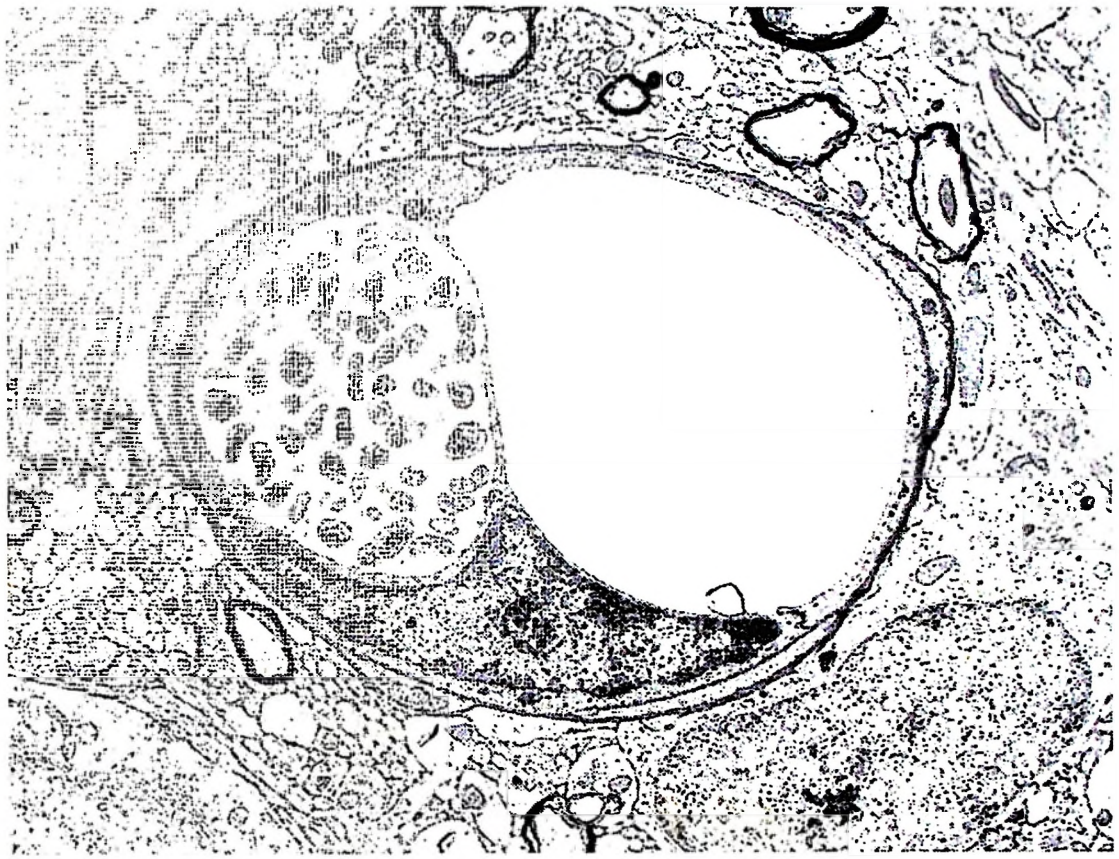
ULTRASTRUCTURAL STUDY OF BRAIN MICROVASCULATURE IN GOATS WITH EXPERIMENTAL *COWDRIA RUMINANTIUM* INFECTION (HEARTWATER)



Ph.D. Thesis

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The Royal Veterinary and Agricultural University
Copenhagen, Denmark, 1991

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Illustration on cover: Electron micrograph showing colony of *Cowdria ruminantium* in endothelial cell of brain capillary in an infected goat.

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1. INTRODUCTION

Heartwater is an infectious, vector-borne, non-contagious disease of ruminants caused by *Cowdria ruminantium*. The etiologic agent is an obligate intracellular parasite infecting phagocytic cells and particularly, vascular endothelial cells. Clinically, the disease is usually characterized by fever and signs of central nervous system disorder. Hydropericardium is a frequent finding at necropsy, hence the name "heartwater". The disease was first reported in South Africa in 1838 (Neitz, 1968) but the first confirmation of its etiology was done by Cowdry (1925b). The organism was named *Rickettsia ruminantium* and classified under the tribe *Rickettsiae*. Later, however, it was moved to the tribe *Ehrlichiae*, together with *Ehrlichia* and *Neorickettsia* due to their similarity with *Ehrlichia*, which is the current classification (Moulder, 1974). Those similarities are discussed by Williams and Vodkin, (1987) and Holland et al. (1987). However, Scott (1987) proposed *C. ruminantium* to be classified together with *Chlamydia* since it fitted best the numerical taxonomy grouping with it, together with *Anaplasma, Ægyptinella, Coxiella and Chlamydia.* Ultrastructural morphologic characteristics relating this organism to the *Chlamydia* group are also reviewed by Avakyan and Popov (1984).

Transmission of *C. ruminantium* is effected by a three host tick of the genus *Amblyomma* (Bezuidenhout, 1987) as they feed on an infected host, mainly domestic ruminants, and move on to feed on the next non-infected host. Following introduction into the host's circulation, *C. ruminantium* are taken up by phagocytic cells and undergo development in reticulum cells, macrophages and neutrophils in lymph nodes (Du Plessis, 1970). The phagocytic cells then enter the circulation, release the parasites and these infect the vascular endothelial cells. Fawi

et al. (1977) used various blood components to infect goats and concluded that it was the red fraction of blood which was infective. In later studies however Sahu et al. (1983) and Sahu (1986) demonstrated *C. ruminantium* in macrophages and neutrophils obtained from infected animals during peak pyrexia. The infectivity of the buffy coat was also confirmed by Ilemobade (1976). It is currently accepted therefore that initial developmental stages in the host take place in phagocytes within lymph nodes and subsequently parasites develop into intracytoplasmic colonies in vascular endothelial cells dividing by binary fission. Du Plessis (1975) observed that the developmental stage of *Cowdria* within leukocytes was represented by dense bodies which continued to subdivide freely within the host cell cytoplasm. These were not enclosed in vacuoles. On the other hand, those in endothelial cells had a double membrane and each organism had electron-dense and electron lucent areas. Colonies in endothelial cells are each enclosed in a vacuole (Du Plessis, 1970).

Hypotheses on the pathogenesis of heartwater have been drawn from observations in different animal species, like all other clinical and pathomorphologic features of heartwater. There is a general agreement on a deranged transport mechanism across the vascular wall in neural tissue and other organs as the basis for the development of the morphologic lesions and clinical signs (Du Plessis et al., 1987). However, the actual mechanism of damage is poorly understood. Elaboration of a toxin by *C. ruminantium* has been suggested (Steck, 1928, Neitz, 1968, Pienaar et al., 1966, Isoun et al., 1974, Ilemobade, 1976) and has formed the basis for discussion of the pathogenesis for many years. Lack of evidence for this hypothesis and that of a possible, direct, necrotizing effect by the organism has made it difficult to resolve the problem (Du Plessis et al., 1987).

In recent years du Plessis (1975) and du Plessis et al. (1987) have tried to investigate the possible involvement of vasoactive substances. Du Plessis et al. (1987) gave histamine and serotonin antagonists some days prior to the onset of clinical signs but failed to prevent the fatal progression of the disease. It was subsequently suggested that possible timing of the chemical antagonism could be critical and therefore this hypothesis could not be ruled out without further investigation. Owen and co-workers (1973) suggested that different pathogenetic mechanisms may apply to different pathomorphologic manifestations of heartwater.

So far investigators have not paid attention to ultrastructural changes around blood vessels and the adjacent neuropil, presumably because no parasites are seen in this location at the light microscopic level; and also because even in target endothelial cells in which *C. ruminantium* are located ultrastructural changes are subtle.

The pathogenesis of heartwater being such an elusive problem yet, a record of ultrastructural changes in this area may be helpful in future investigations. The suggestion that ultrastructural changes exist in endothelial cells other than those parasitized (Pienaar,1970, Prozesky and Du Plessis, 1985) and the observation that mild endothelial ultrastructural changes do not conform with the extent of the corresponding histologic changes (Prozesky and Du Plessis, 1985b, Prozesky, 1987) call for further investigation in order to patch up the gap.

The discrepancy between the tissue pathomorphologic alterations and presence of *C. ruminantium* in endothelium needs to be reconciled in order to pave way for a search for the

pathogenesis of heartwater. Ultrastructural studies have been done in cattle, sheep, goats and mice; but the methods of killing and tissue preparation have varied. Morphologic lesions reflect underlying biochemical disturbances in all pathologic processes. For the recognition and interpretation of such lesions, particularly at the ultrastructural level, it is important to have some consistency in the animals used and ensure minimal tissue artifacts so that the changes can be ascribed to the disease with certainty. The present work aims at establishing and relating pathologic changes at light microscopic and ultrastructural levels while keeping artifacts at a minimum in order to sort out possible pointers to the pathogenesis of heartwater.

The study of control animals was carried out at the Royal Veterinary and Agricultural University in Denmark. The experimental infections , necropsies and collection of tissues were done at Sokoine University of Agriculture, Morogoro, Tanzania. The fixed tissue samples were then transported to Denmark for further processing and examination.

An extensive review of the various aspects of the disease covering the literature until around 1980 is given by Uilenberg (1983). A further review of the disease covering developments up to 1987 is presented by Bigalke (1987, ed.). The present review concentrates on those features that are relevant to the present study and, particularly, on more recent findings.

2. LITERATURE REVIEW

THE PARASITE

Pienaar (1970) found four different forms of *C. ruminantium* in endothelial cells: small forms (diameter = 0.49μm), intermediate (diameter = 0.76μm), large (diameter = 1.04μm) and very large (diameter = 2.7μm). Smaller forms formed large colonies while larger forms formed small colonies. The organisms were very pleomorphic, and large forms were closely packed and had more evenly distributed and less dense matrix than the smaller forms. The life cycle of the parasite takes place in two hosts, ticks of the genus *Amblyomma* which are the main vectors (Cowdry, 1925b, Kocan and Bezuidenhout, 1987, Bezuidenhout, 1987), and in vertebrate hosts, particularly domestic ruminants. Initial bodies are normally dense, while elementary bodies which are the mature form have electron dense and electron light areas (Pienaar, 1970). During development in the host (Kocan et al., 1990) and in the vector (Kocan and Bezuidenhout, 1987) intermediate forms, whose internal structure is reticular in appearance, occur.

Several strains exist with varying pathogenicity (Uilenberg, 1983, Logan et al., 1987, Jongejan et al., 1988) but it has not been possible to establish any relationship between the morphology of the parasites and their pathogenicity or the lesions that are caused by each strain in host tissues. The different forms of *C. ruminantium* described by Pienaar (1970) existed in the same host infected with a single strain.

CLINICAL SIGNS

The incubation period in cattle is 12 days on average following intravenous (i.v.) inoculation with infective blood (Pypekamp and Prozesky, 1987) but is shorter if infective tick homogenate is given in place of blood (van de Merwe, 1979). In goats the incubation period is 9-10 days on average following i.v. inoculation with whole blood. Natural cases show more variation i.e. 9-29 days in cattle and 5-35 days in goats and sheep (Alexander, 1931, Pypekamp and Prozesky, 1987).

Three forms of clinical disease occur in goats: peracute, acute and mild. The onset in each case is marked by sudden pyrexia. Animals with strong innate resistance or immunity may not show signs other than this with subsequent recovery (Alexander, 1931) while with the acute form further signs ensue, including progressive ataxia, basewide posture, holding head down, drooping ears and listlessness. Further development leads to galloping, lateral recumbency, chewing movements, nystagmus and licking of lips (Alexander, 1931, Pypekamp and Prozesky, 1987). Animals with least immunity, particularly exotic breeds, normally develop the peracute form which is marked by a fever followed by sudden collapse and death (Pypekamp and Prozesky, 1987) often accompanied by bleating, tail twitching, regular urination and defecation, forced respiration and terminal convulsions (Spreull, 1922). In most small domestic ruminants clinical signs of the acute form are less pronounced than in cattle (Pypekamp and Prozesky, 1987).

The form and course of the disease are normally determined by the host's immunity and genetic resistance, age and the strain of the infective agent. Goat kids of up to 6 weeks of

age are fairly resistant. Persian breeds and those native to Africa are also resistant, while Angora goats are the most susceptible, followed by the Boer goat (Pypekamp and Prozesky, 1987).

Difficulties in the confirmation of heartwater are due to the fact that only brain tissue examination is dependable. A general clinical diagnostic approach is presented by Camus and Barré (1987). Clinical diagnosis depends on epidemiological information, subinoculation of blood from suspected animals and serology. A brain biopsy technique was developed by Synge (1978), but the method remains a laboratory procedure for experimental animals. Van Amstel (1987) evaluated electro-encephalographic recordings from heartwater cases and found that, although useful, they were not specific. So far the easiest way of confirming heartwater is by brain crash smears taken from dead animals (Purchase, 1945, Schreuder, 1980) and stained with Giemsa or toluidine blue.

Diseases of differential diagnostic importance in goats include: hypophyseal abscess, bacterial meningoencephalitis, plant and heavy metal poisoning and pesticide poisoning (Pypekamp and Prozesky, 1987), but the possibility of rabies should be considered depending on the availability of epidemiologic information.

