgevonden
AVL/CTVM13	+/-			-		-	+
AVL/CTVM17				-			
IDE8	+/-	+				+	
RAN/CTVM3	+	+	-	+/-	-	+	

The decline in infectivity *in vitro* of *Cowdria* EB harvested from BPC cultures was assessed in a single experiment (Appendix 4). Infectivity of *C.ruminantium* (Gardel) EB suspension held on ice declined rapidly, with a 69% loss in the first 30 minutes following harvest, and 97% loss within 3 hours. This result reinforced the view that properly prepared, characterised and administered EB stabilates were preferable to fresh EB for providing a standard experimental challenge in immunisation trials.

EB challenge stabilates were prepared from four *Cowdria* isolates – Gardel (STAB1 made during project R6566), Sankat 430 (STAB2), Ball 3 (STAB3) and Welgevonden (STAB4). All were titrated *in vitro* before and after freezing; loss of infectivity due to cryopreservation ranged between 0 (STAB3) and 80% (STAB1), but sufficient infective EB were present to ensure that every challenge dose would be infective. STABS1-3 were characterised *in vivo* in naïve sheep, at a standard dose of 1ml of a 1:10 dilution of thawed stabilate (see section 4.3.1 below); all infected naïve sheep developed clinical heartwater, confirmed by the presence of *Cowdria* inclusions in post mortem brain smears following euthanasia (section 3.3.1.3).

4.2. CHARACTERISATION OF *COWDRIA* GROWN IN TICK CELL LINES

Morphology: The results of light and electron microscopical examination of *C.ruminantium* (Gardel) in the tick cell lines IDE8 and AVL/CTVM13 are presented in two publications, Bell-Sakyi et al (2000a & b) (Appendix 12). In brief, *Cowdria* organisms *in vitro* in tick cells differed morphologically from those seen in bovine endothelial cells, and resembled those described from *Amblyomma* ticks *in vivo*. In particular, spherical EB were not seen, and the electron-dense inclusion body described in tick stages *in vivo* was also seen *in vitro* by EM; this organelle was often clearly visible in Giemsa-stained cytospin smears of infected tick cells

as a turquoise-staining body within the vacuole containing a *Cowdria* colony. The morphology of the other *Cowdria* isolates in tick cells was similar to that of Gardel, and their behaviour in the other tick cell lines was generally similar to that seen in IDE8 or AVL/CTVM13.

Antigen profile: Western blotting with polyclonal sheep sera and monoclonal antibodies (mab) reactive with the 32kD *Cowdria* MAP1 (Jongejan et al, 1991) revealed that the immunodominant antigen expressed by all *Cowdria* isolates tested in one or more tick cell lines, was not MAP1 but a smaller 29kD antigen. This antigen shared some, but not all epitopes with MAP1, since only one out of the four mab (4F10B4) reacted with it. Sera from sheep immunised with attenuated EB (Gardel or Sankat 430) recognised this antigen in addition to MAP1 in EB, but only the 29kD antigen in tick cell stages. Sera from sheep immunised with tick cell-derived *Cowdria* recognised the 29kD antigen in tick cell stages and EB, but only those sera which gave high titres in ELISA recognised MAP1 in EB. The mab were kindly provided by Frans Jongejan; identification of the 29kD antigen in tick cell stages was due mainly to the work of Stuart Smith. Detailed results of Western blotting with sheep sera are presented in Appendix 5.