EPIDEMIOLOGY

Natural transmission of heartwater is strictly by tick vectors of the genus *Amblyomma* (Bezuidenhout, 1987), and all are 3-host ticks. The transmission is transstadial, i.e. nymphae are infected during feeding on hosts with clinical or subclinical infection and infect new hosts

during subsequent feeding at adult stages. Following feeding on an infected host *C. ruminantium* develops in tick gut and salivary gland epithelial cells. Penetration of mouth parts during feeding is facilitated by saliva which also serves as anticoagulant. As saliva is regurgitated *C. ruminantium* are passed into the host circulation.

The geographic distribution of heartwater is determined by the distribution of *Amblyomma* ticks (Uilenberg, 1983), and in particular the two most important vectors *A. variegatum* and *A. hebraeum* (Provost and Bezuidenhout, 1987, Bezuidenhout, 1987, Walker, 1987, Petney et al. 1987). Heartwater is widespread in Africa South of the Sahara, including Mauritius, and present in some Caribbean islands including Guadeloupe, Antigua and Marie Galante (Camus and Barré, 1982 & 1987, Provost and Bezuidenhout, 1987). The presence of heartwater in the Caribbean islands poses a threat to North and South America (Barré et al. 1987) since the geographic distribution of *Amblyomma* spp. extends to the American continent (Walker, 1987, Barré et al. 1987). According to Uilenberg (1983) heartwater is only second to East Coast Fever and tse-tse-transmitted trypanosomiasis in Africa, but is underreported due to problems of diagnosis (Provost and Bezuidenhout, 1987).

PATHOLOGY

Gross lesions

The majority of cases in sheep and cattle show modest gross changes in the brain in the form of edematous and hyperemic meninges and, in a few cases, hemorrhages (Pienaar et al., 1966). Meninges appear dull and the choroid plexus is thickened and grayish in color.

Hydropericardium is a common feature of heartwater but is more common in sheep and cattle than in goats (Uilenberg, 1983). Angora goats seem to be particularly susceptible, with pronounced clinical signs and lesions as in sheep and cattle (Prozesky and Du Plessis, 1985a). Hydrothorax may also occur together with edema of lungs and mediastinal and perirenal tissues in severe and prolonged cases.

Swelling of kidneys has also been reported in Angora goats by Prozesky and Du Plessis (1985a). Moderate splenomegaly was found in untreated cases but was absent in treated animals. They also reported edema of lymph nodes in both treated and untreated goats and catarrhal enteritis in two treated goats.

Other studies by Prozesky and Du Plessis (1985b) in two Angora goats and two Merino sheep showed that there was interstitial edema of lungs in both sheep and one goat, accompanied by hydrothorax, hydropericardium and kidney swelling, while in the remaining goat these were mild. No mention is made of the amount of fluid found in the body cavities. There was a positive correlation between the severity of the gross and histopathologic lesions in all animals. A mild to moderate splenomegaly was present in all animals. Petechiae of visible mucous membranes (conjunctiva, vagina) and of urinary bladder is often reported in severe cases of heartwater, as well as kidney and heart degeneration (Prozesky, 1987b).

The gross lesions, i.e. hydropericardium; hydrothorax; edema of lung, mediastinum, meninges, brain, and perirenal tissue; mucous membrane petechiae; enteritis; splenomegaly; lymphadenopathy and kidney swelling, form a very variable picture since not all occur in the same animal and to the same degree (Uilenberg, 1983). The gross picture possibly depends

on many factors such as the species and breed of the host, the strain of *Cowdria ruminantium*, the host's innate resistance and immune status, the stage of clinical disease at which necropsy is performed and possibly also other intercurrent infections and nutritional status.

Light microscopic lesions

Pienaar et al. (1966) studied 11 cattle 12 sheep and 1 goat, all having died of naturally acquired heartwater. Their study is one of the most detailed accounts of histologic changes in the brain. They reported necrosis of capillaries and small blood vessels with pyknosis, karyorrhexis and fibrinoid changes as well as perivascular hemorrhages which were more severe in cattle. A mild vasculitis in all cattle and some sheep was noted but no mention is made of the goat. Focal gliosis was seen mainly around small blood vessels in 2 out of 13 sheep.

Enlarged, irregular and vesiculated nuclei of neuroglial cells, particularly astrocytes were commonly seen, with eosinophilic cytoplasm. Intracytoplasmic granules and larger globules were observed around the nucleus and in processes. Similar globules were also found in the perivascular space, and in either case they were PAS- positive. The authors noted that the glial and perivascular space lesions were not uniform and were more obvious in cattle than in sheep. Leukostasis was found in 70% of all cases but again this was more pronounced in cattle. A meningoencephalitis was found in a few cattle.

While comparing treated and untreated Angora goats, Prozesky and du Plessis (1985a) found

no qualitative difference in lesions between the two groups. They reported a mild to moderate edema of the white matter, enlarged neuroglia, swollen axons, and also vasculitis characterized by accumulation of macrophages, lymphocytes and few neutrophils in extravascular spaces. Other changes were edema of lung lymph nodes and lungs, congestion and increased number of alveolar macrophages and intracapillary monocytes. A mild infiltration of interalveolar connective tissue by mononuclear and polymorphonuclear cells was also seen. There were changes in kidney tubules including hydropic degeneration and cloudy swelling of tubular cells as well as dilatation of tubules. Stewart and Howell (1981) found organisms free in the cytoplasm of small blood vessels in the kidney, apart from parasitization of the endothelial cells.

Prozesky and du Plessis (1985b) studied lung lesions in two each of Merino sheep and Angora goats experimentally infected with the Ball 3 strain of *C. ruminantium*. Lungs appeared to be consolidated, with edema, fibrin in alveolar spaces, cellular exudate composed of mononuclear cells in the interstitial tissue. An increase in intravascular monocytes and neutrophils was also noted.

Electron microscopic lesions

Ultrastructural studies on heartwater are limited, and mostly dealing with the morphologic characteristics of the causative agent. Lesions in the choroid plexus of sheep (Pienaar, 1970), reticuloendothelial cells of sheep and mice (Du Plessis, 1975) and lungs of sheep and goats (Prozesky and Du Plessis, 1985b) have been described.

Despite endothelial parasitization there were no noticeable signs of endothelial injury

(Pienaar, 1970, Prozesky and Du Plessis, 1985b) apart from the severe distention of the cytoplasm containing the parasite colony. Phagocytic cells containing the parasites also did not show any cytopathic changes.

However, the authors reported cytopathic changes in endothelial cells other than those parasitized. Those were described by Pienaar (1970) as swelling of the cytoplasm, loss of cytoplasmic organelles and decreased cytoplasmic matrix density. Prozesky and Du Plessis (1985b) observed cell swelling, loss of matrix density, condensation of some mitochondria with loss of cristae and swelling of others with electron-lucent foci or whorled membranes within. The endoplasmic reticulum was dilated; dense intracytoplasmic bodies and membrane bound vacuoles which occasionally contained fine flocculent or amorphous material were seen. They also reported gaps between endothelial cells which contained material similar to plasma. The method of killing is not specified by Prozesky and Du Plessis (1985b). Du Plessis (1975) studied two different strains of *C. ruminantium*, the Ball 3 and Welgevonden strains in reticuloendothelial cells of the host. Colonies were not enclosed in a well defined and distinct vacuole as is the case in vascular endothelial cells. Some forms, considered developmental stages, were dense bodies without a double membrane within the macrophage cytoplasm.

IMMUNOLOGY

The development and duration of immunity in goats is variable. Artificial immunization is done by infection with live, virulent *C. ruminantium*, followed by treatment with oxytetracyclines during the first two days of the febrile period. Du Plessis et al (1983) found

that late treatment during the febrile period resulted in both a high mortality and development of a solid specific immunity among survivors. Similar observations were made by Ilemobade (1976). Animals are immune to subsequent challenge for between 8 weeks (Ilemobade, 1976) and 205 days (Du Plessis et al., 1983). The duration of immunity in cattle and sheep is longer than in goats (Stewart, 1987a). Artificial immunization is achieved by inoculation with fully virulent organisms in whole blood followed by treatment, during peak fever, with long-acting tetracycline, although not without risk of fatality (Du Plessis et al., 1983, Gruss, 1983). However, it has recently been reported that sheep and goats can be protected using an *in vitro* attenuated vaccine (Jongejan, 1991)

Many isolates of *Cowdria ruminantium* show antigenic similarity with some exceptions. Uilenberg et al. (1983) used strains from South Africa, Sudan, Sao Tomé and Nigeria to vaccinate 114 Dutch goats. Subsequent challenge with heterologous strains showed that animals were protected. However, Jongejan et al. (1988) found that Ball 3 and Zambian strains could not protect against a Senegalese strain while the former were cross protective, and that goats immune to the Senegalese strain were also immune to the other two. Logan et al. (1987) compared Kwanyaga and Kümm strains (S. Afr.) and Mali 1 and Gardel (Guadeloupe) strains. Mali 1 protected against Gardel; Gardel protected against Kwanyaga; but Kwanyaga could not protect against Gardel, nor could Gardel, Mali and Kwanyaga singly or together protect against Kümm.

Although the host may mount both humoral and cellular immune responses at the same time against *Cowdria ruminantium,* serum gamma globulins do not protect the animal from homologous challenge (Du Plessis, 1970b). Du Plessis (1982) was able to protect mice from

C. ruminantium by inoculating the mice with lymphocytes from immune ones; while mice inoculated with lymphocytes from non-immune mice remained fully susceptible. While this is so with *C. ruminantium*, other rickettsiae, affecting human beings for example, elicit both cell-mediated (CMI) and antibody-dependent immunity (ADI) as protective (Tringali et al., 1983; Stewart, 1987). Bourgeois et al., (1980) were able to induce transformation of peripheral blood lymphocytes from previously exposed humans by exposing the cells to soluble and membrane fractions of *Rickettsia typhi* and *R. prowazekii*. The specific lymphocyte transformation response was elicited even long after exposure of the individuals to the antigen. They concluded that persistent cell-mediated immunity existed following exposure to the rickettsiae. Shirai et al. (1976) showed that such lymphocytes were actually T lymphocytes.

The variations in host immune responses to different rickettsiae and, in particular, the role of ADI as opposed to CMI mean that there might be major differences too in the pathogenesis. However, the mobilization of CMI appears to be similar in most rickettsial infections (Stewart, 1987b).

3. MATERIALS AND METHODS

CONTROL ANIMALS

Animals

Five, clinically healthy, mature goats, 4 male and 1 female were used. Their weights ranged between 18 and 25 kg. They had been reared in Denmark where heartwater disease does not exist.

Killing and perfusion technique

A perfusion tank containing 3 liters of 3% phosphate-buffered glutaraldehyde adjusted for osmolarity (Appendix 1) was hoisted above the table on which the goat was placed in dorsal recumbency. The tank height was set at 1.5 m.

The hairs on the ventral side of the neck were clipped and a catheter inserted into one of the jugular veins. Pentobarbitone (50 mg/ml) was given at a dose of 25 mg/kg, first half the dose and then the rest after one minute. A few drops of heparin (25,000 i.u/ml) as dilute solution (0.25ml heparin + 2ml physiological saline) were used to flush the catheter by means of a syringe in order to prevent coagulation and blockage; thereafter the catheter was closed. Medical adhesive tape was applied in order to secure the catheter.

An incision was made in the skin overlying the trachea from just posterior to the larynx

caudally for about 10 cm. Dissection was made to expose the trachea and an incision between two tracheal rings was made. A plastic tracheal tube was inserted and surgical thread applied around the trachea to secure the tube. Further dissection was made to expose the carotid arteries on both sides of the trachea and, over a segment of about 4 cm of each carotid artery, the vagal sympathetic and recurrent laryngeal trunk were separated. In order to minimize blood coagulation and blockage of vessels 1.0 ml of heparin, i.e. approximately 1,250 i.u/kg. diluted with the same amount of physiological saline was administered through the catheter and 1 minute allowed for distribution in the blood. The order in which the perfusion process was conducted is given in figure 1. The loose segment of one of the carotids was clamped at both ends using artery forceps and a short longitudinal incision was quickly made between the two clamps. A 2.0 mm calibre perfusion canula was introduced into the artery and the opposite carotid artery was clamped off to prevent back-flow into the perfusion tube. The rostral clamp was removed and subsequently surgical thread applied to secure the canula; the perfusion tube clamps and valve were released followed by removal of the clamp on the opposite carotid artery. The animal died within a few seconds following the introduction of glutaraldehyde solution. The jugular vein was then opened and perfusion allowed for 10-15 minutes until clear fixative appeared at the jugular outflow.

Within one minute of smooth, uninterrupted flow the skin and muscles of the lips started to harden, followed by other facial muscles and ears. The side whose carotid was used in the perfusion hardened first and better. Occasionally, temporary backflow of blood into the canula and tubing resulted in stoppage in which case manual assistance using bladder syringes was necessary with gentle and persistent pressure applied to the plunger just enough to overcome resistance. Perfusion was stopped after 10-15 minutes or when adequate hardening

of skin and muscles of the head was achieved, with a clear perfusate maintained for about 10 minutes at the opposite jugular outflow.

Tissue sampling and processing

The cranium was opened by means of a saw taking care not to damage the brain. Adequately fixed areas of the brain were determined by their yellow color and firmness. From these areas, 1 mm^3 blocks of cerebral and cerebellar cortex were taken. In each case rostral and lateral cerebral and mid- cerebellar cortices were sampled from the same side of the brain.

Tissues for light microscopy were sampled and fixed in 10% neutral, buffered formalin. These included brain, lung, liver, kidney, adrenal gland, pancreas, small and large intestine, abomasum, spleen, heart, thyroid, prescapular lymph node, skeletal muscle, and thymus. They were then processed, embedded in paraffin wax, sectioned at 6 μm, and stained with hematoxylin and eosin.

Tissue blocks for electron microscopy were further fixed by immersion in 3% phosphate-buffered glutaraldehyde solution (pH 7.4) but not necessarily adjusted for osmolarity, and kept overnight at 4°C. Dehydration, infiltration and embedding for electron microscopy were done according to standard procedures (Glauert, 1975) with slight modifications to make five different regimes (Appendices 2.1-2.6), i.e. (1) Standard with post-fixation in osmium tetroxide and uranyl block staining; (2) standard but without uranyl block staining (-UB); (3) Standard with ruthenium red (RR) in place of uranyl; (4) Standard with prolonged

dehydration and without uranyl; (5) Standard with tannic acid (TA) and prolonged dehydration but without uranyl or osmium block staining; (6) Standard with reduced osmium (RO). Semithin sections at 1μm were cut using an LKB NOVA® microtome and stained with toluidine blue.

Appropriate sites were determined from semithin sections in a light microscope and ultrathin sections cut at 60nm using an LKB NOVA® microtome. Sections from blocks processed under the different regimes were then mounted on uncoated 300 mesh copper grids, stained with lead citrate (Reynolds, 1963) and uranyl acetate (Watson, 1958) and examined in a Jeol, JEM 1200EX electron microscope at 60 kV.

Technical variables and discussion

The minimum tank height used with success was 1.5 m. Some stoppages were encountered at tank heights above 1.5 m in some animals, suggesting that blockage was the cause . Aggregates of erythrocytes formed within seconds of contact with glutaraldehyde solution and blockage occurred within the canula if there was back-flow of blood. In perfusion trials using formaldehyde (not included in this thesis) no clogging occurred even in cases of temporary back-flow and fixative flow was unimpeded at tank heights similar to those used with glutaraldehyde solution. This was due to the fact that the cross-linking effect of glutaraldehyde is faster than that of formaldehyde (Vassar et al., 1982). This indicates that the speed of fixation of the canula and the timing of clamping of the opposite carotid artery are crucial when glutaraldehyde solution is the perfusant. When clotting led to blockage in the first animal the heparin dose was raised to 1,250 i.u/kg and this was maintained after the

successful perfusion of the second animal, although it appeared high, since no bad effects were noted.

Brains were normally uniformly yellow-brown, but often large patches of adequately fixed tissue alternated with less well fixed ones. The cerebellum was frequently inadequately fixed. At light microscopic level brain capillaries and larger vessels in fixed areas were devoid of erythrocytes or plasma, and the relative number of vessels devoid of blood cells was used as a qualitative index of the efficiency of the perfusion and such areas were used for electron microscopic examination.

The distribution and size of fixed areas of the brain possibly depended on which part and how much of the vascular network was perfused. Generally, temporal and frontal parts of the cerebral hemispheres were the best fixed areas. Proper selection of well fixed areas was done both by gross examination and light microscopy such that minimum artifacts attributable to inadequate fixation appeared at the electron microscopic level.

Perfusion fixation was successfully done in anesthetized goats with 3% glutaraldehyde solution buffered with sodium phosphate to pH 7.4 and adjusted to approximately 500 mOsm. A tank height of 1.5 m above the table and a heparin dose of 1,250 i.u./kg were found optimal. The most critical step was the time from fixing the canula in the carotid artery and initial fixative flow. The shorter the time for this step the better the chances of a successful perfusion with glutaraldehyde solution.

EXPERIMENTAL ANIMALS

Animals

Fourteen Tanzanian blended goats, 8 male and 6 female, were used. Their ages ranged between 1 and 1½ years and they weighed between 10 and 25 kg. The goats were of mixed breed with their genetic composition including Boer, Kamori and Small East African breeds. All goats had been raised at a government research station at West Kilimanjaro for research purpose and had been regularly dipped for ectoparasites.

Upon transfer to Morogoro where the experimental infections were carried out, the goats were confined indoor in a tickproof house during the rest of the experiment. Guatemala grass was brought in from far out areas not in contact with other animals and fed *ad libitum*. This was supplemented with *Leucaena leucocephala* and goat meal comprised of maize bran, maize and cotton seed cake. Regular physical checks on animals were conducted to make sure that there were no ticks on them, and then 2 months were allowed before the first inoculations were carried out.

Inoculation

Ball 3 strain of *Cowdria ruminantium* in sheep whole blood vaccine was used. The infective material was kindly provided by Dr. Conrad E Yunker, Zimbabwe Heartwater Research Project, Harare. The vials were kept in dry ice during air transport from Zimbabwe to Tanzania. Upon arrival they were placed in liquid nitrogen at -180^{0}C and stored until use.

The vaccine which is issued by the Veterinary Research Institute, Onderstepoort, in the Republic of South Africa contains fully virulent *Cowdria* in whole blood. When used as vaccine, treatment with long-acting oxytetracycline is necessary during the first 2 days of fever, but if used without treatment it results in fatal heartwater (Du Plessis et al., 1983, Gruss, 1983). Each vial contained 10 ml of the inoculum. Prior to inoculation the vaccine was left to thaw at room temperature for 30 minutes. The body temperature of the animal to be infected was recorded and 5 ml of infective material administered intravenously via the jugular vein.

Monitoring

Following inoculation the animals were given access to food and drinking water *ad libitum*. Body temperature was recorded twice daily, once in the morning and once in the afternoon, and the animals were observed for signs of ill health. Two animals were infected each time and since clinical disease appeared about 2 weeks post-inoculation in the first 2 goats, 2 to 3 weeks were allowed between inoculations. Animals were not killed before fever started to drop, but soon after the initial temperature drop, or when central nervous system signs appeared, the animals were taken for perfusion in order not to allow possible recovery or natural death.

Killing and perfusion technique

Animals were killed under general anesthesia by perfusion as described on page 14. There were no major variations to the technique for all 14 animals since the optimal dosage rate for

heparin and tank height had already been tried and fixed at 1,250 i.u/kg and 1.5m respectively with controls. An exception was the first goat to be examined in which methylene blue was given i.v. at a total dose of 5ml five minutes before perfusion as a tracer for hemorrhage in the brain.

Tissue sampling and processing

The head was opened and brain samples taken as described on page 15 under control animals. A complete necropsy was performed and tissues taken for light microscopy from prescapular lymph nodes, spleen, heart, thymus, lung, liver, kidney, adrenal glands, skeletal muscle, small intestine, abomasum, and other organs if they had gross lesions. The tissues were fixed in cold 10% neutral, buffered formalin for at least 48 hours. They were then processed and embedded in paraffin wax, and sections of about 6 μm were cut and stained with toluidine blue, Giemsa, and hematoxylin and eosin.

Brain crash smears were prepared from unfixed areas, fixed on glass slide with 96% ethyl alcohol and stained with Giemsa for 20 minutes. The smears were then examined in the light microscope. In many cases this procedure could not be performed due to total hardening of brain tissue following perfusion.

Adequately fixed areas of the brain were determined by their yellow color and firmness. A uniformly greenish-blue tinge was seen on the brain of the animal which had been given i.v. methylene blue to trace hemorrhage. From these areas, 1 mm^3 blocks of cerebral and cerebellar cortex were taken. In each case rostral and lateral cerebral and mid-cerebellar

cortices were sampled from the same side of the brain. Tissue blocks were further fixed by immersion in 3% buffered glutaraldehyde solution (pH 7.4) but not necessarily adjusted for osmolarity, and kept at 4^0C. The blocks were left to stand in the fixative until just prior to transportation to Denmark. They were rinsed three times in 0.1M sodium cacodylate buffer and then kept in the buffer during transportation.

Brain tissues for light microscopic examination were taken from the same areas as those from which tissues for electron microscopy were taken. The brain tissues and choroid plexus were further fixed in cold 10% neutral, buffered formalin and processed for light microscopy. Blocks of glutaraldehyde-fixed brain tissue and of choroid plexus were processed for electron microscopy according to the procedure given in Appendix 2.7. The modified regimes used on brain tissue from the control animals were not applied to tissues from experimental animals. The reasons for this omission are discussed with the results of electron microscopy of control brains. Semithin sections of 1μm thickness were cut using an LKB NOVA• microtome and stained with toluidine blue. Appropriate sites were determined from semithin sections in a light microscope. Ultrathin sections were cut at a 60nm using an LKB NOVA• microtome. The sections were mounted on uncoated 300 mesh copper grids, stained with lead citrate (Reynolds, 1963) and uranyl acetate (Watson, 1958) and examined in a Jeol, JEM 1200EX electron microscope at 60 kV.

Measurement of size of the parasites was done on electron micrographs. Two approaches were used in taking the measurements. In the first approach it was assumed that some of the organisms are cylindrical and some spherical; and so diameters of nearly round structures were basically the true diameters of the organisms, whether elongate or spherical. Ovoid

structures were assumed oblique sections of the elongate organisms and so measurements across also represented their diameters. Fifty-seven random measurements were taken. In the second approach two measurements were taken for each organism which appeared ovoid but not elongate, one measurement through the middle of the organism along the smaller diameter and another perpendicular to the first, along the greater diameter. This was necessary because a considerable number of organisms were ovoid or not evenly round. Means for the two measurements were calculated and accumulated as diameters for the individual organisms. In both approaches small organisms having a light center were disregarded on assumption that they represented tapering end pieces of the organisms. Also elongate organisms were disregarded since there may be a great variability between organisms in growing, resting, division and newly divided stages. Results were tested using Student's t-test (Snedecor and Cochran, 1967).

4. RESULTS

CONTROL ANIMALS

Light microscopy of brain and other organs

In 1μm sections stained with toluidine blue, capillaries and larger vessels in well fixed areas of the brain were devoid of erythrocytes or contained only few. The relative number of vessels devoid of blood cells was used as a qualitative index of the efficiency of the perfusion at the light microscopic level. Penetration of RR was poor with only a thin peripheral rim assuming a brown color. In 3 goats out of 5 blue-staining intracytoplasmic granules, spherical or oval in shape and variable in size were found in pericytes (Fig. 4). Several of them could be found in one cell but the frequency was one or two cells in a semithin section and not in all blocks. Apart from these the brain tissue appeared normal in both semithin and paraffin sections. There were no pathologic changes in other organs.

Electron microscopy of brain

There were few or no blood cells and plasma solids in vascular lumens in fields where perfusion was partial. Figs. 2 and 3 show a normal capillary. Well fixed endothelial cells encircled an empty lumen and their organelles were intact. Tight junctions joined ends of endothelial cells which were raised into marginal folds. In all cells the cytomembrane was continuous with occasional microvilli projecting into the lumen. There were few cytoplasmic vesicles. Organs encountered were Golgi apparatus, centrioles, microtubules, mitochondria

with lamellar cristae, rough endoplasmic reticulum, nuclei with nucleoli as well as free ribosomes. The cell membrane was in close apposition with the basal lamina on the abluminal surface and the lamina would at some points bifurcate to enclose also a pericyte. Astrocytic feet were in close proximity to the pericytes and endothelial cells, and also within the vicinity were dendrites and axons , with associated organelles such as microtubules and mitochondria in them. Occasionally, electron dense bodies similar to those found in pericytes, to be described later, were found in some neurons but these were fewer and much smaller. Lipofuscin was found in some cells in the neuropil, (fig. 2).

Intracytoplasmic, spherical or oval electron dense bodies were found in the cytoplasm of pericytes. These bodies were enclosed in a membrane. Their size ranged between 0.25μm - 7.6μm and numbers varied from cell to cell, with pericytes of venules having more. Within these bodies were lipid droplets.

There was adequate density and contrast both with and without uranyl block staining but results from ruthenium red and reduced osmium fixation were better in terms of contrast (Fig.3). The extended dehydration incorporated in one of the regimes (Appendix 2.6) was a modification intended to increase membrane contrast by increasing extraction of cell matrix. In this case, however, there was no substantial increase in contrast, possibly due to extraction of membrane components.

The density and contrast of cell membranes and basal lamina were substantially enhanced with RR (fig.3) although the RR could only permeate a small rim of the tissue block. The basal lamina and cell membrane showed sharp contrast. The results with reduced osmium

fixation were similar to those with RR except that there was more contrast with the RR. Membranes of cells and organelles were substantially enhanced and penetration of reduced osmium, although poor, was better than that of RR. Results with tannic acid were not encouraging because there was less density and contrast than with RR and reduced osmium. The idea of using electron dense chemicals in this study was to trace possible endothelial damage in animals with heartwater if such damaged endothelial cells would allow an influx of the chemicals into their cytoplasm as opposed to structurally intact cells.

RR, RO and TA were incorporated into post-fixation regimes because it was evident, that incorporating them in the perfusion solution would require large amounts of them and therefore it would be too expensive. So, in spite of their ability to enhance contrast of normal vascular and neural tissue they were dropped from the processing regime for experimental animals due to their poor tissue penetration. Where perfusion of small organs or small whole animals such as mice is involved the three substances are strongly recommended. Prolonged dehydration was not adopted into the experimental tissue processing because it resulted in uniform extraction of both membranes and matrix, without any contrast improvement.