Molecular characterisation: Presence of *Cowdria* DNA in infected tick cell cultures was confirmed by PCR for the following combinations of isolate and cell line: Gardel in IDE8, AVL/CVTM13 and RAN/CTVM3; Sankat 430 in AVL/CTVM17, AVL/CTVM13 and RAN/CTVM3; Ball 3 in IDE8 and RAN/CTVM3; attenuated Gardel in IDE8 and RAN/CTVM3.
Results from RT-PCR testing of selected combinations of *Cowdria* isolate and tick cell line for transcription of three *map1* gene copies are reported in the publication Bekker et al (2002) (Appendix 12). This work was carried out in collaboration with Utrecht University, by Cornelis Bekker and Edith Paxton. In brief, only one copy of the *map1* multigene family, *map1*, was transcribed in mammalian stage cultures of virulent *Cowdria* isolates, while two copies (*map1* and *map1-1*) were transcribed by *Cowdria* isolates in tick cells *in vitro*. Since the expected size of the protein coded for by *map1-1* is 29kD (C.Bekker, personal communication), it is possible that the 29kD antigen expressed in tick cell stages and recognised by sera from sheep immunised either with EB or with tick cell *Cowdria*, could be MAP1-1, suggesting that this antigen could be an important immunogen.

4.3. *IN VIVO* LABORATORY TRIALS OF THE IMMUNOGENICITY FOR SHEEP OF *COWDRIA* GROWN IN TICK CELL LINES

4.3.1 Experiments carried out at CTVM. A series of experiments was carried out in sheep to test the infectivity and immunogenicity of *C.ruminantium* grown in various tick cell lines. Initial experiments involved the Gardel isolate in IDE8 cells; as additional cell line/isolate combinations became available, they were tested first at a pilot level (one or two sheep), then if applicable or possible, on a larger scale (7 sheep with appropriate control groups). The aim was to identify one or more immunogenic combinations of cell line/*Cowdria* isolate, confirm immunogenicity against homologous challenge, and test ability to protect against heterologous challenge. Sheep were inoculated intravenously with whole, live cultures, and challenged between 28 and 42 days later with virulent *C.ruminantium* elementary bodies (EB) harvested from bovine endothelial cell cultures (as fresh material or cryopreserved stabilates). The results of individual experiments are presented in Appendix 6; a summary is given here.

Characterisation of challenge control EB stabilates: These were prepared and administered as described in sections 3.1.4 and 3.3.1.4 above. Whenever an EB stabilate was used for challenge, all sheep in the experiment received inoculum from the same diluted vial (or 2 pooled vials if more than 10 sheep); test sheep were inoculated first, followed by control(s).

Table 7. Responses of naïve control sheep to inoculation with EB stabilates:

Cowdria isolate	Stabilate number	No of sheep	Mean prepatent period (range)	Mean max temp	Outcome
Gardel	STAB1	11	11.3 (10-13) days	42.04°C	11/11 severe disease*
Sankat 430	STAB2	7	9.6 (9-12) days	41.60°C	7/7 severe disease*
Ball 3	STAB3	7	7.3 (7-8) days	41.75°C	7/7 severe disease*

* All sheep euthanased on third consecutive day of fever >40.8°C, all *Cowdria*-positive brain smears

Infectivity/clinical response resulting from inoculation with *Cowdria*-infected tick cells: Of 105 sheep inoculated with *Cowdria* in tick cells (intravenously as whole tick cell culture, 1-3ml dose) at CTVM and in Africa, 98% showed no clinical response to the inoculum, indicating that the material was generally safe. One sheep inoculated with the Ball 3 isolate in AVL/CTVM13 cells developed mild fever at the time when clinical heartwater would be expected, and was subsequently immune to homologous challenge. One sheep (less than 1% of the total) developed clinical heartwater following inoculation of AVL/CTVM13 cells infected with the Sankat 430 isolate; this isolate was not investigated further as a potential immunogen.