EXPERIMENTAL ANIMALS

Clinical signs

About 13 days post-inoculation all goats started bleating followed by sudden fever, general weakness and loss of appetite. Body temperature rose to peak (range 39.6^{0}C-41.5^{0}C) within 24 hours after the high temperature recording and remained so for 2 days before initial

temperature drop was recorded. During the pyrexia the hair coat was rough, and animals quickly lost condition. Most animals showed abdominal breathing and all had a diminished response to approach by humans. One showed loose fecal matter (semisolid rather than pellets). In most goats coarse head tremors were seen together with pronounced ataxia. Two goats showed severe signs of central nervous disorder including falling into lateral recumbency, followed by paddling of all four limbs with opisthotonus. These attacks followed handling during transportation to the necropsy room and lasted up to 20 minutes after which the animals were unable to rise on their hind legs. Goats infected later in the experiment showed more pronounced signs than those infected at the beginning, 4 months earlier.

Gross lesions

Yellow, clear, pericardial fluid was present in all cases, the amount varying between 2 and 13 ml, measured by drawing by syringe and needle before opening the pericardial sac. The heart was flabby in some cases, but in most cases hardened by fixative during perfusion. There were some subepicardial and subendocardial petechiae, the former mostly located on the ventricles, in most cases near the apex on one or both sides of the heart.

When properly fixed, brains were uniformly firm and yellowish-brown. Vessels were flushed out but partial fixation allowed congestion to be discerned. Most slight color changes were easily obscured by even slight uniform fixation. In the first animal, given methylene blue intravenously prior to perfusion as a tracer for possible hemorrhage in the brain,

a greenish-brown, uniform tinge was imparted without any indication of hemorrhage. This procedure was discarded in the remaining animals.

In all animals the spleen appeared slightly enlarged with rounded edges and a bulging and pulpy cut surface. Follicles were prominent and nodular. Lymph nodes were generally enlarged and wet on the cut surface. The urinary bladder was distended in most cases but the contents were clear, and no obstruction of the urinary tract was observed. Occasionally, a few petechial hemorrhages could be found on the mucosal surface of the bladder.

Distention of the gall bladder was noted in 2 cases but contents were of normal appearance. In 2 cases intestinal contents were loose, corresponding with the observed clinical signs. Fecal matter was not formed in another goat which had not shown signs of diarrhea.

Light microscopy of brain and other organs

In smear preparations infected endothelial cells stained with Giemsa were seen to contain in their cytoplasm purple organisms at the nuclear poles. Their morphology and location in the cells were consistent with that of *C. ruminantium*.

There were varying degrees of infiltration with lymphocytes and macrophages in the subarachnoid space in all 14 goats (Fig. 9). In 12 cases similar infiltrations were observed around medium and small blood vessels including capillaries deeper in the brain substance (Figs. 8,9,10,11). No endothelial cells of these vessels contained recognizable *Cowdria* organisms. Some goats had focal mononuclear cell infiltrations within the grey matter,

although it was difficult to decide if these were extensions of perivascular infiltrations. A few cells could be seen outside the affected vessel within the Virchow-Robin space, but with more pronounced infiltrations inflammatory cells extended beyond this space into the brain substance. In many cases it was possible to locate a capillary or larger vessel in the middle of the inflammatory foci, but often no vessels were demonstrable or they were located eccentrically in which case the inflammatory foci appeared in the neuropil.

Infected endothelial cells had a prominently ballooned cytoplasm filled with parasites. The cytoplasm bulged into the vascular lumen while the nucleus was pressed towards one end or the basal lamina. In semithin sections stained with toluidine blue (Figs. 6, 7) parasites were pale blue against a blue tissue background. Paraffin sections stained with toluidine blue, however, showed purple-stained parasites against a blue background making visibility of parasite colonies better (Fig. 5), although the outline of individual parasites was better in semithin sections. There were large numbers of parasites in each infected endothelial cell, and in all cases without any associated perivascular reaction. In a large number of cases brain smears could not be made due to hardening of tissue following perfusion.

Pericyte inclusions, possibly lysosomal degradation products, were seen with a fairly high frequency compared to those found in control animals. In 1μm semithin sections (Fig.6) stained with toluidine blue these were dark blue in color, but occasionally the larger ones assumed a brownish yellow color. Single, large or small inclusions were found but in most cases inclusions were multiple within one cell. The inclusions were difficult to see in hematoxylin and eosin-stained sections.

Coccidia at various development stages were seen in the epithelium of the small intestinal mucosa of 1 goat. This lesion was focal. Seven animals showed mild multifocal interstitial lymphocytic infiltration of the kidney. Most of the infiltrations were located around vessels in the deeper cortex and were most pronounced at the cortico-medullary junction.

Focal lymphocytic infiltration of the lung with thickening of the interstitial tissue was seen in 4 goats. A large number of hemosiderin-laden macrophages were also found within the inflammatory foci in the interstitium. Cysts of *Sarcocystis spp.* were found in muscle fibers of both skeletal and cardiac muscle in all but one animal. There was no inflammatory reaction around indicating that the cysts were intact. There were focal mononuclear cell infiltrations in subepicardial and subendocardial locations in 6 animals. Most of the inflammatory cells were lymphocytes and macrophages. Some foci were found within the myocardium. Splenic periarteriolar lymphoid sheaths (PALS) were prominently visible in all 14 animals with arterioles assuming an eccentric location within the PALS in which mitotic figures were conspicuous. Periportal lymphocytic infiltrations were found in livers of 4 animals. Most of these infiltrations were mild and without any accompanying bile duct proliferation or thickening.

The clinical, necropsy and histopathologic findings in the experimental animals are summarized in Appendix 3.

Electron microscopy of brain

Vascular changes

Cowdria organisms were located in single, large cytoplasmic vacuoles in endothelial cells. The vacuoles appeared to be membrane-bound (Fig. 12). In between organisms was scant floccular material. Individual organisms were bound by a double membrane and their matrix had electron dense and electron light areas (Fig.13). Light areas appeared to be just under the membrane, surrounding the electron dense center. The size ranged from 0.32μm to 0.6μm in diameter (Mean = 0.43 μm, SD = $\pm$ 0.0676, n = 57) when measured across. When two dimensions perpendicular to each other were used the diameter ranged between 0.38 μm and 0.77 μm (Mean = 0.51μm, SD = $\pm$ 0.1168, n = 24). There was a statistically significant difference between the two means ($t = 3.88$, $p < 0.001$).

Each vacuole containing a colony of *C. ruminantium* displaced the nucleus to one side, compressing it (Fig. 12). A thin strip of cytoplasm remained between the luminal endothelial and vacuolar membranes. In others only the vacuolar membrane and the endothelial membrane on the vacuolar side remained apposed together without discernible cytoplasm in between (Fig. 14). Compression and attenuation of the basal endothelial cytoplasm was also evident but less pronounced (Fig. 15). Mitochondria were sometimes dense in parasitized cells. There appeared to be more cytoplasmic vesicles in some parasitized endothelial cells, but this was difficult to reconcile with the fact that organelles had been displaced in a smaller space. Tight junctions between endothelial cells looked normal.

Electron dense inclusions of various sizes were found in the cytoplasm of pericytes (Fig. 20). The inclusions were composed of electron dense material containing lipid droplets, mainly peripherally. The inclusions were membrane-bound and occurred as single, large bodies, multiple small bodies, or both in the cytoplasm of affected cells. At the light microscopic level the inclusions appeared blue to purple when stained with toluidine blue in 1μm thick sections, while some of the larger inclusions had a yellowish color but both were electron dense at the electron microscopic level. The inclusions were seen in both control and experimental animals, but with a much higher frequency in the latter in every animal.

Intravascular mononuclear cells were observed adhering to endothelial cells (Figs. 16, 17), their cytoplasm pitting deep into the endothelial cells without breaking the endothelial cell membrane (Fig. 18). At some locations large portions of mononuclear cells were seen embedded between endothelial cells and the basement membrane (Fig. 19) presumably in the process of migration to an extravascular location.

Perivascular and paravascular changes

Perivascular areas in the brain were infiltrated with mononuclear cells, mainly lymphocytes and macrophages and occasional plasma cells. Most of the cells were located between the neuropil and the outermost basement membrane around venules, but often with extensions into the neuropil both in lesions around capillaries and around venules (Figs. 21, 24). Sometimes solitary foci of mononuclear cells were seen in the neuropil (Fig. 30) but were believed to represent tangential sections of perivascular infiltrations.

Inclusions reminiscent of lipofuscin granules were commonly found within the cytoplasm of mononuclear cells (Figs. 21, 25). These inclusions were electron dense and contained lipid droplets. The difference between these granules and those seen in pericytes was that those in pericytes were mostly round and much larger, although their size too was variable, and had smooth borders. However, occasionally large inclusions similar in size and appearance to those in pericytes were found in mononuclear cells in neural tissue.

In a number of cases irregular, ovoid or spherical, intracytoplasmic inclusions of variable size were seen in macrophages. These contained several, smaller, round, membrane-bound particles ranging in size from 0.25μm - 0.4μm, closely packed together (Figs. 25, 26). The interphase between these aggregations and the cell cytoplasm sometimes suggested the presence of a delineating membrane while in other places the particles appeared to be lying directly in the cytoplasm (Fig. 26). The internal structure of the particles consisted of electron dense and electron lucent areas, in some cases with a lamellar or cristae-like structure suggestive of mitochondria. A precise identification of these particles was not possible. They may represent aggregations of partly disintegrated mitochondria although the general appearance of these inclusions was not characteristic of autophagic vacuoles.

Plasma cells were mainly located around blood vessels and, in the meninges, also more diffusely. Their cytoplasm showed abundant rough endoplasmic reticulum and prominent Golgi apparatus. Several macrophages contained, apparently phagocytosed, necrotic cells reminiscent of plasma cells (Figs. 22, 23). Such cells had multiple, linear or elongated, partly fragmented, electron dense masses in their cytoplasm, sometimes barely recognizable

mitochondria, and nuclei with markedly condensed chromatin at the periphery and electron lucent centers.

The interphase between the vascular wall and neuropil showed minimal changes apart from the mononuclear cell infiltrations. There was slight dilatation of astrocytic feet with less dense cytoplasm or with fine electron dense granules. In many cases, however, no changes were detectable in this area apart from the presence of mononuclear cells and their inclusions. As in control tissues, some neurons contained lipofuscin granules. They were of much smaller size than those found in mononuclear cells, but otherwise similar in appearance.

Meningeal changes

Mononuclear cells similar to those found around cerebral vessels and in the neuropil were also seen in the subarachnoid space (Fig. 27). Plasma cells were often seen (Fig. 28) and occasionally, eosinophils (Fig. 29). Plasma cells had abundant and prominent rough endoplasmic reticulum and Golgi apparatus. In between inflammatory cells were collagen fibers in oblique, cross or longitudinal sections. Phagocytes containing necrotic cells, similar to those adjacent to blood vessels in the brain, were observed.

Neuropil changes

Neurons, even in the vicinity of mononuclear cell infiltrations, appeared normal (Fig. 31) except for occasional lipofuscin inclusions. In some areas mononuclear cells within the neuropil were seen with various inclusions including some similar to the ones shown in figs. 25 and 26, whereas most appeared to be lipofuscin.

5. DISCUSSION

In this study a compromise was reached where clinical signs of heartwater were left to develop but not allowed to advance to an extent that natural death intervened before perfusion could be performed. Clinical signs appeared 10-13 days post inoculation, slightly longer than the average incubation period stated by Pypekamp and Prozesky (1987). Since animals infected in the beginning of the experiment showed a weaker response than those infected later there is a likelihood that they had partial, waning, naturally acquired immunity. Regular tick control measures are normally taken at the station of origin but tick resistance is not uncommon.

Clinical signs started with bleating especially at night, followed by fever of up to 41.5^{0}C which persisted for about 2 days. During this time animals lost weight dramatically. Signs, including stupor, head tremors and incoordination were consistent in most animals. Few animals progressed to more serious signs of central nervous system disorder, i.e. convulsive signs. All signs conform with those described earlier (Alexander, 1931, Uilenberg 1983, Pypekamp and Prozesky, 1987, Spreull, 1922). If stupor, head tremors and incoordination are considered, all 14 animals developed neurologic signs, although mild. The goats used were blended (Boer, Kamori and Small East African goat), unlike Angora goats which are considered to be more susceptible (Pypekamp and Prozesky, 1987). However, the fact that clinical disease was not left to take its course to the end is another possible reason for the mildness of the signs.

The organisms compared well in size with those described by Pienaar (1970). Values

calculated from two dimensions were significantly higher than single diameter measurements ($t = 3.88$, $p < 0.001$). Basing on values given by Pienaar (1970) it appears that the organisms in this study were of the small forms. It is, however, important to mention here that lengths of organisms in this study were ignored.

Hydropericardium was one of the commonest necropsy findings, appearing in all 14 goats. The amount of the effusion was, however, not striking, ranging from 2 to 13 ml. Mild respiratory distress (abdominal respiration) was noted at clinical examination but no significant signs of cardiac insufficiency were seen at necropsy. Some isolated interstitial mononuclear cell infiltrations were occasionally encountered in the lung. Du Plessis (1986) as cited by Prozesky, (1987b) reported a mild to moderate mononuclear cell infiltration in the lungs of mice infected with the Kümm strain of *C. ruminantium*.

Subendocardial, subepicardial and perivascular mononuclear cell infiltrations were found in the heart. No reports are available on this type of lesion, but since it is mild it is likely to be overlooked. Results in this study are based on single sample examination from each organ. It is therefore difficult to tell whether more animals could have shown a particular lesion or not, if further sampling had been done. Only mild interstitial mononuclear cell infiltration of kidneys was observed in some goats, unlike the more pronounced signs of nephrosis reported by Prozesky and Du Plessis (1985).