Table 8. Clinical response of experimental sheep to inoculation of *Cowdria*-infected tick cells

Tick cell line	*Cowdria* isolate	No of sheep	Clinical response
IDE8	Gardel	6	None
RAN/CTVM3	Gardel	3	None
RAN/CTVM3	Ball 3	2	None
RAN/CTVM3	Attenuated Senegal	2	None
RAN/CTVM3	Pokoase 417	2	None
AVL/CTVM13	Gardel	80	None
AVL/CTVM13	Attenuated Gardel	3	None
AVL/CTVM13	Ball 3	2	1/2 no reaction 1/2 mild reaction*
AVL/CTVM13	Sankat 430	3	2/2 no reaction 1/2 severe disease**
AVL/CTVM17	Sankat 430	2	None
Total		**105**	**103 (98%) no reaction** 1 mild reaction* 1 severe disease**

* fever days 8-12 between 40.5 and 40.8°C, no clinical signs, subsequently immune to homologous challenge

**euthanased on third consecutive day of fever >40.8°C, *Cowdria*-positive brain smear

Immunogenicity: At CTVM, using fresh culture inoculated within 30min of harvest, certain combinations of *Cowdria* isolate and tick cell lines were found to be highly immunogenic, inducing full protection against homologous or antigenically related heterologous EB challenge. The conditions under which the inoculated tick cells were previously cultured appeared to be crucial to the development of immunogenicity; in particular, presence of CO_2 in the atmosphere of the 37°C incubator appeared to be essential for successful immunisation at least with the combination of Gardel and AVL/CTVM13.

Table 9. Clinical response following challenge with virulent EB (stabilate or fresh) of sheep previously inoculated with *Cowdria* in tick cells at CTVM.

Tick cell line	*Cowdria* isolate	Culture condition	No of sheep	Challenge	Outcome
IDE8 or RAN/CTVM3	Gardel	31°C – CO_2 37°C + CO_2	5 2	Gardel	7/7 severe disease*
RAN/CTVM3	Ball 3	37°C + CO_2	2	Ball 3	2/2 severe disease*

RAN/CTVM3	Attenuated Senegal	37°C + CO_2	2	Virulent Senegal	1/2 severe disease* 1/2 fully protected
RAN/CTVM3	Pokoase 417	37°C + CO_2	2	Sankat 430	1/2 severe disease* 1/2 fully protected
AVL/CTVM13 or AVL/CTVM17	Sankat 430	37°C + CO_2	4	Sankat 430	4/4 severe disease*
AVL/CTVM13	Ball 3	37°C + CO_2	2	Ball 3	2/2 fully protected
AVL/CTVM13	Attenuated Gardel	37°C + CO_2	3	Gardel	3/3 fully protected
AVL/CTVM13	Gardel	37°C + CO_2	18	Gardel	2/18 severe disease** 16/18 fully protected
AVL/CTVM13	Gardel	37°C + CO_2	6	Ball 3	6/6 fully protected
AVL/CTVM13	Gardel	37°C – CO_2	14	Gardel	14/14 severe disease*

* euthanased on third consecutive day of fever >40.8°C, *Cowdria*-positive brain smears
** euthanased as above, 1/2 sheep had *Cowdria*-positive brain smears

Serological response of sheep inoculated with *Cowdria*-infected tick cells: The serological response of sheep inoculated with *Cowdria* in tick cells, as measured by cELISA and MAP1-B ELISA, was very variable. Sheep which were not protected against subsequent EB challenge did not mount any detectable serological response following inoculation with infected tick cells. Sheep which were protected against subsequent EB challenge exhibited a variety of serological responses including i) no detectable rise in antibodies following inoculation or challenge, ii) no response following inoculation but post challenge seroconversion, iii) a rise in antibodies following inoculation followed by a decline post challenge, and iv) seroconversion following inoculation with high antibody levels retained post challenge. Sheep immunised naturally by tick transmission or experimentally with *Cowdria* EB generally seroconvert within 3-4 weeks, as measured by the ELISA tests used, and maintain high antibody levels thereafter for many months/years whether or not immunity is boosted by subsequent challenge (Anon, 1997, Van Vliet et al, 1995).

Serological data for all sheep inoculated with *Cowdria*-infected tick cells at CTVM are summarised in Appendix 7. Representative graphs of clinical (daily rectal temperature) and serological (ELISA results) responses of experimental sheep are shown in Appendix 8.