Splenomegaly was present in all animals. Du Plessis (1985) made similar observations in Angora goats and Merino sheep. In this study the cut surface was only slightly pulpy, but splenic follicles were prominent. Light microscopic examination showed enlargement of

PALS, marked mitotic activity within germinal centers and an eccentric location of arterioles, which is an indication of an active immune response (Wheater et al., 1979). However, the splenomegaly as recorded at necropsy in this study may have been partly caused by the pentobarbitone administered prior to perfusion.

Spreull (1922) reported regular urination and defecation in goats with heartwater. Animals in this study had a full bladder at necropsy without any apparent physical urinary tract obstruction. Occasionally, petechial hemorrhage was noted in the bladder. Spreull's clinical observation and findings in this study indicate that there is an underlying central urination control loss.

Pericyte lysosomal inclusions found in brains of experimental animals were similar in structure to those found in normal control animals. The role of pericytes is still not clear. Crocker et al., (1970) observed contacts between pericytes and endothelial cells during wound healing and suggested that pericytes probably regulate capillary proliferation in non-neural tissue. Pericytes in neural tissue have been considered to play a phagocytic role (van Deurs, 1976, Cancilla et al., 1972, Murabe and Sano, 1981). It has also been observed that pericytes have F_c and complement receptors (Oehmichen, 1981). While pericyte lysosomal inclusions are commonly found in neural tissues of healthy individuals (Peters et al., 1976) there was a difference in the frequency of their occurrence between control and heartwater goats in this study, being only occasional in control goats and very frequent in the latter. Whether this has to do with heartwater or not, cannot be decided here, since control goats had been reared in a geographically far-off country from where experimental goats were. It is only important to point out that heartwater goats showed a tendency to having more blood

vessels with such "loaded" pericytes than controls.

Cellular infiltrations of leptomeninges were frequently observed. Lymphocytes, macrophages, plasma cells and occasional eosinophils were seen. Pienaar et al. (1966) described a diffuse meningitis with mainly macrophages in some cattle, while Prozesky and Du Plessis (1985a) did not mention any meningeal involvement in the 10 goats they studied. The lesion was seen in 11 out of the 14 goats in this study. It can be speculated here that perhaps the immune status of the individual animal has a significant bearing on the histologic picture since even among the many cattle studied by Pienaar et al. (1966) only few showed this change.

In the brain mononuclear cell infiltration was the commonest finding. The mononuclear cells were seen adhering to and migrating through the vascular walls, indicating that at least some of the mononuclear cells in extravascular locations were derived from the circulation, a view also held by other investigators (Murabe and Sano, 1983, Oehmichen, 1983, Torvik, 1975). The infiltration was strictly perivascular. Pienaar et al. (1966) and Prozesky (1985a) reported vasculitis, but in this study the infiltrations did not appear to involve any subendothelial vascular structures. Intramural infiltrations are caused by the presence of antigen or immune complexes in vascular structures (Cheville, 1983, Tizzard, 1987). Considering the size of the parasites in this case, were they the antigen it should be possible to see them. Furthermore, they show specific tropism for phagocytic and endothelial cells. Any presence of antigen, in this case *C. ruminantium* or their metabolic products, in intramural locations would presumably attract inflammatory cells. But this was not apparent here. Instead, in view of the electron microscopic findings it seems reasonable to view antigen presence in

extravascular structures outside vascular structures as the stimulus for the inflammatory reaction. This poses a question as to how the antigen might have migrated. There is no doubt that *C. ruminantium* during their initial stages multiply in phagocytic cells of lymph nodes and spleen (Du Plessis, 1970). Further parasitization of endothelial cells takes place from within the lumen. Any antigen located extravascularlly must have crossed the vascular wall.

Parasitized segments had no cellular exudation, and segments with perivascular exudation had no endothelial parasitization. The observation, at the electron microscopic level, of the non-membrane-bound aggregations of particles in macrophages in infiltrated areas is the closest suggestion of a possible parasite-induced lesion in proximity to the exudate. It is logical not always to expect the presence of parasitized endothelial cells in segments where infiltrations are, since the infiltrations cover larger areas, chances being that a section will miss one infected endothelial cell and still include a cuff. However, if the two processes should occur concurrently parasitized segments would be expected to be cuffed, which they were not.

Kocan et al. (1990) found that it took 48 hours between initial entry of *C. ruminantium* into a host cell and formation of reticulated forms, and a further 8 hours to mature. Sixteen more hours lapsed before final release of the parasite. This means that, at least *in vitro*, a cycle is 72 hours. Du Plessis (1975) also found that organisms became clearly distinguishable in peritoneal macrophages at 72-96 hours. Animals in this study were killed 2-3 days after the onset of fever, which falls within this period. It is likely that the inflammatory process in this study started towards the end of parasitization since intact parasitized endothelial cells had no cuffs.

A considerable proportion of the cellular exudate observed in neural tissue and around blood vessels in this study consisted of macrophages when viewed in the electron microscope, including many cells which light microscopically looked like lymphocytes. No obvious necrotic neural damage was present in this study. According to Fujita and Kitamura (1976), once monocytes cross into extravascular sites they are transformed into one of two forms: a form with a lymphoid appearance in case of non-necrotic neural damage, or distinct macrophages when necrotic neural damage has occurred, with some intermediate forms. Findings in this study are in accordance with the observation by the 2 authors. The fact that antigen did not elicit any vasculitis while in transit suggests that it was possibly shielded from the immune system up to this point.

It may be desirable to try to draw a hypothetical sequence of events. Parasites appear in endothelial cells after the onset of pyrexia and begin to disappear when fever has subsided (Cowdry, 1925). During this time also monocytes and lymphocytes emigrate from the vascular lumen into the extravascular locations. At the end parasites are cleared and animals recover or die depending on the seriousness of the infection.

Assuming that parasites released into the lumen are taken up by circulating monocytes, it is possible that such monocytes may find their way into extravascular sites where parasites fail to elude the immune system as they did during the incubation period. As it appears from observation in this study the incubation period is long enough for development of an effective immune reaction such that the organisms fail to repeat their developmental cycle without seriously being hampered, but also causing the deleterious effects of the inflammatory reaction to the host. Possibly through lymphokines, more cells are recruited from the blood

stream, resulting in the observed morphologic picture. Findings in this study could conceivably represent an aborted attempt by *C. ruminantium* to repeat a developmental cycle in monocytic macrophages outside convenient sites and after the host has mounted a strong immune response.

The possibility of *C. ruminantium* crossing the neurovascular endothelium into the neuropil has not been investigated. Since multiplication in early infection takes place in reticulum cells and macrophages (Du Plessis, 1970) and the infective component of blood is the buffy coat (Sahu, 1986, Ilemobade, 1976) it is not unlikely that the organisms may be carried by monocytes into the neuropil. This "Trojan Horse" concept of spread of organisms is reviewed by Williams and Blakemore (1990).

It is argued that microglia originate from circulating monocytes in neonatal life (Ling, 1978, Perry and Gordon, 1988, Hickey and Kamura, 1988, Ling, 1981) and that they are capable of presenting antigen (Hickey and Kamura, 1988). However, Fujita and Kitamura (1976) view microglial and blood derived macrophages as ontogenically unrelated and do not consider microglial cells to play any role in antigen processing and presentation. The origin and relationship of various cells of the macrophage class in the brain have been reviewed recently by Jordan and Thomas (1988). Whatever opinion is true, recruitment of circulating monocytes into the central nervous system during infection, including heartwater, arouses interest in investigating the possible involvement of direct presence of antigen in the neuropil in the pathogenesis of heartwater.

The severe distention of cytoplasm of parasitized endothelial cells seen in this study, without

other significant ultrastructural alterations, concurs with what has been described by others (Pienaar, 1970, Prozesky and Du Plessis, 1985b). *C. ruminantium* appear to be able to survive and multiply in endothelial cells while at the same time causing no significant metabolic disturbance, which would be reflected in morphologic changes. The physical displacement/compression of organelles seems to be tolerable to a large extent, and probably only physical disintegration of the cytomembrane marks the end of parasitization.

Non-parasitized endothelial cells appeared completely normal even though they were directly in contact with parasitized ones. This finding contradicts those reported by Pienaar (1970) and Prozesky and Du Plessis (1985b) who observed non-parasitized endothelial cell swelling and inter-endothelial cell gaps. This study was designed to eliminate possible sources of artifacts. Prozesky and Du Plessis (1985b) did not specify the method of killing or the time lapse between death and sampling. It is also not clear whether electrocution could influence the anatomical relationships in the capillary endothelium. It is, however, important to note that animals in this experiment were killed before becoming critically ill, except for 2 which did not show such lesions either.

Interesting is the fact that in the absence of endothelial alterations and protein droplets in perivascular spaces as reported by Pienaar et al. (1966), signs of central nervous system disorder were present. The presence of eosinophilic droplets around capillaries and the observation of inter-endothelial gaps have been the basis for the hypothesis that a vasoactive substance plays a key role in the pathogenesis of heartwater. Du Plessis et al. (1987) were not able to alter the course of clinical disease by administering serotonin and histamine antagonists. Morphologic alterations in this study, including perivascular and neuropil

leukocytic accumulations, seem to be the basic alteration since even the mildest inflammatory process in the brain substance may result in neurologic signs (Cheville, 1983). It would seem most probable that signs of vasoactive substance activity are a later development of an inflammatory reaction rather than being pure and basic, at least in the cerebral vasculature.

Although mitochondria in parasitized endothelial cells appeared normal several particles in fragmented inclusions were frequently found in phagocytic cells in the perivascular locations, occurring in clusters. These particles appeared faintly lamellated as if they were mitochondria. If those inclusion particles are mitochondria, then mitochondrial inclusions could be a newly observed phenomenon in the host. Mitochondrial associations with *C. ruminantium* have been reported in cell cultures (Kocan et al., 1990). These authors noticed a close association between *C. ruminantium* growing in vacuoles in mouse macrophage cell cultures and host mitochondria. The mitochondria were intact and surrounded reticulated forms which are apparently an intermediate stage between an initial stage which infects the host cell and dense forms which represent mature organisms.

The particles within the inclusions seen in this study appeared degenerate but with recognizable outlines, and interspaced with non-identifiable debris. Much as an external ATP source may be needed at this stage of development (Kocan et al., 1990), and the host cell ATP being available, it appears that the environment in presumably armed macrophages in a reacting host is unfavorable for the development of the organism. No clear reticulated forms were discernible but there is no other obvious explanation for the presence of mitochondrial aggregations in the phagocyte cytoplasm apart from a possible link between them and reticulated forms of *C. ruminantium* as observed by Kocan et al. (1990).

Sequestration of defective mitochondria occurs as part of a normal turnover process or in pathologic conditions (Ghadially, 1988) with or without other organelles. However, the extent and features of such sequestrations do not correspond with the findings in this study.

On the other hand, Du Plessis (1975) observed some developmental stages of *C. ruminantium* in peritoneal macrophages. The organisms were aggregated in what he called "fragmented bodies". The fragmented bodies did not appear to be in vacuoles, and had particles recognizable as developmental stages of *C. ruminantium*, their size varying between 0.41μm - 0.43μm. There are similarities between these "organisms" and the ones described in this study, namely that they often appeared to be lying directly in the cell cytoplasm, that they have been found in macrophages and that the size is between 0.25μm - 0.4μm. However, the internal structure, though not very clear, is suggestive of mitochondria. Observations by Kocan et al. (1990) indicate the presence of both, mitochondria and organisms, while Du Plessis (1975) observed only organisms. However, the observations in this study are not conclusive. It is believed that the particles represent one of the two, but more studies are needed. Whichever is the case, there are suggestions of the presence of *C. ruminantium* in phagocytic cells outside vessels in neural tissue. Future investigations into the pathogenesis of heartwater may benefit from inquests into this possibility.

The presence of macrophages in the central nervous system parenchyma is usually associated with clearance of degenerate myelin and native cells. Phagocytes in such missions are in later stages often laden with lipid. In this study no obvious necrotic processes were observed (myelin breakdown products or degenerating cells). Although such processes may occur later, phagocytes were there in the vicinity of blood vessels with an accompanying monocytic cell

accumulation, none accumulating in intramural sites. Phagocytic cells may be recruited for antigen processing and presentation purpose primarily rather than myelin clearance, and since such cells did not appear within vascular walls it is plausible that the antigen is not soluble and has no particular affinity for vascular structures except for the prior establishment and replication of *Cowdria* in endothelial cells. Early macrophage and lymphocyte dominance, and previous findings by Du Plessis (1982) concerning the role of cell-mediated immunity strongly suggest an interaction between the macrophages and lymphocytes.

6. CONCLUSIONS

No morphologic evidence of abnormal vascular permeability or intramural damage was observed in the study. Apart from the presence of *C. ruminantium* in otherwise unaltered, vascular endothelial cells, the primary lesion in the brain of goats with heartwater appears to be perivascular accumulations of mononuclear cells, variably extending into the adjacent neuropil.

The unique type of cytoplasmic inclusion encountered in several perivascular macrophages suggests an extravascular presence of *C. ruminantium*, possibly representing abortive developmental stages.

The findings further suggest the possibility of a direct involvement of circulating monocytes in the pathogenesis of the brain lesions. Such cells may carry and shield the antigen through the vessel wall into extravascular sites. Once there, a cell-mediated immune response, developed during and after the incubation period, limits a second developmental cycle of the parasite and results in the perivascular mononuclear cell infiltrations observed.