4.3.2 Laboratory trials carried out by collaborators in laboratories in Ghana, The Gambia and Kenya using infected tick cells produced at CTVM

The aim of these experiments was twofold – to test whether or not the candidate immunogen (Gardel in AVL/CTVM13), produced at CTVM, would retain its immunogenicity following travel by courier to Africa, and to assess its ability to protect local sheep against local, heterologous *Cowdria* isolates. In September and October 2000, *Cowdria*-infected tick cell cultures were dispatched to Ghana, The Gambia and Kenya (NVRC/KARI) by courier with journeys lasting 7-10 days. The cultures were then incubated at 37°C for 4-7 days prior to inoculation. Challenge was heterologous with isolates local to the area; Sankat 430 (Ghana) and Senegal (The Gambia) were the only suitably pathogenic West African isolates available in culture, and unfortunately do not have good cross-protectivity with Gardel. Kathiani 972 (Kenya) is partially cross-protective with Gardel (K.Sumption, pers. comm.), but the viability of the available blood stabilates was suspect. In these laboratory trials, groups of sheep immunised with inactivated Gardel EB were included for comparison (EB inactivated with sodium azide, administered as two doses of 250μg emulsified in Montanide ISA 50 adjuvant, intramuscularly at 2-week intervals).

In July 2001, cultures grown at CTVM were carried by air to Kenya (ILRI), with care taken to maintain an incubation temperature as near as possible to 37°C; they were then incubated at 37°C for 4 days prior to inoculation. A control group of sheep inoculated with uninfected AVL/CTVM13 cells was included in this trial.

In the trials carried out in 2000, the infected tick cells were grown at CTVM in sealed flasks in a CO_2-gassed humidified incubator; in the ILRI trial, the tick cells were grown at CTVM in sealed flasks in a dry incubator without CO_2.

The detailed results are presented in Appendices 9 and 10 ; a summary is given in Table 10.

Table 10. Summary of results of laboratory trials in Africa of immunisation of sheep with *C.ruminantium* (Gardel) grown in AVL/CTVM13 cells.

Location	Treatment	No of sheep	Challenge isolate	Outcome
Ghana	Inactivated Gardel EB	7	Sankat 430 BPC culture supernate	1/7 died of heartwater 5/7 severe disease* 1/7 no response
	Gardel in AVL/CTVM13 grown with CO_2	7		2/7 died of heartwater 2/7 severe disease* 3/7 mild disease**
	Naïve control	7		2/7 died of heartwater 4/7 severe disease* 1/7 no response
The Gambia	Inactivated Gardel EB	7	Senegal BPC culture supernate	2/7 severe disease* 5/7 mild disease**
	Gardel in AVL/CTVM13 grown with CO_2 (inoculated s/c instead of i/v)	7		1/7 severe disease* 5/7 mild disease** 1/7 no response
	Naïve control	7		1/7 euthanased (heartwater) 2/7 severe disease* 4/7 mild disease**
Kenya (NVRC)	Inactivated Gardel EB	8	Kathiani 972 blood stabilate	4/8 died/euthanased (heartwater) 1/8 mild disease** 3/8 no response
	Gardel in AVL/CTVM13 grown with CO_2	8		4/8 mild disease** 4/8 “protected” (no disease)
	Naïve control	8		2/8 died/euthanased (heartwater) 3/8 mild disease** 3/8 no response
Kenya (ILRI)	Uninfected AVL/CTVM13	10	Gardel STAB1	7/10 died/euthanased (heartwater) 3/10 reacted and recovered***
	Gardel in AVL/CTVM13 grown without CO_2	10		6/10 died/euthanased (heartwater) 4/10 reacted and recovered***
	Naïve control	10		9/10 died/euthanased (heartwater) 1/10 reacted and recovered***

*3 or more days >40.8°C and/or clinical signs **1-3 days >40.5°C, no clinical signs
*** detailed clinical data not provided by ILRI