7. SUMMARY

Heartwater is an infectious, vector-borne, non-contagious disease of ruminants caused by *Cowdria ruminantium*. The etiologic agent is an obligate, intracellular parasite infecting phagocytic cells and, particularly, vascular endothelium. Transmission is effected by a three-host tick of the genus *Amblyomma*. Clinically, the disease is characterized by fever and signs of central nervous system disorder. There is general agreement on a deranged transport mechanism across the vascular wall as the basis for the development of morphologic lesions and clinical signs, but the actual mechanism of damage is poorly understood. Previous ultrastructural studies are few and have mainly been concerned with the parasite itself. The present morphologic study of the cerebral microvasculature is an attempt to elucidate the pathogenesis of the central nervous system changes.

Five, clinically healthy, adult, Danish goats were used to establish the optimal procedure for perfusion fixation of the brain and, at the same time, to serve as normal controls. The animals were put under general anesthesia with pentobarbitone intravenously at 25 mg/kg and placed in dorsal recumbency. Following dissection of the ventral neck region intubation was performed and carotid arteries were isolated. Heparin (1250 i.u./kg) was injected and perfusion done through one of the carotid arteries using 3% phosphate-buffered glutaraldehyde solution at pH 7.4 and 500 mOsm. The best perfusion results were achieved with the tank placed 1.5 m above the heart. The brain was removed and 1 mm^3 samples were diced from the best fixed areas of the cerebral and cerebellar cortices and further fixed i 3% glutaraldehyde solution. Some of the samples were routinely processed and stained for transmission electron microscopy. In an attempt to improve fixation and enhance demon-

stration of cellular membranes and other structures, other samples were subjected to alternative processing and staining regimes including reduced osmium tetroxide, ruthenium red, tannic acid, and uranyl block staining. On balance, however, these regimes did not produce entirely satisfactory results and were therefore omitted in the experimental studies. Corresponding pieces of brain tissue were post-fixed in 10% neutral, buffered formalin and routinely processed for light microscopy. A complete necropsy was performed and various tissues were taken and similarly processed for light microscopy.

Fourteen Tanzanian blended goats were experimentally infected by intravenous inoculation with the Ball 3 strain of *Cowdria ruminantium*. Following an incubation period of about 2 weeks the animals developed temperatures up to 41.7°C. Other clinical signs included bleating, loss of appetite, loss of body condition, listlessness, abdominal respiration, stupor, head tremors and ataxia. In 2 goats, fits, opisthotonus, paddling movements and lateral recumbency were also observed. When temperatures started to drop the animals were killed and subjected to the same procedures as the controls.

Necropsy revealed mild hydropericardium (2-13 ml) and splenomegaly in all 14 goats. Other, inconstant findings were urine retention in bladder, subepi- or subendocardial petechiae and flabbiness of the heart. In areas of incomplete perfusion leptomeninges appeared hyperemic.

Light microscopically, *Cowdria* organisms were found in vascular endothelium of the brain in 8 goats. Focal perivascular mononuclear cell infiltrations, sometimes extending into the adjacent neuropil, were present in the brains of 12 animals. More extensive mononuclear cell infiltrations, occasionally with a few eosinophils, were in the leptomeninges of 11 goats.

Focal mononuclear cell infiltrations were also found, although variably, in pulmonary and renal interstitium and in subepi- and subendocardial locations. All experimental animals exhibited moderate hyperplasia of splenic lymphoid tissue.

The only ultrastructural change of parasitized endothelial cells was compression and displacement of cytoplasm and organelles by membrane-bound colonies of *Cowdria* organisms. Perivascular accumulations of cells, consisting of macrophages, lymphocytes and occasional plasma cells, were invariably associated with non-parasitized segments of capillaries or venules.

Often intravascular mononuclear cells were seen adhering to the endothelium with portions of their cytoplasm dipping into the endothelial cell membrane or interposed between the endothelial cell and the basement membrane. Large, spherical, electron-dense inclusions, often several in the same cell, were found in pericytes, both in control and experimental animals but with a notably higher incidence in the latter. Similar inclusions as well as smaller inclusions with the features of lipofuscin were seen in perivascular macrophages. An additional and unique type of cytoplasmic inclusion was encountered in perivascular macrophages. These inclusions presented as aggregations of irregularly round, membrane-bound particles, 0.25-0.4 μm in diameter, in some cases with an internal structure reminiscent of partly degraded mitochondria. However, the aggregations were not convincingly enclosed within membranes as would be expected in case of autophagocytosis. Another, hypothetical, interpretation is that they represent abortive stages of *Cowdria ruminantium* attempting to develop extravascularly and that possibly cell-mediated immunity, developed during and after the incubation period, limits this second cycle within the host and results in

the perivascular mononuclear cell infiltrations observed.

The findings in this study suggest the possibility of a direct involvement of circulating monocytes in the pathogenesis of the brain lesions. Such cells may carry and shield the antigen through the vessel wall into extravascular sites since no signs of vasculitis or abnormal vascular permeability were found, making it unlikely that soluble antigens are involved. The finding of a morphologically intact endothelium and vessel wall, even under perfusion conditions, also casts doubt on a possible role of vasoactive substances in the development of the lesions. If and when permeability changes of the brain microvasculature occur, they apparently represent a later development of an inflammatory process rather than a primary mechanism in the pathogenesis of cerebral heartwater.

8. SAMMENDRAG

Heartwater er en infektiøs, vektor-båren, ikke-smitsom lidelse hos drøvtyggere forårsaget af *Cowdria ruminantium*. Agens er en obligat intracellulær parasit, som inficerer fagocyter og, specielt, karendothelceller. Overførsel sker via en 3-værts flåt tilhørende slægten *Amblyomma*. Sygdommen er karakteriseret klinisk ved feber og centralnervøse forstyrrelser. I almindelighed anses forstyrrelser af vaskulære transportmekanismer for at ligge til grund for udviklingen af morfologiske forandringer og kliniske symptomer, men den egentlige mekanisme herfor er ikke afklaret. Der foreligger kun få ultrastrukturelle undersøgelser, og disse har hovedsagelig vedrørt selve agens. Nærværende morfologiske undersøgelse af hjernens mikrocirkulation er et forsøg på, at belyse patogenesen af forandringerne i centralnervesystemet.

Fem, klinisk sunde, voksne, danske geder blev brugt til at fastlægge den optimale procedure for perfusionsfiksering af hjernen og tjente samtidig som kontroldyr i forsøget. Dyrene blev lagt i totalanæstesi med pentobarbital, 25 mg/kg intravenøst, og anbragt i rygleje. De ventrale halsorganer blev fridissekeret og der foretoges intubering og isolering af a. carotis i begge sider. Efter injektion af heparin (1250 i.u./ kg) perfunderedes gennem den ene a. carotis med 3% glutaraldehydopløsning i fosfatstødpude ved pH 7,4 og 500 mOsm. Det bedste resultat opnåedes med tanken placeret 1,5 m over hjertet. Hjernen blev udtaget, og 1 mm^3 store stykker skåret ud fra de bedst fikserede områder af cortex af cerebrum og cerebellum og yderligere fikseret i 3% glutaraldehyd. Nogle af vævsstykkerne blev rutinemæssigt viderebehandlet med henblik på transmissions-elektronmikroskopi. I et forsøg på at forbedre fikseringen og fremme visualiseringen af membraner og andre cellestrukturer, blev andre af

vævsstykkerne underkastet alternative procedurer, herunder anvendelse af reduceret osmium tetroxyd, ruthenium-rødt, garvesyre og "en bloc"-farvning med uranyl. Da disse metoder, som helhed, imidlertid ikke gav helt tilfredsstillende resultater, blev de ikke anvendt på væv fra de eksperimentelt inficerede dyr. Parallelle stykker hjernevæv blev udtaget, viderefikseret i 10% neutral stødpudeformalin og rutinemæssigt behandlet med henblik på lysmikroskopi. Der foretoges total sektion og udtagning af forskellige væv til lysmikroskopisk undersøgelse.

Fjorten Tanzanianske geder af blandet race blev eksperimentelt inficeret ved podning intravenøst med "Ball 3 strain" af *Cowdria ruminantium*. Efter en inkubationstid på ca. 2 uger udviklede dyrene temperaturstigninger på op til 41,7°C. De øvrige kliniske symptomer omfattede brægen, anorexi, vægttab, abdominal respiration, slaphed, sløvhed, hovedrystelser og ataxi. Hos 2 geder iagttoges krampeanfald, opisthotonus og travebevægelser liggende i sideleje. Når temperaturen begyndte at falde, blev dyrene aflivet og behandlet som kontrollerne.

Ved sektion fandtes let hydropericardium (2-13 ml) og splenomegali hos alle 14 geder. Andre, variable, fund var urinretention i blæren, subepi- og subendocardiale petechier og mangelfuld kontraktion af hjertet. Leptomeninges fremtrådte hyperæmiske i områder af hjernen, hvor fikseringen var ufuldstændig.

Cowdria-organismer påvistes lysmikroskopisk i hjernens karendothelceller hos 8 geder. I hjernen hos 12 dyr fandtes fokale, mononukleære, perivaskulære celleinfiltrationer, nogle gange strækkende sig ind i den omgivende neuropil. Hos 11 dyr fandtes mere udbredte mononukleære celleinfiltrationer, lejlighedsvis tilblandet nogle få eosinofile granulocyter, i

leptomeninges. Fokale, mononukleære celleinfiltrationer sås også, omend varierende, interstitielt i lunger og nyrer samt subepi- og subendocardialt. Alle eksperimentelt inficerede dyr havde moderat hyperplasi af miltens lymfoide væv.

Den eneste ultrastrukturelle forandring af inficerede endothelceller var kompression og forskydning af cytoplasma og organeller forårsaget af de membranafgrænsede kolonier af *Cowdria*-organismer. Perivaskulære celleansamlinger, bestående af makrofager, lymfocyter og enkelte plasmaceller, fandtes udelukkende i forbindelse med ikke-inficerede segmenter af kapillærer eller venoler.

Intravaskulære mononukleære celler sås ofte adhæreret til endothelet med dele af deres cytoplasma beliggende i dybe impressioner i endothelcellerne eller mellem disse og basalmembranen. I pericyter fandtes store, kuglerunde, elektrontætte inklusioner, ofte flere i samme celle, hos både kontroller og eksperimentelt inficerede dyr men med en betydelig hyppigere frekvens hos de sidstnævnte. Lignende inklusioner, samt mindre lipofuscinansamlinger, sås i perivaskulære makrofager. Derudover fandtes, ligeledes i perivaskulære makrofager, yderligere en speciel type cytoplasmatiske inklusioner bestående af ansamlinger af uregelmæssige, afrundede, membranbegrænsede partikler, omkring 0.25-0.4 μm i diameter og med en indre struktur, som nogle gange kunne minde om delvis nedbrudte mitochondrier. Ansamlingerne var imidlertid ikke tydeligt omgivet af membraner, som det var at vente, hvis det drejede sig om autofagocytose. En anden, hypotetisk, forklaring kunne være, at de repræsenterede abortive udviklingsstadier af extravaskulære *Cowdria ruminantium* og at et cellemedieret immunrespons, udviklet under og efter inkubationsperioden, har bremset en sådan anden cyklus hos værtsdyret og resulteret i de

iagttagne perivaskulære celleinfiltrationer.

Resultatet af undersøgelsen peger på muligheden af, at cirkulerende monocyter er involveret direkte i patogenesen af hjerneforandringerne. Det foreslås, at sådanne celler beskytter antigenet og transporterer det extravaskulært gennem karvæggen, eftersom der ikke blev påvist tegn på vasculitis eller abnorm karpermeabilitet, hvilket gør det usandsynligt, at opløselige antigener er involveret. Tilstedeværelsen af morfologisk intakte endothelier og karvægge, selv efter perfusionsfiksering, gør det også tvivlsomt, om vasoactive substanser spiller en rolle for udviklingen af forandringerne. Hvis og når der optræder permeabilitetsforstyrrelser i hjernens mikrocirkulation, repræsenterer disse snarere senere manifestationer af en inflammatorisk proces end en primær mekanisme ved patogenesen af forandringerne i centralnervesystemet.

9. REFERENCES

Alexander, R.A., 1931. Heartwater. The present state of our knowledge of the disease. 17th Report of the Director of Veterinary Services and Animal Industry, Union of South Africa. 89-149.

Avakyan, A.A. and V.L. Popov, 1984. *Rickettsiaceae and Chlamydiaceae*: Comparative electron microscopic studies. Acta Virol. **28**, 159-173.

Barré, N., G. Uilenberg, P.C. Morel and E. Camus, 1987. Danger of introducing heartwater onto the American mainland: Potential role of indigenous and exotic *Amblyomma* ticks. Onderstepoort J. Vet. Res. **54**, 405-417.

Bezuidenhout, J.D., 1987. Natural transmission of heartwater. Onderstepoort J. Vet. Res. **54**, 349-351.

Bjørkman, N., V. Dantzer, E. Hasselager, H. Holm and P. Kjærsgaard, 1981. Perfusion in vivo of porcine placenta. Fixation for electron microscopy. Placenta **2**, 287-302.

Bourgeois, A.L., G.A. Dash and D.M. Strong, 1980. In vitro stimulation of human peripheral blood lymphocytes by soluble and membrane fractions of renograpfin-purified typhus group rickettsiae. Infection and immunity **27**, 483-491.