4.3.3 Conclusion

Results of experiments carried out up to March 2001 indicated that freshly harvested *Cowdria* (Gardel) in AVL/CTVM13 cells gave excellent protection against experimental challenge with mammalian tissue culture derived EB stabilates, both homologous and heterologous (Ball 3, known to fully cross-protect with Gardel). Results using "travelled" material were less conclusive; in an experiment at CTVM (Appendix 6), the material was administered immediately following the simulated travel, with resultant decrease in vaccine "take" (measured by serology) and level of protection against homologous challenge. The material sent to Africa was allowed to recover for 4-7 days before administration, with resultant improvement in vaccine "take" to levels seen with fresh vaccine at CTVM; however the availability of appropriate and suitably pathogenic challenge material was limited, and the results reflect the low levels of cross-protectivity with Gardel of heterologous challenge isolates (Ghana and The Gambia), and problems with viability of blood stabilates (Kenya).

All tick cells grown at 37°C and used as immunogens during the first two years of the project were maintained in sealed containers in a CO_2-gassed humidified incubator. Inevitably some CO_2 entered the sealed cultures, as indicated by the lower pH of the culture medium compared with that of cultures maintained in dry incubators. For example, AVL/CTVM13 cultures were approx pH 6.8 without CO_2, and pH 6.5 with CO_2. During the third year of the project, all cultures were maintained in dry incubators, without CO_2, since this was believed to be preferable for the tick cells. There was no apparent difference in morphology or growth rate of tick cells or *Cowdria* between cultures with or without CO_2. Indeed, presence of CO_2 and/or low pH (below 7.0) are harmful to other ehrlichial pathogens growing in tick cells (U.Munderloh, personal communication). However, all laboratory trials carried out during the third year, involving Gardel in AVL/CTVM13 grown at 37°C without CO_2, resulted in failure, with no indication of seroconversion or protection in immunised animals. In addition to the trials reported here (ILRI and CTVM), Gardel-infected AVL/CTVM13 cells grown without CO_2 were sent to CIRAD-EMVT, Guadeloupe, in April 2001, where they were inoculated into 5 goats. None of the goats were protected against virulent homologous challenge. Thus it appears that CO_2 may play a role in the development of immunogenicity in *Cowdria* grown in tick cell lines.

The variability of the serological response of sheep successfully immunised with *Cowdria*-infected tick cells suggests that the immune response engendered by this material differs from that induced during experimental immunisation with mammalian stages of *Cowdria*. It was not possible to investigate the nature of this immune response within the confines of the present project, but it is likely to be largely cell-mediated. Both the ELISA tests used are based on antigens from the mammalian endothelial cell stage (EB) of *Cowdria*. The absence of a detectable response to these antigens in some sheep which were nonetheless fully protected against EB challenge suggests that, in these sheep at least, the tick cell-derived *Cowdria* may not have developed as far as the EB stage, and thus that protective immunity can develop without exposure to the endothelial cell stage.

5. Contribution of Outputs

The project has demonstrated that the *Cowdria*/tick cell culture system provides an alternative/complementary source of immunogenic material with the potential for greatly improving existing heartwater vaccines and diagnostic tools.

The outputs have contributed to the project goal "Performance of livestock of poor people improved through the control of disease - simpler, cheaper, safer and more effective vaccines for heartwater and improved diagnostic tools" in the following ways:

Under certain conditions, the *Cowdria*l tick cell culture system produces highly immunogenic material, different from mammalian stages, which protects sheep against lethal experimental challenge in the absence of clinical disease. So far, protection has been achieved with four *Cowdria* isolates using a single i/v dose of live culture, comparing favourably with other live vaccines which are limited by number of isolates (attenuated vaccines – only two isolates available) or the need for monitoring and treatment (Ball 3 blood vaccine, infection-and-treatment using virulent EB). Incorporation of tick cell-derived *Cowdria* antigens in inactivated vaccines may improve their currently poor protectivity.