Camus, E. and N. Barré, 1987. Epidemiology of Heartwater in Guadeloupe and in the Caribbean. Onderstepoort J. Vet. Res. **54**, 419-426.

Camus, E. and N. Barré, 1987. Diagnosis of heartwater in the live animal: Experiences with goats in Guadeloupe. Onderstepoort J. Vet. Res. **54**, 291-294.

Cancilla, P.A., R.N. Baker, P.S. Pollock and S.P. Frommes, 1972. The reaction of pericytes of the central nervous system to exogenous protein. Lab. Invest. **26**, 376-383.

Cheville, N.F., 1983. *Cell Pathology*. 2.ed. The Iowa State University Press, Ames.

Cowdry, E.V., 1925a. Studies on the etiology of heartwater. I. Observation of a rickettsia, *Rickettsia ruminantium* (N.Sp) in the tissues of infected animals. J. Exp. Med. **42,** 231-252.

Cowdry, E.V., 1925b. Studies on the etiology of heartwater. II. Rickettsia ruminantium (N. Sp.) in the tissues of ticks transmitting the disease. J. Exp. Med. **42,** 253-274.

Cowdry, E.V., 1926. Studies on the etiology of heartwater. III. Multiplication of *Rickettsia ruminantium* within the endothelial cells of the infected animals and their discharge into the circulation. J. Exp. Med. **44,** 803-814.

Crocker, D.J., T.M. Murad and J.C. Geer, 1970. Role of the pericyte in wound healing: An ultrastructural study. Exp. & Mol. Pathol. **13,** 51-65.

Du Plessis, J.L., 1970a. Immunity in heartwater: I. A preliminary note on the role of serum antibodies. Onderstepoort J. Vet. Res. **37,** 147-150.

Du Plessis, J.L., 1970b. Pathogenesis of heartwater: I. *Cowdria ruminantium* in the lymph nodes of domestic animals. Onderstepoort J. Vet. Res. **37,** 89-96.

Du Plessis, J.L., 1975. Electron microscopy of *Cowdria ruminantium* infected reticulo-endothelial cells of the mammalian host. Onderstepoort J. Vet. Res. **42,** 1-14.

Du Plessis, J.L., 1982. Mice infected with a *Cowdria ruminantium*-like agent as a model in the study of heartwater. D.V.Sc. thesis, University of Pretoria.

Du Plessis, J.L., B.C. Jansen and L. Prozesky, 1983. Heartwater in Angora goats. 1 Immunity subsequent to artificial infection and treatment. Onderstepoort J. Vet. Res. **50,** 137-143.

Du Plessis, J.L., L. Malan and Z.E. Kowalski, 1987. The pathogenesis of heartwater. Onderstepoort J. Vet. Res. **54,** 313-318.

Elling, F., E. Hasselager and C. Friis, 1977. Perfusion fixation of kidneys in adult pigs for electron microscopy. Acta anat. **98,** 340-342.

Fujita, S. and T. Kitamura, 1976. Origin of brain macrophages and the nature of microglia. In Zimmerman, H. (Ed.): Progress in Neuropathology **3,** 1-50. Grune & Stratton. New York.

Ghadially, F.N., 1988. *Ultrastructural Pathology of The Cell and Matrix.* 3.ed. Butterworths. London.

Glauert, A.M., 1975. Fixation, dehydration and embedding of biological specimens Vol. 3. In Glauert A.M. (Ed.): *Practical Methods in Electron Microscopy.* North Holland. Amsterdam.

Gruss, B., 1983. The danger of immunizing Boer goats against heartwater. J. S. Afr. Vet. Assoc. **54,** 67-68.

Hickey, W.F. and H. Kamura, 1988. Perivascular microglial cells of the central nervous system are bone marrow-derived and present antigen in vivo. Science **239,** 290-292.

Holland, C.J., L.L. Logan, C.A. Mebus and M. Ristick, 1987. The serological relationship between *Cowdria ruminantium* and certain members of the genus *Ehrlichia.* Abstr. Onderstepoort J. Vet. Res. **54,** 331.

Ilemobade, A.A., 1976. Study of heartwater and its causative agent *Cowdria ruminantium* (Cowdry, 1925) in Nigeria. PhD. thesis, Ahmadu Bello University, Zaria.

Isoun T.T., J.U. Akpokodje, B.O. Ikede and O. Fayemi, 1974. Heartwater in imported Brown Swiss breed of cattle in Western Nigeria. Bull. Epizoot. Dis. Afr. **22,** 331-334.

Jongejan, F, G. Uilenberg and J. Fransen, 1988. Antigenic differences between stocks of *Cowdria ruminantium.* Res. Vet. Sci. **44,** 186-189.

Jongejan, F, G., 1991.Protective immunity to heartwater (*Cowdria ruminantium* infection) is acquired after vaccination with in vitro-attenuated Rickettsiae. Infection and Immunity. **59,** 729-731.

Jordan, F.L. and W.E. Thomas, 1988. Brain macrophages: Questions of origin and interrelationship. Brain Res. Rev. **13,** 165-178.

Kocan, K. and J.D. Bezuidenhout, 1987. Morphology and development of *C. ruminantium* in *Amblyomma* ticks. Onderstepoort J. Vet. Res. **54,** 177-182.

Kocan, C.M., T.B. Crawford, P.M. Dilbec, J.F. Evermann and T. C. McGuire, 1990. Development of Rickettsia isolated from an aborted bovine fetus. J. Bacteriol. **172,** 5949-5955.

Ling, E.A., 1978. Brain macrophages in rats following intravenous labelling of mononuclear leukocytes with colloidal carbon. J. Anat. **125,** 101-106.

Ling, E.A., 1981. The origin and nature of microglia. In Federoff and L. Hertz (Eds.): Advances in Cellular Neurobiology, Vol. 2, 33-82. Academic Press, New York.

Logan, L.L., E.F. Birnie and C.A. Mebus. 1987. Cross immunity between isolates of *Cowdria ruminantium*. Onderstepoort J. Vet. Res. **54,** 345.

Moulder, J.W., 1974. The Rickettsias. pp. 882-928. In Buchanan, R.E. and N.E. Gibbons, (eds.): *Bergeys Manual of Determinative Bacteriology*. 8.ed. The Williams & Wilkins Company, Baltimore.

Murabe, Y. and Y. Sano, 1983.Morphological studies on neuroglia. VII. Distribution of "brain macrophages" in brains of neonatal and adult rats, as determined by means of immunohistochemistry. Cell & Tissue Res. **229,** 85-95.

Murabe, Y. and Y. Sano, 1981. Morphologic studies on neuroglia. I. Electron microscopic identification of silver-impregnated glial cells. Cell & Tissue Res. **216,** 557-568.

Neitz, W.O, 1968. Heartwater. Bull. Office Internatl. Epizootics. **70,** 329-336.

Oehmichen, M, 1983. Inflammatory cells in the nervous system: An integrating concept based on recent research in pathology, immunology and forensic medicine. Prog. Neuropathol. **5,** 277-335.

Owen, N.C, A. Littlejohn, J.M. Kruger and B.J. Erasmus, 1973. Physiopathological features of heartwater in sheep. J. S. Afr. Vet. Assoc. **44,** 397-403.

Perry, V. and S. Gordon, 1988. Macrophages and microglia in the nervous system. Trends Neurosci. **11,** 273-277.

Peters A., S.L. Palay and H. de F. Webster. 1976. *The Fine Structure of the Nervous System: The Neurones and Supporting Cells.* W.B Saunders & Co., Philadelphia.

Petney, T.N., I.G. Horak and Y. Rechav, 1987. The ecology of the African vectors of heartwater, with particular reference to *Amblyomma hebraeum* and *Amblyomma variegatum.* Onderstepoort J. Vet. Res. **54,** 381-395.

Pienaar J.G., P.A. Basson and J.L. de B. van der Merwe, 1966.
Studies on the pathology of heartwater [*Cowdria (Rickettsia) ruminantium, Cowdry,* 1926]. I. Neuropathologic changes. Onderstepoort J. Vet. Res. *33,* 115-138.

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

Provost, A. and J.D. Bezuidenhout, 1987. The historical background and global importance of heartwater. Onderstepoort J. Vet. Res. **54,** 165-169.

Prozesky, L. and J.L. Du Plessis, 1985a. Heartwater in Angora goats. II. A pathological study of artificially infected, treated and untreated goats. Onderstepoort J. Vet. Res. **52,** 13-19.

Prozesky, L. and J.L. Du Plessis, 1985b. The pathology of heartwater. II. A study of lung lesions in sheep and goats infected with the Ball 3 strain of *Cowdria ruminantium*. Onderstepoort J. Vet. Res. **52**, 81- 85.

Prozesky, L., 1987a. The diagnosis of Heartwater at post mortem and the confirmation of *Cowdria ruminantium* in mice. Onderstepoort J. Vet. Res. **54**, 301-303.

Prozesky, L, 1987b. The pathology of heartwater. A review. Onderstepoort J. Vet. Res. **54**, 281-286.

Purchase, H.S., 1945. A simple and rapid method for demonstration of *Rickettsia ruminantium* (Cowdry, 1925) in heartwater brains. Vet. Rec. **57**, 413-414.

Pypekamp, H.E. van de, and L. Prozesky, 1987. Heartwater: An overview of the clinical signs, susceptibility and differential diagnosis of the disease in domestic ruminants. Onderstepoort J. Vet. Res. **54**, 263-266.

Reynolds, E.S., 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J. Cell Biol. **17**, 208-212.

Robinson, W.F. and M.G. Maxie, 1985. The Cardiovascular System. In Jubb, K.V.F., P.C. Kennedy and N. Palmer (Eds.): *Pathology of Domestic Animals*. 3.ed. Vol.3. 60-63. Academic Press, Orlando.

Sahu, S.P., A.H. Dardiri and S.H. Wool. 1983. Observation of *Rickettsia ruminantium* in cell cultures from heartwater-infected goats, sheep and cattle. Am. J. Vet. Res. **44**, 1093-1097.

Schreuder, B.E.C., 1980. A simple technique for the collection of samples for the diagnosis of heartwater. Trop. Anim. Hlth.Prod. **12**, 25-29.

Scott, G.R., 1987. The taxonomic status of the causative agent of heartwater. Onderstepoort J. Vet. Res. **54**, 257-260.

Shirai, A, P.J. Catanzaro, S.M. Phillips and J.V. Osterman, 1976. Host defenses in experimental scrub typhus: Role of cellular immunity in heterologous protection. Infection and Immunity **14**, 39-46.

Snedecor, G.W. and W.G. Cochran, 1967. Statistical Methods. 6'th ed., Iowa State University Press, Ames.

Spruel, J., 1922. Heartwater. Joernaal van die Department van der Landbou. 11, 1-12. Cit.

Pypekamp, H Van de, and L. Prozesky, 1987. An overview of the clinical signs, susceptibility and differential diagnosis of the disease in domestic ruminants. Onderstepoort J. Vet. Res. **54**, 263-266.

Steck, W., 1928. Pathological studies on heartwater. 13th-14th Rep. Dir. Vet. Educ. Res. Un. S. Afr., 283-305.

Stein, O. and Y. Stein, 1971. Light and electron microscopic radioautography of lipids. Techniques and biological applications. Adv.Lipid Res. **9**, 1-72.

Stewart, C.G., 1987a. Specific immunity in farm animals to heartwater. Onderstepoort J. Vet. Res. **54**, 341-342

Stewart, C.G., 1987b. Specific immunity in mice to heartwater. Onderstepoort J. Vet. Res. **54**, 343-344.

Stewart, C.G. and P.G. Howell, 1981. Electron microscopic studies on *Cowdria ruminantium*. Proc. Internatl. Conf., 27-29 January, Grahamstown, South Africa.

Synge, B.A., 1978. Brain biopsy for the diagnosis of heartwater. Trop. Anim. Hlth. & Prod. **10**, 45-48.

Tizzard, I., 1987. *Veterinary Immunology*. 3rd.ed. W.B. Saunders Co. Philadelphia.

Torvik, A., 1975. The relationship between microglia and brain macrophages: Experimental investigations. Acta Neuropathol. **S6,** 297-300.

Uilenberg , G., 1983. Heartwater (*Cowdria ruminantium* infection). Current status. Adv. Vet. Sci. Comp. Med. **27,** 428-480.

Uilenberg, G., D. Zivkovic, R.H. Dwinger, A.A.H.M Ter Huurne and N.M. Perié. 1983. Cross immunity between strains of *Cowdria ruminantium*. Res. Vet. Sci. **35,** 200-205.

van Amstel, S.R., 1987. The use of electro-encephalography and brain biopsy in the clinical diagnosis of heartwater. Onderstepoort J. Vet. Res. **54,** 295-299.

van de Merwe, L., 1979. Field experience with heartwater (*C.ruminantium*) in cattle. J. S. Afr. Vet. Assoc. **50,** 323-325.

Van Deurs, B, 1976. Observations on the blood-brain barrier in hypertensive rats, with particular reference to phagocytic pericytes. J. Ultrastruct. Res. **56,** 65-77.

Vassar, P.S., J.M. Havards, D.E. Brooks, B. Hagenberger and G.V.F. Seaman, 1972. Physiochemical effects of aldehydes on the human erythrocytes. J. Cell Biol. **53,** 809-818.

Walker, J.B. and A. Olwage, 1987. The tick vectors of *Cowdria ruminantium* (Ixodoidea, Ixodidae, Genus *Amblyomma*) and their distribution. Onderstepoort J. Vet. Res. **54,** 353-379.