Because of the limited spectrum of cross-protection exhibited by individual *Cowdria* isolates, it will be essential to test the immunogenicity of additional *Cowdria* isolates in tick cells. If several antigenically distinct isolates (from different geographic areas) can be demonstrated to induce protection against disease to the level already shown by the combination of Gardel and AVL/CTVM13 cells, options will increase for tailoring vaccines to particular disease situations by using local isolates alone or in combination. Since all six *Cowdria* isolates tested so far have been successfully established in tick cell lines, it seems probable that any isolate which can currently be cultivated in mammalian endothelial cells will also grow in tick cells.

At present, immunogenicity of *Cowdria* in tick cells has been demonstrated using a crude preparation containing *Cowdria*, tick cells and culture medium. Additional research is required to identify the active immunogen(s) and develop methods for purification and removal of unnecessary components. As with any potential vaccine, factors including optimum dose level, duration of immunity, extent of cross-protectivity and ability to protect against natural (tick) challenge should be investigated, as well as infectivity of immunised animals for ticks. The nature of the immune response engendered by *Cowdria* in tick cells deserves further study, since it apparently differs in some respects from that induced by other forms of heartwater immunisation.

Initial characterisation of tick cell-derived *Cowdria* indicates that the immunodominant antigen, which may play a role in protection, is different from the mammalian stage MAP1, and could be the more highly conserved MAP1-1. Gene transcription studies show that the *map1-1* gene is transcribed in tick cells *in vivo* and *in vitro*, and in attenuated, but not virulent, mammalian stage cultures; because of its conserved nature this gene, and its associated protein, could contribute to improved diagnostic tests.

Although DFID have declined to fund further research on *Cowdria* in tick cell lines, studies will continue as part of the ongoing European Community-funded project "Integrated diagnostic and recombinant vaccine development for cowdriosis and anaplasmosis" in which CTVM is a partner. Aspects to be investigated in collaboration with partner institutes include characterisation of the immune response in goats immunised with *Cowdria*-infected tick cells, differential stage-specific transcription of the *map*1 multigene family (now known to comprise 15 gene copies), and further characterisation of antigens expressed by tick cell stages of *Cowdria*.

In March 2002, the technology for production of *Cowdria*l tick cell vaccine was transferred by the project to the Ghana Veterinary Services Department in Accra (Dr E.B.M.Koney), who have secured local funding for laboratory immunisation trials in sheep. The combination of the Ghanaian Pokoase 417 isolate and RAN/CTVM3 cells will be particularly appropriate, since Pokoase 417 has already been shown to cross-protect against at least one, more pathogenic local isolate (Sankat 430), and the tick *Rhipicephalus appendiculatus* does not occur in West Africa, thus minimising the potential risk of adverse reactions to inoculation of RAN/CTVM3 cells in previously tick-exposed animals. If protection is demonstrated at the laboratory level, it is hoped that field trials will be carried out at sites with high heartwater challenge, previously identified by the Ghana Government/CTVM DFID (ODA) project R5971CB (Anon, 1997). Backup support for the tissue culture element of this study will continue to be provided by CTVM through the EU Cowdriosis network.

Lesley Bell-Sakyi will attend the fourth Tick and Tick-borne Pathogens Conference in Banff, Canada in July 2002, where she will present a paper on some aspects of the project work "Immunogenicity of *Ehrlichia ruminantium* grown in tick cell lines" (Bell-Sakyi, Paxton, Wright & Sumption). A poster to be presented at the same conference, "Kinetics of experimental infection of sheep with *Ehrlichia ruminantium* cultivated in tick and mammalian cell lines" (Postigo, Bell-Sakyi, Paxton & Sumption) incorporates some data generated by the project. Two manuscripts are in preparation - "*Ehrlichia ruminantium* grows in cell lines from four Ixodid tick genera" and "*Ehrlichia ruminantium* grown in tick cell lines protects sheep against experimentally induced heartwater".

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