Watson, M.L., 1958. Staining of tissue sections of electron microscopy with heavy metals. J. Biophysi. Biochem. Cytol. **4,** 475-478.

Wheater, P.R., H.G. Burkitt and V.G. Daniels, 1979. *Functional Histology: A text and Color Atlas.* Churchill Livingstone. Edinburgh.

Williams, A.E. and W.F. Blakemore, 1990. Monocyte-mediated entry of pathogens into the central nervous system. Neuropathology and Applied Neurobiology **16,** 377-392.

Williams, J.C. and H. Vodkin, 1987. Metabolism and genetics of Chlamydias and Rickettsias. Onderstepoort J. Vet. Res. 54, 211-221.

10. APPENDICES

APPENDIX 1: Recipe for 3% glutaraldehyde (pH 7.4, 500 mOsm) used for perfusion of goat brains. Polyvidon® is polyvinyl pyrrolidone.

Constituent chemicals		Amount
Na H_2PO_4 .H_2O	(g)	11.00
Na_2 HPO_4 .$2H_2O$	(g)	60.56
Dist. water	(ml) brought to	2000.00 (a)
Polyvidon®	(g)	300.00
Dist. water	(ml) brought to	2000.00 (b)
Glutaraldehyde 25%	(g)	720.00
Dist. water	(ml) brought to	2000.00 (c)
Total ml (a+b+c) adjusted to pH 7.4		6000.00

APPENDIX 2.1: Processing with reduced osmium

Recipe for osmium tetroxide.

1) 10 ml 2% osmium tetroxide aqueous solution
2) 100 mg potassium ferrocyanide
3) 0.7 g sucrose

Processing regime

1) Rinsed 2× in 0.1 sodium cacodylate buffer
2) Rinsed 2× in 7% sucrose water
3) 2 hours with agitation in reduced osmium at 22°C
4) Rinsed 2× in 7% sucrose water
5) Rinsed 2× in 0.1M cacodylate buffer
6) Rinsed 3× in distilled water
7) Stained in 0.5% uranyl acetate aqueous solution
8) Rinsed 3× in distilled water

APPENDIX 2.2: Ruthenium red

1) Rinsed in 0.1M cacodylate buffer 2 × 5 minutes
2) Stained in ruthenium red for 2 hours at 20°C in darkness.

APPENDIX 2.3: Tannic acid

1) Rinsed in 0.1M cacodylate buffer 2× 15 minutes
2) Stained in 1% tannic acid/.1M cacodylate buffer for 30 minutes at 21°C
3) Rinsed in 0.1M cacodylate buffer 2× 5 minutes
4) Post-fixed in 1% osmium tetroxide/0.1M cacodylate buffer for 2 hours at 21°C and left to stand in buffer overnight.

APPENDIX 2.4: Osmium tetroxide

1) Rinsed in 0.1M cacodylate buffer 3× 10 minutes
2) Post-fixed in 1% osmium tetroxide/sodium cacodylate buffer for 2 hours (pH 7.4).
3) Rinsed in sodium cacodylate buffer 2 × 5 minutes
4) Rinsed in distilled water 1 × 5 minutes

APPENDIX 2.5: Standard processing regime for electron miccroscopy of brain from control goats.

	Time	Temperature °C
0.1 M cacodylate buffer rinse	2×10min	4
1% osmium in cacodylate buffer	3hours	20
Distilled water	1min	20
70% alcohol	10min	20
96% alcohol	10min	20
96% alcohol	15min	20
0.5% uranyl acetate/96% alcohol	2hours	4
99% alcohol	3×20min	20
Propylene oxide (PO)	2×15min	20
PO/Epon 2+1	30min	20
PO/Epon 1+1	30min	20
PO/Epon 1+2	30min	20
Epon	2hours	20
Epon[1]	8hours	15

[1] Epon: 14.22 g MNA + 39.47 g DDSA + 46.31 g Epon

APPENDIX 2.6: Prolonged dehydration regime

	Time	Temperature °C
70% alcohol	2×10min	20
96% alcohol	2×10min	20
99% alcohol	15min	20
99% "	3×20min	20
99% "	4×60min[3]	20
PO	2×15min	20
PO/Epon 2+1	40min	20
PO/Epon 1+1	40min	20
PO/Epon 1+2	40min	20
Epon	2hours	20
Epon[2]	8hours	20

APPENDIX 2.7: Processing procedure for electron microscopy of brain tissues from experimental goats 1-14.

1) Rinsed 2× in .1M Na. cacodylate buffer
2) Post-fixed for 1½ hours. in 1% Osmium tetroxide + .1M Na. cacodylate buffer at 21°C
3) Rinsed 2× in .1M Na. cacodylate buffer
4) Rinsed 1× in distilled water
5) 70% alcohol 5 minutes
6) 96% alcohol 5 minutes
7) 99% alcohol 5 minutes
8) 99% alcohol 2 hours
9) 99% alcohol 20 minutes
10) 99% alcohol 20 minutes
11) P.O 1+0 10 minutes
12) P.O 1+0 10 minutes
13) P.O/EP 2+1 20 minutes
14) P.O/EP 1+1 20 minutes
15) P.O/EP 1+2 20 minutes
16) EP 1+0 overnight, without stoppers
17) Embedding
18) Polymerization at 60°C for 3 days.

[2] Epon = Standard (1.5% DMP)

[3] Prolonged dehydration

APPENDIX 3: Summary of clinical, necropsy, and histopathologic findings in 14 goats experimentally infected with *Cowdria ruminantium*.

Animal no. 1. Male (tag no. 3048). Killed on post-inoculation day 15.

Clinical signs	Fever (recorded peak temperature: 41°C) Rough hair coat Listlessness Loss of body condition Stupor Ataxia Head tremors
Necropsy findings	Hydropericardium (8 ml) Flabby heart Splenomegaly Urine retention in bladder
Histopathology	*C.ruminantium* in smears and/or sections of brain Mononuclear leptomeningitis Focal perivascular mononuclear cell infiltration in brain Splenic lymphoid hyperplasia

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Animal no. 2. Male (tag no. 2913). Killed on post-inoculation day 18.

Clinical signs	Fever (recorded peak temperature: 39.7°C) Listlessness Loss of body condition Stupor
Necropsy findings	Hydropericardium (4 ml) Flabby heart Splenomegaly Urine retention in bladder
Histopathology	Focal pulmonary, subepi- and subendocardial mononuclear cell infiltration Splenic lymphoid hyperplasia

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Animal no. 3. Male (tag no. 2953). Killed on post-inoculation day 13.

Clinical signs	Fever (recorded peak temperature: 41.3°C) Inappetence Listlessness Loss of body condition Stupor Head tremors
Necropsy findings	Hydropericardium (6 ml) Subepicardial petechiae Splenomegaly Congestion of meninges and small intestine
Histopathology	Focal perivascular and parenchymal mononuclear cell infiltration in brain and meninges Focal renal interstitial and pulmonary mononuclear cell infiltration Splenic lymphoid hyperplasia

Animal no. 4. Male (tag no. 2950). Killed on post-inoculation day 15.

Clinical signs	Fever (recorded peak temperature: 40.9°C) Rough hair coat Inappetence Loss of body condition Listlessness Stupor Ataxia Head tremors
Necropsy findings	Hydropericardium (9 ml) Subepicardial petechiae Splenomegaly Congestion of small intestine and meninges Loose colonic contents
Histopathology	Focal renal interstitial, subepi- and subendocardial mononuclear cell infiltration Splenic lymphoid hyperplasia Coccidia in small intestinal epithelium

Animal no. 5. Male (tag no. 2969). Killed on post-inoculation day 15.

Clinical signs	Fever (recorded peak temperature: 41.5°C) Rough hair coat General weakness Inappetence Rapid loss of body condition Abdominal respiration Loose stool Stupor and ataxia Head tremors
Necropsy findings	Hydropericardium (8 ml) Splenomegaly Bladder ecchymoses Urine retention in bladder Congestion of meninges Feces not formed
Histopathology	*C.ruminantium* in smears and/or sections of brain Mononuclear leptomeningitis Focal perivascular mononuclear cell infiltration in brain Focal renal interstitial mononuclear cell infiltration Splenic lymphoid hyperplasia

Animal no. 6. Male (tag no. 2994). Killed on post-inoculation day 16.

Clinical signs	Fever (recorded peak temperature: 41.5°C) Rough hair coat Listlessness Inappetence Rapid loss of body condition Abdominal respiration Stupor Ataxia Head tremors
Necropsy findings	Hydropericardium (10 ml) Splenomegaly Watery small intestinal contents Congestion of meninges
Histopathology	*C. ruminantium* in smears and/or sections of brain Mononuclear leptomeningitis Focal perivascular mononuclear cell infiltration in brain Splenic lymphoid hyperplasia

Animal no. 7. Male (tag no. 2943). Killed on post-inoculation day 17.

Clinical signs	Fever (recorded peak temperature: 41.2°C) Listlessness Inappetence Rapid loss of body condition Abdominal respiration Stupor Ataxia Head tremors
Necropsy findings	Hydropericardium (3 ml) Subepicardial petechiae Splenomegaly Urine retention in bladder
Histopathology	*C. ruminantium* in smears and/or sections of brain Mononuclear leptomeningitis Focal perivascular mononuclear cell in- filtration in brain Focal renal interstitial, subepi- and subendocardial mononuclear cell infiltration Splenic lymphoid hyperplasia

Animal no. 8. Female (tag no. 2877). Killed on post-inoculation day 15.

Clinical signs	Fever (recorded peak temperature: 41.3°C) Listlessness Inappetence Loss of body condition Abdominal respiration Stupor Ataxia Head tremors
Necropsy findings	Hydropericardium (6 ml) Flabby heart Subendocardial petechiae Splenomegaly
Histopathology	*C. ruminantium* in smears and/or sections of brain Mononuclear leptomeningitis Focal perivascular mononuclear cell in-filtration in brain Focal subepi-and subendocardial mononuclear cell infiltration Splenic lymphoid hyperplasia

Animal no. 9. Female (tag no. 2903). Killed on post-inoculation day 18.

Clinical signs	Fever (recorded peak temperature: 40.7°C) Rough hair coat Listlessness Inappetence Loss of body condition Abdominal respiration Stupor Ataxia Head tremors Severe fits, opisthotonus, paddling movements, and lateral recumbency during transportation prior to killing
Necropsy findings	Hydropericardium (13 ml) Subepicardial petechiae Splenomegaly Urine retention in bladder
Histopathology	*C. ruminantium* in smears and/or sections of brain Mononuclear leptomeningitis Focal perivascular and parenchymal mononuclear cell infiltration in brain Focal pulmonary mononuclear cell infiltration Splenic lymphoid hyperplasia

Animal no. 10. Female (tag no.2925). Killed on post-inoculation day 18.

Clinical signs	Fever (recorded peak temperature: 40.9°C) Rough hair coat Inappetence and listlessness; Loss of body condition Stupor, ataxia and head tremors; Developed fits, paddling movements, posterior paralysis, and lateral recumbency
Necropsy findings	Hydropericardium (4 ml) Splenomegaly Congestion of liver Urine retention in bladder
Histopathology	*C. ruminantium* in smears and/or sections of brain Mononuclear leptomeningitis Focal perivascular and parenchymal mononuclear cell infiltration in brain Focal renal interstitial, subepi- and subendocardial mononuclear cell infiltration Splenic lymphoid hyperplasia

Animal no. 11. Female (tag no. 3019). Killed on post-inoculation day 18.

Clinical signs	Fever (recorded peak temperature: 39.6°C) Rough hair coat Inappetence Loss of body condition Listlessness Stupor Head tremors
Necropsy findings	Hydropericardium (3 ml) Splenomegaly Urine retention in bladder
Histopathology	Focal perivascular and parenchymal mononuclear cell infiltration in brain Focal renal interstitial mononuclear cell infiltration Splenic lymphoid hyperplasia

Animal no. 12. Male (tag no. 2892). Killed on post-inoculation day 19.

Clinical signs	Fever (recorded peak temperature: 41.2°C) Listlessness Inappetence oss of body condition Stupor Ataxia Head tremors
Necropsy findings	Hydropericardium (2 ml) Flabby heart Splenomegaly Urine retention in bladder
Histopathology	*C. ruminantium* in smears and/or sections of brain Mononuclear leptomeningitis Focal perivascular and parenchymal mononuclear cell infiltration in brain Splenic lymphoid hyperplasia

Animal no. 13. Male (tag no. 2944). Killed on post-inoculation day 19.

Clinical signs	Fever (recorded peak temperature: 40.6°C) Listlessness Loss of body condition Stupor Ataxia
Necropsy findings	Hydropericardium (3 ml) Subepicardial petechiae Splenomegaly Mucosal petechiae in bladder Urine retention in bladder
Histopathology	Mononuclear leptomeningitis Focal perivascular and parenchymal mononuclear cell infiltration in brain Splenic lymphoid hyperplasia

Animal no. 14. Male (tag no. 3007). Killed on post-inoculation day 19.

Clinical signs	Fever (recorded peak temperature: 40.5°C) Listlessness Abdominal respiration Loss of body condition Stupor Ataxia
Necropsy findings	Hydropericardium (6 ml) Subepicardial petechiae Splenomegaly Urine retention in bladder
Histopathology	Mononuclear leptomeningitis Focal perivascular and parenchymal mononuclear cell infiltration in brain Focal renal interstitial, subepi- and subendocardial mononuclear cell infiltration Splenic lymphoid hyperplasia